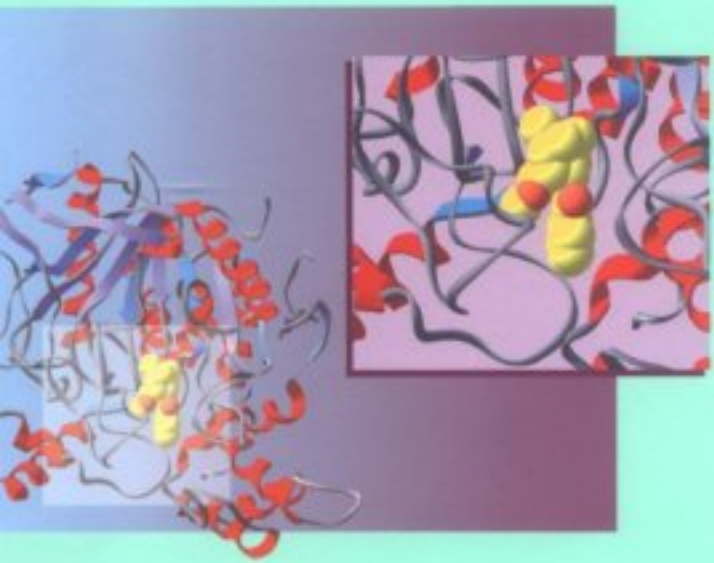
Bernard Testa, Joachim M. Mayer

Hydrolysis in Drug and Prodrug Metabolism

Chemistry, Biochemistry, and Enzymology



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Dedicated to the fond memory of
Joachim M. Mayer (1949 – 2001) and *Nabil El Tayar* (1957 – 2001),
former graduate students, colleagues, and friends in eternity

Foreword

Most drugs and other xenobiotics (compounds foreign to an organism) are metabolized to inactive and excretable compounds by an uncounted arsenal of enzymes. Among these enzymes are the hydrolases reviewed in this book. Xenobiotics enter the organism *via* the intestine, lung, skin, and other barriers between the organism and its environment, at which sites they may already be biotransformed. Clearly, however, the major organ of drug metabolism is the liver. It is a major 'filter' between the gut and systemic circulation, pointing to the importance of dietary xenobiotics that have to be 'detoxified'. Defense against the invasion of small lipid-soluble molecules in the diet and the air most probably has been the evolutionary force in the proliferation of this large number of enzymes of drug metabolism from genes that previously coded for enzymes involved in the metabolism of endogenous compounds such as fatty acids, sterols, bile acids, vitamins, *etc*. This scenario also offers a conceptual framework for the overlapping substrate specificities and low-affinity reactions of these enzymes, which together can metabolize thousands of xenobiotic substrates and, also, many endogenous molecules. Thus, xenobiotic metabolism has become a subject of broad interdisciplinarity, encompassing physiology, pathophysiology, pharmacology in all its aspects, toxicology including environmental issues, biochemistry, genetics, medicinal chemistry, computational (bio)chemistry, analytical chemistry, *etc*.

Bernard Testa is very well-known for his capacity to conceptualize reactions of drug metabolism. After his *Citation Classic Award* volume in 1976 on chemical and biochemical aspects of drug metabolism, written with *Peter Jenner* [1], he has edited another classic on '*Concepts in Drug Metabolism*' [2]. Continuing with his exceptional gift to synthesize and present the enormous amount of new data and concepts in the field, *Bernard Testa* published in 1995 a single-author book dedicated to the biochemistry of redox reactions in the metabolism of drugs and other xenobiotics [3]. The prosaic title is misleading, however, since this book contains a thoughtful 'global view' of xenobiotic metabolism, presenting the author's vision of the history, evolution, and impact of drug metabolism, providing much general knowledge and structure for the whole field.

In the present volume, somewhat delayed by the unfortunate and untimely death of *Joachim Mayer*, a comprehensive review of all metabolic reac-

tions and enzymes involved in the hydrolysis of drugs and prodrugs is presented. Nothing of this sort has been written before, as hydrolysis together with some other reactions of drug metabolism have long been in the shadow of the 'P450 hype', the preoccupation of the drug-metabolism community with the superfamily of over 2000 *CYP* genes coding for oxidative enzymes of drug metabolism. Recent medicinal developments such as peptide drugs or inhibitors of cholinesterases now put new emphasis on these 'other' important enzymes of drug metabolism. *Bernard Testa*'s fundamental knowledge of this field and his commitment to conceptualization, clear structure, and precise language make this book another important addition to his contributions that make us understand the principles of biotransformation of xenobiotics.

November 2002

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Biozentrum
University of Basel

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Preface

This book was originally conceived as a single chapter, then as a series of two to three chapters. However, it rapidly became obvious that any coverage of metabolic reactions of hydrolysis aiming at some degree of comprehensiveness would require a complete volume at least. Two persons were critical in the completion of the project. First, I was happy to secure the collaboration of Dr. *Joachim M. Mayer*, a Reader in Biopharmacy, who agreed with enthusiasm to join me as co-author. The second person to trust me was Dr. *M. Volkan Kisakürek*, the Managing Director of the publishing house *Verlag Helvetica Chimica Acta* and the Editor-in-Chief of the *Helvetica Chimica Acta*. The agreement *Joachim* and I signed with the publisher in October 1998 set us on course, with Dr. *Kisakürek* offering advice and encouragement ever since.

The brutal death from heart failure of *Joachim* in October 2001 was a severe blow to the progress of our project, but so much work had already been dedicated to it that there was no option but to bring it to completion, and to do so singlehandedly. Tragically, Dr. *Nabil El Tayar*, another of my closest friends, had known the same fate a few weeks earlier. The book is dedicated to the memory of these two bright and creative scientists.

The structure of the book you now hold in your hands is as logical as we could have it, with an *Introduction* (*Chapt. 1*) and a very brief *Conclusion* (*Chapt. 12*) framing its main body. The ten chapters of the main body are ordered in four groups, as follows:

a) The first group (*Chapt. 2* and *3*) treats the various hydrolytic enzymes involved in amide and ester hydrolysis.
b) The next group of three chapters presents the hydrolysis of amides, namely acyclic amides in *Chapt. 4*, lactams in *Chapt. 5*, and peptides in *Chapt. 6*.
c) This is followed by three chapters on the hydrolysis of esters, namely carboxylic acid esters (*Chapt. 7* and *8*), and esters of inorganic acids (*Chapt. 9*).
d) The final two main chapters cover epoxide hydrolases and their reactions (*Chapt. 10*), followed, in *Chapt. 11*, by a number of unclassifiable reactions of hydration and dehydration, some enzymatic and others not.

A number of people have helped in this endeavor and deserve my gratitude. Dr. *Kisakürek*, as already mentioned, has played a major role in guid-

ing us and in ensuring that the printed work will be of the highest possible technical quality. It is a unique privilege to be able to collaborate with a publisher who combines scientific competence, friendly dedication, and professional excellence.

Dr. *Giulia Caron*, Dr. *Georgette Plemper van Balen*, Dr. *Frédéric Ooms*, and (the soon-to-be Dr.) *Xiangli Liu* helped me in the preparation of the numerous structures and figures. These post-doctoral fellows and graduate students were dedicated to quality and generous with their time. They deserve my warmest appreciation and thanks.

Finally, I am in debt beyond words to my wife *Jacqueline* for her unfailing support and understanding. Evening after evening, she encourages me as I face screen and keyboard, struggling to make sense of the avalanche of facts and data that our colleagues generate and publish with ever increasing efficiency. Perhaps the present book goes a little way toward this objective and brings a semblance of order in an important area of xenobiotic metabolism. Such are the readers' expectations and the authors' hope.

January 2003 *Bernard Testa*

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Chapter 8 The Hydrolysis of Carboxylic Acid Ester Prodrugs

Chapter 1

Introduction: Metabolic Hydrolysis and Prodrug Design

Contents

1.1. Hydrolysis and Hydration in Xenobiotic Metabolism

1.1.1. Metabolic Hydrolysis, a Neglected Field?

As demonstrated by the innumerable references contained in this book, hydrolysis reactions are a major focus of interest in the study of the metabolism of drugs, prodrugs, and other xenobiotics. In contrast and to the best of our knowledge, no book has ever been published with such reactions as its main or single theme. Even recent monographs on drug metabolism tend to neglect or minimize the great chemical, biochemical, pharmacological, and toxicological significance of metabolic hydrolysis. To take but one example, a recently edited book of broad scope [1] contains 13 comprehensive chapters dedicated to all oxidoreductases and transferases that play a major or modest role in xenobiotic metabolism. There is also a very nice chapter on epoxide hydrolases and another on sulfatases and glucuronidases, but practically nothing about other hydrolases of great and ever increasing importance in xenobiotic metabolism. The objective of the present book is to correct this intriguing neglect and to demonstrate the diversity of hydrolases and the richness of their substrates.

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1.1.2. Functionalization or Conjugation?

The metabolism of drugs and other xenobiotics is often a biphasic process in which the compound may first undergo a *functionalization reaction* (phase-I reaction). This introduces or unveils a functional group, such as a hydroxy or amino group, suitable for coupling with an endogenous molecule or moiety in a second metabolic step known as a *conjugation reaction* (phase-II reaction). In a number of cases, phase-I metabolites may be excreted prior to conjugation, and there are many xenobiotics that can be directly conjugated. Furthermore, reactions of functionalization may follow some reactions of conjugation, *e.g.*, some conjugates are hydrolyzed and/or oxidized prior to excretion [2 – 11].

Xenobiotic biotransformation thus produces two types of metabolites, namely *functionalization products* and *conjugates*. But, with the growth of knowledge, biochemists and pharmacologists have progressively come to recognize the existence of a third class of metabolites, namely *xenobiotic–macromolecule adducts*, also called macromolecular conjugates. These peculiar metabolites are formed when a xenobiotic becomes covalently bound to a biological macromolecule, usually following metabolic activation (*i.e.*, post-enzymatically). Both functionalization products and conjugates have been found to bind covalently to biological macromolecules, the reaction often being toxicologically relevant.

Whereas metabolic reactions of oxidation and reduction are universally recognized as being functionalization reactions, there has been some debate over whether reactions of hydrolysis should be classified as conjugations. This is a view we strongly oppose, as argued below.

Conjugation reactions are characterized by a number of *criteria*:

a) They are catalyzed by enzymes known as *transferases*.
b) They transfer to the substrate a molecule or moiety that is synthesized endogenously and linked covalently to a *cofactor*.
c) This endogenous molecule or moiety is highly polar (*hydrophilic*) and of a *size* comparable to that of the substrate.

It is important from a biochemical and practical viewpoint to note that each criterion taken individually is neither sufficient nor necessary to define a conjugation reaction. No single criterion is sufficient, since, for example, hydrogenation reactions (*i.e.*, typical reactions of functionalization) involve the transfer of a hydride anion from a cofactor (NADPH or NADH). Further, no single criterion is necessary, since all the above criteria know some important exceptions. As examples, we mention that *a*) glutathione conjugation sometimes occurs nonenzymatically; *b*) the conjugation of a hydrazine with an endogenous ketone or aldehyde also occurs nonenzymatically and does

not involve a cofactor; and c) some endogenous molecules or moieties being transferred are either lipophilic and small (*e.g.*, a neutral methyl group), lipophilic and large (*e.g.*, glycerides, cholesterol), or hydrophilic and large (*e.g.*, glutathione).

While some conjugation reactions fail to totally meet the above criteria, they all satisfy at least one criterion. But what about the reactions of hydrolysis? On the one hand, these fulfil none of the three above criteria, with the partial exception that the molecule (water) that reacts with the substrate is a polar one. On the other hand, the metabolic reactions of hydrolysis modify pre-existing functional groups of the substrates and, thus, meet the definition of functionalization reactions.

1.1.3. Hydrolysis, Hydration, Dehydration

The water molecule, either as a reactant or a product, plays an important role in the metabolism of innumerable endogenous and exogenous compounds. Water is, of course, a by-product of some metabolic redox reactions, *e.g.*, reactions catalyzed by monooxygenases [7]. However, this book focuses on non-redox reactions involving water as a reactant or a product.

As a reactant, the water molecule can be added to a substrate molecule either to cleave it into two molecules, or to yield a single product. Perhaps arbitrarily, we designate cleavage into two molecules as *hydrolysis* (*e.g.*, the production of an acid and an alcohol from an ester), and incorporation of water to form a single metabolite as *hydration*. However, such a neat nomenclature is not always followed in practice, witness the ring opening of lactones resulting from the addition of a water molecule, which is commonly referred to as hydrolysis rather than hydration in analogy with the same reaction occurring in acyclic carboxylic acid esters.

Non-redox reactions where water is formed as a product are reactions of *dehydration*. Such reactions can occur between two substrate molecules, or they can involve two functional groups in a single substrate, either creating a new bond (*e.g.*, lactone formation), or transforming a single into a double bond. In xenobiotic metabolism, dehydration is usually in dynamic equilibrium with hydrolysis or hydration and is of relatively modest significance (*Chapt. 11*).

1.2. Principles of Prodrug Design

Prodrugs will receive much attention in this book (*Chapt. 4*, *6*, *8*, *9*, and *11*). It is, therefore, appropriate in this introductory chapter to clarify some definitions and review various aspects of prodrug design and research [11 – 22].

1.2.1. Definition and Objectives

Prodrugs are defined as therapeutic agents that are inactive *per se* but are predictably transformed into active metabolites. As such, prodrugs must be contrasted with soft drugs, which are active *per se* and yield inactive metabolites. And, in a more global perspective, prodrugs and soft drugs appear as the two extremes of a continuum of possibilities where both the parent compound and the metabolite(s) contribute in a large or small proportion to the observed therapeutic response.

Prodrug design aims to overcome a number of barriers to a drug's usefulness (*Table 1.1*). Based on these and other considerations, the major *objectives* of prodrug design can be listed as also shown in *Table 1.1*.

1.2.2. Complementary Viewpoints in Prodrug Design

The successes of prodrug design are many, and a large variety of such compounds have proven their therapeutic value. When discussing this multidisciplinary field of medicinal chemistry, several complementary viewpoints can be adopted, as listed in *Table 1.2*.

Table 1.1. *Prodrugs: A Concept to Overcome Barriers and Enhance a Drug's Usefulness* [15][21]

Barriers to a drug's usefulness
Pharmaceutical
Insufficient chemical stability
Poor solubility
Offensive taste or odor
Irritation or pain
Pharmacokinetic
Poor oral absorption
Marked presystemic metabolism
Short duration of action
Unfavorable distribution in the body
Pharmacodynamic
Toxicity
Major objectives of prodrug design
Improved formulation (*e.g.*, increased hydrosolubility)
Improved chemical stability
Improved patient acceptance and compliance
Improved bioavailability
Prolonged duration of action
Improved organ selectivity
Decreased side-effects
Marketing considerations, 'me-too' or 'me-better' drugs

Table 1.2. *Complementary Viewpoints when Considering Prodrugs* [21]

Chemical classification? [a])
Bioprecursors
Classical carrier-linked prodrugs
Site-specific chemical delivery systems
Macromolecular prodrugs
Drug-antibody conjugates
Mechanisms of activation? [b])
Enzymatic: biologically variable but difficult to optimize
Nonenzymatic: no biological variability but easier to optimize
Mechanisms of tissue/organ selectivity?
Tissue-selective activation of classical prodrugs
Site-specific delivery of *ad hoc* chemical systems
Toxic potential
Of a metabolic intermediate (for bioprecursors)?
Of the carrier moiety or a metabolite thereof?
Gain in therapeutic benefit?
Prodrugs of marketed drugs (*post hoc* design): modest to marked benefit
Prodrugs of difficult candidates (*ad hoc* design): marked to significant benefit

[a]) Overlapping classes. [b]) Not mutually exclusive.

In a *chemical perspective*, it may be convenient to distinguish between carrier-linked prodrugs, *i.e.*, drugs linked to a carrier moiety by a labile bridge, and bioprecursors, which do not contain a carrier group and are activated by the metabolic creation of a functional group [13]. In *carrier-linked prodrugs*, the carrier moiety is often and conveniently linked to a hydroxy, an amino, or a carboxy group. Derivatization of the latter is often particularly rewarding in terms of lipophilicity, since a highly polar carboxylate group becomes masked inside an ester group whose properties can be broadly modulated.

Bioprecursors provide relevant examples of chemotherapeutic agents whose activation occurs by reduction in oxygen-deprived cells. Bioprecursors certainly appear as a viable class of prodrugs, since they avoid potential toxicity problems caused by the carrier moiety (see below). In contrast, attention must be given here to metabolic intermediates.

A special group of carrier-linked prodrugs are the site-specific chemical delivery systems [23]. Macromolecular prodrugs are synthetic conjugates of drugs covalently bound (either directly or *via* a spacer) to proteins, polypeptides, polysaccharides, and other biodegradable polymers [24].

Prodrug activation occurs enzymatically, nonenzymatically, or, also, sequentially (an enzymatic step followed by a nonenzymatic rearrangement). As much as possible, it is desirable to reduce biological variability, hence the particular interest currently received by nonenzymatic reactions of hydrolysis or intramolecular catalysis [18][20]. Reactions of cyclization–elimination appear quite promising and are being explored in a number of studies.

The problem of *tissue or organ selectivity* (targeting) is another important aspect of prodrug design. Various attempts have been made to achieve organ-selective activation of prodrugs, particularly *via* dermal and brain delivery. A promising approach appears to be the *site-specific chemical delivery systems*, which may be the 'magic bullets' of drug design, their selectivity being based on some enzymatic or physicochemical peculiarities characteristic to a given tissue or organ.

The *toxic potential* of metabolic intermediates, of the carrier moiety, or of a fragment thereof, should never be neglected. For example, some problems may be associated with formaldehyde-releasing prodrugs such as *N*- and *O*-[(acyloxy)methyl] derivatives or *Mannich* bases. Similarly, arylacetylenes assayed as potential bioprecursors of anti-inflammatory arylacetic acids proved many years ago to be highly toxic due to the formation of an intermediate ketene.

The *gain in therapeutic benefit* provided by prodrugs is a question that has no general answer. Depending on both the drug and its prodrug, the therapeutic gain may be modest, marked, or even significant. As suggested in *Table 1.2*, a trend is apparent when comparing marketed drugs and candidates in research and development. In the case of marketed drugs endowed with useful qualities but displaying some unwanted properties that a prodrug form should ameliorate, the therapeutic gain is usually modest yet real, but may become marked if good targeting is achieved.

In the case of difficult candidates that show excellent target properties but suffer from some severe physicochemical and/or pharmacokinetic drawback (*e.g.*, high hydrophilicity that restricts bioavailability), a marked to significant benefit can be obtained. Here, indeed, a prodrug form may prove necessary, and its design will be integrated into the iterative process of lead optimization. This possibility is aptly illustrated by the recently marketed neuraminidase inhibitor *oseltamivir*, the ethyl ester prodrug of RO-64-0802, which showed very high *in vitro* inhibitory efficacy toward the enzyme but low oral bioavailability due to its high polarity. Following intestinal absorption, the prodrug undergoes rapid enzymatic hydrolysis and produces high and sustained plasma levels of the active parent drug. It is interesting to compare *oseltamivir* with *zanamivir*, which, like RO-64-0802, is poorly absorbed orally and is administered to humans by means of a dry powder inhaler (see *Chapt. 8*).

1.2.3. Prodrugs in Research and Development

The global benefit brought forth by a prodrug relative to the corresponding active agent may range from considerable to negligible. The gain will be

considerable when the development of an innovative and very promising agent is blocked by a major pharmacokinetic or pharmaceutical defect that appears surmountable by a prodrug strategy. In contrast, the gain will be negligible when the drug's defect is tolerable or barely improved by transformation to a prodrug.

What remains to be discussed, however briefly, are specific difficulties encountered in the design and development of prodrugs, as related to the viewpoints discussed above (*Table 1.2*). These difficulties may range from fair to prohibitive and can occur at all stages of the research and development process (*Table 1.3*).

Careful *prodrug design* is required to minimize the number of proposed candidates and maximize the explored space of physicochemical and pharmacokinetic properties. The ability to predict target properties (*e.g.*, solubility, extent of absorption, and rate of activation) is a major need in rational prodrug design, but global quantitative models simply do not exist, despite

Table 1.3. *Specific Difficulties in Prodrug Design and Development* (modified from [22]) [a])

Objectives	Strategies and useful tools	Problems and dubious tools
Minimize the number of candidates; maximize the explored space of physicochemical and PK properties	Careful design based on physicochemical and PK properties; use of local quantitative models and global qualitative models (*e.g.*, 'rule of 5')	Global quantitative models
Minimize the additional synthetic work	Careful weighing of costs and benefits	Limited acceptance of additional costs and efforts
Reach target physicochemical profile	HT physicochemical profiling and virtual screening	Limited relevance of some HT techniques; dubious predictive capacity of some statistical models
Reach target PK profile	HT-PK profiling and virtual screening	Limited *in vivo* relevance of some HT screens; dubious predictive capacity of some statistical models
Reach target metabolic behavior, in particular target rate of activation	Fast *in vitro* metabolic assessment and use of expert systems	Dubious extrapolation to *in vivo* situations; limited predictive capacity of some expert systems
Lack of toxicity of prodrug, pro-moiety or fragment thereof	Toxicity screens and knowledge-based design	Limited *in vivo* relevance of screens; incomplete knowledge

[a]) Abbreviations: HT = high-throughput; PK = pharmacokinetic.

some claims to the contrary. At present, prodrug designers can rely on some local models or rules to make semi-quantitative or even quantitative predictions. In contrast, quantitative predictions of rates of biotransformation remain an elusive goal.

One or several additional *synthetic step(s)* are needed for each prodrug candidate being prepared. This implies additional synthetic work and increased production costs that may prove prohibitive.

As far as molecular properties are concerned, a first issue is the *physicochemical profile* of the prodrug candidate and its adequacy within the goals of the project. Some physicochemical properties can be calculated or estimated at the design stage, but experimental verification cannot be omitted. For other properties, such as solubility, quantitative predictions are more difficult and experimental assessment is mandatory.

A truly critical difficulty is the *pharmacokinetic behavior* (absorption, distribution, *etc.*) of the prodrug candidate and its adequacy within the goals of the project, first *in vitro* and, ultimately, *in vivo*. High-throughput methods are necessary to rapidly assess the *in vitro* pharmacokinetic profile of many prodrug candidates, and to verify or falsify the predictions of the prodrug designers. As with drug candidates, a major hurdle is extrapolation to humans. The danger is real, indeed, that prodrug candidates selected during *in vitro* PK screening programs may prove disappointing *in vivo*.

The truly critical difficulty is the *metabolic behavior* and particularly the *rate of activation* of prodrug candidates. The huge diversity of drug-metabolizing enzymes and the large interspecies variations that exist make rational optimization of the rate of activation in humans an impossible task. Human liver microsomes have become a common tool for metabolic profiling, but even results so obtained may give a misleading preview of *in vivo* metabolism.

Another problem is *toxicity* relative to the underivatized active agent. By influencing the distribution and tissular concentrations of the active agent they deliver, prodrugs may elicit toxic effects not displayed by the active agent itself. Furthermore, the carrier moiety may generate toxic fragments (*e.g.*, formaldehyde). Additional and careful *in vitro* (in the presence of activating enzymes) and *in vivo* toxicological investigations are, therefore, unavoidable and costly steps in prodrug development, whatever the lack of toxicity of the active agent.

The above problems appear to be the major sources of difficulty in prodrug research and development, not to mention possible complications during registration. No wonder, therefore, that so many medicinal chemists are critical of prodrugs. However, a lucid view cannot ignore the sunny side, in this case the mere existence of a number of successful prodrugs. *Nabumetone*, *oseltamivir*, and *pivampicillin* are just a few examples that come to mind.

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Chapter 2

Classification, Localization, and Some Physiological Roles of Hydrolytic Enzymes

Contents

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2.1. Introduction

The hydrolysis or hydration of endogenous and exogenous compounds is catalyzed by an extremely large variety of enzymes collectively known as *hydrolases*. This chapter is concerned with the presentation, and, principally, with the *classification*, *localization*, and *some physiological roles* of the hydrolases that catalyze the hydrolysis of ester and amide (including peptide) bonds. The *catalytic mechanisms* of these hydrolytic enzymes will be examined again in *Chapt. 3*. The various enzyme *substrates* (amides, lactams, peptides, esters, and lactones) will be presented in subsequent chapters (*Chapt. 4–9*) in their turn, with the focus almost exclusively on drugs, prodrugs, and other xenobiotics. The reactions of hydration involving the ring opening of *epoxides* will be discussed in *Chapt. 10*, and the *hydration* of multiple bonds and some cases of *dehydration* will be considered in *Chapt. 11*.

2.2. Classification of Peptidases

Enzymes that act on peptide bonds (*i.e.*, *peptidases* and *proteases*) hydrolyze peptide bonds in peptides *and* proteins. We examine first their classification before outlining their localizations and some physiological roles.

2.2.1. *IUBMB* Classification

The classification adopted by the *Nomenclature Committee* (*NC*) of the *International Union of Biochemistry and Molecular Biology* (*IUBMB*) divides peptidases into classes and subclasses according to the positional specificity in the cleavage of the peptide link of the substrate. The last publication of the complete printed version of the *Enzyme Nomenclature* was in 1992 [1][2], but a constantly updated version with supplements is available on the World Wide Web at http://www.chem.qmul.ac.uk/iubmb/enzyme/. Similarly, all available *Protein Data Bank* (*PDB*) entries classified as recommended by the *NC-IUBMB* can be found on the WWW at http://www.biochem.ucl.ac.uk/bsm/enzymes/.

The *NC-IUBMB* classifies peptidases (EC 3.4) into *exopeptidases* (EC 3.4.11–19), which remove one or a few amino acids, and *endopeptidases* (proteinases, EC 3.4.21–99), which catalyze the cleavage of peptide bonds away from either end of the polypeptide chain (*Fig. 2.1*). Exopeptidases are further subdivided into enzymes that carry out hydrolysis at the N-terminus or the C-terminus (*Figs. 2.1* and *2.2*). Thus, *aminopeptidases* (EC 3.4.11) cleave a single amino acid from the N-terminus [3]; those removing a dipep-

tide or a tripeptide are the *dipeptidyl-peptidases* and *tripeptidyl-peptidases*, respectively (EC 3.4.14). *Carboxypeptidases* (EC 3.4.16–18) hydrolyze a single amino acid from the C-terminus of the peptide chain. The peptidases that split off a dipeptide unit from the C-terminus are classified as *peptidyl-dipeptidases* (EC 3.4.15). Carboxypeptidases are separated into three groups based on catalytic mechanism, *i.e.*, serine-type carboxypeptidases (EC 3.4.16), metallocarboxypeptidases (EC 3.4.17), and cysteine-type carboxypeptidases (EC 3.4.18). Exopeptidases specific for dipeptide substrates are classified as *dipeptidases* (EC 3.4.13). *Omega peptidases* (EC 3.4.19) are enzymes that remove terminal residues that are substituted, cyclized, or linked by isopeptide bonds (peptide linkages other than those of α-carboxyl to α-amino groups).

Endopeptidases (proteinases, EC 3.4.21–99) are subdivided into five classes based on catalytic site and mechanism (see *Chapt. 3*), *i.e.*, serine endopeptidases (EC 3.4.21), cysteine endopeptidases (EC 3.4.22), aspartic endopeptidases (EC 3.4.23), metalloendopeptidases (EC 3.4.24), and the newly created threonine endopeptidases (EC 3.4.25; *Fig. 2.2*) [4–6]. A sixth class exists containing the endopeptidases of yet unknown catalytic mechanism (EC 3.4.99).

The *NC-IUBMB* has introduced a number of changes in the terminology following the proposals made by *Barrett*, *Rawlings* and co-workers [7][8]. The term '*peptidase*' should now be used as a synonym for '*peptide hydrolase*' and includes all enzymes that hydrolyze peptide bonds. Previously the term 'peptidases' was restricted to 'exopeptidases'. The terms 'peptidase' and '*protease*' are now synonymous. For consistency with this nomenclature, the term '*proteinases*' has been replaced by 'endopeptidases'. To complete this note on terminology, we remind the reader that the terms 'cysteine endopeptidases' and 'aspartic endopeptidases' were previously called 'thiol proteinases' and 'acid or carboxyl proteinases', respectively [9].

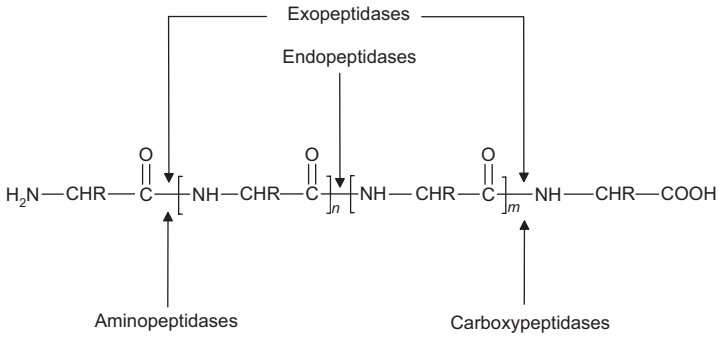


Fig. 2.1. *Classification of peptidases according to the site along the polypeptide chain cleaved*

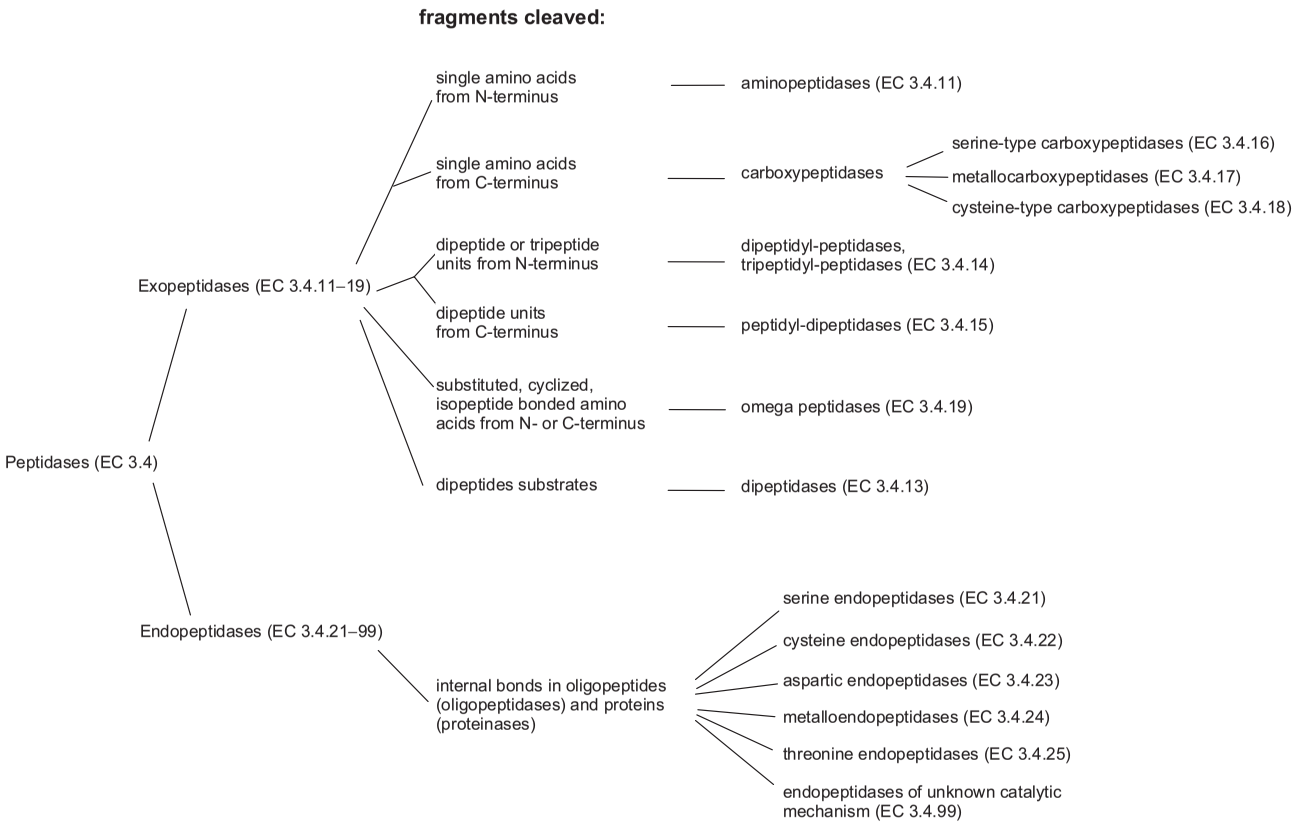


Fig. 2.2. *Scheme for the classification of peptidases*

One of the general principles of the *Nomenclature Committee* is that enzymes should be classified and named according to the reaction they catalyze. However, the overlapping specificities of and great similarities in the action of different peptidases render naming solely on the basis of function impossible [10]. For example, some enzymes can act as both endo- and exopeptidases. Thus, cathepsin H (EC 3.4.22.16) is not only an endopeptidase but also acts as an aminopeptidase (EC 3.4.11), and cathepsin B (EC 3.4.22.1) acts as an endopeptidase as well as a peptidyl-dipeptidase (EC 3.4.15). The actual classification of peptidases is, therefore, a compromise based not only on the reaction catalyzed but also on the chemical nature of the catalytic site, on physiological function, and on historical priority.

2.2.2. Classification Based on Sequence

Another, more recent and more general way to classify peptidases is based on amino acid sequence, *i.e.*, on evolutionary relationships [7a][8]. Evolutionary relationships are established by means of algorithms that compare the amino acid sequences and then characterize similarities.

- The peptidases were separated into *catalytic types* according to the chemical nature of the group responsible for catalysis. The major catalytic types are, thus, Serine (and the related Threonine), Cysteine, Aspartic, Metallo, and As-Yet-Unclassified. An in-depth presentation of catalytic sites and mechanisms, based on this classification, is the subject of *Chapt. 3*.
- The term *family* designates a group of enzymes '*in which every member shows a statistically significant relationship in amino acid sequence to at least one other member of the family in the part of the molecule that is responsible for peptidase activity*'. The statistical criteria applied in this analysis give confidence '*that any two peptidases placed in the same family have evolved from a common ancestor and are, thus, homologous by the definition of* Reeck et al. [11]'. Each peptidase family is named with a letter denoting the catalytic type (*e.g.*, S, T, C, A, M, or U) followed by an arbitrarily assigned number.
- A *clan* designates '*a group of families the members of which have evolved from a single ancestral gene, but have diverged so far that we can no longer prove their relationship by comparing the primary structures*' of the proteins. The name of a clan is formed from the letter of the catalytic type (*i.e.*, for the family) followed by an arbitrary second letter.

Thus, more than 500 peptidases are listed in the *Handbook of Proteolytic Enzymes* [7a], this classification being summarized in part in *Table 2.1*.

Table 2.1. *Examples of Clans and Families of Peptidases, with Focus on Eukaryotes* (taken from [7a]) [a])

Clan	Types of families	Catalytic residue(s)	3D Structure
Serine-type (and threonine-type) peptidases			
SA	9 Families (endopeptidases including trypsin in family S1)	His, Asp, Ser [b])	Double β-barrel
SB	1 Family of endo- and exopeptidases (including proprotein convertases)	Asp, His, Ser	Parallel β-sheet
SC	6 Families of endopeptidases (oligopeptidases) and exopeptidases (including lysosomal carboxypeptidase A in family S10)	Ser, Asp, His	α,β-Hydrolase
SE	3 Families (including many penicillin-binding proteins)	Ser, Lys	Helices and $\alpha+\beta$ sandwich
SF	4 Families of endopeptidases (including signal peptidases in family S26)	Ser, Lys (His)	Single β-barrel
TA	2 Families of endopeptidases (including proteasomes in family T1)	Thr	$\alpha,\beta,\beta,\alpha$ Sandwich
–	Several unclassified families and enzymes		
Cysteine peptidases			
CA	5 Families of mostly endopeptidases (including papain and some cathepsins in family C1, and calpains in family C2)	Gln, Cys, His, Asn/Asp	
CD	1 Family of endopeptidases (including caspases)	His, Cys	α/β
–	Several unclassified families and enzymes		
Aspartic peptidases			
AA	2 Families of endopeptidases (including pepsin, renin and some cathepsins in family A1) and several other families	Asp	Two lobes from separate genes
–	Several unclassified families and enzymes		
Metallopeptidases			
MA	9 Families of exo- and endopeptidases (including several aminopeptidases in family M1, and enkephalinase in family M13)	Zn^{2+} Bound to *His*-Glu-Xaa-Xaa-*His* (HEXXH motif)	α/β
MB	6 Families of endopeptidases (including collagenases in family M10, and the ADAM metalloproteinases in family M12)	Zn^{2+} Bound to *His*-Glu-Xaa-Xaa-*His*-Xaa-Xaa-Gly-Xaa-*His/Asp*	α/β
MC	1 Family (including various carboxypeptidases)	Zn^{2+} Bound to *His*-Xaa-Xaa-*Glu* and another His	$\alpha+\beta$ sandwich

Table 2.1 (cont.)

Clan	Types of families	Catalytic residue(s)	3D Structure
ME	2 Families (including nardilysin in family M16)	Zn^{2+} Bound to *His*-Xaa-Xaa-Glu-*His* (HXXEH motif)	
MF	1 Family of aminopeptidases	2 Co-catalytic Zn^{2+}	
MG	1 Family of aminopeptidases and dipeptidases	2 Co-catalytic ions of Co or Mn	
MH	6 Families (aminopeptidases and carboxypeptidases)	2 Co-catalytic Zn^{2+}	
–	Several unclassified families and enzymes		
Others			
–	Many peptidases of unknown catalytic type		

[a]) For more information and further updates, see the MEROPS Database at http://www.bi.bbsrc.ac.uk/Merops/. [b]) Catalytic residues are listed in their order in the sequence.

The evolutionary classification has a rational basis, since, to date, the catalytic mechanisms for most peptidases have been established, and the elucidation of their amino acid sequences is progressing rapidly. This classification has the major advantage of fitting well with the catalytic types, but allows no prediction about the types of reaction being catalyzed. For example, some families contain endo- and exopeptidases, *e.g.*, SB-S8, SC-S9 and CA-C1. Other families exhibit a single type of specificity, *e.g.*, all families in clan MB are endopeptidases, family MC-M14 is almost exclusively composed of carboxypeptidases, and family MF-M17 is composed of aminopeptidases. Furthermore, the same enzyme specificity can sometimes be found in more than one family, *e.g.*, D-Ala-D-Ala carboxypeptidases are found in four different families (SE-S11, SE-S12, SE-S13, and MD-M15).

2.3. Localization and Some Physiological Roles of Peptidases

Proteolytic activity can be detected in all biological tissues and fluids, but there is considerable heterogeneity in both the type and number of peptidases associated with different tissues, cells, and organelles. The activities of peptidases may be divided into two categories. The first category is characterized by limited proteolysis, *i.e.*, only a limited number of peptide bonds are split. In the second category, the target protein is extensively degraded through the hydrolysis of many peptide bonds [12]. The latter mechanism promotes complete elimination of proteins, whereas limited proteolysis regulates the maturation and function of proteins.

In recent years, an increasing number of studies have demonstrated the role of peptidases in numerous physiological functions and particularly as biological regulators. Because the scope of this book is the metabolism of xenobiotics, it cannot offer a comprehensive presentation of the biology of peptidases. The following short overview gives only some representative examples of peptidases grouped according to their localization.

2.3.1. Extracellular Peptidases

Extracellular peptidases are relatively easy to isolate and are, therefore, well-studied. In higher organisms, they perform a variety of different functions such as food digestion, control of blood pressure and blood clotting, and complement activation, among others. A few examples are compiled in *Table 2.2*.

Table 2.2. *Location and Function of Some Extracellular Peptidases*

Enzyme(s), function	EC Number(s)	Location
Digestion		
Carboxypeptidases A and B	3.4.17.1 – 2	Pancreas
Chymotrypsin	3.4.21.1	Pancreas
Trypsin	3.4.21.4	Pancreas
Pancreatic elastase	3.4.21.36	Pancreas
Pepsin A and B	3.4.23.1 – 2	Gastric juice
Chymosin	3.4.23.4	Gastric juice
Blood pressure homeostasis		
Peptidyl-dipeptidase A	3.4.15.1	Lung, kidney, vascular wall
Kallikrein	3.4.21.34 – 35	Plasma, kidney
Renin	3.4.23.15	Plasma, kidney
Blood coagulation		
Thrombin	3.4.21.5	plasma
Coagulation factor Xa	3.4.21.6	plasma
Coagulation factor VIIa	3.4.21.21	plasma
Coagulation factor IXa	3.4.21.22	plasma
Fibrinolysis		
Plasmin	3.4.21.7	plasma
u-Plasminogen activator	3.4.21.73	kidney, plasma
Complement activation		
Classical-complement-pathway C3/C5 convertase	3.4.21.43	plasma
Complement factor I	3.4.21.45	plasma

The digestion of food proteins is essentially undertaken by *gastric* and *pancreatic peptidases.* These enzymes are synthesized as inactive precursors (*zymogens*), which are activated extracellularly under appropriate physiological conditions [13]. Pepsinogen, the zymogen of pepsin secreted by the gastric mucosa, is spontaneously activated at low pH by an autocatalytic intramolecular reaction. In contrast to gastric peptidases, pancreatic peptidases, which are synthesized by acinar cells of the pancreas, need other proteases for activation. The pancreatic zymogens (chymotrypsinogen, proelastase, procarboxypeptidase, and even trypsinogen) have trypsin as a common activator [14]. The activation of trypsinogen is specifically initiated by the enteropeptidase enterokinase (EC 3.4.21.9), an enzyme located in the intestinal brush border cell membranes. Premature activation of pancreatic zymogens is prevented by natural trypsin inhibitors, small proteins that bind covalently to the active site of trypsin.

Plasma peptidases can be subdivided into permanent and transient circulating peptidases. *Permanent circulating peptidases* shorten the effect of active peptides and proteins in the blood. Thus, plasma aminopeptidases convert somatostatin to des-Asp^1-somatostatin [15], angiotensin I to des-Asp^1-angiotensin I [16], and enkephalin to des-Trp^1-enkephalin [17]. The circulating form of peptidyl-dipeptidase A (angiotensin I converting enzyme, EC 3.4.15.1) inactivates bradykinin and activates angiotensin I [18]. Plasma amidases such as acylamidase (EC 3.5.1.4) and ω-amidase (EC 3.5.1.3) are responsible for the inactivation of thyrotropin-releasing hormone and melanotrophin-release-inhibiting hormone [19].

Transient circulating plasma peptidases are enzymes whose action tends to be very short-lived due to rapid inhibition by soluble protein inhibitors. The enzymes that control blood coagulation, fibrinolysis, blood-pressure homeostasis, and the complement system belong to this category. The enzymatic controls for these processes owe their effectiveness to the amplification of a physiological signal by a cascade of consecutive zymogen activations and fine-tuning by protease inhibitors (*Fig. 2.3*). Thus, blood coagulation involves at least seven consecutive zymogen-activation steps [20]. Another important cascade system operates for the complement system, which belongs to the humoral-response system, and, when activated, causes chemotaxis, phagocytosis, and lysis of foreign cells and bacteria [21].

2.3.2. Intracellular Peptidases

The isolation and investigation of intracellular tissue peptidases is more difficult than that of extracellular peptidases, and, as a result, less is known about such peptidases. Illustrative examples of peptidases with their intracellular localization are presented in *Table 2.3* [7a].

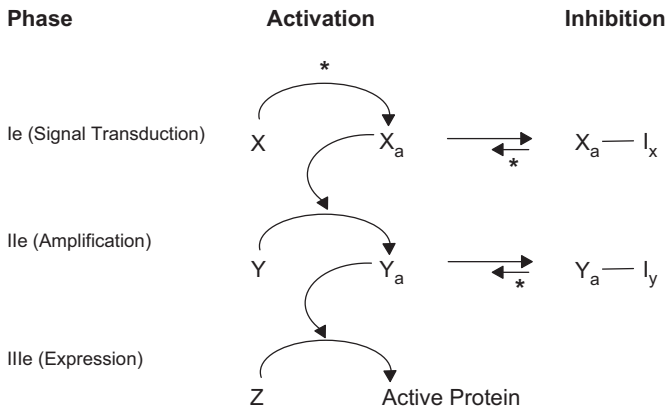


Fig. 2.3. *Schematic representation of consecutive zymogen activation reactions (cascade).* Following initiation by a physiological signal (*), the zymogens X, Y, and Z are sequentially activated. The resulting peptidases (X_a and Y_a) are inactivated by specific inhibitors (I_x and I_y) to limit their action (modified from [20a]).

2.3.2.1. *Membrane-Bound Peptidases*

Ectopeptidases are a class of enzymes that are attached to the cell membrane with their catalytic site directed outward. Their distribution is specific and certainly of crucial physiological significance [22]. The *kidney brush border membranes* have very high peptidase content. These enzymes degrade peptides and proteins present in the glomerular filtrate to amino acids that are re-absorbed renally. Another important role of membrane-bound peptidases in the kidney is the regulation of bioactive peptides. Thus, neprilysin (EC 3.4.24.11) participates in clearing circulating atrial natriuretic peptide from the plasma. Membrane peptidases in the *intestinal brush border* participate in protein digestion both directly and indirectly through activation of the pancreatic zymogens. The transformation of trypsinogen to trypsin by the membrane-bound enteropeptidase (EC 3.4.21.9) is an example of the latter case.

Membrane alanyl aminopeptidase (microsomal aminopeptidase, aminopeptidase M, EC 3.4.11.2) and peptidyl-dipeptidase A (angiotensin I converting enzyme, EC 3.4.15.1) located in the *vascular endothelium* and *smooth muscle cell surface* modulate the levels of vasoactive peptides [23]. One of the roles of membrane-bound enzymes is to switch off the action of peptides in the vicinity of the target or to prevent them from gaining access to a region containing receptors that are activated only by locally released peptides.

Ectopeptidases of the *alveolar epithelium* are also involved in the regulation of pulmonary surfactants [24]. The cell-surface enzymes on monocyte-

Table 2.3. *Subcellular Location of Some Intracellular Peptidases*

Enzyme, subcellular compartment	EC Number
Membrane-bound	
Membrane alanyl aminopeptidase	3.4.11.2
X-Trp Aminopeptidase	3.4.11.16
Membrane dipeptidase	3.4.13.19
Dipeptidyl-peptidase IV	3.4.14.5
Peptidyl-dipeptidase A	3.4.15.1
Membrane Pro-X carboxypeptidase	3.4.17.16
Enteropeptidase	3.4.21.9
Neprilysin	3.4.24.11
Cytosolic	
Leucyl aminopeptidase	3.4.11.1
Tripeptide aminopeptidase	3.4.11.4
Prolyl aminopeptidase	3.4.11.5
X-Pro Dipeptidase	3.4.13.9
Cytosol nonspecific dipeptidase	3.4.13.18
Prolyl oligopeptidase	3.4.21.26
Calpain	3.4.22.17
Thimet oligopeptidase	3.4.24.15
Proteasome endopeptidase complex	3.4.25.1
Lysosomal	
Dipeptidyl-peptidase I	3.4.14.1
Dipeptidyl-peptidase II	3.4.14.2
Lysosomal Pro-X carboxypeptidase	3.4.16.2
Cathepsin X	3.4.18.1
Cathepsin B	3.4.22.1
Cathepsin H	3.4.22.16
Cathepsin D	3.4.23.5

lineage cells have been shown to participate in inflammatory events [25]. Thus, dipeptidyl-peptidase IV (EC 3.4.14.5) and another tripeptidyl endopeptidase regulate extracellular concentrations of tumor necrosis factor-α (TNF-α), a protein implicated in a wide variety of physiological and pathological processes including immunoregulation and inflammation. Dipeptidyl-peptidase IV also plays a role in antigen-induced activation of T lymphocytes probably *via* control of interleukin 2 (IL2) production [26].

2.3.2.2. *Cytosolic Peptidases*

High-molecular-weight complex peptidases composed of a series of low-molecular-weight subunits are found in the cytosol [27]. These enzymes were

collectively known as the multicatalytic endopeptidases complex (EC 3.4.99.46) but have been renamed the *proteasome endopeptidases complex* (EC 3.4.25.1) [28–31]. The complex contains three cooperative catalytic components belonging to the class of serine endopeptidases that have chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide-hydrolyzing activities. Multicatalytic peptidases operate in the nonlysosomal pathway of intracellular protein degradation and represent an important factor in intracellular protein turnover. They are also characterized by a very broad specificity in peptide bonds cleaved.

The proteolytic activity of some multicatalytic peptidases is stimulated by ATP, whereas that of others is not influenced by ATP [32]. The *ATP-dependent proteolytic system* first found in reticulocytes requires the presence of a heat-stable polypeptide called *ubiquitin*, one of the roles of which is to mark particular proteins for subsequent degradation [33]. *ATP-Independent multicatalytic peptidases* can degrade proteins that have a free amino or an *N*-acetylated terminus, as well as oxidatively altered or phosphorylated proteins [34]. The small peptides generated are resistant to multicatalytic peptidases and are further degraded by cytoplasmic endopeptidases and exopeptidases.

During the last ten years, it has become apparent that calcium-dependent papain-like peptidases called *calpains* (EC 3.4.22.17) represent an important intracellular nonlysosomal enzyme system [35][36]. These enzymes show limited proteolytic activity at neutral pH and are present in virtually every eukaryotic cell type. They have been found to function in specific proteolytic events that alter intracellular metabolism and structure, rather than in general turnover of intracellular proteins. Calpains are composed of two nonidentical subunits, each of which contains functional calcium-binding sites. Two types of calpains, *i.e.*, μ-calpain and m-calpain (formerly calpain I and calpain II, respectively), have been identified that differ in their Ca^{2+} requirement for activation. The activity of calpains is regulated by intracellular Ca^{2+} levels. At elevated cytoplasmic calcium concentrations, the precursor procalpain associates with the inner surface of the cell membrane. This interaction seems to trigger autoproteolysis of procalpain, and active calpain is released into the cytoplasm [37].

A large and diverse group of proteins, including enzymes, cytoskeleton, contractile proteins, and receptors, have been shown to be modified by calpains. Thus, a number of enzymes such as tyrosine hydrolase, tryptophan hydrolase, transglutaminase, protein kinase C, and membrane Ca^{2+}-ATPase are activated by calpain proteolysis [38]. Several receptor proteins, in particular receptors for steroid hormones, growth factors, and adrenaline, are modulated by calpains, which participate also in platelet activation, cell fusion, and mitosis [39]. Although the physiological roles of calpains continue to be un-

raveled, it appears that altered calpain and calpastatin expression is basis for a number of disease states, such as muscular dystrophy, muscle denervation and tenotomy, hypertension, and platelet abnormalities [35].

2.3.2.3. *Lysosomal Peptidases*

Lysosomes are a major site of intracellular protein degradation. These organelles contain a large variety of peptidases and other hydrolases at relatively high concentrations (*ca.* 1 mM). Lysosomal peptidases (*Table 2.3*) comprise both endopeptidases and exopeptidases active at acidic pH [40]. The low pH (*ca.* 5) necessary for enzyme activity is maintained by an ATP-dependent proton pump in the lysosomal membrane. The bulk of intracellular proteins are delivered to lysosomes for degradation by a process called *autophagy*, the first step of which is the formation of a vacuole (prolysosome) that contains a portion of the cell cytoplasm separated from the remainder of the cell by a membranous border [41]. After a series of transformations, the vacuole fuses with a lysosome to form an autolysosome in which digestion takes place. Extracellular material taken up into the cell by endocytosis can be digested by a process known as *heterophagy* [42]. The protein breakdown in lysosomes is initiated by endopeptidases in the rate-limiting step, and continued by lysosomal exopeptidases. Many of the dipeptides so produced can diffuse through the lysosomal membrane for final hydrolysis by dipeptidases elsewhere in the cell.

Lysosomal protein degradation is probably important for a number of biological processes, including elimination of abnormal or denaturized proteins, regulation of enzyme levels, posttranslational processing of newly synthesized proteins, regulation of cytoplasmic and tissue growth, supply of amino acids for essential metabolic reactions, organ involution, and alteration of cellular phenotype [42].

2.3.2.4. *Signal and Processing Peptidases*

The site of synthesis of numerous proteins is remote from their site of function. During transfer from one site to the other, proteins must, therefore, cross cellular membranes [43][44]. Proteins are usually synthesized as precursors containing an amino terminal extension, called the *signal (leader) peptide*, the sequence of which contains the necessary information to guide the protein to and across a specific membrane. After transmembrane transport (called *translocation*), the signal peptide is cleaved off by specific signal peptidases, which are found in the rough endoplasmic reticulum, and the

inner membrane of mitochondria [45]. *Signal peptidases* of the endoplasmic reticulum play a central role in the secretion of proteins, such as serum proteins (including immunoglobulins), milk proteins, precursors of peptide hormones, digestive enzymes, and lysosomal enzymes [37][46]. Mitochondrial signal peptidases are involved in the import of proteins into the mitochondria. The translocation of ornithine transcarbamoylase, of precursors of cytochrome c peroxidase, and of F_1-ATPase are all examples of mitochondrial uptake dependent on signal peptidases.

Following the removal of the signal peptide, further proteolysis is usually necessary to generate active proteins [37]. These post-translational modifications are promoted by *processing peptidases*. The enzymes located in secretory granules process proalbumin and prorenin and are involved in the maturation of protein hormones, growth factors, and neuropeptides. Processing peptidases are also found in the mitochondria, where they contribute to the maturation of translocated precursor proteins.

2.3.3. Viral and Bacterial Peptidases

Peptidases encoded by many *viruses* play essential roles at various stages of viral replication, including the coordinated assembly and maturation of virons [7a]. Viral peptidases have become important drug targets in the treatment of viral infections. Of note are inhibitors of proteases of the human immunodeficiency virus (HIV), particularly HIV-1 protease (HIV-1 retropepsin, EC 3.4.23.16) and HIV-2 protease [47–50]. Drugs in this class, which include *indinavir*, *ritonavir*, and *saquinavir*, are useful in the treatment of AIDS, especially when administered as a cocktail together with one of the drugs that act on the viral retrotranscriptase (*e.g.*, *didanosine*, *stavudine*, and *zidovudine* (*AZT*)).

Bacteria possess a very large number of proteolytic enzymes located in their cytoplasm, inner membrane, and periplasm. Although the functions of individual enzymes have not been completely elucidated, it can be concluded that these enzymes have housekeeping and regulatory roles similar to those described for corresponding mammalian enzymes [51]. Protective roles are assumed by *β-lactamases*, which will be presented in *Chapt. 5* [52–54]. Many bacteria also secrete a variety of toxins, some of which are proteolytic enzymes. For example, staphylococci produce streptokinase, an enzyme that digests fibrin and other proteins; clostriadial neurotoxins, including the botulinum and tetanus toxins, are zinc endopeptidases that block neurotransmitter release by selectively cleaving synaptobrevin, a membrane protein of synaptic vesicles [55].

2.4. Classification of Esterases

2.4.1. *IUBMB* Classification

The classification system of the *Enzyme Nomenclature Committee* divides esterases into classes according to the type of ester bond they cleave (*Table 2.4*; see also *Sect. 2.2.1*) [1]. The enzymes within these classes are further divided according to the nature of their preferred substrate(s).

Table 2.4. *Classification of Esterases Based on the Type of Ester Bond They Cleave*

Enzyme	EC Number(s)
Carboxylic ester hydrolases	3.1.1
Thiolester hydrolases	3.1.2
Phosphoric monoester hydrolases	3.1.3
Phosphoric diester hydrolases	3.1.4
Triphosphoric monoester hydrolases	3.1.5
Sulfuric ester hydrolases	3.1.6
Diphosphoric monoester hydrolases	3.1.7
Phosphoric triester hydrolases	3.1.8
Nucleases	3.1.11 – 31

A selection of carboxylic ester hydrolases (EC 3.1.1) of major or more-modest significance in xenobiotic metabolism is given in *Table 2.5*. The recommendations of the *Enzyme Nomenclature Committee* on the classification of esterases cannot be considered completely satisfactory, but, even after decades of debate, a more satisfactory classification system remains to be proposed [56][57]. The main difficulties with esterase classification have been summarized as follows [58].

First, the true *physiological substrates of most esterases are unknown*. It is, therefore, hardly practicable to systematically name esterases according to the recommendations of the *Enzyme Nomenclature Committee* [1], *i.e.*, based on the definite (physiological) role of the enzyme. The difficulty is that the use of nonphysiological substrates during purification and in characterization assays does not contribute to discovering the physiological role of an enzyme.

Second, esterases have broad (or even very broad) and *overlapping substrate specificities*. For example, carboxylesterase (EC 3.1.1.1) also catalyzes reactions characteristic of a number of other hydrolases. The discovery that individual isoenzymes of carboxylesterases may be identical to or closely related to acylglycerol lipase, acylcarnitine hydrolase, and palmitoyl-CoA hydrolase (see *Sect. 2.4.3*) has increased the confusion surrounding esterase classification [59]. Many esterases are able to hydrolyze amides, thiolesters,

Table 2.5. *A Selection of Carboxylic Ester Hydrolases* (EC 3.1.1) *Having Some Role in Xenobiotic Metabolism*

EC Number	Common name	Other names	Typical substrates
3.1.1.1	Carboxylesterase	Carboxylic ester hydrolase, nonspecific carboxylesterase, ali-esterase, B-esterase	Aliphatic esters
3.1.1.2	Arylesterase	Aryl-ester hydrolase, A-esterase	Aromatic esters
3.1.1.3	Triacylglycerol lipase	Lipase, triglyceride lipase	Triglycerides
3.1.1.6	Acetylesterase	Acetic-ester acetylhydrolase, C-esterase	Acetic acid esters
3.1.1.7	Acetylcholinesterase	Acetylcholine acetylhydrolase	Acetylcholine
3.1.1.8	Cholinesterase	Acylcholine acylhydrolase, butyrylcholinesterase, pseudocholinesterase	Choline esters and other esters
3.1.1.10	Tropinesterase	Atropine acylhydrolase	Tropine esters
3.1.1.13	Sterol esterase	Steryl-ester acylhydrolase, cholesterol esterase	Steryl esters
3.1.1.55	Acetylsalicylate deacetylase	Aspirin esterase, aspirin hydrolase	Acetyl esters of aryl alcohols, negatively charged esters
3.1.1.60	Bis(2-ethylhexyl)-phthalate esterase	DEHP esterase	Acts also on long-chain 4-nitrophenyl esters

and phosphates, as well as carboxylic acid esters. Depending on the choice of substrate used during enzyme purification, the hydrolase may, therefore, be classified as an esterase, amidase, thiolesterase, or phosphatase.

Third, the *difficulty of obtaining pure enzyme preparations* makes characterization of esterases problematic. Very often, the cleavage of esters is investigated in complex systems such as subcellular fractions, homogenates, or cells, or *in vivo*. All too often, the observed degradation attributed to the esterase is based largely or entirely on evidence of the overall chemical change, and not on an isolated reaction and catalytic mechanism. The shortcomings associated with this approach are obvious.

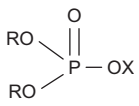
Fourth, most esterases are *highly polymorphic enzymes*. Many of the purified carboxylesterases are mixtures of isoenzymes that have different substrate specificities [60][61]. For example, the substrate(s) used to isolate pig liver carboxylesterase influences the isoenzyme composition, and, hence, the substrate specificity of the resulting esterase preparation.

Fifth, esterase activity can be due to *enzymes with nonesterase main activities*. Thus, lysophospholipase (EC 3.1.1.5) and carbonic anhydrase (EC

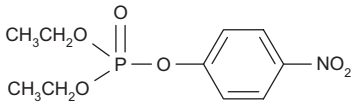
4.2.1.1), both of which exhibit considerable hydrolyzing activity toward simple esters, can be classified unambiguously according to their physiological roles [62][63]. Nevertheless, both enzymes are also included among the numerous *nonspecific esterases* described in the literature. It is clear that improvement of the classification will depend on progress made in the isolation, purification, and sequencing of these enzymes.

2.4.2. Classification Based on Interactions with Organophosphates

A classification based on the interaction of esterases with organophosphates (**2.1**) has been introduced by *Aldridge* [64]: class A-esterases hydrolyze organophosphate esters while B-esterases are irreversibly inhibited by them. Another, lesser-used criterion, is the effect of sodium 4-(hydroxymercurio)benzoate or Hg^{2+}, which inhibits A-esterases but has little effect on B-esterases. Class C-esterases, enzymes that do not interact at all with organophosphates, have been added to the classification system [64][65].



2.1



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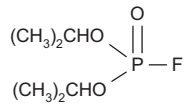

2.3

Organophosphates phosphorylate the OH group of the catalytic serine at the active site of *B-esterases* (see *Sect. 3.3*). The rate of dephosphorylation of the enzyme is very slow, thus, the organophosphate acts as a mechanism-based inactivator. B-Esterases are classified as carboxylesterases (EC 3.1.1.1).

The mechanism by which *A-esterases* hydrolyze organophosphates is not completely understood. Involvement of a phosphorylated active-site cysteine and displacement of an activated H_2O molecule are two possible hypotheses (see *Sect. 3.7.1*) [56]. A-Esterases comprise enzymes that hydrolyze aryl esters, paraoxon (**2.2**) and related organophosphate pesticides, and diisopropylfluorophosphate (DFP, diisopropyl phosphorofluoridate, **2.3**) and related compounds, including nerve gases. These enzymes are found in the current nomenclature listed under arylesterases, aryldialkylphosphatase, and diisopropyl-fluorophosphatase.

The distinction between *aryldialkylphosphatase*, also called *paraoxonase* (*Sect. 2.5.6* and *3.7*), and *arylesterase* was introduced in the last printed revision of the nomenclature recommendations [1]. Arylesterases (EC 3.1.1.2) act on many phenolic esters [66][67]; aryldialkylphosphatases (aryltriphos-

phate dialkylphosphohydrolase, EC 3.1.8.1) include A-esterases that hydrolyze paraoxon and other neutral organophosphorus triesters, but are inactive toward phenylacetate substrates of arylesterases [56][67]. These Ca^{2+}-dependent esterases exist in multiple forms and bear some resemblance to phospholipases C (EC 3.1.4.3) and D (EC 3.1.4.4). In mammalian blood, these enzymes are particularly associated with high-density lipoproteins. The hydrolysis of paraoxon was previously attributed to arylesterase, but there is evidence that the same esterase hydrolyzes both paraoxon and phenylacetates [68]. Further studies have revealed that this enzyme exists as two closely related isozymes, form A and B, and that isoenzyme B has a higher turnover rate for paraoxon than isoenzyme A [69–72].

The A-esterases now classified as *diisopropyl fluorophosphatases* (diisopropyl-fluorophosphate fluorohydrolase, DFPase, somanase, EC 3.1.8.2) were previously listed under EC 3.8.2.1. These enzymes, which hydrolyze P–F and P–CN bonds such as those of nerve gases, should be described as organophosphorus acid anhydrolases rather than phosphatases [56]. Diisopropyl-fluorophosphatases exist in different forms with contrasting substrate specificities. One form is able to hydrolyze paraoxon at a low rate, while others have no paraoxonase activity. The different forms differ in their molecular weights and in their requirements for bivalent cations for activity [56].

C-Esterases are found in the *IUBMB* classification under the entry acetylesterase (acetic ester acetylhydrolase, EC 3.1.1.6). Doubts have been expressed about the existence of C-esterases [56]. Indeed, their activation by sodium 4-(hydroxymercurio)benzoate is not reproducible, and paraoxon has at least slight inhibitory effects on these esterases.

2.4.3. Classification Based on p*I* Value

The esterases have also been classified according to p*I* value, *i.e.*, the isoelectric points assessed by electrophoretic mobility expressed in pH units. For many years, this was one of the major criteria used to distinguish esterases. The drawback of the classification based on p*I* values is that the isoelectric points can vary between species and even between strains of the same species.

A number of rat liver carboxylesterases identified by their p*I* values are listed in *Table 2.6* [73]: five nonspecific carboxylesterases were purified from rat liver and were characterized according to their p*I* values [61]. They appeared to be isoenzymes, since they had similar substrate specificities toward phenyl and naphthyl esters and monooleylglycerol. Subsequent studies, however, revealed different specificities with respect to their physiological substrates. The p*I* 5.2 and 5.6 enzymes were shown to be acylcarnitine hydrolases (EC 3.1.1.28), and a p*I* 6.0 enzyme an octanoylglycerol lipase. The p*I*

Table 2.6. *Classification of Rat Liver Carboxylesterases Based on pI Value* (taken from [73])

p*I*	Subunit M_r (kDa) [a])	Quaternary structure	Other name(s)
5.0	57–59	Monomer	Esterase ES15
5.2	58	Monomer	Acylcarnitine hydrolase
5.5	61	Monomer	Carboxylesterase RL2
5.5	57–60	Monomer	Esterase pI 5.5
5.6	61	Monomer	Hydrolase pI 5.6
5.6	61 (M)	Monomer	Acylcarnitine hydrolase
5.7	*ca.* 75	Dimer	Esterase e_1
5.82	70	Monomer	Esterase I
6.0	58	Trimer	Esterase ES10
6.0	58	Trimer	Carboxylesterase RH1
6.0	58	Trimer	Octanoylglycerol lipase
6.1	57–60	Trimer	Esterase pI 6.1
6.2	61	Monomer	Hydrolase pI 6.2
6.2/6.4	61	Monomer	Esterase ES4; palmitoyl-CoA hydrolase
6.32	80	Dimer	Esterase II
6.4	61	Monomer	Hydrolase pI 6.4
6.4	57–60	Monomer	Esterase ES4B
6.5	61	Monomer	Carboxylesterase RL1
6.6	70	Monomer	Esterase e_2
6.8	62	Monomer	Monoacylglycerol lipase
6.9	59	Monomer	Long-chain acyl-CoA hydrolase

[a]) Estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

5.2 esterase was reported to hydrolyze 1,2-diglycerides of long-chain fatty acids. The p*I* 6.2/6.4 enzyme was identified as a palmitoyl-CoA hydrolase (EC 3.1.2.2) and an acylglycerol lipase (EC 3.1.1.23) [59][74].

Three isoenzymes of carboxylesterase were purified from rat liver microsomes and were named RL1, RL2, and RH1. These differ from each other in their response to hormone treatment, inducibility, substrate specificity, and immunological properties [75]. It was shown that RL1, RL2, and RH1 resemble hydrolases p*I* 6.2/6.4, p*I* 6.0, and p*I* 5.6, respectively. Enzyme RL2 was found to be identical to *egasyn*, a protein with esterase activity found in the endoplasmic reticulum [76]. The role of egasyn is to stabilize glucuronidase (EC 3.2.1.31) by noncovalent binding to the microsomal membrane.

2.4.4. Classification Based on Sequence

All classification systems mentioned above are empirical, *i.e.*, they are based on some functional characteristic (substrate specificity, inhibition, or

electrophoretic mobility) of the enzymes. A number of authors have recommended the creation of a phylogenetic classification of esterases as has been made for cytochromes P450 and other drug-metabolizing enzymes [77][78].

The first esterases to be characterized by sequence were identified by 'ES' numbers [79][80]. Important efforts are now devoted to sequencing, and, as a result, correspondence to the p*I* classification has appeared. Thus, microsomal carboxylesterase ES10 is probably identical to p*I* 6.1, and esterases ES4 and ES15 correspond to the p*I* 5.0 and p*I* 6.2/6.4 enzymes, respectively [74a].

Many genes that code for esterases have been identified. In the *rat*, for example, the esterase ES1 found in the plasma, intestinal lymph, and small intestine seems to be involved in the uptake of fatty acid esters [81]. Rat liver esterase ES3 has shown high sequence specificity to mouse egasyn (ES22), whereas ES4 seems to correspond to palmitoyl-CoA hydrolase (EC 3.1.2.2) [82]. The enzyme ES6 appears to be an acetylesterase (EC 3.1.1.6), and the liver microsomal ES10 hydrolyzes medium-chain acylglycerols. Esterases ES12 and ES13 have been described as butyrylesterases, and ES14 (also called ES-SI) is a female-specific serum esterase, the concentration of which is regulated by levels of both testosterone and estradiol [83]. The enzyme ES15 seems to correspond to acylcarnitine hydrolase (EC 3.1.1.28) and ES17 to arylesterase (EC 3.1.1.2) [61]. Many such reports have accumulated (see below).

In the *rabbit*, esterase loci that code for three types of esterases, *i.e.*, erythrocyte esterases (ES1, ES2, ES3), serum esterases (Est1, Est2, Est3), and tissue esterases (Est4, Est5, Est6) have been described [80][84]. In the *mouse*, there is also evidence for numerous genetically distinct loci for carboxylesterases, which are even used as markers for genotyping and characterizing different strains [85]. The largest number of esterases is found in liver and kidney: ES17 has tentatively been classified as an acetylesterase (EC 3.1.1.6), whereas ES19 is reserved for arylesterase (EC 3.1.1.2).

In *humans*, erythrocytes contain an esterase that displays genetic polymorphism [86]. This esterase has been called esterase D (ES-D), a name without connection to the above-presented A-, B-, and C-classification. Three carboxylesterases named HU1, HU2, and HU3 have been found in human liver microsomes. Other tissues where esterases have been found include brain, plasma, stomach, small intestine, and colon [79].

In analogy to other enzyme systems, the superfamily of carboxylesterases has been divided into families and subfamilies based on sequence homology (*Table 2.7*) [79]. The major family, CES 1, all exhibit >60% homology with human carboxylesterase HU1. This family is divided into subfamilies, namely CES 1A, 1B, and 1C. As shown in *Table 2.7*, the enzyme CES 1A1 includes the major forms of human carboxylesterases (≥99.5% homology).

CES 1A2 includes the major isoforms of rat, dog, rabbit, and mouse carboxylesterase (77–78% homology with HU1). Given the considerable degree of homology, the various forms of CES 1A2 may be considered orthologues.

Some aspects of the sequence classification of esterases need clarification [87]. In the case of ES4, two separate forms were purified and found to have virtually identical specificity and chemical properties. The enzymes ES8 and ES10 appear to be a monomer and a dimer, respectively, of the same enzyme, and ES9 is probably a combination of ES7 and ES8/10 [88]. For esterase ES15, different p*I* values have been reported by different authors.

Table 2.7. *Classification and Nomenclature for the Carboxylesterases* (taken from [79])

Gene symbol	Species	Trivial name of enzyme
CES 1A1	Human	HU1 hCE Human CE CE Macrophage hCEv
CES 1A2	Dog Rabbit Mouse Rat	D1 Rabbit 1 MH1 pI 6.1 Hydrolase A RH1
CES 1A3	Mouse Rat	Egasyn ES3 (Egasyn)
CES 1B	Rat Mouse	ES4 Hydrolase C Hydrolase B RL1 ML1
CES 1C	Rat Mouse	E1 (Sec) Rat E Hydrolase S RS1 Es-N MS1
CES 2	Rabbit Hamster	Rabbit 2 AT-51
CES 3	Mouse	ES-Male
CES 4	Human	46.5 kDa

2.5. Localization and Some Physiological Roles of Esterases

2.5.1. Carboxylesterases

2.5.1.1. *Localization of Carboxylesterases*

Carboxylesterases (EC 3.1.1.1) can be detected in most mammalian tissues. Besides organs with high carboxylesterase activity such as liver, kidney, and small intestine, esterase activity is present, *e.g.*, in the brain, nasal mucosa, lung, testicle, and saliva. Compared to rat plasma, human plasma contains little carboxylesterase, its esterase activity being essentially due to cholinesterase [61][73][79][89–91].

Most tissues have well-defined patterns of esterase activity. Thus, the ES1 synthesized in mouse liver constitutes the major esterase activity in murine plasma. It has been postulated that the ES2 present in the lymph plays an essential role in fat resorption [92]. Four esterases designated ES5, SEI, SEII, and SEIII are restricted to serum and have not been identified in other tissues. The content of ES6 is highest in organs with active fat metabolism, but is absent in serum and erythrocytes. ES7 is present in erythrocytes, lung, tongue, testis, and most other tissues but not in plasma and brain.

The intracellular localization of carboxylesterases is predominantly *microsomal*, the esterases being localized in the endoplasmic reticulum [73][79][93]. They are either free in the lumen or loosely bound to the inner aspect of the membrane. The carboxylesterases in liver *mitochondria* are essentially identical to those of the microsomal fraction. In contrast, carboxylesterases of liver *lysosomes* are different, their isoelectric point being in the acidic range. Carboxylesterase activity is also found in the *cytosolic fraction* of liver and kidney. It has been suggested that cytosolic carboxylesterases are mere contaminants of the microsomal enzymes, but there is evidence that soluble esterases do not necessarily originate from the endoplasmic reticulum [94]. In guinea pig liver, a specific cytosolic esterase has been identified that is capable of hydrolyzing acetylsalicylate and that differs from the microsomal enzyme. Also, microsomal and cytosolic enzymes have different electrophoretic properties [77]. Cytosolic and microsomal esterases in rat small intestinal mucosa are clearly different enzymes, since they hydrolyze *rac*-oxazepam acetate with opposite enantioselectivity [95]. Consequently, studies of hydrolysis in hepatocytes reflect more closely the *in vivo* hepatic hydrolysis than subcellular fractions, since cytosolic and microsomal esterases can act in parallel.

2.5.1.2. *Some Physiological Functions of Carboxylesterases*

The physiological functions of carboxylesterases are still partly obscure but these enzymes are probably essential, since their genetic codes have been preserved throughout evolution [84][96]. There is some evidence that microsomal carboxylesterases play an important role in *lipid metabolism* in the endoplasmic reticulum. Indeed, they are able to hydrolyze acylcarnitines, palmitoyl-CoA, and mono- and diacylglycerols [74a][77][97]. It has been speculated that these hydrolytic activities may facilitate the transfer of fatty acids across the endoplasmic reticulum and/or prevent the accumulation of membranolytic natural detergents such as carnitine esters and lysophospholipids. Plasma esterases are possibly also involved in fat absorption. In the rat, an increase in dietary fats was associated with a pronounced increase in the activity of ES1. In the mouse, the infusion of lipids into the duodenum decreased ES1 levels in both lymph and serum, whereas an increase in ES2 levels was observed. In the lymph, the levels of ES2 paralleled triglyceride concentrations [92][98].

Seven carboxylesterase isoenzymes were purified from liver microsomes of mouse, hamster, guinea pig, rabbit, and monkey, and found to be glycoproteins hydrolyzing long-chain monoglycerides. Marked physical, enzymatic, and immunological similarities were found among these carboxylesterases, except for the monkey isoenzyme MK2 [99].

Esterases play a role in regulating the *platelet-activating factor* (PAF), a lipid with hypotensive properties [96]. Phospholipase A_2 (EC 3.1.1.4) is involved in this pathway by hydrolyzing a precursor to lyso-PAF and a free fatty acid. The activity of PAF, formed by acetylation of lyso-PAF, is controlled by an esterase hydrolyzing the acetate moiety [100].

The overall metabolism of *vitamin A* in the body is regulated by esterases. Dietary retinyl esters are hydrolyzed enzymatically in the intestinal lumen, and free retinol enters the enterocyte, where it is re-esterified. The resulting esters are then packed into chylomicrons delivered *via* the lymphatic system to the liver, where they are again hydrolyzed and re-esterified for storage. Prior to mobilization from the liver, the retinyl esters are hydrolyzed, and free retinol is complexed with the retinol-binding protein for secretion from the liver [101]. Different esterases are involved in this sequence. Hydrolysis of dietary retinyl esters in the lumen is catalyzed by pancreatic sterol esterase (steryl-ester acylhydrolase, cholesterol esterase, EC 3.1.1.13) [102]. A bile salt independent retinyl-palmitate esterase (EC 3.1.1.21) located in the liver cell plasma hydrolyzes retinyl esters delivered to the liver by chylomicrons. Another neutral retinyl ester hydrolase has been found in the nuclear and cytosolic fractions of liver homogenates. This enzyme is stimulated by bile salts and has properties nearly identical to those observed for

pancreatic sterol esterase (EC 3.1.1.13). However, it is not clear whether these enzymes are relevant to the metabolism of retinyl esters, or whether this activity reflects only a broad substrate specificity of the enzyme, all the more so since hepatic microsomal carboxylesterases (EC 3.1.1.1) can also catalyze the hydrolysis of long-chain retinyl esters [77]. Retinyl-palmitate esterase is also found in the lysosomal fraction of the retinal epithelium [103]. The function of this esterase is believed to be mobilization of retinol by hydrolysis of retinyl palmitate to serve in visual pigment synthesis.

Research on delayed neuropathy caused by organophosphates has led to the discovery of the *neuropathy target esterase* (NTE) in the vertebrate nervous system [104]. This phenyl valerate hydrolyzing enzyme is probably a serine esterase. NTE is tightly bound to membranes, which makes its isolation very difficult [105]. Its inhibition by organophosphates can lead to the degeneration of myelin and associated paralysis over a period of weeks or months following exposure. The development of delayed neuropathy depends on the aging of the phosphorylated esterase, defined as the loss of an alkyl or aryl group to leave a phosphorylated serine residue in the catalytic site with a negative charge on the phosphoryl group. The physiological role of NTE is poorly known, but it may function in the maintenance and/or repair of nerve axons [106].

Carboxylesterases are well-represented in *insects* and are sometimes important in the development of resistance to insecticides. Thus, a well-characterized carboxylesterase E4 is responsible for resistance to organophosphorus insecticides in the aphid (*Myzus persicae*) [107]. In the California *Culex* mosquito, the esterase B1 is 500-fold more abundant in organophosphate-resistant than in susceptible insects. The increase of esterase levels is the result of gene amplification, *i.e.*, the resistant animals have an increased number of copies of the structural esterase gene [108].

Finally, and most importantly, esterases play an essential role in the biotransformation and detoxification of xenobiotic esters and amides, a major theme of this book.

2.5.2. Cholinesterases

Cholinesterases are subdivided into acetylcholinesterase and cholinesterase, one with a narrow, the other with broad substrate specificity [109–112]. Both enzymes exist in multiple molecular forms distinguishable by their subunits association (*Fig. 2.4*). The hydrodynamic properties of these associations have allowed globular (G) and asymmetric (A) forms to be distinguished. The G forms can be hydrophilic (water-soluble, and excreted into body fluids) or amphiphilic (membrane-bound). The *homomeric class* exists

as monomers (G1), dimers (G2), and tetramers (G4), and can be subdivided into hydrophilic (water-soluble) and amphiphilic (membrane-bound) forms. The G2 amphiphilic forms of erythrocytes have a glycophospholipid anchor. The *heteromeric class* exists as amphiphilic G4 and as asymmetric forms containing one to three tetramers (*e.g.*, A12 in *Fig. 2.4*).

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 1.1.1.7) has a high specificity for acetylcholine, a marked reduction in catalysis being seen between propionylcholine and butyrylcholine. The enzyme is mainly associated with nerve tissue and, particularly, with cholinergic synapses. It is membrane-bound notably on postsynaptic membranes, but it is also found in striated muscle and erythrocytes. Acetylcholinesterase plays a central role in neuromuscular transmission by hydrolyzing the acetylcholine released upon depolarization of the presynaptic nerve terminal [112]. A number of acetylcholinesterase inhibitors are used in clinical practice, *e.g.*, *donepezil* and *galanthamine* in *Alzheimer*'s disease, and *physostigmine* in the management of *myasthenia gravis*. The function of acetylcholinesterase in red blood cells is not clear, but it might be correlated with cell differentiation.

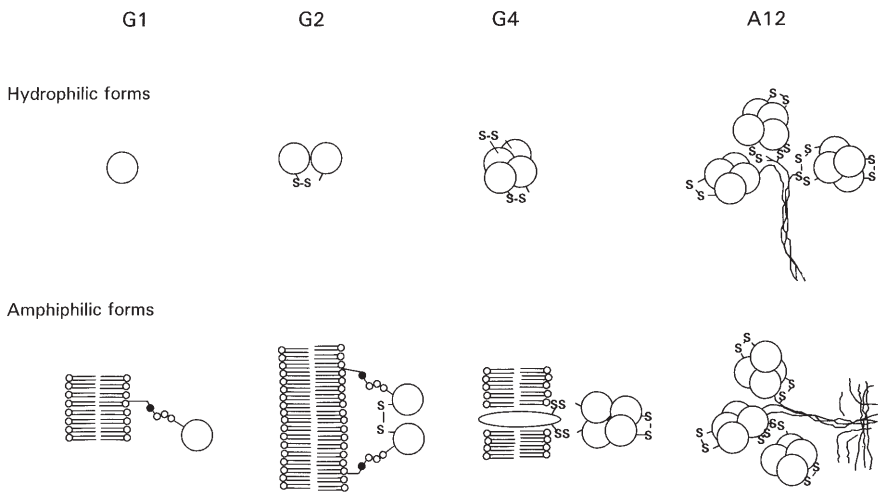

Fig. 2.4. *Schematic model of the molecular polymorphism of acetylcholinesterase and cholinesterase* [110][112a]. Open circles represent the globular (G) catalytic subunits. Disulfide bonds are indicated by S–S. The *homomeric* class exists as monomers (G1), dimers (G2), and tetramers (G4) and can be subdivided into hydrophilic (water-soluble) and amphiphilic (membrane-bound) forms. The G2 amphiphilic forms of erythrocytes have a glycophospholipid anchor. The *heteromeric* class exists as amphiphilic G4 and as asymmetric forms (A) containing one to three tetramers. Thus, heteromeric G4 forms found in brain are anchored into a phospholipid membrane through a 20 kDa anchor. The asymmetric A12 forms have three hydrophilic G4 heads linked to a collagen tail *via* disulfide bonds.

In contrast to acetylcholinesterase, *cholinesterase* (acylcholine acylhydrolase, butyrylcholinesterase, EC 3.1.1.8) exhibits relatively unspecific esterase activity toward choline esters, with a broad specificity relative to the size of the acyl group. The enzyme is synthesized in the liver and can be found in smooth muscle, adipocytes, and plasma. Its physiological role remains partly obscure, but there is evidence that it is present transiently in the embryonic nervous system, where it is replaced in later stages of development by acetylcholinesterase. It has, therefore, been suggested that cholinesterase functions as an embryonic acetylcholinesterase.

2.5.3. Sterol Esterase

The intestinal absorption of dietary cholesterol esters occurs only after hydrolysis by sterol esterase steryl-ester acylhydrolase (cholesterol esterase, EC 3.1.1.13) in the presence of taurocholate [113][114]. This enzyme is synthesized and secreted by the pancreas. The free cholesterol so produced then diffuses through the lumen to the plasma membrane of the intestinal epithelial cells, where it is re-esterified. The resulting cholesterol esters are then transported into the intestinal lymph [115]. The mechanism of cholesterol re-esterification remained unclear until it was shown that cholesterol esterase EC 3.1.1.13 has both bile-salt-independent and bile-salt-dependent cholesterol ester synthetic activities, and that it may catalyze the net synthesis of cholesterol esters under physiological conditions [116–118]. It seems that cholesterol esterase can switch between hydrolytic and synthetic activities, controlled by the bile salt and/or proton concentration in the enzyme's microenvironment. Cholesterol esterase is also found in other tissues, *e.g.*, in the liver and testis [119][120]. The enzyme is able to catalyze the hydrolysis of acylglycerols and phospholipids at the micellar interface, but also to act as a cholesterol transfer protein in phospholipid vesicles independently of esterase activity [121].

2.5.4. Lipases

Triacylglycerol lipase (triacylglycerol acylhydrolase, lipase, EC 3.1.1.3) is a key enzyme in the digestion and storage of lipids [122–124]. This enzyme has, in contrast to other esterases, a turnover rate that is fastest at substrate–water interfaces. It also displays activity toward molecular aggregates, and exhibits weak activity toward monomeric carboxylic acid esters. The natural and most-specific substrate for lipases are long-chain fatty acid triesters of glycerol (triacylglycerides). Short-chain fatty acid esters of glycerol are

hydrolyzed even faster than long-chain esters, but the various lipases are less specific toward them. The rate of hydrolysis is fastest for triglycerides, followed by di- and monoglycerides. Lipases are regiospecific in that triglyceride esters are split only at C(1) and C(3) of the glyceryl moiety [125].

Serum lipase is synthesized and stored in the granules of pancreatic acinar cells and is excreted from the apical poles of the acinar cells into the duct system of the gland. Lipases are produced not only in the pancreas but also at various sites in the human digestive tract [126][127]. Lipases are also found in leucocytes, adipose tissue, lung, and milk.

2.5.5. Thiolester Hydrolases

Thiolester hydrolases (EC 3.1.2) play an important role in the biochemistry of lipids. They catalyze the hydrolysis of acyl-coenzyme A thiolesters of various chain lengths to free fatty acids and coenzyme A. The current list of over 20 specific enzymes includes acetyl-CoA hydrolase (EC 3.1.2.1), palmitoyl-CoA hydrolase (EC 3.1.2.2), and an acyl-CoA hydrolase (EC 3.1.2.20) of broad specificity for medium- to long-chain acyl-CoA [128].

Thiolester hydrolases are present in most tissues and cell compartments. High concentrations are found in liver microsomes and in brown adipose tissue mitochondria and peroxisomes. Several acyl-CoA hydrolases have shown a close relationship to the 'nonspecific' carboxylesterases EC 3.1.1.1. Thus, palmitoyl-CoA hydrolase purified from rat liver microsomes was found to be identical to esterase p*I* 6.2/6.4 (ES4 type). An acyl-CoA hydrolase was isolated that showed high similarity to esterase p*I* 6.1 [74a][129][130]. These few examples are further illustrations of the unsatisfying situation of the traditional classification of esterases.

Fatty-acid synthase (acyl-CoA:malonyl-CoA *C*-acyltransferase, EC 2.3.1.85) is a multifunctional transferase that also has the capacity to hydrolyze thiolesters. The role of its thiolesterase domain is to terminate the growth of fatty acids by hydrolyzing acyl-CoA intermediates [131].

2.5.6. Hydrolases of Esters of Inorganic Acids

2.5.6.1. *Phosphatases*

Phosphatases of documented or potential interest in the context of this book include phosphoric monoester hydrolases (EC 3.1.3), phosphoric diester hydrolases (EC 3.1.4), and phosphoric triester hydrolases (EC 3.1.8).

Aryldialkylphosphatase (aryltriphosphate dialkylphosphohydrolase, paraoxonase, PON, EC 3.1.8.1) is an A-esterase that cleaves aryldialkylphosphate

esters such as paraoxon (**2.2**), previously regarded as identical to EC 3.1.1.2 [71][78][132][133]. Two enzymes (PON1 and PON2) have been characterized [134]. They require the presence of Ca^{2+} and have an optimal pH in the alkaline range (*Sect. 3.7*). In mammals, paraoxonase is found in a variety of organs, such as the liver, and, particularly, in plasma, where it is tightly bound to high-density lipoproteins (HDL) and prevents the damaging oxidation of low-density lipoproteins (LDL). Paraoxonase shows polymorphism in human populations, and several pathologies are associated with genetically based low activity of this enzyme in plasma [56][135–139]. This seems to be the case of non-insulin-dependent diabetes, Tangier disease (a condition where the plasma levels of apolipoproteins are drastically decreased), and cystic fibrosis (where gastric mucus contains excessive amounts of fatty acids probably attached to it by ester bonds). Paraoxonase polymorphism, and the resulting low activity in the serum of some individuals, is also associated with the risk of *coronary heart disease*. The possibility of using serum enzymes as biomarkers of atherosclerosis development is of considerable interest.

Phosphates of pharmaceutical interest are often monoesters (*Sect. 9.3*), and the enzymes that are able to hydrolyze them include alkaline and acid phosphatases. *Alkaline phosphatase* (alkaline phosphomonoesterase, EC 3.1.3.1) is a nonspecific esterase of phosphoric monoesters with an optimal pH for catalysis of *ca.* 8 [140]. In the presence of a phosphate acceptor such as 2-aminoethanol, the enzyme also catalyzes a transphosphorylation reaction involving transfer of the phosphoryl group to the alcohol. Alkaline phosphatase is bound extracellularly to membranes and is widely distributed, in particular in the pancreas, liver, bile, placenta, and osteoplasts. Its specific functions in mammals remain poorly understood, but it seems to play an important role in modulation by osteoplasts of bone mineralization.

Acid phosphatase (acid phosphomonoesterase, EC 3.1.3.2) also catalyzes the hydrolysis of phosphoric acid monoesters but with an acidic pH optimum. It has broad specificity and catalyzes transphosphorylations. Acid phosphatases are a quite heterogeneous group with monomeric, dimeric, larger glycoprotein, and membrane-bound forms. Acid phosphatase activity is present in the heart, liver, bone, prostate, and seminal fluid. Prostate carcinomas produce large quantities of acid phosphatase, and the enzyme is, therefore, used as a biomarker [141].

2.5.6.2. *Sulfatases*

Some sulfatases (sulfuric ester hydrolases, EC 3.1.6) play a pharmacological role in the hydrolysis of sulfuric acid ester metabolites and the few such esters that are used as prodrugs. *Arylsulfatase* (sulfatase, arylsulfohydrolase,

EC 3.1.6.1) is a lysosomal enzyme that hydrolyzes sulfuric acid ester bonds. The enzyme exists in two forms, arylsulfatases A and B, that differ in substrate specificity and in sensitivity toward inhibitors [142][143]. Human tissues contain more arylsulfatase A than arylsulfatase B. The natural substrates of these enzymes are complex lipids such as cerebroside 3-sulfate, and glycosaminoglycans such as chondroitin 4-sulfate and derman sulfate [144]. Deficiencies of these enzymes are associated with a number of lysosomal disorders.

Another enzyme in this class is *steryl sulfatase* (steroid sulfatase, steryl-sulfate hydrolase, EC 3.1.6.2). The typical substrate of this enzyme is the endogenous metabolite 3β-hydroxyandrost-5-en-17-one 3-sulfate, but the enzyme also hydrolyzes some related steryl sulfates.

2.6. Nonhydrolytic Enzymes That Act as Hydrolases

A few nonhydrolytic enzymes are able to catalyze the hydrolysis of some xenobiotics. These are briefly mentioned in the present context, and will be given greater attention in *Chapt. 3*, in regard to catalytic mechanism.

Aldehyde dehydrogenase (EC 1.2.1.3) catalyzes the oxidation of aldehydes to acids (see *Sect. 3.7.2*). The enzyme is ubiquitously distributed, but has mainly been characterized in brain and liver, where it is found in the cytoplasm, mitochondria, and microsomes. It is not clear whether its esterase activity has a physiological role or is a surviving activity inherited from an evolutionary thiolesterase precursor.

Carbonate anhydrase (carbonic anhydrase, EC 4.2.1.1) catalyzes the reversible interconversion of CO_2 and HCO_3^- (see *Sect. 3.7.3*). The enzyme is found in erythrocytes, and in kidney and gastric juices where it contributes to the control of the acid-base balance. The esterase activity of carbonic anhydrase is probably due to the similarity between its active site and that of the zinc proteases. A possible physiological role of the esterase activity of this enzyme remains to be established.

The primary function of *hemoglobin* is the transport of oxygen. This protein carries oxygen by means of its prosthetic heme group, a porphyrin ring system with a central Fe^{2+} cation. The esterase activity of hemoglobin is located in the polypeptide chains (see *Sect. 3.7.4*). At present, no physiological role can be attributed to the esterase activity of hemoglobin.

Serum albumin is the most abundant protein in blood plasma. Its primary function is to control the colloidal osmotic pressure in blood, but is also important for its buffering capacity and for its ability to transport fatty acids and bilirubin, as well as xenobiotic molecules. The physiological implications of its esterase-like activity are unknown (see *Sect. 3.7.5*).

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Chapter 3

Catalytic Mechanisms of Hydrolytic Enzymes

Contents

3.1. Introduction

The previous chapter offered a broad overview of peptidases and esterases in terms of their classification, localization, and some physiological roles. Mention was made of the classification of hydrolases based on a characteristic functionality in their catalytic site, namely serine hydrolases, cysteine hydrolases, aspartic hydrolases, and metallopeptidases. What was left for the present chapter, however, is a detailed presentation of their *catalytic site* and *mechanisms*. As such, this chapter serves as a logical link between the preceding overview and the following chapters, whose focus is on *metabolic reactions*.

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3.2. An Overview of the Hydrolysis of Ester and Amide Bonds

Before discussing the mechanism of cleavage of carboxylic acid esters and amides by hydrolases, some chemical principles are worth recalling. The *chemical hydrolysis* of carboxylic acid derivatives can be catalyzed by acid or base, and, in both cases, the mechanisms involve addition–elimination *via* a *tetrahedral intermediate*. A general scheme of ester and amide hydrolysis is presented in *Fig. 3.1*; the chemical mechanisms of ester hydrolysis will be discussed in greater detail in *Chapt. 7*.

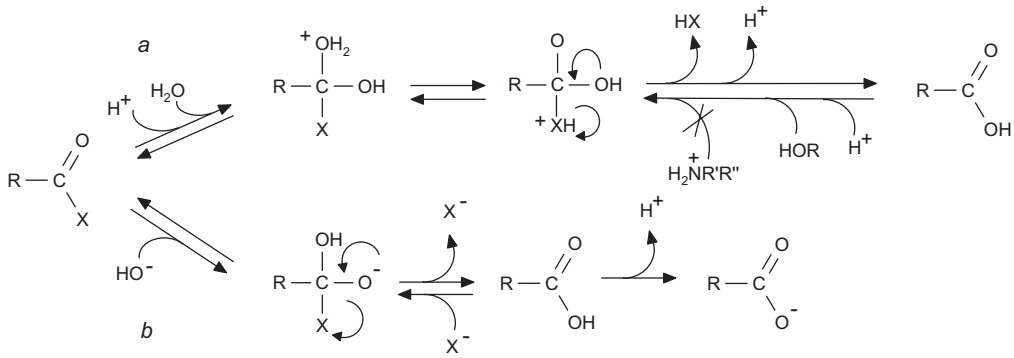


Fig. 3.1. *General scheme for chemical hydrolysis of carboxylic acid esters and amides. Pathway a:* Proton (general acid) catalyzed hydrolysis. *Pathway b:* HO^- (general base) catalyzed hydrolysis.

In *proton-catalyzed hydrolysis* (specific acid catalyzed hydrolysis), protonation of the carbonyl O-atom leads to polarization of the carbonyl group, facilitating addition of the nucleophile, *i.e.*, a H_2O molecule (*Fig. 3.1, Pathway a*). The acid-catalyzed hydrolysis of esters is reversible because the neutral alcohol or phenol released is nucleophilic, whereas hydrolysis of amides is irreversible because the amine released is protonated in the acidic medium and, hence, has considerably reduced nucleophilicity.

In *HO^--catalyzed hydrolysis* (specific base catalyzed hydrolysis), the tetrahedral intermediate is formed by the addition of a nucleophilic HO^- ion (*Fig. 3.1, Pathway b*). This reaction is irreversible for both esters and amides, since the carboxylate ion formed is deprotonated in basic solution and, hence, is not receptive to attack by the nucleophilic alcohol, phenol, or amine. The reactivity of the carboxylic acid derivative toward a particular nucleophile depends on *a*) the relative electron-donating or -withdrawing power of the substituents on the carbonyl group, and *b*) the relative ability of the –OR′ or –NR′R″ moiety to act as a leaving group. Thus, electronegative substituents accelerate hydrolysis, and esters are more readily hydrolyzed than amides.

The *enzymatic hydrolysis* of carboxylic acid derivatives is far more effective than chemical hydrolysis. For example, subtilisin (EC 3.4.21.62) accelerates the hydrolysis of amide bonds at least 10^9- to 10^{10}-fold. The reason for the overall rate enhancement by enzymes, *i.e.*, the decrease in the *Gibbs* energy of the transition state, has already been presented (see Chapt. 1 in [1]). The hydrolases include the following three catalytic features at the active site that enormously accelerate the rate of hydrolysis. First, each contains an *electrophilic component*, which increases the polarization of the carbonyl group in the substrate ($\mathbf{Z}^+$ in *Fig. 3.2*). Second, each has a *nucleophile* (**Y:** in *Fig. 3.2*) to attack the carbonyl C-atom, leading to the formation of a tetrahedral intermediate. And, finally, each has a *proton donor* (H–**B** in *Fig. 3.2*) to transform the –OR′ or –NR′R″ moiety into a better leaving group.

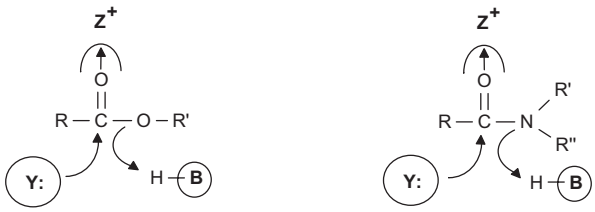


Fig. 3.2. *Common catalytic groups of hydrolases involved in ester and amide bond hydrolysis* ($\mathbf{Z}^+$ = electrophilic component polarizing the carbonyl group; **Y:** = nucleophilic group attacking the carbonyl C-atom; H–**B** = proton donor transforming the –OR′ or –NR′R″ moiety into a better leaving group)

These three catalytic functionalities are similar in practically all hydrolytic enzymes, but the actual functional groups performing the reactions differ among hydrolases. Based on the structures of their catalytic sites, hydrolases can be divided into five classes, namely *serine hydrolases*, *threonine hydrolases*, *cysteine hydrolases*, *aspartic hydrolases*, and *metallohydrolases*, to which the similarly acting calcium-dependent hydrolases can be added. Hydrolases of yet unknown catalytic mechanism also exist.

Furthermore, two major catalytic and mechanistic classes can be distinguished, and, in both, a H_2O molecule is involved as a critical partner in the reaction.

- The serine hydrolases, threonine hydrolases, and cysteine hydrolases, the attacking nucleophile of which is a serine or threonine *OH group* or a cysteine *thiolate group*, respectively, and which form an intermediate covalent complex (*i.e.*, the acylated enzyme). Here, an activated H_2O molecule enters the catalytic cycle in the second step, *i.e.*, hydrolysis of the covalent intermediate to regenerate the enzyme.

- The aspartic hydrolases, metallohydrolases, and calcium-dependent hydrolases, which activate (*i.e.*, render more nucleophilic) a *H_2O molecule* and allow it to attack the substrate. Here, no covalent complex is formed with the enzyme.
- Peptide hydrolases (*peptidases* or proteases, *i.e.*, enzymes hydrolyzing peptide bonds in peptides *and* proteins, see *Chapt. 2*) have received particular attention among hydrolases. As already described in *Chapt. 2*, peptidases are divided into *exopeptidases* (EC 3.4.11 – 19), which cleave one or a few amino acids from the N- or C-terminus, and *endopeptidases* (proteinases, EC 3.4.21 – 99), which act internally in polypeptide chains [2]. The presentation of enzymatic mechanisms of hydrolysis in the following sections will begin with peptidases and continue with other hydrolases such as esterases.

3.3. Serine Hydrolases

3.3.1. Serine Peptidases

The mechanism by which serine peptidases, particularly *serine endopeptidases* (EC 3.4.21), hydrolyze peptide bonds in peptides and proteins has been extensively investigated by X-ray crystallography, site-directed mutagenesis, detection of intermediates, chemical modification, ^{1}H-NMR spectroscopy, and neutron diffraction [2 – 14]. These studies revealed that all serine peptidases possess a *catalytic triad*, composed of a serine, a histidine, and an aspartate residue, and a so-called *oxyanion hole* formed by backbone NH groups.

The major steps in the *catalytic process*, namely reversible binding, acylation, and deacylation, are schematically represented in *Fig. 3.3* with chymotrypsin as an example.

a) Formation of a noncovalent *Michaelis* complex between enzyme and substrate (*Fig. 3.3,a*).

b) Nucleophilic attack of the substrate C-atom by the OH group of Ser^{195}, leading to the formation of a covalent tetrahedral intermediate (*Fig. 3.3,b*). The transfer of the Ser^{195} OH proton to an imidazole N-atom of His^{57} is essential in this step. The carboxylate group of Asp^{102} markedly facilitates proton transfer by correctly orienting His^{57} to ensure formation of the appropriate tautomeric form for acceptance of a proton from Ser^{195}. Finally, Asp^{102} stabilizes the positively charged form of His^{57} in the transition state. The backbone NH groups of Gly^{193} and Ser^{195} polarize the carbonyl group, further facilitating nucleophilic attack and then stabilizing the resulting oxyanion.

c) The imidazolium proton is transferred to the N-atom of the amide bond, which is then cleaved, leaving an acyl–enzyme intermediate (*Fig. 3.3,c*).
d) Deacylation, the next step in this sequence, is essentially the reverse of acylation, with H_2O substituting for the amine component (*Fig. 3.3,d*). Thus, imidazole activates a H_2O molecule by general base catalysis, forming another tetrahedral intermediate (*Fig. 3.3,e*), which decomposes in turn and liberates the carboxylic acid and the free enzyme (*Fig. 3.3,f*).

The mechanism schematized above is a summary of the current knowledge. The role of Asp^{102} has long been controversial [10]. Indeed, the catalytic triad has been depicted as a *charge-relay system*, meaning that the activation of the serine residue involves a concerted transfer of two protons, *i.e.*, from serine to histidine and then to aspartic acid. More recent studies have shown that aspartic acid remains ionized and serves to stabilize the ionic transition state [6][14–16].

It is interesting to note that serine peptidases can, under special conditions *in vitro*, catalyze the reverse reaction, namely the formation of a peptide bond (*Fig. 3.4*). The overall mechanism of peptide-bond synthesis by peptidases is represented by the reverse sequence *f–a* in *Fig. 3.3*. The nucleophilic amino group of an amino acid residue competes with H_2O and reacts with the acyl–enzyme intermediate to form a new peptide bond (*Steps d–c* in *Fig. 3.3*). This mechanism is not relevant to the *in vivo* biosynthesis of proteins but has proved useful for preparative peptide synthesis *in vitro* [17]. An interesting application of the peptidase-catalyzed peptide synthesis is the enzymatic conversion of porcine insulin to human insulin [18][19].

3.3.2. Stereoelectronic Factors That Control the Fate of the Tetrahedral Intermediate in Serine Peptidases

The tetrahedral intermediate in *Fig. 3.3,b* can break down by two distinct mechanisms, namely by elimination of $R'–NH_2$ (*Fig. 3.5*, *Pathway a*) or of ROH (*Pathway b*).

The nature of the products can be predicted by a theory in which it is suggested that the cleavage of the C–N (forward reaction) or C–O bond (backward reaction) occurs only when the two other heteroatoms each have a nonbonded electron pair antiperiplanar to the leaving group [20]. According to this theory and the principle of *microscopic reversibility*, the nonbonded electron pairs of the amide N-atom and of the serine OH group are antiperiplanar in the tetrahedral intermediate formed during nucleophilic attack of Ser^{195} on the carbonyl group of the peptide bond [21]. In this conformation of the tetrahedral intermediate, the N–H bond is directed toward the imida-

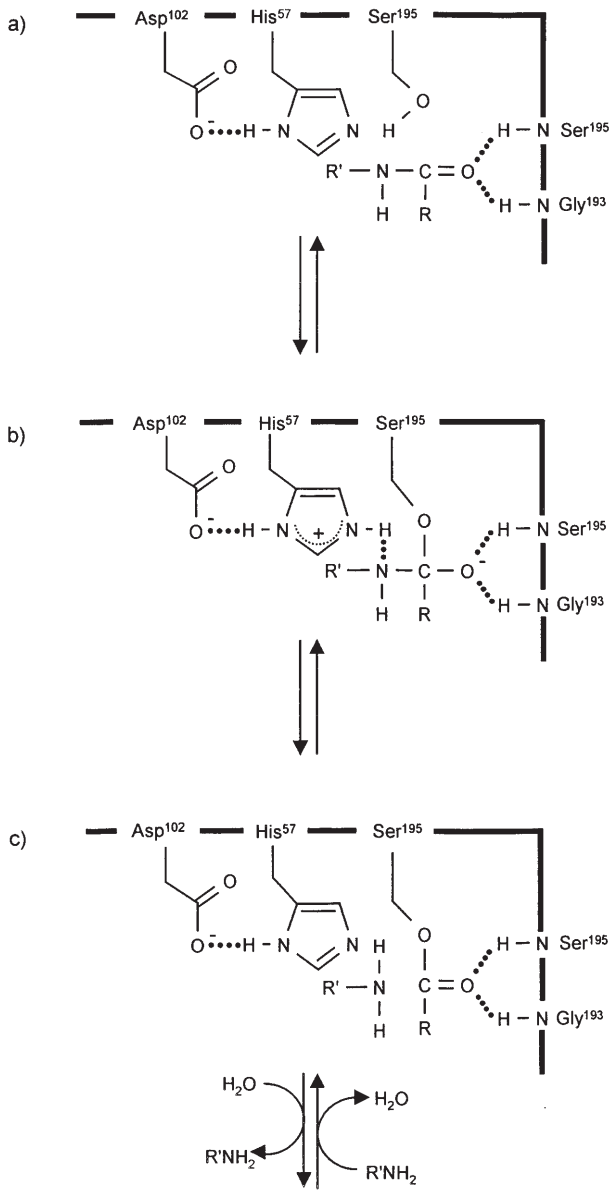


Fig. 3.3. *Major steps in the hydrolase-catalyzed hydrolysis of peptide bonds, taking chymotrypsin, a serine hydrolase, as the example.* Asp[102], His[57], and Ser[195] represent the 'catalytic triad'; the NH groups of Ser[195] and Gly[193] form the 'oxyanion hole'. *Steps a–c*: acylation; *Steps d–f*: deacylation. A possible mechanism for peptide bond synthesis by peptidases is represented by the reverse sequence *Steps f–a*.

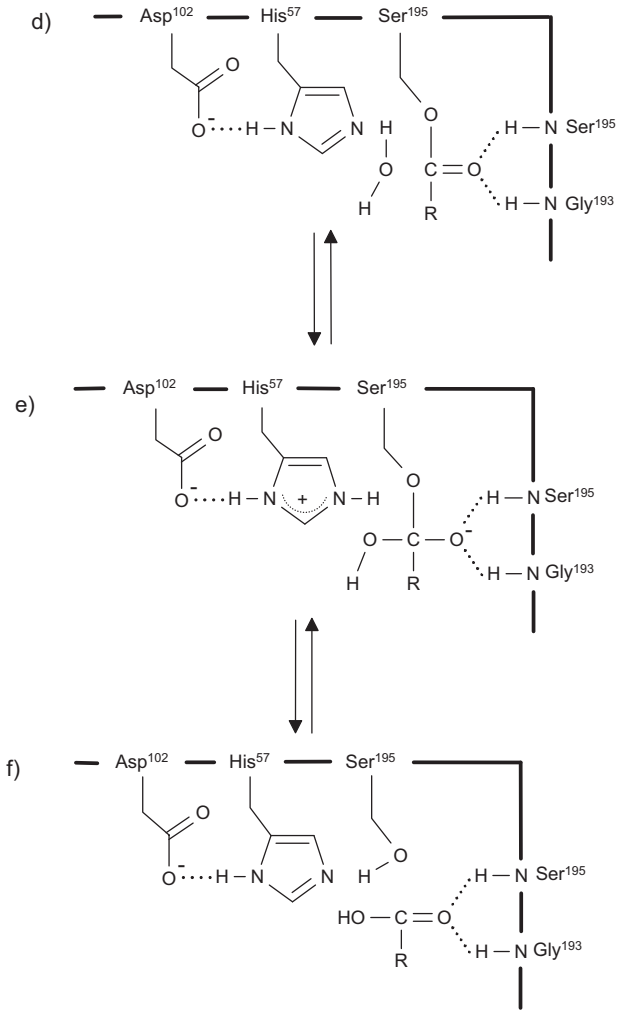


Fig. 3.3 (cont.)

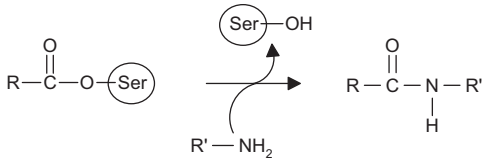


Fig. 3.4. *Formation of peptide bonds by peptidases under special* in vitro *conditions*

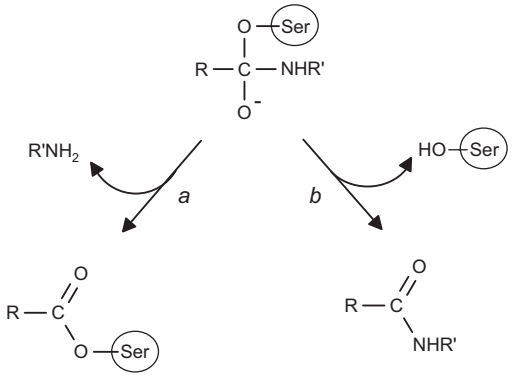


Fig. 3.5. *Two mechanisms of breakdown of the tetrahedral intermediate*, i.e., *elimination of the amino group* (*Pathway a*) *or of the serine OH group* (*Pathway b*)

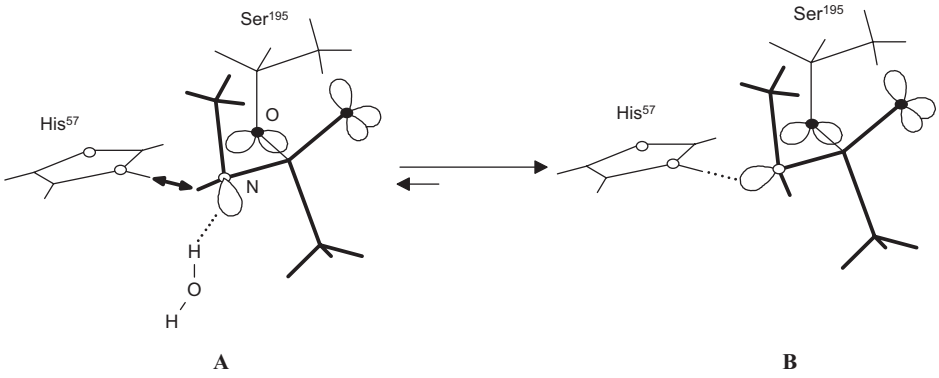


Fig. 3.6. *Stereoelectronic control of the cleavage of the tetrahedral intermediate during hydrolysis of a peptide bond by a serine hydrolase.* The thin lines represent the reactive groups of the enzyme (serine, imidazole ring of histidine); the thick lines represent the tetrahedral intermediate of the transition state. The full circles are O-atoms; open circles are N-atoms. The dotted lines represent H-bonds; the thick double arrow indicates an unfavorable dipole–dipole interaction [21]. **A**: (*R*)-configured N-center; **B**: (*S*)-configured N-center.

zole ring of His^{57}, whereas the nonbonded electron pair points toward the H_2O molecule (*Fig. 3.6*, **A**).

The *elimination of the amino moiety* requires that it first is protonated. Because an internal proton transfer can occur only when the nonbonded electron pair of the leaving N-atom is oriented toward His^{57}, the leaving N-atom must undergo inversion of configuration (*Fig. 3.6*, **B**). Inversion of a pyramidal N-atom is normally a fast process. Here, the equilibrium is likely to favor the (*S*)-configured N-center (*Fig. 3.6*, **B**), since the (*R*)-configured N-center (*Fig. 3.6*, **A**) is destabilized by an unfavorable dipole–dipole interaction

between the N–H of His^{57} and the N–H of the leaving group, whereas the (*S*)-configured form is stabilized by a H-bond between the imidazolium proton and the lone-pair orbital of the N-atom of the leaving amino group. Due to this equilibrium, the tetrahedral intermediate is locked into a configuration in which return to the enzyme–substrate complex is no longer possible, since the nonbonded electron pair of the N-atom is now periplanar to the bond between the serine O-atom and the carbonyl C-atom of the substrate. Thus, N-inversion blocks the cleavage of the C–O bond but allows cleavage of the C–N bond *via* N-protonation.

3.3.3. Kinetics of Hydrolysis by Serine Hydrolases

Serine peptidases can hydrolyze both esters and amides, but there are marked differences in the kinetics of hydrolysis of the two types of substrates as monitored *in vitro*. Thus, the hydrolysis of *4-nitrophenyl acetate* by α-chymotrypsin occurs in two distinct phases [7][22 – 24]. When large amounts of enzyme are used, there is an initial rapid burst in the production of 4-nitrophenol, followed by its formation at a much slower steady-state rate (*Fig. 3.7*). It was shown that the initial burst of 4-nitrophenol corresponds to the formation of the acyl–enzyme complex (acylation step). The slower steady-state production of 4-nitrophenol corresponds to the hydrolysis of the acetyl–enzyme complex, regenerating the free enzyme. This second step, called deacylation, is much slower than the first, so that it determines the overall rate of ester hydrolysis. The rate of the deacylation step in ester hydrolysis is pH-dependent and can be slowed to such an extent that, at low pH, the acyl–enzyme complex can be isolated.

For *amide substrates*, however, no burst phase can be observed. It must, therefore, be concluded that the rate-limiting step is not the same for amides

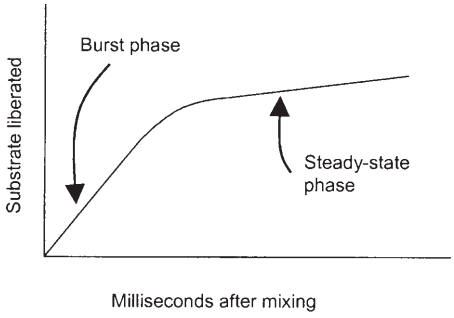
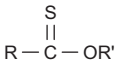


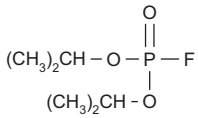
Fig. 3.7. *Two phases in the formation of 4-nitrophenol from 4-nitrophenyl acetate by α-chymotrypsin*

and esters. In the hydrolysis of amides, the deacylation step is more rapid than the formation of the acyl–enzyme complex, which, therefore, does not accumulate.

Carbothioic O-*acid esters* (**3.1**; to be distinguished from carbothioic *S*-acid esters, R–CO–SR′, discussed in *Chapt. 7*) are not hydrolyzed by serine peptidases. It has been postulated that the thiocarbonyl S-atom, which is larger and less prone to H-bonding than the carbonyl O-atom, cannot interact effectively with the oxyanion hole. The resulting inability to stabilize the transition state considerably reduces the rate of hydrolysis.



3.1



3.2

3.3.4. Other Serine Hydrolases

Other serine hydrolases such as *cholinesterases*, *carboxylesterases*, *lipases*, and *β-lactamases* of classes A, C, and D have a hydrolytic mechanism similar to that of serine peptidases [25 – 27]. The catalytic mechanism also involves an acylation and a deacylation step at a serine residue in the active center (see *Fig. 3.3*). All serine hydrolases have in common that they are inhibited by covalent attachment of diisopropyl phosphorofluoridate (**3.2**) to the catalytic serine residue. The catalytic site of esterases and lipases has been less extensively investigated than that of serine peptidases, but much evidence has accumulated that they also contain a catalytic triad composed of serine, histidine, and aspartate or glutamate (*Table 3.1*).

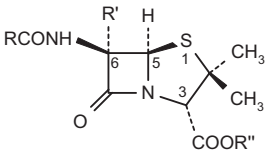
Table 3.1. *Catalytic Triad of Some Serine Hydrolases*

Enzyme	Catalytical triad	Ref.
Torpedo acetylcholinesterase	Ser^{200}; Glu^{327}; His^{440}	[28]
Human acetylcholinesterase	Ser^{203}; Glu^{334}; His^{447}	[29]
Human butyrylcholinesterase	Ser^{198}; Glu^{325}; His^{438}	[30]
Carboxylesterases	Ser^{203}; Glu^{335}; His^{448}	[26]
Cholesterol esterase	Ser^{194}; Asp^{320}; His^{435}	[31]
Human pancreatic lipase	Ser^{152}; Asp^{176}; His^{263}	[32]
Triacylglycerol lipase	Ser^{144}; Asp^{203}; His^{257}	[33]
Bovine lipoprotein	Ser^{134}; Asp^{158}; His^{243}	[34]
Lipase (*Geotrichum candidum*)	Ser^{217}; Glu^{354}; His^{463}	[35]
β-Lactamase C (*Citrobacter freudii*)	Ser^{64}; Lys^{67}, Lys^{315}; Tyr^{150}	[36]

This is, in particular, the case for *acetylcholinesterase* (EC 3.1.1.7) [25][28][37 – 40]. Investigations of the active site of acetylcholinesterase from *Torpedo californica* have provided evidence for the involvement of Ser^{200} and His^{440}, and showed that the third element of the catalytic triad is glutamic acid (Glu^{327}) rather than aspartic acid. The oxianion hole in this case is comprised of Gly^{119}, Gly^{120}, and Ala^{201}. Among the *carboxylesterases* (EC 3.1.1.1) of great importance in the metabolism of xenobiotic esters (see *Chapt. 7* and *8*), many have the same Ser–Glu–His catalytic triad (*Table 3.1*), plus Gly^{123} and Gly^{124} as part of an oxyanion hole [26]. A similar triad has been found in human *cholinesterase* (butyrylcholinesterase, EC 3.1.1.8) and in a lipase isolated from the fungus *Geotrichum candidum* [30][35]. Most mammalian *lipases* (triacylglycerol lipases, EC 3.1.1.3) and *cholesterol esterase* (sterol esterase, EC 3.1.1.13), however, have a Ser–His–Asp triad composition (*Table 3.1*) [27][31][41].

To help the reader gain a better understanding of the three-dimensional structure of the catalytic site of an esterase, *Fig. 3.8* presents the 3D structure of human butyrylcholinesterase (EC 3.1.1.8) obtained by homology modeling [42]. The overall structure of the enzyme is shown in *Fig. 3.8,a*, while *Fig. 3.8,b* shows a closeup of the active site with the catalytic triad highlighted and the close spatial relationship of the Ser–His–Glu residues revealed.

β-Lactamases (EC 3.5.2.6) constitute a group of bacterial enzymes that hydrolyze the four-membered β-lactam ring of penicillins, cephalosporins, and related antibiotic agents (see *Chapt. 5*) [43 – 47]. Four classes of β-lactamases, *i.e.*, A, B, C, and D, have been identified. The β-lactamases that belong to serine hydrolases have been subdivided into classes A, C, and D based on amino acid sequence, whereas class B β-lactamases are Zn^{2+}-containing metallohydrolases (see *Sect. 3.6*). The mechanism of hydrolysis of penicillins (**3.3**) by *Escherichia coli* class A β-lactamase has been unraveled [47 – 53]. The lactam carbonyl is bound and polarized by an oxyanion hole made of the NH groups of Ser^{70} and Ala^{237}. In addition, the lactam N-atom accepts a H-bond from Ser^{130}. Catalysis is initiated by the nucleophilic attack of the Ser^{70} OH group on the lactam carbonyl. The amino group of Lys^{73} (itself H-bonded to the backbone C=O of Ser^{130} and the terminal C=O of Asn^{132}) acts as a



3.3

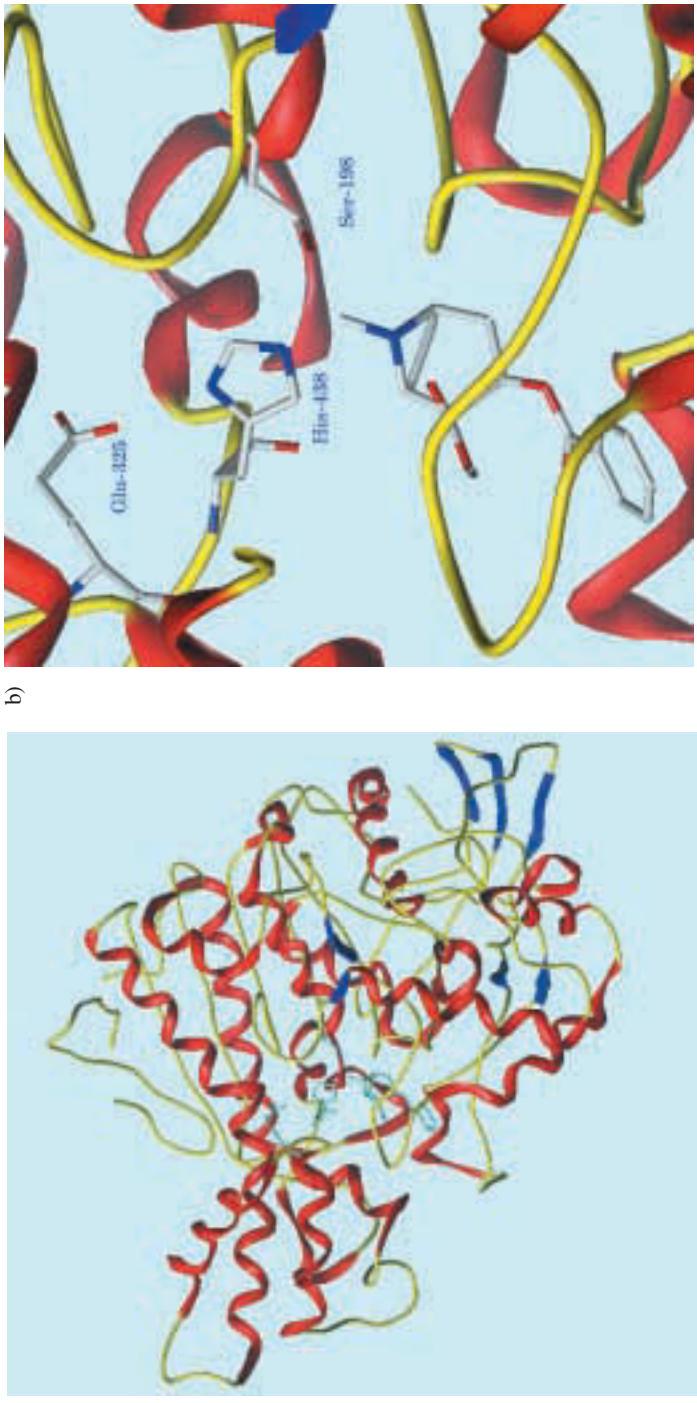


Fig. 3.8. *The 3D structure of butyrylcholinesterase obtained from 1eho.pdb* [42]. *a)* Structure of the enzyme, with the Ser–His–Glu catalytic triad shown in blue. *b)* Closeup of the catalytic triad, revealing the close spatial proximity of the three catalytic residues.

general base and assists this nucleophilic attack by abstracting the proton from Ser^{70}. The protonated amino group of Lys^{73} then facilitates a proton transfer from Ser^{130} to the lactam N-atom during cleavage of the lactam bond. In the deacylation step, a H_2O molecule assisted by Glu^{166} carries out the nucleophilic attack on the penicilloyl carbonyl C-atom.

Class C β-lactamases (from *Citrobacter freudii*) also feature a serine residue at the active site [36], but an anionic tyrosine (Tyr^{150}) acts as a general base in a way similar to histidine in serine peptidases. The basicity and spatial position of the phenolic O-atom of tyrosine are determined by H-bonding with lysine residues Lys^{67} and Lys^{315}. The polarization of the carbonyl group is provided by the NH groups of Ser^{64} and Ser^{318}. In contrast to class A β-lactamases, in class C β-lactamases the same amino acid plays the role of general base during both the acylation and deacylation steps. Thus, Tyr^{150} is also involved in deacylation by activating the H_2O molecule for nucleophilic attack on the carbonyl group of the acyl–enzyme intermediate.

There are a few reported cases of esterases that catalyze not only hydrolysis but also the reverse reaction of *ester formation*, in analogy with the global reaction described for serine peptidases (*Fig. 3.4*). Thus, *cholesterol esterase* can catalyze the esterification of oleic acid with cholesterol and, more importantly in our context, that of fatty acids with haloethanols [54]. Esterification and transesterification reactions are also mediated by carboxylesterases, as discussed in greater detail in *Sect. 7.4*.

3.4. Cysteine Hydrolases

The mechanism of hydrolysis of cysteine peptidases, in particular *cysteine endopeptidases* (EC 3.4.22), shows similarities and differences with that of serine peptidases [2][3a][55 – 59]. Cysteine peptidases also form a covalent, acylated intermediate, but here the attacking nucleophile is the SH group of a cysteine residue, or, rather, the deprotonated thiolate group. Like in serine hydrolases, the imidazole ring of a histidine residue activates the nucleophile, but there is a major difference, since here proton abstraction does not appear to be concerted with nucleophilic substitution but with formation of the stable *thiolate–imidazolium ion pair*. Presumably as a result of this specific activation of the nucleophile, a H-bond acceptor group like Glu or Asp as found in serine hydrolases is seldom present to complete a catalytic triad. For this reason, cysteine endopeptidases are considered to possess a *catalytic dyad* (*i.e.*, $Cys–S^-$ plus $H–His^+$). The active site also contains an oxyanion hole where the terminal NH_2 group of a glutamine residue plays a major role.

Fig. 3.9 shows the amino acids of the catalytic site of *papain* (EC 3.4.22.2), one of the most studied cysteine hydrolases [58][60 – 62]. The cat-

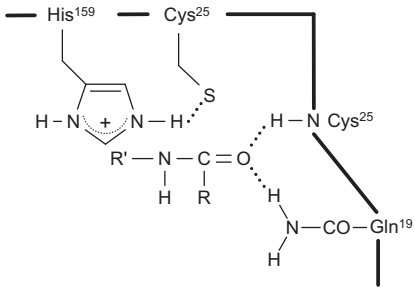


Fig. 3.9. *The Cys–thiolate/His–imidazolium catalytic dyad and the oxyanion hole of papain, a cysteine peptidase*

alytic dyad is formed by Cys^{25} and His^{159}, and the oxyanion hole is formed by the backbone NH group of Cys^{25} and the side-chain NH_2 group of Gln^{19} [63]. Not shown are other H-bonding sites that help tether the substrate, such as the backbone NH of Gly^{66} and Asp^{158}. Note that the latter residue was once thought to be part of a catalytic triad.

Cysteine endopeptidases can hydrolyze not only peptide bonds but also *esters* and *amides* such as anilides. It has been suggested that the formation of the tetrahedral intermediate is rate-determining for esters, while its breakdown is rate-determining for anilides and other amides [64]. In contrast to serine endopeptidases, cysteine endopeptidases are able to hydrolyze carbothioic *O*-acid esters (**3.1**). This was taken as evidence against the contribution of an oxyanion hole interaction in the catalytic mechanism of cysteine peptidases [65]. Indeed, the inability of serine endopeptidases to hydrolyze carbothioic *O*-acid esters had been attributed to a perturbation of the oxyanion hole interactions by the thiocarbonyl S-atom. However, there is direct evidence of a mechanistic requirement for oxyanion stabilization in the hydrolysis of proteins by papain. Indeed, the replacement of Gln^{19} by site-directed mutagenesis reduced considerably the rate of substrate hydrolysis [62]. It is, therefore, necessary to seek other explanations to account for the difference between serine and cysteine peptidases in their reactions with carbothioic *O*-acid esters.

Cysteine endopeptidases, like serine endopeptidases, can also catalyze *peptide synthesis* under preparative conditions [66 – 68]. Thus, papain has been used to synthesize enkephalins and angiotensin.

3.5. Aspartic Hydrolases

Aspartic endopeptidases (EC 3.4.23) are the best-known aspartic hydrolases and the only ones to be presented here. These enzymes were formerly called *acid proteinases* because most of them are active at low pH. In con-

trast to serine and cysteine peptidases, they catalyze the cleavage of peptide bonds without the formation of a covalently bound enzyme–substrate intermediate [69][70].

Aspartic endopeptidases contain two catalytically essential aspartic residues. Their postulated catalytic mechanism is summarized in *Fig. 3.10* with *pepsin A* (EC 3.4.23.1) as an example [2]:

a) In a concerted reaction, Asp^{215} in anionic form activates a H_2O molecule to become more nucleophilic and attack the carbonyl C-atom of the pep-

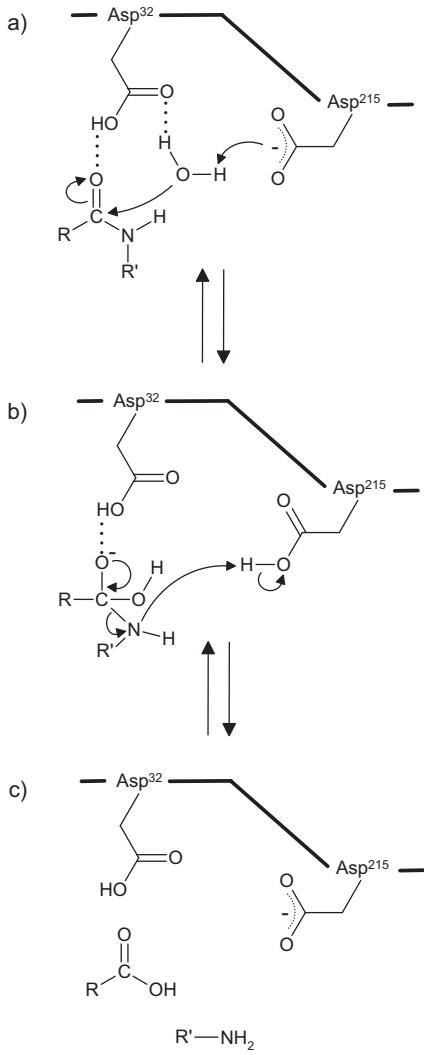


Fig. 3.10. *Mechanism of peptide bond hydrolysis by pepsin, an aspartic endopeptidase* [2]

tide bond. Asp^{32} in neutral form assists this process by polarizing the carbonyl group and presumably also activating the H_2O molecule (*Fig. 3.10,a*).

b) The tetrahedral intermediate in the transition state is stabilized by H-bonds. After configurational inversion, the peptide N-atom is protonated by Asp^{215} (*Fig. 3.10,b*).

c) The tetrahedral intermediate breaks down (*Fig. 3.10,c*) in a manner consistent with the stereoelectronic hypothesis (see *Sect. 3.3.2*) [20].

Enzymes of the pepsin family rarely catalyze the hydrolysis of esters, with the exceptions of, for example, esters of L-β-penicillactic acid and some sulfinic acid esters. Under suitable conditions, *i.e.*, low pH, high enzyme concentration, and formation of an insoluble peptide, aspartic peptidases are able to catalyze the synthesis of peptides [71][72].

3.6. Metallopeptidases

Like aspartic peptidases, metallopeptidases act by activating a H_2O molecule, and they do not form a covalent intermediate with the substrate. Here, the *activation of a H_2O molecule* is mediated by a residue that acts as general base (*e.g.*, Glu, His, Lys, Arg, or Tyr), with a divalent cation (usually Zn^{2+} but sometimes Co^{2+} or Mn^{2+}) perhaps also contributing. The major role of the metal cation, however, is to act as an electrophilic catalyst by *coordinating the carbonyl (or phosphoryl) O-atom in the substrate* and orienting the latter for nucleophilic attack by the HO^- ion generated from H_2O by the general base.

The metal ion is held in place by amino acid residues, generally His, Glu, Asp, or Lys. In many metallopeptidases, which may be exopeptidases or endopeptidases, only one zinc ion is required. In all Co^{2+} or Mn^{2+}-dependent, and in some Zn^{2+}-dependent metallopeptidases, two metal ions are present and act cocatalytically; these enzymes are exopeptidases [2][73][74].

One of the best-known metallopeptidases is pancreatic *carboxypeptidase A* (EC 3.4.17.1), which cleaves the C-terminal peptide or ester bond of peptides having a free C-terminal carboxy group. The structure of the active site of carboxypeptidase A has been determined by X-ray crystallography of the complex formed between the enzyme and glycyl-L-tyrosine (*Fig. 3.11*), a very poor substrate [75]. The catalytically important Zn^{2+} ion is ligated by the side-chains of His^{69}, Glu^{72}, and His^{196} (carboxypeptidase A numbering scheme). This His–Glu–His zinc-binding motif allows polarization of the carbonyl group *via* coordination to the metal ion, thus playing the role of the oxyanion hole present in serine and cysteine hydrolases. The catalytically essential Glu^{270} is H-bonded by its carboxylate group to a H_2O molecule that is itself bound to the Zn^{2+}

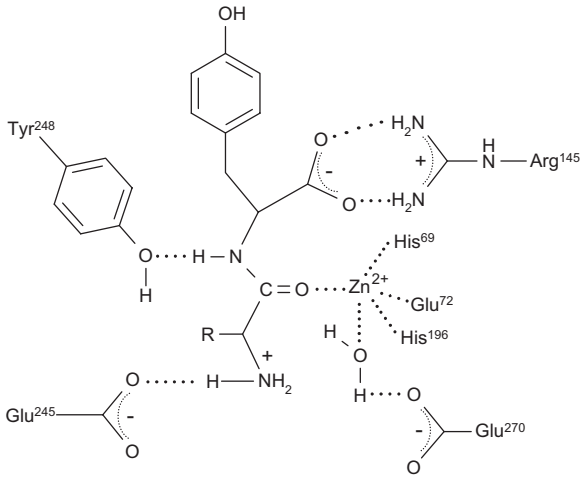


Fig. 3.11. *Simplified representation of the complex between carboxypeptidase A and glycyl-*L*-tyrosine* [76]

ion. A number of residues bind the substrate, whose C-terminal carboxylate group interacts with the guanidinium group of Arg^{145}. Some of the other substrate-binding residues include Glu^{245} and Tyr^{248}, as shown.

A simplified representation of the postulated *mechanism* of carboxypeptidase is shown in *Fig. 3.12* [2][76]. This mechanism involves the following steps:

a) Glu^{270} initiates a general-base catalyzed attack of the active-site H_2O molecule on the carbonyl C-atom of the amide bond (*Fig. 3.12,a*);
b) This produces a tetrahedral intermediate (*Fig. 3.12,b*), whose NH will be protonated, and its OH group deprotonated, by the OH group of Tyr^{248};
c) The resulting tetrahedral zwitterion (*Fig. 3.12,c*) undergoes cleavage of the C–N bond, release of the shortened peptide, and protonation plus release of the C-terminal amino acid (*Fig. 3.12,d*).

The finding that the hydrolytic activity of the enzyme is retained after replacement of a tyrosine residue by phenylalanyl challenges the notion that a tyrosine acts as a general acid catalyst in peptide hydrolysis. It has been suggested that either the protonated Glu^{270} moiety or the zinc–water complex could perform the proton transfer [77].

Neprilysin (enkephalinase, Endopeptidase-24.11, neutral endopeptidase, NEP, EC 3.4.24.11) bears considerable resemblance to other zinc-containing metallopeptidases: it is an oligopeptidase that hydrolyzes enkephalins and a range of other active peptides. Enkephalins are endogenous ligands of opiate receptors, and the prolongation of their action *via* inhibition of enkephalinase

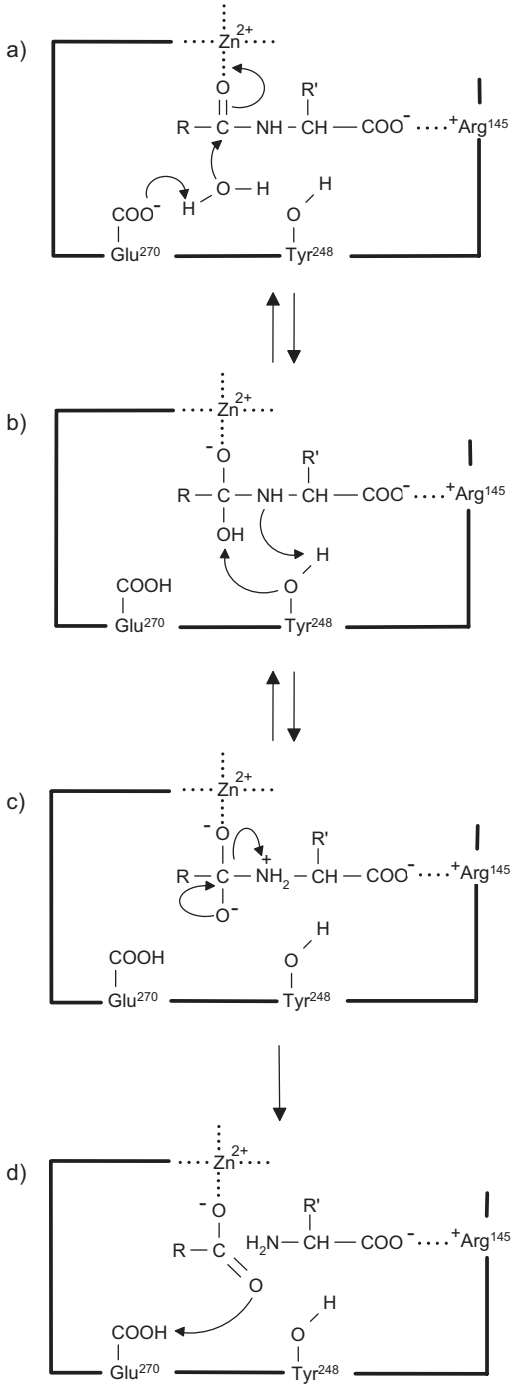
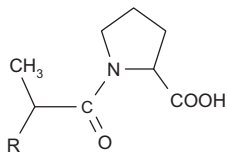


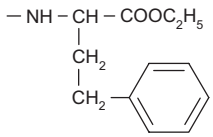
Fig. 3.12. *Mechanism of peptide bond hydrolysis by carboxypeptidase A*

has been extensively explored as an approach for pain treatment [78]. A 3D image of human enkephalinase is shown in *Fig. 3.13* to reveal the spatial arrangement of the Glu and the two His residues of the His–Glu–Xaa–Xaa–His zinc-binding motif.

Peptidyl-dipeptidase A (angiotensin-I converting enzyme, ACE, EC 3.4.15.1) plays a pivotal role in the control of blood pressure [80]. It has been established that its active site contains an essential Zn-atom that functions like that of carboxypeptidase A [2]. ACE is inhibited by peptides having a proline or aromatic amino acid at the C-terminal position. These observations as well as the similarities with the active site of carboxypeptidase A have allowed a rational design of effective inhibitors of ACE (*e.g.*, captopril (**3.4**) and enalapril (**3.5**)) used in the treatment of hypertension [81].



3.4 R = —CH_2—SH

3.5 R = 

β-Lactamases of classes A, C, and D are serine peptidases and as such have been discussed in *Sect. 3.3*. Class B β-lactamases, in contrast, are metallohydrolases. For example, a class B β-lactamase isolated from *Bacillus cereus* was shown to contain two Zn-atoms per protein molecule, of which only one is essential for catalysis. Three histidine residues act as ligands for the first Zn^{2+} ion, and a fourth histidine contributes to the binding of the second Zn-atom [82][83].

Metallopeptidases display an equal or greater *catalytic efficiency* toward *peptides* than toward the corresponding *esters* [84]. This behavior is the opposite of that observed with serine peptidases and is unexpected, since the ester bond is chemically more labile than an amide bond. It has been postulated that the difference in catalytic efficiency is due to difference in product release, meaning that this step could be rate-limiting for esters but not for amides [85].

3.7. Other Hydrolases and Hydrolase-Like Activities

3.7.1. Calcium-Dependent Hydrolases

The hydrolytic mechanism of *phospholipase* A_2 (EC 3.1.1.4) seems to represent a special case compared to serine hydrolases (*Fig. 3.14*). The active site

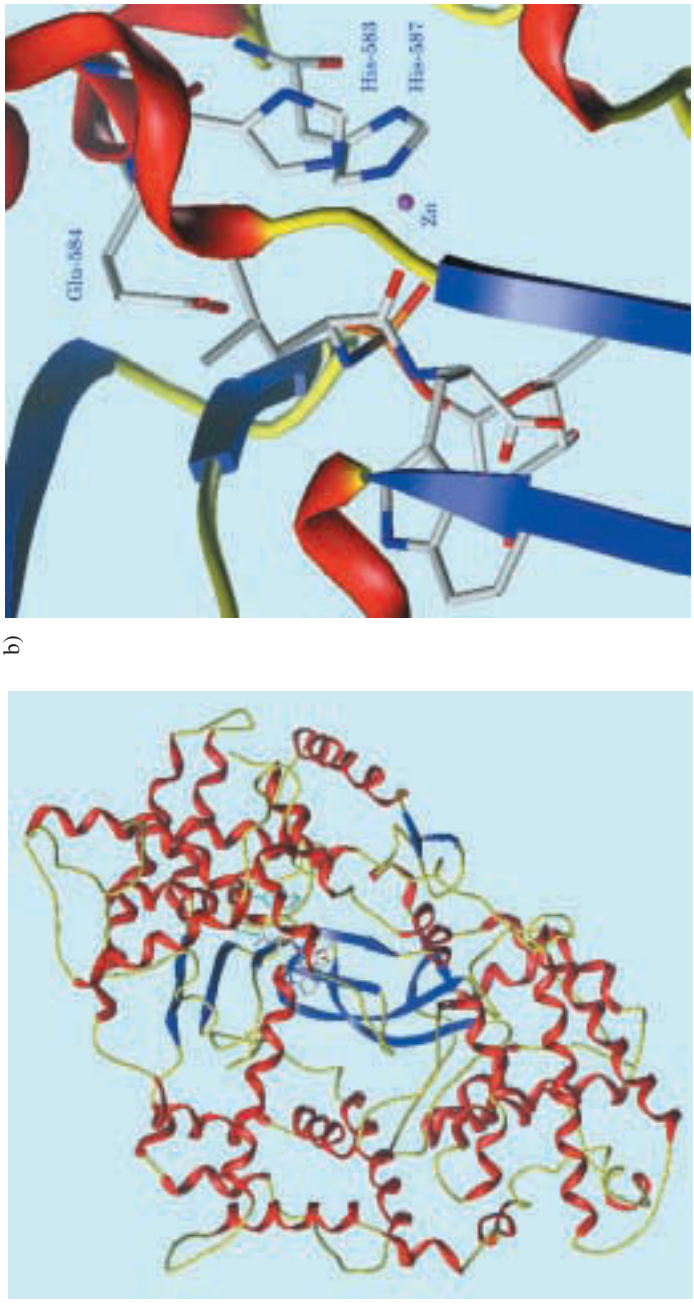


Fig. 3.13. *The 3D structure of neprilysin* (enkephalinase, EC 3.4.24.11) *obtained from 1dmt.pdb* [79]. This structure was determined for the enkephalinase–phosphoramidon complex, but the inhibitor has been removed to unmask the catalytic center. *a)* Structure of the enzyme, with the Glu and the two His residues of the HEXXH zinc-binding motif shown in blue. *b)* Zoom on the catalytic center, revealing the spatial arrangement of the zinc-binding residues.

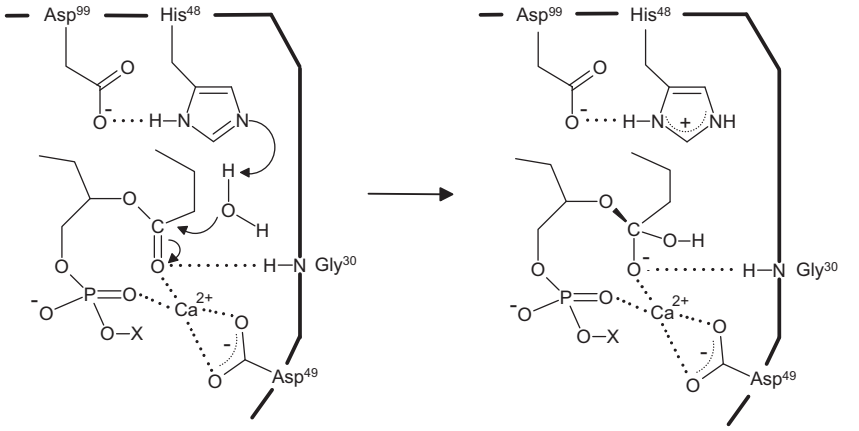


Fig. 3.14. *Mechanism of the formation of a noncovalently bound tetrahedral intermediate in the hydrolysis of phospholipids by phospholipase A_2* [86]

of this enzyme contains a histidine–aspartate pair but no active serine residue could be detected. A H_2O molecule held in place by H-bonds is believed to take over the role played by the serine residue. In addition, a Ca^{2+} ion appears to be essential to polarize the carbonyl group and stabilize the tetrahedral intermediate [86]. In contrast, platelet-activating factor (PAF) acetylhydrolase, another phospholipase A_2, has a Ser–Asp–His catalytic triad characteristic of esterases and neutral lipases (*i.e.*, Ser^{273}–Asp^{296}–His^{351}) [87].

Paraoxonases (PON, *arylesterases*, A-esterases, EC 3.1.8.1; previously regarded as identical to EC 3.1.1.2 arylesterase) are closely related enzymes that catalyze the hydrolysis of numerous aromatic esters (*e.g.*, phenyl acetate) and organophosphates (*e.g.*, paraoxon) [88]. These enzymes contain a conserved cysteine and for many years have been thought to belong to cysteine hydrolases. However, site-directed mutagenesis of the human PON has shown that a free SH group is not required for either paraoxonase or arylesterase activity [89]. What is essential for activity of the mammalian enzyme, however, is the presence of Ca^{2+} [90]. There are, indeed, two distinct calcium-binding sites, one required for stability and the other for catalytic activity. A catalytic mechanism similar to that of phospholipase A_2 has, therefore, been proposed, with Ca^{2+} acting as an electrophilic catalyst by coordinating the carbonyl group in the substrate and orienting the latter for nucleophilic attack by HO^- ion generated from H_2O by a histidinyl imidazole acting as general base [89].

Thus, calcium-dependent hydrolases show clear structural and catalytic similarities with metallohydrolases, both in the role played by a divalent cation and in the mechanism of H_2O activation.

3.7.2. Aldehyde Dehydrogenase

Aldehyde dehydrogenase (EC 1.2.1.3) oxidizes aldehydes to acids ([91][92]; Chapt. 2 in [1]) but is also able to catalyze the hydrolysis of 4-nitrophenyl esters and some other esters [93 – 95]. After being long debated, it is now known that both activities are mediated by the same active site [96][97]. Furthermore, Cys^{302} is the essential enzymatic nucleophile for both esterase and dehydrogenase activities [98][99]. It may be expected that the esterase mechanism of aldehyde dehydrogenase is similar to that of cysteine peptidases. Indeed, there exists a large cluster of matching residues around the catalytic Cys^{302} of aldehyde dehydrogenase and the active site Cys^{25} in papain; based on this finding, it has been proposed that cysteine peptidases and aldehyde dehydrogenases have evolved from a common thiolesterase precursor [100].

3.7.3. Carbonic Anhydrase

Carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1) is a small, monomeric zinc-containing metalloenzyme that catalyzes the reversible hydration of CO_2 to bicarbonate [101][102]. In addition to this activity, carbonic anhydrase also catalyzes the hydrolysis of many aromatic esters [103].

The structure of carbonic anhydrase determined by X-ray crystallography has shown that the catalytic Zn^{2+} ion is coordinated by His^{94}, His^{96}, His^{119}, and a HO^- ion [104]. The steps involved in the catalysis of CO_2 hydration by carbonic anhydrase are outlined in *Fig. 3.15,a – d* [105]. A H-bonding network involving Glu^{106}, a tyrosine, and Zn^{2+} keeps the nucleophilic HO^- properly oriented for attack of the C=O group (*Fig. 3.15,a*). This step is common to carbonic anhydrase and zinc-containing peptidases (*Fig. 3.12*). However, in CO_2 hydration, the adduct is a product (*i.e.*, bicarbonate, *Fig. 3.15,b* and *c*), whereas in proteolysis by zinc peptidases, the nucleophilic adduct is an intermediate. A network of H-bonds between the zinc-bound H_2O and residues such as His^{64} mediates the regeneration of zinc hydroxide, the active catalytic species, from zinc-bound H_2O (*Fig. 3.15,d*) [102].

The first step in the esterase activity of carbonic anhydrase (*Fig. 3.15,e*) is analogous to the first step in CO_2 hydration (*Fig. 3.15,a*). The tetrahedral intermediate so formed (*Fig. 3.15,f*) necessitates the participation of a proton donor for the departure of the leaving alcohol group (*Fig. 3.15,f* and *g*). It is possible that the Thr^{200} residue plays an important role in the esterase activity of carbonic anhydrase. Indeed, its replacement by other amino acids enhances the esterase activity but has no significant effect on the rate of CO_2 hydration [106].

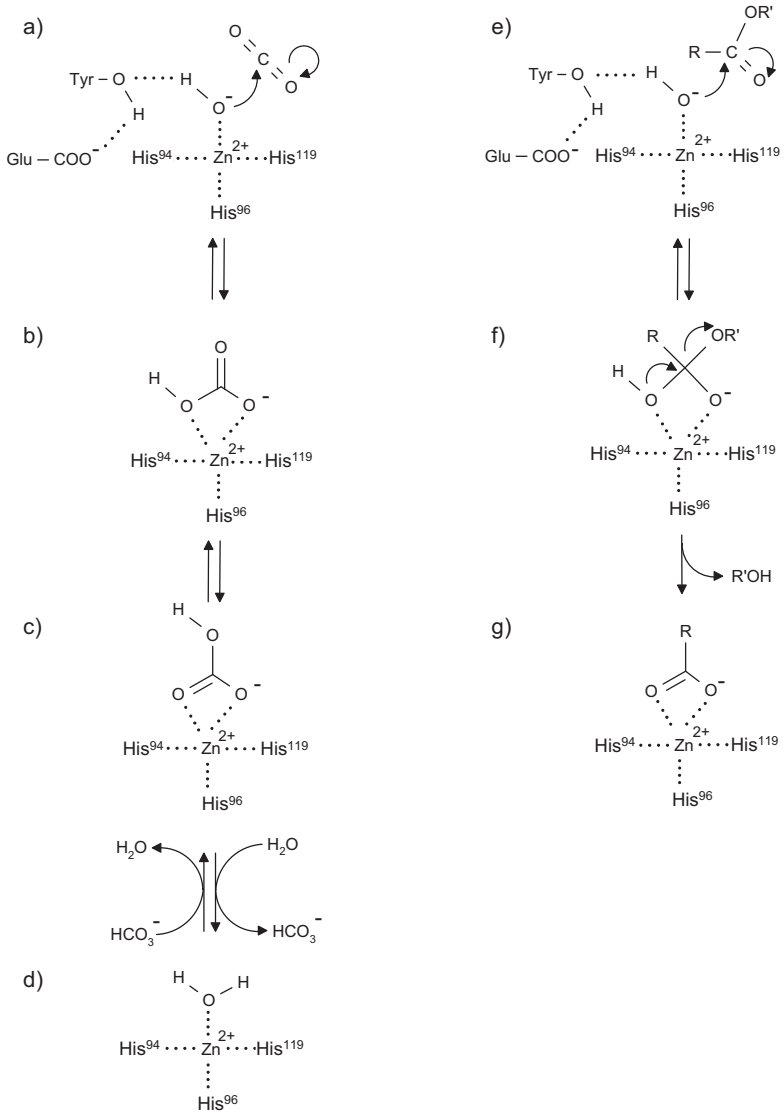


Fig. 3.15. *Schematic mechanism of CO_2 hydration (Steps a – d) and ester hydrolysis (Steps e – g) by carbonic anhydrase II*

3.7.4. Hemoglobin

Human oxyhemoglobin (oxyHb A) hydrolyzes 4-nitrophenyl acetate at a higher rate than bovine serum albumin [107]. It has been proposed that imidazole catalysis by β-His^2 is primarily responsible for the esterase activity, and

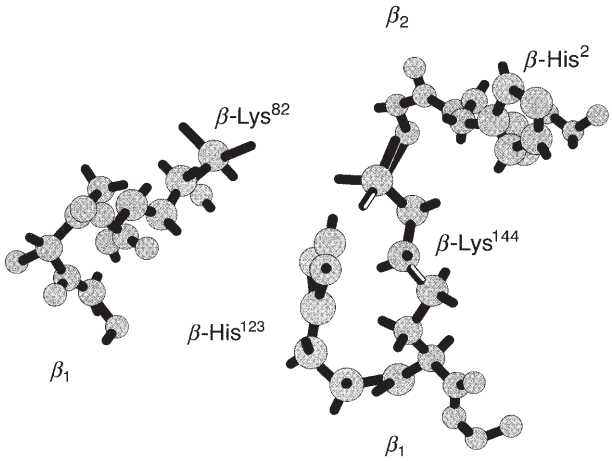


Fig. 3.16. *3D Representation of the esterase site in hemoglobin A* (oxy-Hb A, modified from [108])

that the neighboring β-Lys^{82} becomes acylated as the reaction progresses. Also, the reactivity of 4-nitrophenyl esters is in decreasing order with respect to increasing length of the acyl group [108]. A 3D representation of the esterase site on Hb A is given in *Fig. 3.16*. Sickle-cell oxyhemoglobin (oxy-Hb S) hydrolyzes 4-nitrophenyl acetate at approximately half the rate of normal oxyhemoglobin (oxy-Hb A). An explanation for this difference in catalytic activities lies probably in conformational changes between Hb A and Hb S [108]. The esterase activity of hemoglobin is low compared to that of true carboxylesterases (EC 3.1.1.1) but may be compensated by the high concentration of hemoglobin inside erythrocytes. The possible physiological role of the esterase activity of hemoglobin remains to be better understood.

3.7.5. Serum Albumin

Serum albumin is not an enzyme but a transport protein, yet it has demonstrated hydrolytic activity against a variety of xenobiotic substrates. This 'esterase-like' activity has been known for years, but there is still confusion in the literature regarding its nature and mechanism. Indeed, it was not clear whether this activity is intrinsic to the albumin molecule or results from contamination of albumin preparations by one or more hydrolytic enzymes. More-recent studies with highly purified human serum albumin (HSA) have confirmed that the protein has an intrinsic esterase activity toward several substrates, but that activity due to contaminants and particularly serum cholinesterase is involved

in other cases. For example, it was found that HSA cleaves various esters (4-nitrophenyl esters and acetylsalicylic acid), and some amides and phosphates [109 – 111] (see also *Chapt. 7* and *8*). Indeed, 4-nitrophenyl acetate is still the most commonly used substrate to determine the esterase-like activity of HSA. Although the rates of hydrolysis by HSA are much lower than those obtained with typical carboxylesterases (EC 3.1.1.1), considering the high plasma concentrations of serum albumin, its esterase-like activity may play an important role in the overall disposition of some esters [109a][112][113].

It appears that HSA has one markedly reactive site and a few less-reactive ones. The primary reactive site, known as the *R-site*, corresponds to *Sudlow*'s binding site II. It contains Tyr^{411} and a histidine residue. Various 4-nitrophenyl esters and various other substrates are cleaved at this site. The reaction involves the acylation of the OH group of Tyr^{411}, followed by slow deacylation. In the crystal structure of HSA, the reactive phenolic group of Tyr^{411} is located in the binding pocket in *subdomain IIIA* (see *Fig. 3.17*), in close proximity to Arg^{410}, perhaps explaining the enhanced reactivity of Tyr^{411} toward nucleophilic substitution. The second reactive group in the R-site is a reactive histidine residue, since histidine ethoxycarbonylation by diethylpyrocarbonate markedly decreases the esterase activity of HSA toward some substrates [109a][110][111][114 – 116]. Drugs that inhibit the hydrolysis of 4-nitrophenyl acetate by HSA are referred to as R-type drugs (*e.g.*, *acetohexamide*, *clofibric acid*, *diazepam*, and *ibuprofen*). Since the R-type drugs bind to the site known as Site II [117][118], the R-site and Site II are thought to overlap.

Another reactive site, called the *T-site*, makes a modest contribution to the overall hydrolytic activity of the protein (*ca.* 11%), and a lysine residue has been suggested as the catalyst. The position of the T-site might be in the *subdomain IIA* of HSA (*Fig. 3.17*), since there is evidence that Lys^{220} in subdomain IIA could belong to an esterase site [119][120].

The hydrolysis of *acetylsalicylic acid* (*aspirin*) has also been described, and it involves the rapid acetylation of Lys^{199} [123]. This reactive site also involves Trp^{214} and has been called the *U-site* [124]. Drugs that inhibit the hydrolysis of substituted acetylsalicylic acids by HSA and decrease the fluorescence intensity of Trp^{214} are referred to as U-type drugs (*e.g.*, *sulfinpyrazone* and *warfarin*). Because U-type drugs also bind to the site know as site I (which overlaps with *subdomain IIA*), the U-site and I-site are believed to overlap. Lysine residues also appear to be involved in the β-lactamase activity of HSA [125].

It is important to note that acetylsalicylic acid and some 4-nitrophenyl esters are quite reactive species that easily acylate nucleophiles. With such compounds, albumin indeed behaves as a catalyst, but it is simultaneously a target, and the term 'esterase-like activity' can only be understood with this restriction in mind.

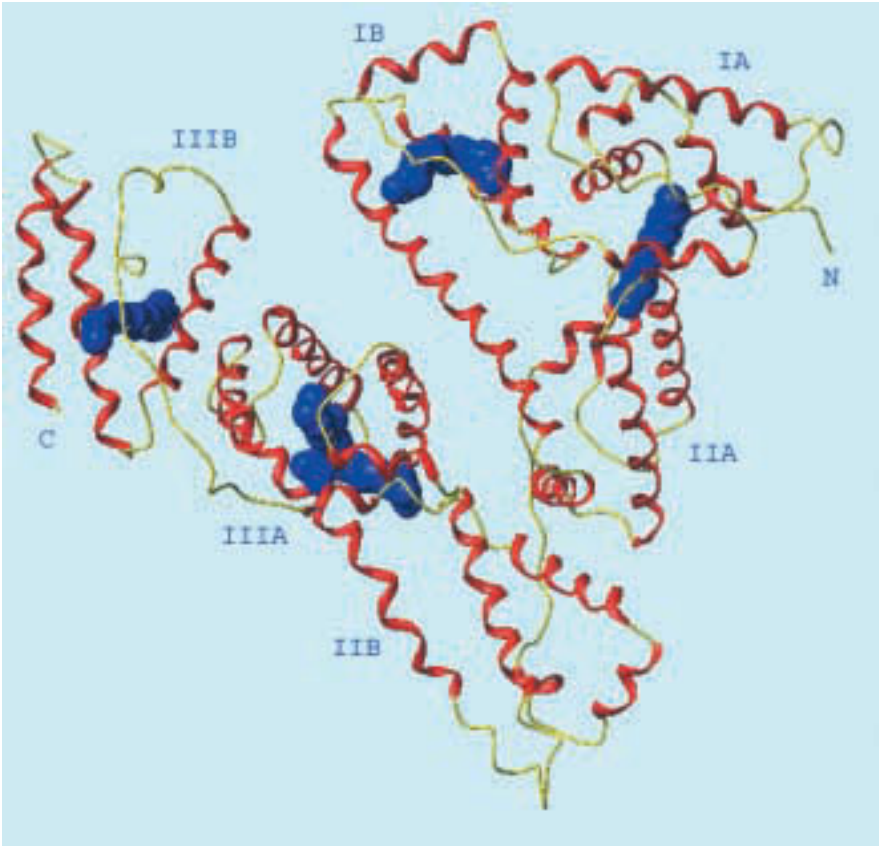


Fig. 3.17. *The crystal structure of human serum albumin* (HSA) *complexed with four molecules of myristic acid* (from 1bj5.pdb [121][122]). The picture shows the domains (I – III) and subdomains (A and B) of HSA. The primary hydrolytic site is located in subdomain IIIA, and two others probably in subdomain IIA.

Whereas the above evidence clearly points to a catalytic activity of serum albumin, it does not exclude an activity toward less-reactive substrates due to contamination of some HSA preparations. Indeed, the hypothesis of a *contamination by plasma cholinesterase* (EC 3.1.1.8) has been raised [126][127]. The efficient hydrolysis of nicotinate esters by HSA (see *Chapt. 8*) [128][129] could be due to contamination by cholinesterase in samples of a commercially available, essentially fatty acid free albumin. Support for this hypothesis was obtained when HSA contaminated with cholinesterase was resolved into two peaks by affinity chromatography, and the esterase activity toward nicotinate esters was found exclusively in the cholinesterase fraction [130].

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Chapter 4

The Hydrolysis of Amides

Contents

4.1. Introduction

The amide bond occurs in a great variety of chemical environments, as shown in *Fig. 4.1.* The chemical diversity of carboxamides is even greater than that of carboxylic acid esters (see *Chapt. 7* and *8*). Variations at the amide

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bond (R–CO–NR′R″) can exist in both moieties, namely the acyl group (R–CO–) and the amino group (–NR′R″). The acyl group can originate from aliphatic or aromatic acids. As for the –NR′R″ moiety, it can originate not only from ammonia, or aliphatic or aromatic amines, but also from hydrazines and ureas. The amide linkage can also produce a large variety of cyclic structures, which can be classified into three categories: cyclic imides, cyclic ureides, and lactams. Sulfonamides, which are formally analogous to amides of carboxylic acids, are resistant to chemical and enzymatic cleavage under the usual conditions. In contrast, the sulfonylurea linkage, particularly when situated between two aryl groups, can undergo both chemical and enzymatic hydrolysis.

From a chemical point of view, amide and ester bonds have comparable structural and spectroscopic features and are hydrolyzed by the same general mechanism, *i.e.*, a nucleophilic acyl substitution involving an addition–elimination sequence (see *Chapt. 3*). However, in a given structure, the amide

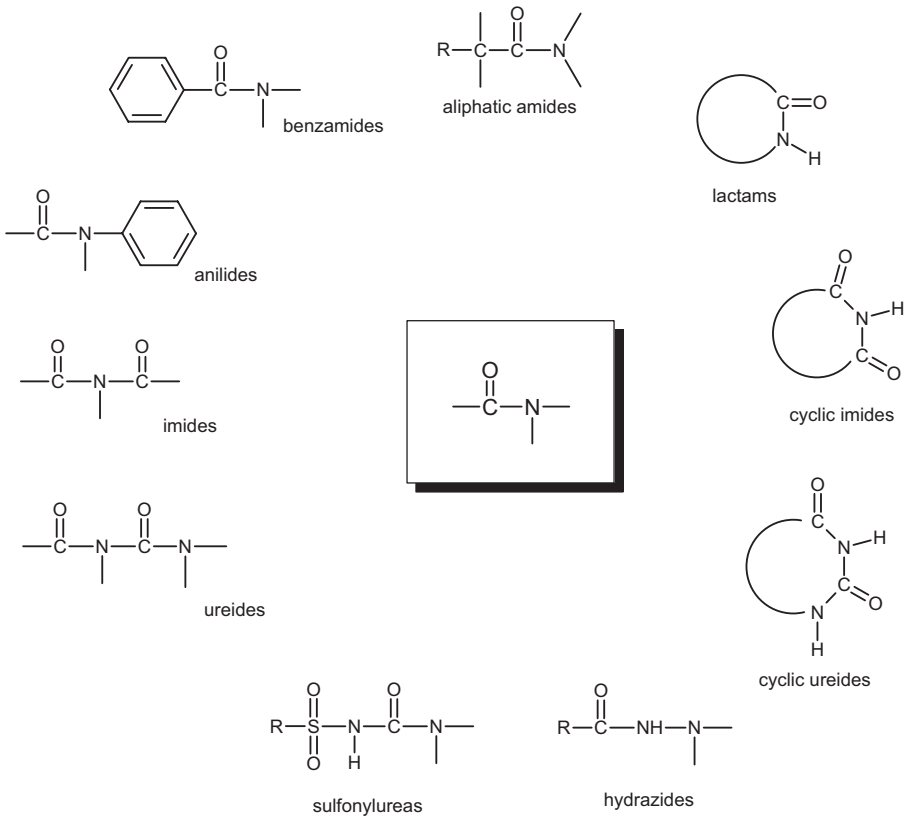


Fig. 4.1. *Chemical diversity of the amide bond*

bond is more resistant than the ester bond to chemical hydrolysis. The reason for this difference is that the amide bond is less polarized than the ester bond due to the lower electronegativity of the N-atom compared to the corresponding O-atom.

Both amides and esters are often hydrolyzed by the same hydrolases (see *Chapt. 2* and *3*). As a general rule, amides are hydrolyzed more slowly than esters, due to the stereoelectronic and catalytic properties of the enzymes. The greater stability of amides has, therefore, been used in the development of drugs that possess longer biological half-lives than their carboxylate analogues. For the same reason – *i.e.*, the relative stability of amides *in vivo* – *N*-acylation of amines to give amide prodrugs has been used to only a limited extent. It should be noted that various cyclic imides and ureides are not hydrolyzed by the classical esterase/amidase but by a particular enzyme, namely *dihydropyrimidine aminohydrolase* (DHPase, EC 3.5.2.2).

In this chapter, we confine our discussion to *xenobiotic amides*, mostly drugs, agrochemicals, and model compounds. Whenever possible, emphasis will be given to the existing relationship between hydrolysis and structure. *Endogenous amides*, mostly peptides, are examined in *Chapt. 2* and *6*, in which the physiological role of proteases is discussed. *Chapt. 6* is also devoted to compounds such as *xenobiotic peptides* and analogues, and amides formed by conjugation of the carboxy group with amino acids. The *lactam* structure can be found in many drugs of great significance in medicinal chemistry, thus, the hydrolysis of these compounds will be considered separately in *Chapt. 5*.

There is another mechanism leading to the cleavage of the amide bond, namely hydrolysis by intramolecular catalysis, as discussed in *Chapt. 11*.

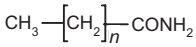
4.2. Aliphatic Amides

The following sections examine the hydrolysis of amides originating from aliphatic acids. The presentation is organized according to the degree of substitution on the amide nitrogen – *i.e.*, primary, secondary, and tertiary amides.

4.2.1. Primary Aliphatic Amides

The first systematic study of the metabolic hydrolysis of *primary aliphatic amides* was carried out by *Bray et al.* in 1950 [1]. The substrates were incubated in rabbit liver preparations for 5 h at 37°. In *Fig. 4.2*, the effect of chain length on the degree of hydrolysis of amides containing 1 to 18 C-atoms (**4.1**) is shown. The extent of hydrolysis was very small for the first three homo-

logues, then increased markedly to reach a maximum with hexanamide (**4.1**, $n=4$) and heptanamide (**4.1**, $n=5$), and then fell off rapidly. Amides with eleven or more C-atoms were hydrolyzed to only a small extent, like the lowest homologues. *Branching* of the side chain, moreover, reduced the extent of hydrolysis: isohexanamide (**4.2**), a branched-chain amide with six C-atoms, was hydrolyzed less than the corresponding linear amide. *ω-Phenyl substitution*, however, facilitated the hydrolysis of amides with four or fewer aliphatic C-atoms (**4.3**). The first member of this series showed a notably higher percentage of hydrolysis than the second member. The reason for this is that the aromatic ring influences the amide group of benzamide located only one C-atom away, and, therefore, shows reactivity similar to that of benzamide (see below). The authors had also shown earlier that the hydrolysis of these amides does not involve the peptidases glutaminase or asparaginase, nor does it involve the proteases pepsin, trypsin, or cathepsin [2].



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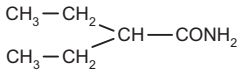

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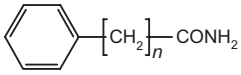

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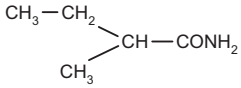

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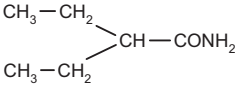

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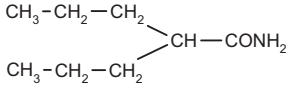

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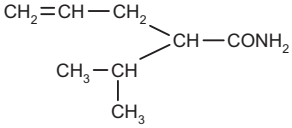

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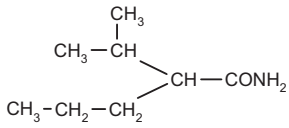


4.8

Later studies in chick embryo liver cell cultures reexamined the hydrolysis of a series of primary aliphatic amides toward elucidation of their *toxicity* [3]. This work was based on the observation that some amides are potent porphyrin-inducing agents, while others are devoid of this toxicity. Since the corresponding free acids were in all cases inactive, it was speculated that hydrolysis could be one of the factors reducing the toxicity of amides. To verify this hypothesis, the hydrolysis of unbranched and branched amides was studied in the presence and absence of an inhibitor of amidases/esterases. In the unbranched series of amides, hexanamide (**4.1**, $n=4$) and heptanamide (**4.1**, $n=5$) were most effectively hydrolyzed, whereas amides with shorter or longer chain lengths were less efficiently hydrolyzed. These results are similar to those reported by *Bray et al.* [1]. Furthermore, it was confirmed that *branching in the alkyl chain* significantly reduces hydrolysis of the amide group [2]. Thus, amides branched at C(2) (2-methylbutyramide (**4.4**), 2-ethylbutyramide (**4.5**), and dipropylacetamide (**4.6**)) were poorer substrates for hydrolases than the corresponding linear compounds. Further branching

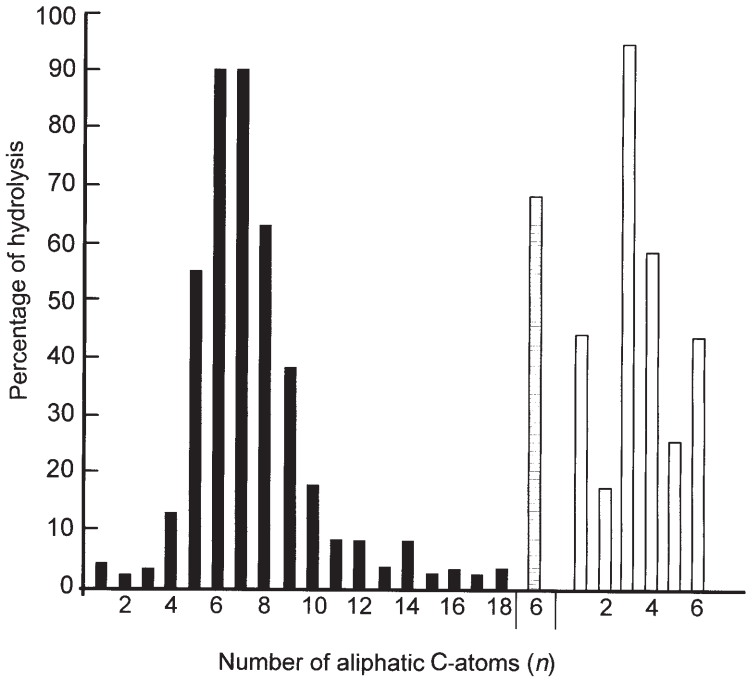


Fig. 4.2. *The effect of chain length* (*n*) *on the degree of hydrolysis* [%] *of primary amides by rat liver preparations* (5 h at pH 7.4). Black bars: amides of normal saturated fatty acids; hatched bar: isohexanamide; unshaded bars: amides of ω-phenyl-substituted saturated fatty acids [1].

completely impeded hydrolysis: amides branched at both C(2) ((allyl)(isopropyl)acetamide, **4.7**) and C(3) ((isopropyl)(propyl)acetamide, **4.8**) were not hydrolyzed at all. The study also demonstrated that the addition of bis(4-nitrophenyl) phosphate (BNPP), a typical inhibitor of liver carboxylesterase, suppressed amide hydrolysis to various degrees. The porphyrin-inducing activity of sterically unhindered aliphatic amides increased when the hydrolase was inhibited by BNPP. Furthermore, the toxicity of linear amides in the presence of BNPP could be correlated with their lipophilicity. It was concluded that the porphyrin-inducing activity of aliphatic amides in cultures of chick embryo liver cells depends both on lipophilicity and resistance to rapid hydrolysis.

Investigations on the structure–pharmacokinetic relationships of valpromide derivatives produced further information concerning the hydrolysis of branched primary aliphatic amides. *Valpromide* (dipropylacetamide; **4.6**) is a commonly used antiepileptic drug. Following intravenous administration to humans, valpromide is rapidly and almost completely metabolized (80%) to valproic acid [4]. Since direct administration of valproic acid (instead of its amide) produces an anticonvulsant effect, the amide might be considered a prodrug for the acid. However, valpromide does not correspond to the usual definition of prodrugs in that it is itself pharmacologically active. Tests of anticonvulsant activity in mice have shown that valpromide is 2 to 5 times more potent than valproic acid [5]. Furthermore, valpromide is largely responsible for sedative side effects.

A series of *branched aliphatic amides* were prepared to evaluate the role of amide hydrolysis on the pharmacokinetics and anticonvulsant activity of valpromide analogues. *Table 4.1* summarizes the structures investigated, the fraction of amide hydrolyzed (f_m), and the stability in blood. These results were obtained after intravenous administration to dogs [6]. The structures are classified here in order of decreasing f_m.

The first group of amides discussed here are those that underwent complete hydrolysis in dogs. Octanamide (**4.1**, $n=6$), (methyl)(pentyl)acetamide (**4.9**), and (methyl)(neopentyl)acetamide (**4.10**) were also found to be completely transformed into their corresponding acids. That these three amides were hydrolyzed in the blood contributed to their high clearance values. Here, in contrast to the previously discussed study [3], hydrolysis of the amide moiety was not affected by the presence of a Me group at C(2). Because these studies were carried out with different types of biological models (*in vivo vs. in vitro*), one is tempted to explain the discrepancy as being due to different species- and/or organ-specific requirements of the amidases.

3-Ethylpentanamide (**4.11**) and (allyl)(propyl)acetamide (**4.12**) were also completely biotransformed to the corresponding acids *in vivo*, but they were

Table 4.1. *Hydrolysis and activity of valpromide analogues* [6a,c][7]

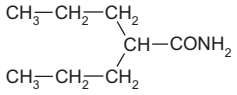
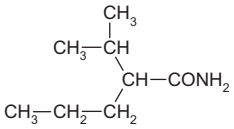
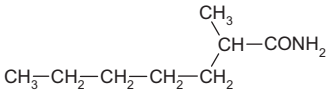
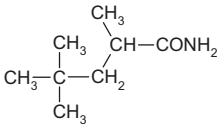
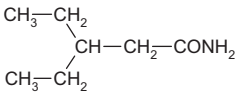
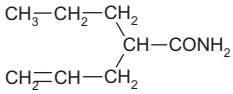
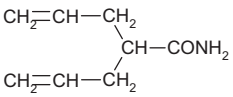
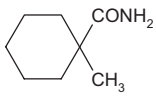
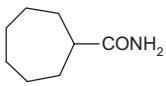
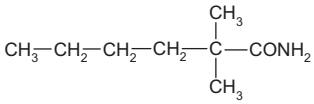
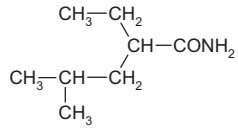
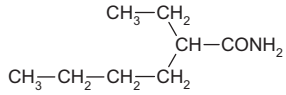
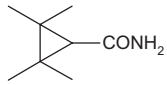
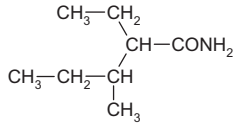
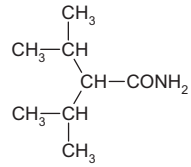
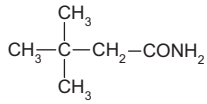
Amide	Structure	f_m [%][a]	Stability in blood	Pharmacological activity	
				ED_{50}[b] [mg/kg]	TD_{50}[c] [mg/kg]
4.1		100	unstable	–	–
4.6		*ca.* 30		56	81
4.8		0	stable	58	99
4.9		100	unstable	167	205
4.10		100	unstable	–	–
4.11		85	stable	–	–
4.12		100	stable	67	96
4.13		79	stable	125	182
4.14		60	unstable	–	–
4.15		44	unstable	–	–
4.16		36	unstable	–	–

Table 4.1 (cont.)

Amide	Structure	f_m [%] [a])	Stability in blood	Pharmacological activity	
				ED_{50} [b]) [mg/kg]	TD_{50} [c]) [mg/kg]
4.17		34	stable	–	–
4.18		16	stable	78	116
4.19		0		>120	94
4.20		0	stable	58	81
4.21		0	stable	87	57
4.22		0	stable	–	–

[a]) Fraction metabolized in dogs to the corresponding acid. [b]) ED_{50}: effective protective dose in 50% of the animals (maximal electroshock test in mice). [c]) TD_{50}: neurotoxic dose in 50% of the animals (rotorod ataxia test in mice).

stable in blood. This may indicate that the liver is the principal site of metabolism for these amides. Furthermore, it can be concluded that liver amidases have different substrate specificities than blood amidases.

The next group of amides shown in *Table 4.1* are those that undergo partial hydrolysis. Thus, a partial conversion to the corresponding acid was ob-

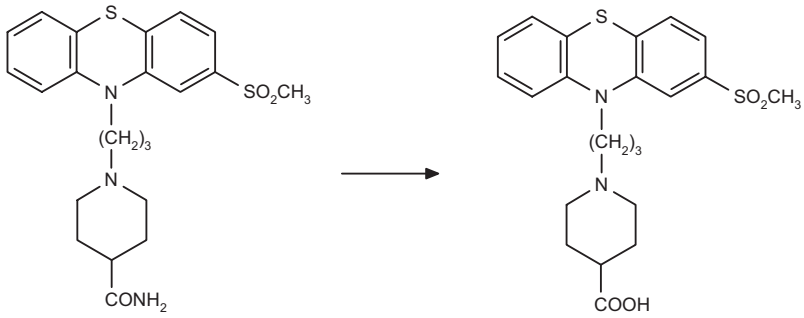
served for diallylacetamide (**4.13**). This compound was stable in blood and was metabolized mainly in the liver. The cyclic analogues 1-methylcyclohexanecarboxamide (**4.14**) and cycloheptanecarboxamide (**4.15**), as well as the α-substituted compound (butyl)(dimethyl)acetamide (**4.16**), had high clearance values that were partly due to hydrolysis in blood. Substitution at the γ-position, as in (ethyl)(isobutyl)acetamide (**4.17**), rendered the amide stable in blood and further reduced biotransformation to the corresponding acid. In dogs, the extent of the biotransformation of valpromide (**4.6**) into valproic acid was only half that observed in humans. This difference in f_m might be caused by a species difference in metabolism, since, in dogs, metabolic oxidation appears to be more important than in humans.

Finally, there is a group of amides that were completely resistant to hydrolysis (*Table 4.1*). Compounds carrying an alkyl substituent at the β-position, *e.g.*, 2-ethyl-3-methylpentanamide (**4.20**), (isopropyl)(propyl)acetamide (**4.8**), and diisopropylacetamide (**4.21**), were stable in blood and were not transformed to the corresponding acid in the liver. The presence of two Me moieties in the β-position in the case of (*tert*-butyl)acetamide (**4.22**) also prevented hydrolysis. These amides were eliminated from the body mainly through oxidative pathways. 2,2,3,3-Tetramethylcyclopropanecarboxamide (**4.19**), a cyclic compound with two substitutions in the β-position, was resistant both to hydrolysis and to other metabolic transformations [7]. This metabolic stability explains its low clearance rate and relatively long half-life. In summary, a stable valpromide analogue must have either two Me moieties at the β-position or substituents at both α- and β-positions.

Pharmacological screening showed that the amides were more active as anticonvulsants than the corresponding acids. It was, therefore, concluded that stable analogues of valpromide have the potential to be less toxic and more potent anticonvulsants than valpromide itself [6a,b]. The greater potency may be due to the amides acting as such, and not as prodrugs. That amides, as neutral compounds with moderate plasma protein binding, exhibit better brain penetration than their corresponding acids may contribute to their greater pharmacological activity.

Apart from valpromide derivatives, there are only few other drugs with a primary amide group that undergo metabolic hydrolysis. One example is that of *metopimazine* (**4.23**), a phenothiazine with antiemetic properties. Its carboxylic acid **4.24** was the major urinary metabolite in rabbits, but was not formed in dogs [8].

Medicinal chemists are interested not only in hydrolysis of amides by mammalian amidases as exemplified above, but also in *bacterial amidases* as useful biosynthetic tools. Of particular interest is the enantioselective hydrolysis of chiral amides by various bacterial amidases. Some of these



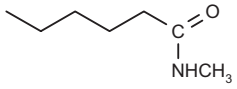
4.23 **4.24**

amides have been studied with the view to produce optically active D- or L-amino acids or for the enantioselective synthesis of α-methyl- and α-methoxyacetic acids used in synthons in the preparation of drugs or herbicides [9][10]. Recently, an amidase isolated from *Rhodococcus erythropolis* was found to hydrolyze enantioselectively racemic ketoprofenamide and naproxenamide to the corresponding (*S*)-acid (enantiomeric excess >99%) [11].

4.2.2. Secondary Aliphatic Amides

We continue our presentation of amides with a subsection devoted to secondary aliphatic amides, *i.e.*, having one alkyl substituent on the N-atom. There have been very few investigations on the relationship between enzymatic hydrolysis and the structure of *N*-monosubstituted aliphatic amides. *Chen* and *Dauterman* [12] showed that an amidase isolated from sheep liver microsomes was unable to hydrolyze *N*-Me-substituted amides having fewer than four C-atoms in the acyl chain. Starting with *N*-methylvaleramide, the extent of hydrolysis increases with increasing length of the acyl chain; the maximum is reached with *N*-methylcaproamide (**4.25**), and, thereafter, decreases with increasing chain length.

N-*Methyl substitution* does not seem to influence dramatically the hydrolysis of the amide bond. Indeed, a similar relationship between hydrolysis and chain length has been described for unsubstituted aliphatic amides



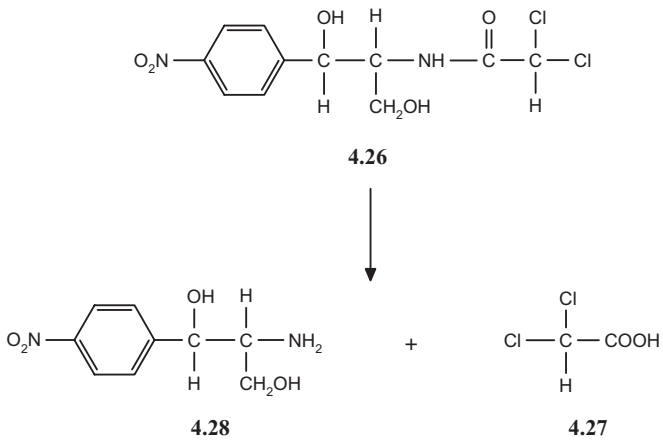
4.25

(see *Sect. 4.2.1*). In secondary amides, *chain length* can be subject to modification not only on the acyl side but also on the *N*-alkyl side of the bond. The size of the *N*-alkyl group influences the affinity of the molecule for the enzyme [12]: *N*-propylcaproamide has a K_m value almost 27-fold higher than that of *N*-methylcaproamide. However, comparison of the enzymatic $t_{1/2}$ values (which are proportional to the ratio K_m/V_{max}) revealed that *N*-methyl-, *N*-ethyl-, and *N*-propylcaproamide were hydrolyzed at almost the same rate.

Like the simple aliphatic secondary amides discussed above, structurally more-complex compounds may also be expected to undergo hydrolysis. However, very few such results are available, implying either that xenobiotics are relatively stable, or that they have been insufficiently studied. It seems that the former reason is the more likely, since the amide bond, in general, is chemically stable and is metabolized over only a narrow range of structures (see, *e.g.*, the *N*-alkyl-substituted amides discussed above). Some of the few reported examples of structurally complex xenobiotics that undergo amide hydrolysis are discussed below.

The hydrolysis of the amide bond in *chloramphenicol* (**4.26**), which liberates dichloroacetic acid (**4.27**) and the primary amine (**4.28**), has been shown in bacteria, rodents, and humans [13–15]. In the microsomal fraction of guinea pig liver, moreover, the enzyme responsible for hydrolysis has been identified as one of the B-type carboxylesterase isoenzymes [16].

In humans, *ca.* 10% of the urinary metabolites of *epicainide* (**4.29**, *Fig. 4.3*), an antiarrhythmic agent, is accounted for by the carboxylic acid derivative **4.32**. Three distinct pathways are possible for the formation of this metabolite, namely direct hydrolysis of the secondary amide function of epicainide (*Fig. 4.3*, *Pathway a*), hydrolysis of the primary amide (**4.30**) resulting



from oxidative *N*-dealkylation (*Fig. 4.3*, *Pathway b*), and hydrolysis of the intermediate metabolite (**4.31**) formed by *N*-deethylation and pyrrolidine oxidation (*Fig. 4.3*, *Pathway c*). The relative contributions of these three processes in the formation of the acidic metabolite have not been determined. Rats displayed a metabolic pattern completely different from that of humans: no hydrolysis occurred and the main metabolites resulted from hydroxylation of the Ph rings [17].

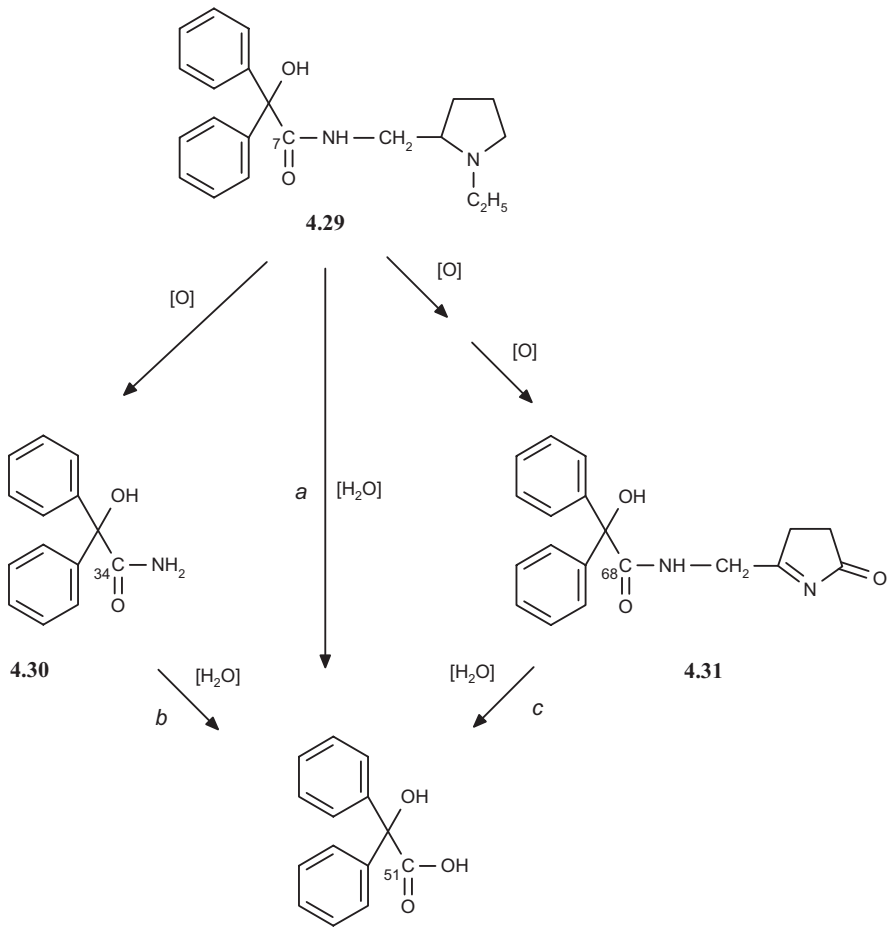
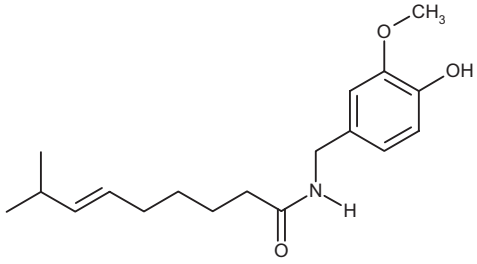


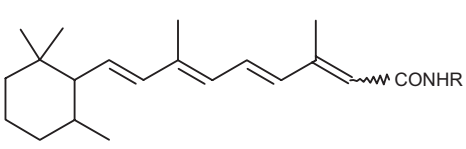
Fig. 4.3. *Hydrolysis pathways in the metabolism of epicainide* (**4.29**). *Pathway a*: direct hydrolysis of the secondary amide function. *Pathway b*: hydrolysis of the primary amide (**4.30**) formed by oxidative *N*-dealkylation. *Pathway c*: hydrolysis of the intermediary metabolite (**4.31**) formed by *N*-deethylation and subsequent oxidation of the pyrrolidine moiety [17].

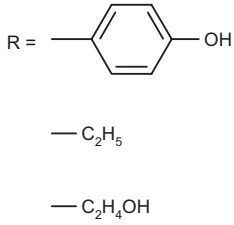
The biotransformation of *clofexamide* (**4.33**, *Fig. 4.4*), a compound with anti-inflammatory and antidepressant activities, was investigated in rats [18]. About 15% of the dose administered was found in urine as 2-(4-chlorophenoxy)acetic acid (**4.37**). This metabolite was formed *via* the secondary amine **4.34**, the primary amine **4.35**, and the acid **4.36** resulting from oxidative deamination. However, direct formation of 2-(4-chlorophenoxy)acetic acid (**4.37**) from the parent compound (**4.33**) cannot be excluded. Clofexamide and its metabolite **4.34** also underwent hydroxylation on the aromatic ring, but these hydroxylated metabolites did not appear to be hydrolyzed.

Capsaicin (**4.38**), an anti-arthritic phytochemical and also the active ingredient in pepper sprays, is found in various hot peppers. *Park* and *Lee* [19] showed that this compound is hydrolyzed by hydrolases p*I* 5.6 and p*I* 6.1 isolated from rat liver microsomes. Several *retinamides* (**4.39**) tested as anticancer agents showed the desired biological effect and were generally less toxic than the corresponding retinoic acid. It has, therefore, been speculated that the amides act as prodrugs, *i.e.*, that they are hydrolyzed by amidases to release the biologically active parent acid. The amide bond of these compounds can, indeed, be hydrolyzed to some extent by rat liver microsomes, *N*-ethylretinamides (**4.39**, R=Et) being the best substrate. However, no free retinoic acids have been detected in *in vivo* studies in rat liver [20]; thus, it is unlikely that these retinamides are extensively hydrolyzed in animals. More detailed studies are required to determine the extent and biological significance of the hydrolysis of retinamides.



4.38





4.39

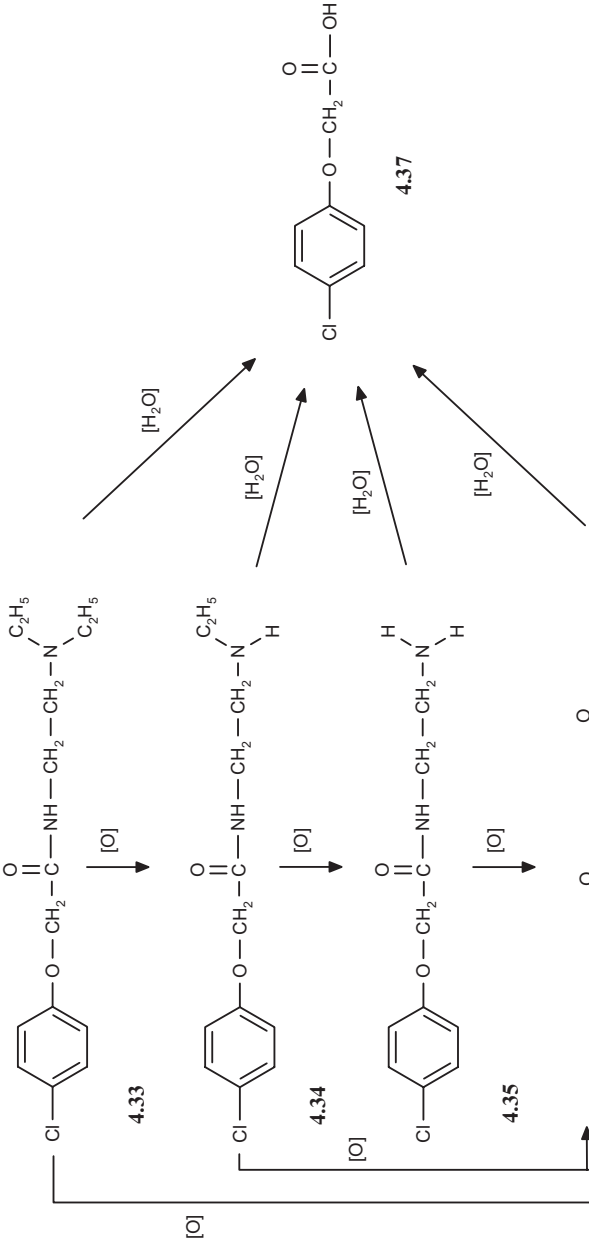
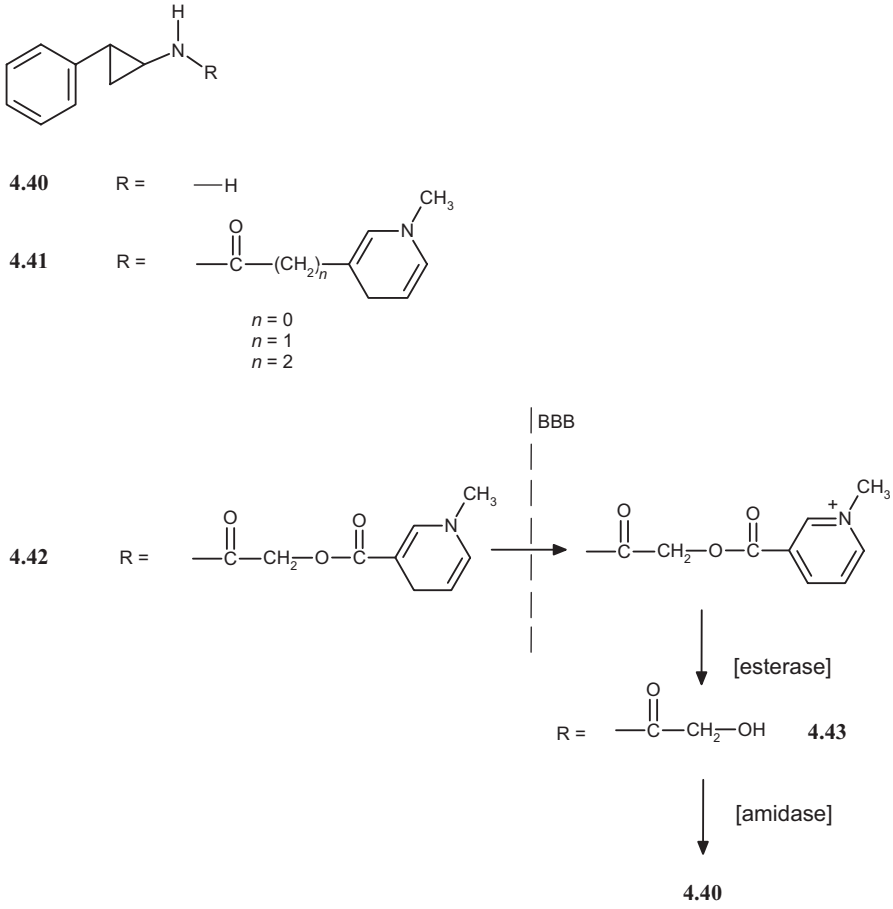


Fig. 4.4. *Pathways of clofexamide* (**4.33**) *biotransformation that yield 2-(4-chlorophenoxy)acetic acid* (**4.37**) [18]

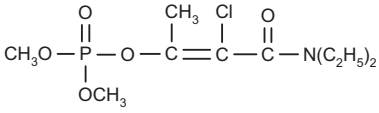
Replacement of an ester link by an amide link in the design of *dihydropyridine-based chemical delivery systems* (CDSs) was also investigated. The principles by which these dihydropyridine-based prodrugs can deliver drugs to the brain have been discussed elsewhere (Chapt. 13 in [21]), and the ester type CDSs are presented in this volume (*Chapt. 8*). The application of this brain-targeting strategy to amines can be illustrated by a study performed with the monoamine oxidase (MAO) inhibitor *tranylcypromine* (**4.40**) [22]. The dihydropyridine/pyridinium redox system was attached to the amino group of tranylcypromine by an amide (**4.41**) or carbamate linkage (**4.42**) [22]. After permeating the blood–brain barrier, the transport group must be removed by enzymatic hydrolysis. *In vitro* studies showed that both the dihydropyridine and the pyridinium derivative were stable toward chemical and enzymatic hydrolysis. In preliminary *in vivo* studies performed in the rat, only compound **4.42** had some MAO-inhibitory activity. This observation may be explained



by a two-step activation. First, the ester bond was hydrolyzed by esterases, yielding a 2-hydroxyacetamide intermediate (**4.43**), which, in turn, rapidly released the parent drug. It appears that the amide bond was, in most cases, too stable to be used in prodrug design. The substituted carbamates also investigated were more-rapidly hydrolyzed and had significant biological activity [22].

Amidases can be found in all kinds of organisms, including insects and plants [24]. The distinct activities of these enzymes in different organisms can be exploited for the development of selective insecticides and herbicides that exhibit minimal toxicity for mammals. Thus, the low toxicity in mammals of the malathion derivative *dimethoate* (**4.44**) can be attributed to a specific metabolic route that transforms this compound into the nontoxic acid (**4.45**) [25–27]. However, there are cases in which toxicity is not species-selective. Indeed, in the preparation of these organophosphates, some contaminants that are inhibitors of mammalian carboxylesterase/amidase may be present [28]. Sometimes the compound itself, and not simply one of its impurities, is toxic. For example, an insecticide such as *phosphamidon* (**4.46**) cannot be detoxified by deamination since it is an amidase inhibitor [24].

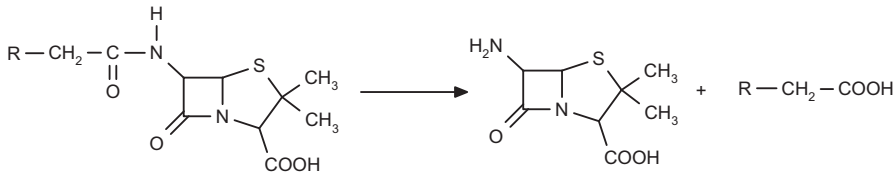
4.44 **4.45**



4.46

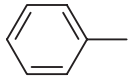
Like with primary amides (see *Sect. 4.2.1*), *bacterial amidases* can be useful for the transformation of secondary amides in drug synthesis. Bacterial amidases have been extensively studied in the presence of *penicillins* and other β-lactam antibiotics, for which two hydrolysis reactions are possible. One of these is carried out by enzymes known as penicillinases or β-lactamases that open the β-lactam ring; this aspect will be discussed in *Chapt. 5*. The second type of hydrolysis involves cleavage of the side-chain amide bond (**4.47** to **4.48**) and is carried out by an enzyme called penicillinacylase (penicillin amidohydrolase, EC 3.5.1.11). Both types of hydrolysis inactivate the antibiotic [29–31].

Penicillinacylase is used industrially to catalyze the hydrolytic removal of the side chain in naturally occurring penicillins such as benzylpenicillin (**4.49**) and phenoxymethylpenicillin (**4.50**). The nucleus 6-aminopenicillic acid (**4.48**) is then used as the starting material for the preparation of semi-synthetic penicillins. Appropriate acylation of the 6-NH_2 group leads to β-lactamase-stable and broad-spectrum penicillins.

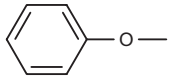


4.47 **4.48**

4.49 R =



4.50 R =



The penicillinacylase produced by *Escherichia coli* has a narrow substrate specificity, its activity being limited to compounds containing the acyl group R–CH_2–CO–, where R is an aryl group or an unbranched chain of three or four C-atoms. Substitution of the α-C-atom with an NH_2 or OH group reduces the rate of hydrolysis, whereas the introduction of an α-carboxy group blocks the hydrolysis of the side-chain. 4-Hydroxybenzyl-penicillin, however, is hydrolyzed more rapidly than benzylpenicillin. The insertion of an O-atom between the phenyl and –CH_2–CO– groups markedly reduces the rate of hydrolysis [30]. Enzymes from other microorganisms generally have different substrate specificities, and changes to the structure of the side chain often have the opposite effect than that observed with *E. coli.*

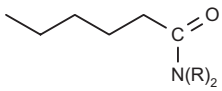
4.2.3. Tertiary Aliphatic Amides

The last group of aliphatic amides to be discussed are the tertiary amides, which, by definition, carry two alkyl substituents on the N-atom. Investigations of their chemical stability have disclosed a surprising difference between tertiary and secondary amides, since the rate of acid-catalyzed hydrolysis of N,N-*dimethyl amides* is higher than that of *N*-methyl amides. If steric fac-

tors were predominant, tertiary amides would be less reactive. *Challis* and *Challis* [32] have proposed that this phenomenon may result from a higher stability of the protonated intermediate. A comparable rule would appear to apply also to enzymatic hydrolysis. Indeed, investigation of the activity of amidase in sheep liver microsomes have shown that *N*,*N*-dimethylcaproamide (**4.51**) was hydrolyzed faster than the mono-*N*-methyl analogue [12].

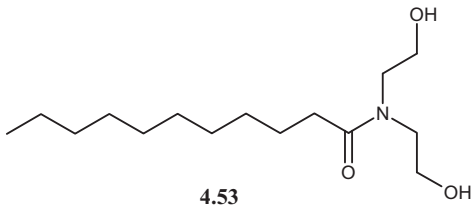
Alkyl groups larger than Me can be expected to prevent hydrolysis, as observed for *N*,*N*-diethylcaproamide (**4.52**). That *N*,*N*-bis(2-hydroxyethyl)lauramide (**4.53**), which is used in cosmetics as an emollient, thickener, and foam stabilizer, is not converted to 2,2′-iminodiethanol in rats may be rationalized by steric factors. This *N*,*N*-bis(2-hydroxyethyl)amide of a fatty acid is mainly metabolized *via* hydroxylation [33].

Another example in which *N*,*N*-diethyl substitution protects the amide linkage is *propanidid* (**4.54**), an anesthetic containing both an ester and an amide bond. In humans, only the ester function was hydrolyzed to form the

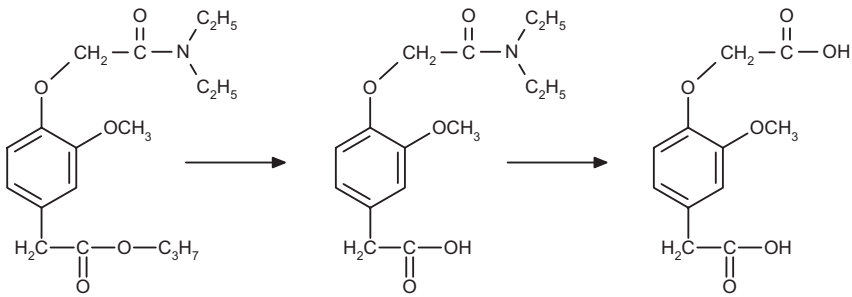


4.51 R = —CH_3

4.52 R = —C_2H_5



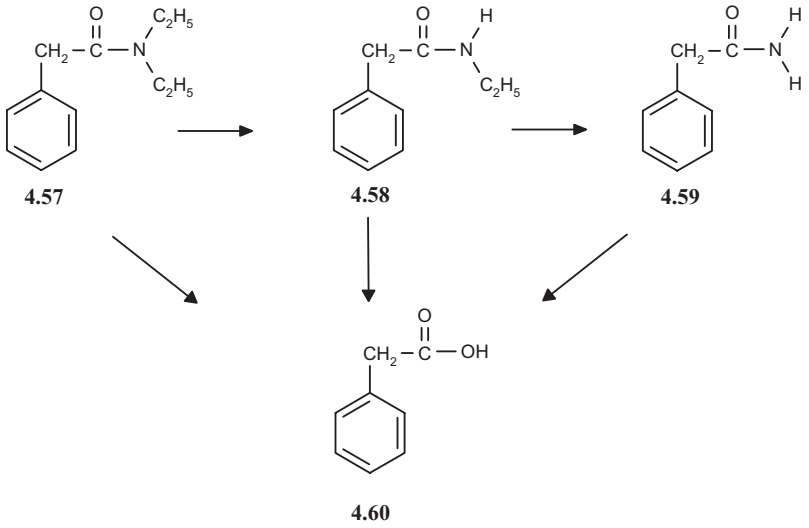
4.53



4.54 **4.55** **4.56**

monoacid **4.55**, although a minor amount of the diacid metabolite (**4.56**) was detected in the rat [34].

In rats dosed orally with the insect repellent N,N-*diethyl-2-phenylacetamide* (**4.57**), *N*-ethyl-2-phenylacetamide (**4.58**), 2-phenylacetamide (**4.59**), and 2-phenylacetic acid (**4.60**) were found as metabolites in the blood, liver, and kidney [35]. Hydrolysis of this tertiary amide is, perhaps, facilitated by the presence of the aromatic ring. Indeed, a similar metabolic pattern has been found for the aromatic amide *N*,*N*-diethyl-3-methylbenzamide (**4.82**) (see *Sect. 4.3.1*).



4.3. Aromatic Amides

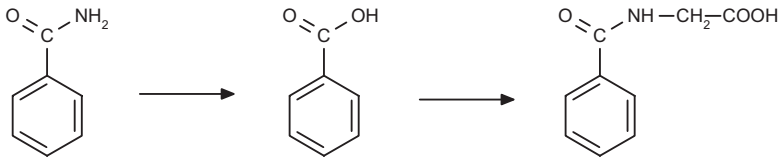
In the following sections, we discuss compounds whose amide group is adjacent to an aromatic system. A distinction is made between aromatic amides derived from aromatic acids (*e.g.*, benzamides) or from aromatic amines (*e.g.*, anilides). The discussion will be completed with compounds in which the amide bond links two aromatic systems.

4.3.1. Benzamides and Analogues

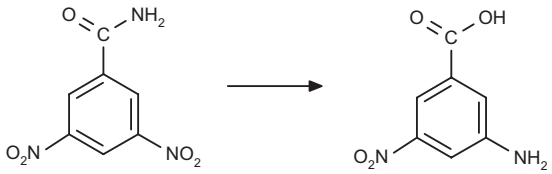
4.3.1.1. *Primary Benzamides and Analogues*

That *benzamide* itself (**4.61**) undergoes hydrolysis was shown many years ago. Indeed, it is converted in rabbits to benzoic acid, which is then excret-

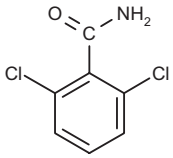
ed mainly as hippuric acid (**4.62**) [2]. Subsequently, it was found that *ring substitution* affects the rate of hydrolysis, depending upon the nature and position of the substituent. Thus, a study of the fate of ring-substituted benzamides in rabbit liver extracts showed that the presence of NH_2 or OH in any position prevented hydrolysis. With the other substituents examined (NO_2, Me, Cl), substitution in the *para*-position enhanced and in the *ortho*-position diminished the extent of hydrolysis. In the case of fluorobenzamides, the effect of position was less pronounced [1].



4.61 **4.62**



4.63 **4.64**

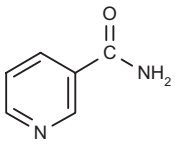


4.65

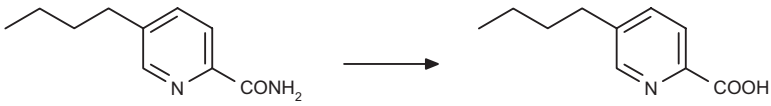
In contrast to the aforementioned monosubstitutions, much less is known about the effect of disubstitution. It appears that such benzamides are resistant to hydrolysis. The metabolic fate of *nitromide* (3,5-dinitrobenzamide, **4.63**), a chicken-feed additive for the prevention of coccidosis, was investigated in rats. The metabolite 3-amino-5-nitrobenzoic acid (**4.64**), formed by nitro reduction and hydrolysis, was excreted in only trace amounts. Nitromide was metabolized mainly *via* nitro reduction [36]. Similarly, no hydrolysis was

observed after oral administration to rats of *2,6-dichlorobenzamide* (**4.65**), a major environmental metabolite of the herbicide 2,6-dichlorobenzonitrile [37]. In this case, the major metabolic route was gluthathione conjugation.

But what about the case of *aromatic heterocyclic amides*? In primary amides of pyridine carboxylic acids, only the *ortho* compound underwent marked hydrolysis in rabbit liver extracts [1]. In other biological systems, however, the *meta* compound *nicotinamide* (**4.66**) was well-hydrolyzed [38]. This hydrolysis appears to be of physiological importance, since the resulting nicotinic acid serves as a precursor in the synthesis of pyridine nucleotides [39]. It would seem that nicotinamide is hydrolyzed by a specific enzyme whose activity is regulated by the intracellular concentration of diphosphopyridine nucleotide. A study with *Torula cremoris*, a microorganism with a high 'nicotinamidase' activity, revealed that the presence of a Me group on either the ring or the amide N-atom prevented hydrolysis [40].



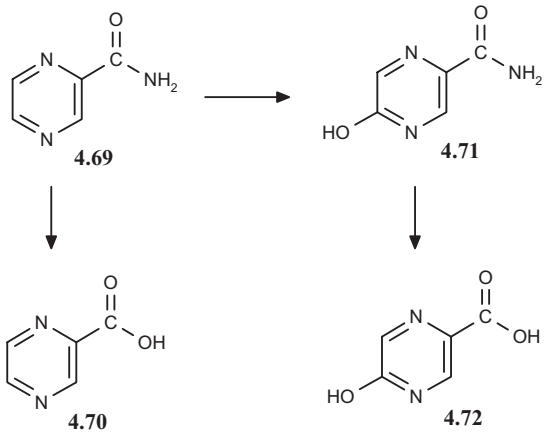
4.66



4.67 **4.68**

5-Butylpicolinic acid (fusaric acid, **4.68**) is a major metabolite of *5-butylpicolinamide* (SCH 10985, **4.67**) in the plasma of rats, dogs, and humans. The biological activity of this compound (an inhibitor of dopamine-β-hydroxylase) is mainly due to its metabolite fusaric acid, indicating that, in this example, the hydrolysis of the amide group is bioactivating [41].

In many other cases, hydrolysis results in drug inactivation, as with the tuberculostatic agent *pyrazinamide* (**4.69**). In humans and rats, the metabolism of this compound involves deamidation to produce pyrazinoic acid (**4.70**). Ring oxidation is another major pathway, leading to 5-hydroxypyrazinamide (**4.71**), which, in turn, is hydrolyzed to 5-hydroxypyrazinoic acid (**4.72**). The rates of hydrolysis were found to determine the apparent half-life of pyrazinamide [42][43].



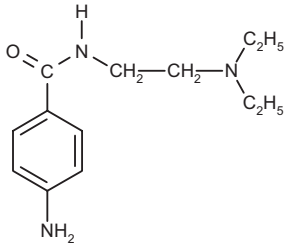
It is interesting to note that the tuberculostatic activity of pyrazinamide also involves hydrolysis. A bacterial enzyme, pyrazinamidase (also called pyrazinamide amidohydrolase), hydrolyzes pyrazinamide to form pyrazinoic acid and ammonia. The resistance of *Mycobacterium tuberculosis* to pyrazinamide can, in most cases, be explained by the loss of pyrazinamide hydrolase activity [44]. *Frothingham et al.* [45] have identified and characterized a pyrazinamidase gene from *E. coli*. Furthermore, they found that a single enzyme in *E. coli* hydrolyzes both pyrazinamide and nicotinamide. Examination of mycobacterial species differing in pyrazinamide hydrolase activity and susceptibility to pyrazinamide revealed a second mechanism of resistance. Indeed, the pyrazinamidase enzyme is not exposed on the bacterial surface, which implies that the drug must enter the cells before being activated to pyrazinoic acid. Because permeation of the cell membrane involves a transport system, mutation can prevent the penetration and activation of the drug. It, thus, appears that susceptibility to pyrazinamide in mycobacteriae requires the presence of both a functional pyrazinamidase and a pyrazinamide-transport system [46].

4.3.1.2. *Secondary Benzamides*

The simplest structures in this series are *N*-methyl- and *N*-ethylbenzamide (**4.73** and **4.74**, respectively). When administered intraperitoneally in rats, these amides yielded methylamine and ethylamine, respectively, plus benzoic acid, which was detected in the urine as hippuric acid [47]. An alternative metabolic pathway is possible, involving *N*-dealkylation to the primary amide followed by hydrolysis; its contribution, if any, must be minor, since benzamide levels in urine were negligible.

4.73 R = —CH_3

4.74 R = —C_2H_5

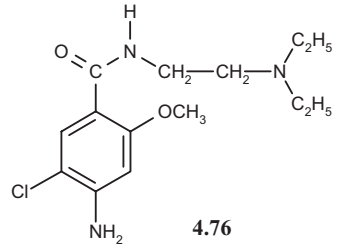


4.75

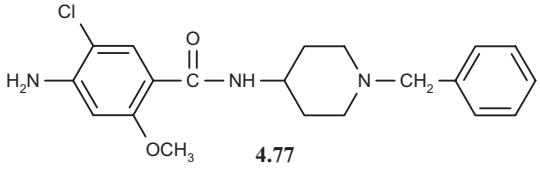
As shown by the above examples, secondary benzamides indeed undergo hydrolysis. However, their rate of hydrolysis is, in general, much slower than that of the corresponding esters. The stability of amides has, therefore, been used in drug design to obtain drugs with half-lives longer than those of their carboxylate analogues. The difference between the stability of these bonds can be illustrated by comparing procaine, a local anesthetic, with its amide analogue *procainamide* (**4.75**), an antiarrhythmic agent. In contrast to its ester analogue, procainamide is hydrolyzed only slowly, with less than 1% of the dose being transformed to 4-aminobenzoic acid in humans [48].

Other examples of secondary benzamides include the therapeutic class of *orthopramides*, which are, again, markedly resistant to hydrolysis. Thus, hydrolysis of the amide bond is a minor metabolic pathway in humans for the antiemetic drug metoclopramide (**4.76**) [49]. *Clebopride* (**4.77**), an antidopaminergic agent, was found to be hydrolyzed to a limited extent in rabbit liver homogenates and in dogs to 4-amino-5-chloro-2-methoxybenzoic acid (**4.78**) and to the amine **4.79** [48][50]. Attempts to detect *in vivo* formation of this metabolite in rats, rabbits, or humans were not successful [50].

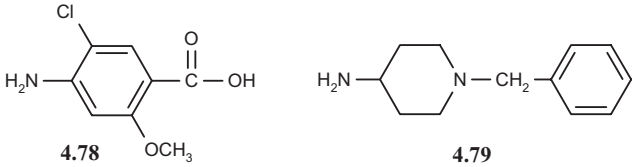
Other orthopramides have been shown to be resistant to *in vivo* hydrolysis. Thus, the gastrokinetic drug *cisapride* (**4.80**) was not hydrolyzed after oral administration to dogs and humans [51]. Similarly, *sulpiride* (**4.81**), an antidepressant and antipsychotic drug, did not undergo hydrolysis in humans and laboratory animals. These compounds are metabolized by other routes, and hydrolysis of the amide bond, when it occurs at all, is only a minor pathway.



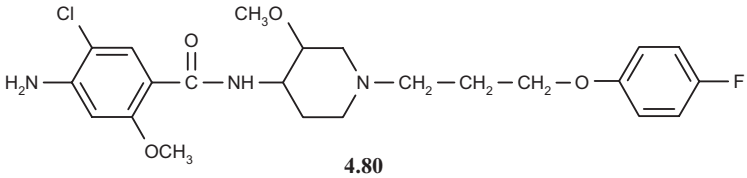
4.76



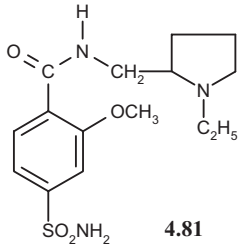
4.77



4.78 **4.79**



4.80

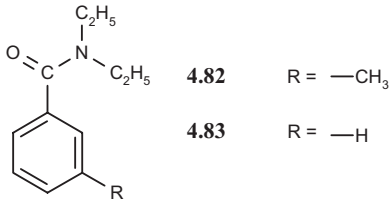


4.81

4.3.1.3. *Tertiary Benzamides*

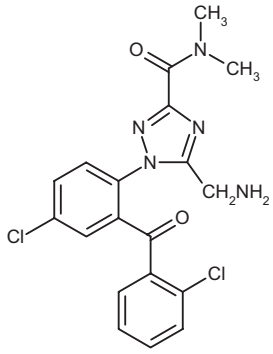
A simple example in this class with which to begin is *N*,*N*-diethyl-*m*-toluamide (*N*,*N*-diethyl-3-methylbenzamide, DEET, **4.82**), an extensively used topical insect repellant. The hydrolysis product 3-methylbenzoic acid was detected in the urine of rats dosed intraperitoneally or topically with DEET. However, amide hydrolysis represented only a minor pathway, the major metabolites resulting from methyl oxidation and *N*-dealkylation [52]. Treatment of rats with *N*,*N*-diethylbenzamide (**4.83**), a contaminant in DEET, produced the same urinary metabolites as its secondary analogue, *N*-ethylbenzamide (see *Sect. 4.3.1.2*). This observation can be explained by invoking a metabolic pathway that involves initial oxidative mono-*N*-deethylation followed by enzymatic hydrolysis of the secondary amide to form ethylamine and benzoic acid [47]. Since diethylamide was not detected in these experiments, it appears that *N*,*N*-diethylbenzamide cannot be hydrolyzed by amidases, perhaps due to the increased steric bulk of the tertiary amido group.

A *N*,*N*-dimethylcarboxamido group attached to other aromatic systems can be hydrolyzed enzymatically as demonstrated by the metabolism of the ring-opened 1,4-benzodiazepine derivative **4.84** in dogs and rats [53]. Here also, hydrolysis was shown to proceed *via* the secondary and primary amide formed by *N*-demethylation.



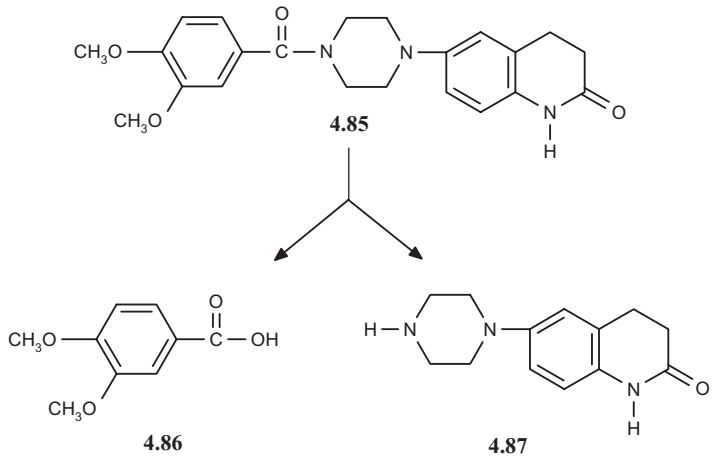
4.82 R = —CH_3

4.83 R = —H



4.84

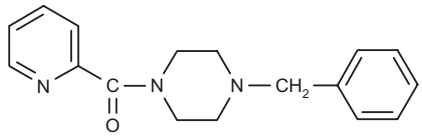
Tertiary benzamides whose *N-atom is part of a cyclic system* can also be hydrolyzed metabolically as shown in the following examples. The hydrolysis of the amide group in 6-[4-(3,4-dimethoxybenzoyl)piperazin-1-yl]-3,4-dihydro-1*H*-quinolin-2-one (OPC-8212, **4.85**), an inotropic agent, occurred in rats, mice, dogs, monkeys, and humans [54]. After oral administration to rats, both products of hydrolysis, namely 3,4-dimethoxybenzoic acid (veratric acid, **4.86**) and piperazine-1*H*-quinolin-2-one (**4.87**) were detected in the plasma, urine, and feces.



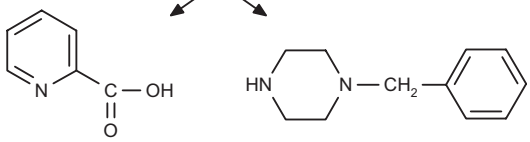
N-*Benzyl*-N′-*picolinoylpiperazine* (EGYT-475, **4.88**), a compound with potential antidepressant activity, underwent similar hydrolysis. After intravenous administration, picolinic acid (**4.89**) was one of its major urinary metabolites in rats; the other product, *N*-benzylpiperazine (**4.90**) was also detected, but at much lower levels, since it was further transformed by *N*-debenzylation [55]. Since the products of direct hydrolysis of these cyclic tertiary amides (*i.e.*, the corresponding secondary amines) were found at substantial levels, it appears that oxidative *N*-monodealkylation is not an essential step for hydrolysis in these compounds, in contrast to the findings for *N*,*N*-diethylbenzamide. This contradicts the hypothesis [52] (see above) that the steric bulk of the tertiary amide group impedes direct hydrolysis. Here, although the degree of steric bulk is at least comparable, direct hydrolysis clearly takes place.

Delavirdine (**4.91**), a selective inhibitor of HIV-1 reverse transcriptase, also contains an amido N-atom as part of a piperazine ring. In rats and monkeys, cleavage of the amide bond constituted only a minor pathway [56].

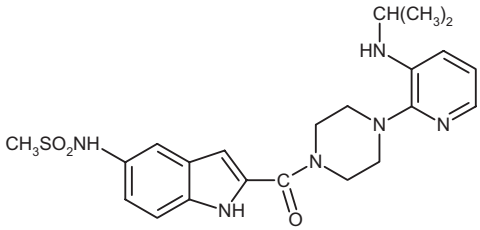
In humans, the principal metabolic pathway of the anti-inflammatory agent *indomethacin* (**4.92**) proceeds through *O*-demethylation to yield **4.94**,



4.88



4.89 **4.90**

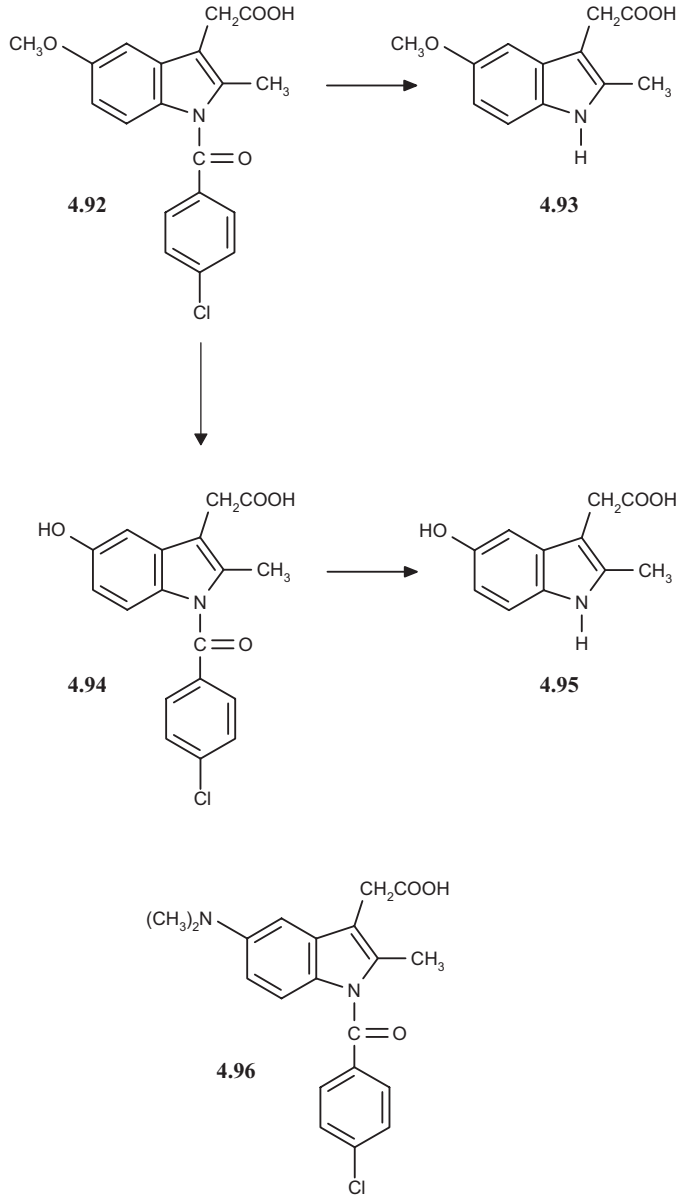


4.91

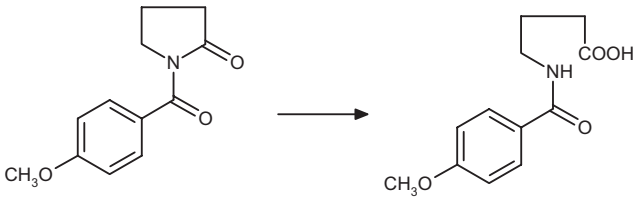
followed by deacylation to yield 5-hydroxy-2-methyl-1*H*-indole-3-acetic acid (**4.95**). Direct deacylation leading to 5-methoxy-2-methyl-1*H*-indole-3-acetic acid (**4.93**) also occurs, but as a minor pathway [57]. More recently, amide hydrolysis of indomethacin has been demonstrated in rabbit skin [58]. Given the low turnover rate of hydrolysis, the limited efficacy of topically applied indomethacin does not appear to be due to extensive metabolic inactivation by the skin.

Replacing the 5-MeO substituent of indomethacin with a Me_2N group produced a compound with higher water solubility. This analogue (MK-825, **4.96**) was extensively metabolized (*ca.* 40% in humans) by hydrolysis to 4-chlorobenzoic acid, which was excreted in the urine as 4-chlorobenzoylglycine [59].

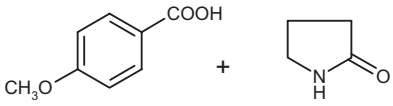
The indomethacin-hydrolyzing enzyme from pig liver microsomes was purified and partially characterized [60]. The enzyme was found to be different from known pig liver esterases, since it did not hydrolyze naphth-1-yl-acetate and (4-nitrophenyl)acetate, which are typical substrates for these carboxylesterases. The amino acid sequence of the enzyme showed high homology with the mouse carboxylesterase isoenzyme ES-male. Human liver car-



boxyl-esterases p*I* 5.3 and p*I* 4.5 have no catalytic activity toward indomethacin, yet this drug is hydrolyzed in human liver homogenates. Based on these observations, it was proposed that indomethacin is hydrolyzed in humans by an enzyme similar to the one isolated from pig liver [60].



4.97 **4.98**



4.99 **4.100**

The nootropic agent *aniracetam* (**4.97**) is discussed here, despite its being not only an amide but also a lactam and an imide. This compound can be hydrolyzed by two routes, *i.e.*, cleavage of the amide bond and cleavage of the lactam ring. In humans, 70% of the administered dose was transformed to anisamidobutyrate (*N*-anisoyl-GABA, **4.98**), while the pathway leading to *p*-anisilic acid (**4.99**) and 2-pyrrolidinone (**4.100**) accounted for the remaining 30% [61]. Two different enzymes were characterized: the indomethacin-hydrolyzing enzyme (see above) hydrolyzed the endocyclic amide linkage but not the exocyclic amide bond, while, in contrast, carboxylesterase hydrolyzed both amide bonds [60]. Recently, it has been demonstrated that both metabolites anisamidobutyrate and *p*-anisilic acid play an important role in the central cholinergic activation elicited by aniracetam [62].

4.3.2. Anilides

This subsection is devoted to the metabolic reactivity of the amide bond in anilides, *i.e.*, compounds whose amino moiety is attached to an aromatic ring. Based on the nature of the acyl moiety, a number of classes of anilides exist, three of which are of particular interest here, namely arylacetamides, acylanilides, and aminoacylanilides. The first group contains several analgesic-antipyretic drugs, the second N^4-acyl derivatives of sulfonamides, and the third a number of local anesthetics. Particular attention will be paid to structure–metabolism relationships in the hydrolysis of these compounds. Cases where hydrolysis leads to toxification will be summarized in the last part of the chapter.

4.3.2.1. *Arylacetamides*

Acetanilide (**4.101**) represents the simplest structure in this series of compounds. This obsolete analgesic and antipyretic agent, also known as antifebrin, was abandoned long ago because of its excessive toxicity and low activity. Deacylation plays an important role in the disposition of this compound, and was believed at one time to be a determining factor in its toxicity (*Sect. 4.3.2.4*). The hydrolysis of acetanilide is mediated by carboxylesterase. Pretreating rats with the inhibitor bis(4-nitrophenyl) phosphate (BNPP; see *Chapt. 3*) greatly reduced the formation of aniline (**4.102**) [63]. On the other hand, it is interesting to note that endopeptidases such as chymotrypsin and trypsin, which hydrolyze amide bonds in peptides, did not hydrolyze acetanilide [64].

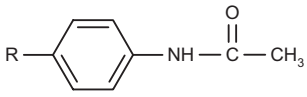
4.101 R = —CH_3 **4.102**

4.103 R = —C_2H_5 to —C_5H_{11}

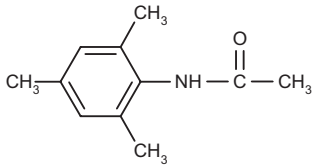
A number of studies were undertaken to investigate the influence of structural changes in the amino or acyl moiety. Lengthening the acyl chain increased the rate of cleavage. Thus, *N*-valeroylanilide was hydrolyzed 40 times faster than acetanilide by pig liver esterase [64]. A more recent study investigating the hydrolysis of a series of *fatty acyl anilides* (**4.103**) by two isoenzymes of carboxylesterase isolated from rat liver revealed a complex situation. In the case of esterase 6 (E1), the maximal rate of hydrolysis increases with increasing chain length of the acyl residue, whereas in the case of esterase 5.6 (EA) the opposite dependence was found [65].

Modifications of the arylamino moiety profoundly influence the rate of hydrolysis. Thus, the hydrolysis of *N*-acetyl-4-aminobenzoic acid was *ca.* 1000-fold slower than that of acetanilide [66]. *Sorci* and *Macalady* [67] investigated the influence of ring substitution on the hydrolysis of *para*-substituted acetanilides (**4.104**) in alkaline solution and in soil bacteria. No correlation was found between alkaline and biotic hydrolysis, which appeared to be controlled by different physicochemical properties. Bacterial hydrolysis was best correlated with the *Van der Waals* radius of the substituent, whereas chemical hydrolysis was correlated with the *Hammett* constant characterizing the electron-withdrawing capacity of the substituent. Other studies confirmed that a correspondence between bacterial and mammalian esterases

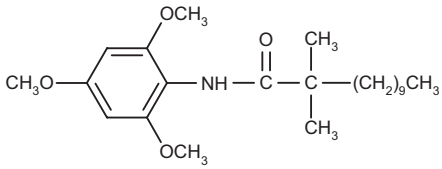
does not always exist. Thus, 4-nitroacetanilide was a good substrate for esterases from *Pseudomonas acidivorans* but not from rat liver [68][69]. Finally, studies of the rate of hydrolysis of acetanilide and its derivatives in mammals revealed large species differences [70].



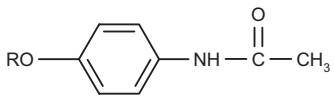
4.104 R = — H; — Br; — CN; — F; — OCH_3; — CH_3; — NO_2



4.105



4.106



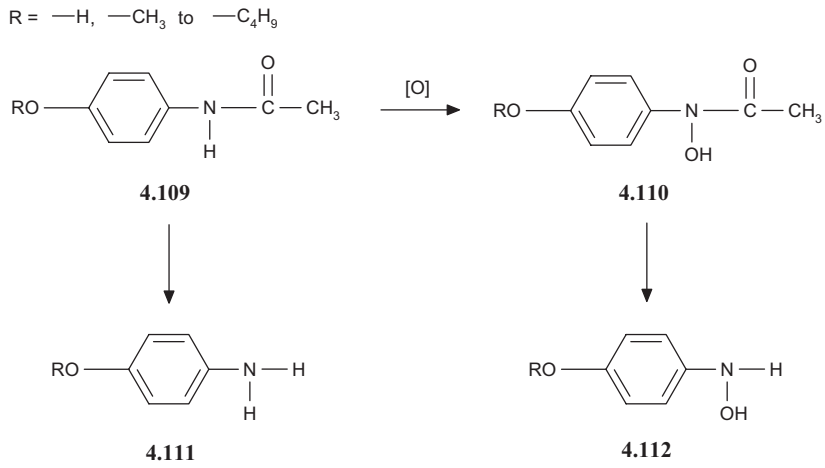
4.107 R = —C_2H_5

4.108 R = —H

Methylation in both *ortho*-positions on the aniline ring appears to impede hydrolysis of the amide bond. Thus, 2,4,6-trimethylacetanilide (**4.105**) was not hydrolyzed by rat liver homogenates, perhaps due to steric hindrance by the 2- and 6-Me groups [71]. Similarly, the amide bond of the hypocholesterolemic agent 2,2-dimethyl-*N*-(2,4,6-trimethoxyphenyl)dodecanamide (**4.106**) is protected by the flanking MeO groups. The metabolism of this molecule occurred mainly by ω-oxidation of the alkyl side chain followed by chain-shortening β-oxidation [72].

A toxicologically important *para*-substituted acetanilide is *phenacetin* (**4.107**), an obsolete antipyretic/analgesic agent originally introduced to replace acetanilide. In contrast to acetanilide, deacetylation is a minor metabolic reaction for phenacetin, its major pathway being oxidative *O*-dealkylation to form acetaminophen (*N*-acetyl-4-aminophenol, paracetamol, **4.108**).

The effect of the *length of the alkoxy group* on deacetylation was investigated in a homologous series (MeO to BuO) of phenacetin analogues (**4.109**) and their *N*-hydroxy derivatives (**4.110**), which represent one of their potential metabolites [73]. In microsomal fractions isolated from mouse liver and kidney, the rates of deacylation of **4.109** to form **4.111** increased with increasing chain length of the alkoxy group. Deacetylase activity was five to

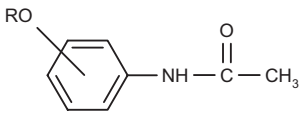


six times greater in the liver than in the kidney. The rates of deacylation of the *N*-hydroxy derivatives (**4.110**) to form **4.112** also increased with increasing chain length, at rates *ca.* 100-fold greater than those observed for the parent amides [73]. *N*-Hydroxylation is a primary step in the metabolic process leading to the carcinogenic and mutagenic effects of aromatic amides (see *Sect. 4.3.2.4*).

The hydrolysis of the *positional isomers* of acetaminophen (**4.113**) and phenacetin (**4.114**) were compared in homogenates of rat brain, liver, and kidney [74]. Hydrolytic activity in the liver was higher than in the kidney and very low in the brain. The phenacetin analogues were better substrates than the acetaminophen analogues, as were the *ortho*-substituted compounds relative to their corresponding *meta*- or *para*-isomers. The hydrolysis of *meta*-acetophenetidine was comparable to that of acetanilide. No convincing arguments can be formulated to explain these observations.

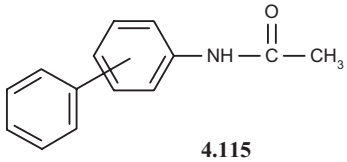
The *ortho*-effect has also been observed for the deacylation of isomeric acetamidobiphenyls (**4.115**) by hepatic microsomes from mouse, hamster, guinea pig, rat, and rabbit [75]. In all species investigated, the rate of deacylation of the *ortho*-isomer was greatest, whereas the rates of hydrolysis of the *para*- and *meta*-isomers were comparable. Hydrolysis was shown to be mediated by B-esterases, since the reaction was inhibited by organophosphates. With selective inhibitors, the esterase was identified as the ES-3 carboxylesterase in hamster, guinea pig, rat, and rabbit. In mouse, the hydrolyzing activity was less sensitive to the same inhibitors, suggesting that an enzyme distinct from ES-3 carboxylesterase was involved.

Methylation of the amido N-atom appears to reduce hydrolysis. Thus, the tertiary amide *N*-methylphenacetin (**4.116**) was deacetylated by purified pig liver esterase at a rate much lower than observed for phenacetin [64].



4.113 R = —H

4.114 R = —C_2H_5



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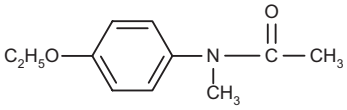
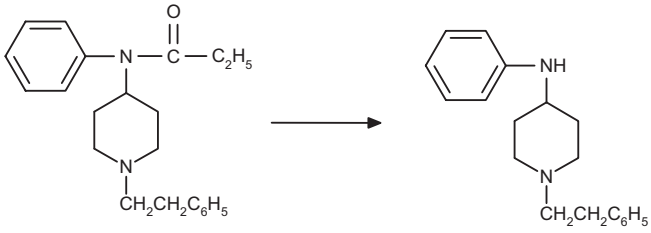
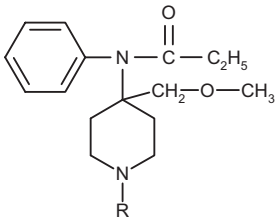


4.116

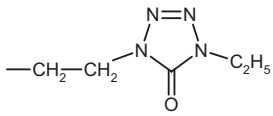
Tertiary arylacetamides appear to undergo hydrolysis to a very limited extent only. Hydrolysis of the synthetic opioid fentanyl (**4.117**) to despropanoylfentanyl (**4.118**) was a very minor pathway in humans [76]. No metabolites resulting from amide hydrolysis were detected for the fentanil analogues alfentanil (**4.119**) and sufentanil (**4.120**) [77], for which oxidative *N*-dealkylation was the main metabolic pathway.



4.117 **4.118**



4.119 R = —CH_2—CH_2



4.120 R = —CH_2—CH_2

4.3.2.2. N^4-*Acylated Sulfonamides*

Antibacterial sulfonamides contain two N-atoms, the sulfonamido (N^1) and the *para* primary amino (N^4). The *sulfonamido group*, in contrast to a carboxamido group, is chemically and metabolically stable. In other words, hydrolytic cleavage of sulfonamides to produce a sulfonic acid and an amine has never been observed. We, therefore, focus our discussion on the primary amino group, acetylation of which is one of the major metabolic pathways for some sulfonamides. Hydrolysis of the N^4-*acetylated metabolites* back to the parent sulfonamide can occur in the liver, kidney, and intestinal tract. The reaction is strongly influenced by the structure of the parent amine: *e.g.*, N^4-acetylsulfisoxazole (**4.121**) was deacetylated by intestinal bacteria whereas N^4-acetylsulfanilamide (**4.122**) under identical conditions was not [78][79].

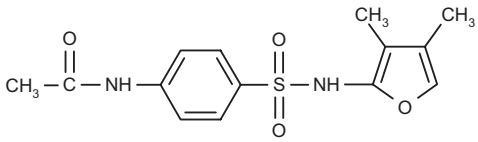
The influence of the acyl moiety on hydrolysis was investigated in mice with a homologous series of N^4-acylsulfadiazines (**4.123**). Hydrolysis was negligible for N^4-acetylsulfadiazine, became extensive for the N^4-propanoyl and N^4-butanoyl derivatives, then decreased with increasing chain length, and was negligible for the N^4-lauryl and N^4-palmitoyl derivatives [78].

The N^4-acylated sulfonamides, succinylsulfathiazole (**4.124**) and phthalylsulfathiazole (**4.125**), are poorly absorbed by the gastrointestinal tract. These compounds, which were once used as intestinal antiseptics, are inactive *in vitro* and must be activated by hydrolysis to sulfathiazole by bacterial hydrolases in the large intestine [78].

N^4-Acylation usually leads to reduced water solubility compared to the parent drug. However, other substituents may increase solubility. Thus, *N*-(propylaminoacetyl)sulfonamides were prepared to improve the water solubility of sulfonamides for intravenous application [80]. Some of these *N*-(propylaminoacetyl) derivatives were well-soluble in water as salts. Enzymatic hydrolysis of the potential prodrug bis{4-[2-(propylamino)acetamido]phenyl}sulfone (**4.126**) was rapid and liberated bis(4-aminophenyl)sulfone (*dapsone*, **4.127**), a major antileprosis drug. More recently, amino acid derivatives of dapsone were synthesized as water-soluble, chemically stable prodrugs. In this case, peptidases were responsible for *in vivo* cleavage to the parent drug (*Sect. 6.2*).

4.3.2.3. *Aminoacylanilides*

Lidocaine (lignocaine, **4.128**, *Fig. 4.5*) is a typical representative of the class of aminoacylanilides. Lidocaine is a local anesthetic and an antiarrhythmic agent that is very resistant to chemical hydrolysis even in strongly acidic or basic media. Only at higher temperature does lidocaine



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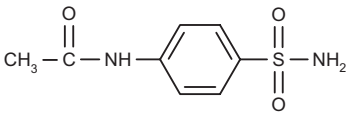

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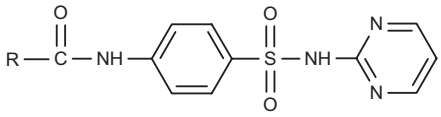

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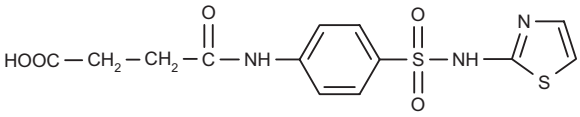

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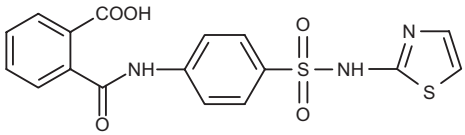

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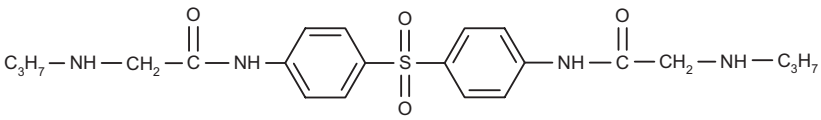

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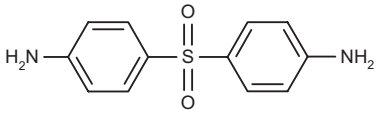

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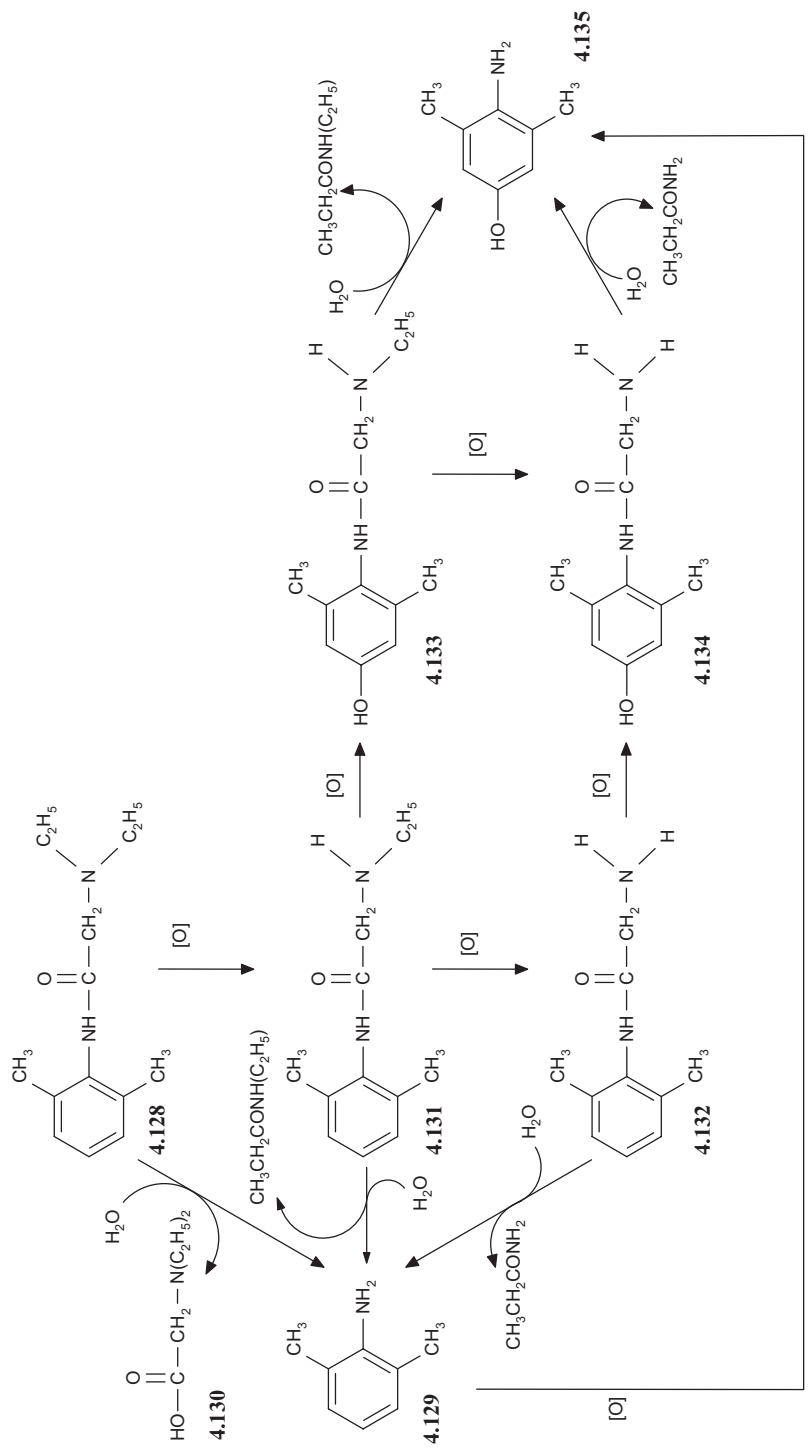
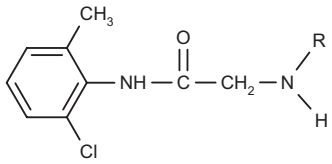


Fig. 4.5. *Major routes of metabolism for lidocaine* (**4.128**). Direct hydrolysis yields 2,6-dimethylaniline (2,6-xylidine, **4.129**) and 2-(diethylamino)acetic acid (diethylglycine, **4.130**). Hydrolysis of the metabolites monoethylglycinexylidine (**4.131**) and glycinexylidine (**4.132**) also yields **4.129**. The metabolites **4.131** and **4.132** also undergo hydroxylation at the aromatic ring to form **4.133** and **4.134**, respectively, which, in turn, are hydrolyzed to 4-hydroxy-2,6-dimethylaniline (**4.135**). This compound can also be formed by oxidation of **4.129**.

degrade slowly to 2,6-dimethylaniline (2,6-xylidine, **4.129**) and 2-(diethylamino) acetic acid (diethylglycine, **4.130**). The rate of hydrolysis is increased by the presence of metal ions. It has been proposed that lidocaine reacts *via* an intramolecular reaction between the carboxy group and the tertiary amino group in a mechanism involving general base catalysis and a H_2O molecule [81].

Despite the high chemical stability of the amide bond of lidocaine, hydrolysis represents a major pathway of its metabolism in mammals. Considerable interspecies differences in the extent of metabolic hydrolysis have been observed. Thus, rats excreted *ca.* 15% of a dose as products of hydrolysis (4-hydroxy-2,6-dimethylaniline (**4.135**) and 2,6-xylidine (**4.129**) as their glucuronic acid conjugates), and guinea pigs and dogs *ca.* 35%. In humans, hydrolysis of the amide bond represents the major metabolic pathway, accounting for *ca.* 75% of the amount excreted in urine [82]. The possible routes of formation of 4-hydroxy-2,6-dimethylaniline (**4.135**) and 2,6-xylidine (**4.129**) involve hydrolysis of lidocaine and/or monoethylglycinexylidine (**4.131**) and/or glycinexylidine (**4.132**). Interestingly, the latter metabolites were hydrolyzed to a greater extent than the parent compound [64][80]. 4-Hydroxy-2,6-dimethylaniline (**4.135**) can be formed either by oxidation of 2,6-xylidine (**4.129**) or by hydrolysis of the hydroxylated metabolites (**4.133** and **4.134**). Using human liver slices, *Parker et al.* [83] provided evidence that 2,6-xylidine is produced by direct hydrolysis of lidocaine. Their data also show that the conversion of 2,6-xylidine to 4-hydroxy-2,6-dimethylaniline represents a saturable pathway. The possible toxicological consequences of the formation of 2,6-xylidine will be discussed in *Sect. 4.3.2.4*.

The local anesthetic *butanilicaine* (**4.136**) is used by enzymologists largely to study liver carboxylesterases. Indeed, its amide bond is split by isoenzymes from rat, pig, and human liver at high rates, similar to those measured with good ester substrates. *Eckert et al.* [80] carried out a systematic study of the relationship between the chemical structure of a large number of butanilicaine homologues (**4.137**) and their rates of hydrolysis by pig liver esterases. The maximal velocity was found to rise steeply from C_1 to C_3, to



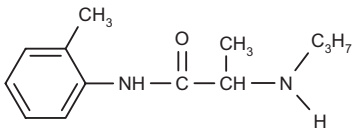
4.136 R = $—C_4H_9$

4.137 R = $—CH_3$ to $—C_6H_{13}$

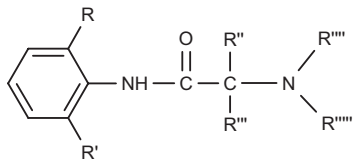
reach a maximum at C_3 and C_4 and then to decrease for C_5 and C_6. The K_m values decreased continuously but leveled off at the *N*-pentyl derivative.

Further studies on structure–metabolism relationships allowed the identification of other factors that decrease the maximal rate of hydrolysis of aminoacylanilide analogues [64]. Thus, replacement of the benzene ring with a cyclohexene ring, introduction of a CH_2 group between the aromatic ring and the amino group, introduction of a second *N*-alkyl group, as well as substitution of the Cl-atom at C(2) by a Me group, all decreased the rate of enzymatic hydrolysis.

Prilocaine (**4.138**), a chiral local anesthetic, was hydrolyzed stereoselectively at its amide bond. Indeed, the plasma concentrations of the (–)-(*R*)-enantiomer were lower than those of the (+)-(*S*)-enantiomer after *i.v.* administration in the cat. *In vitro* studies of liver preparations from various mammals confirmed that the (*R*)-isomer was hydrolyzed at much higher rates than the (*S*)-form [84].



4.138



4.139

The structural elements that determine amide cleavage in compounds of type **4.139** were investigated in mouse liver homogenates [84] and can be summarized as follows: Increasing the size of the *N*-substituent (the R′′′′ and R′′′′′ groups) increased the rate of hydrolysis, whereas it was decreased by the presence of substituents surrounding the amide bond (R to R′′′). The R′′ and R′′′ groups were critical, and reactivity was considerably reduced for R′′=Me and R′′′=H. Compounds substituted in both positions (R′′ = R′′′ = Me) were resistant to amidase.

The anticonvulsant agent 4-{[(diethylamino)acetyl]amino}-*N*-(2,6-dimethylphenyl)benzamide (**4.140**, *Fig. 4.6*) has a side chain resembling that of local anesthetics. The compound was rapidly metabolized by *N*-dealkylation to form the monodeethylated (**4.141**) and dideethylated metabolite (**4.142**). The three compounds could then undergo amide hydrolysis to form *ameltolide* (**4.143**). The parent compound and its dealkylated metabolites must be considered prodrugs of ameltolide, since mice pretreated with the reversible esterases/amidases inhibitor bis(4-nitrophenyl) phosphate (BNPP) were not protected by **4.140** against seizure [86]. The amide link between the two benzene rings was not hydrolyzed. Other compounds with an amide bridge between two aromatic systems will be discussed in *Sect. 4.3.3.*

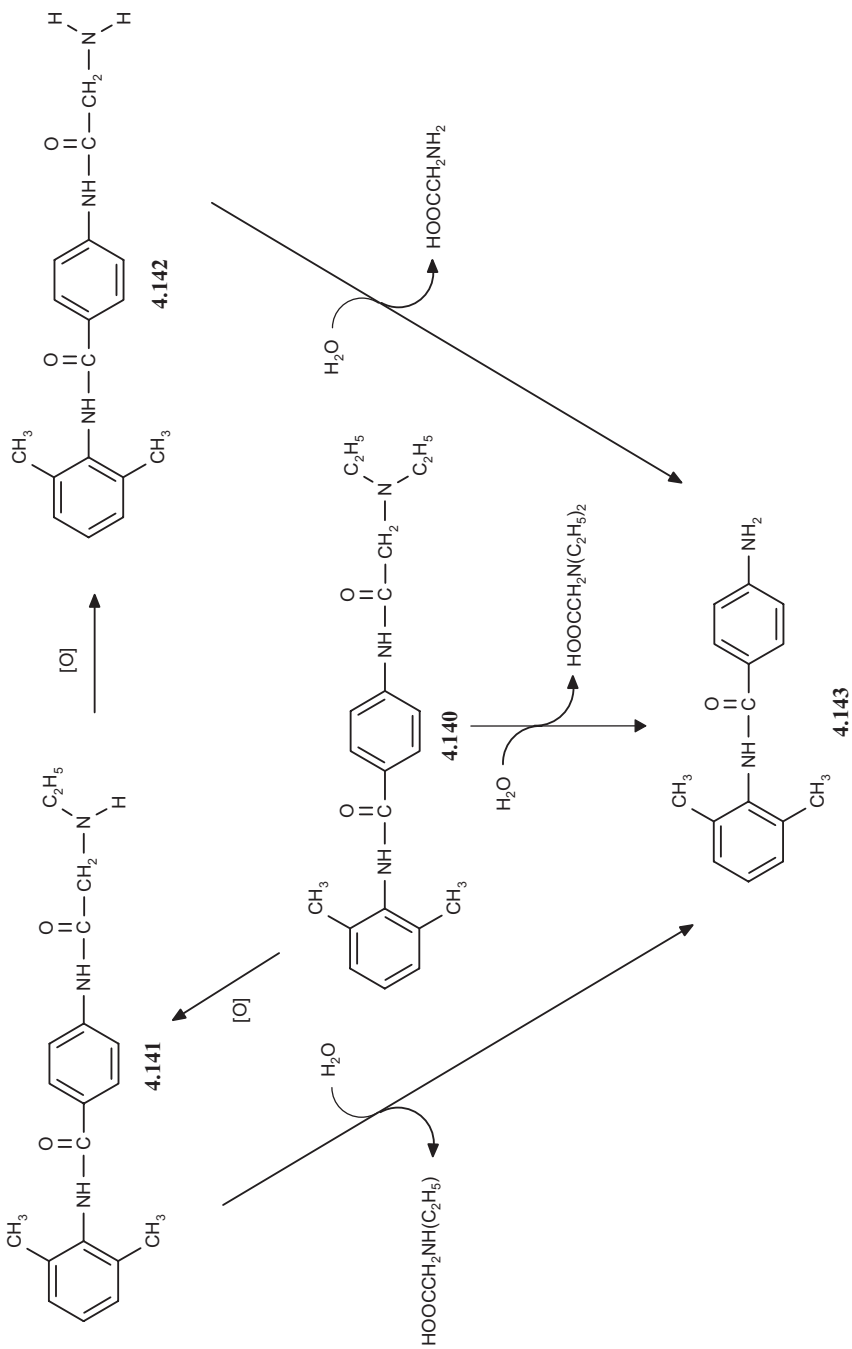
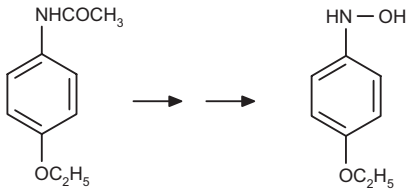


Fig. 4.6. *Metabolic pathways of 4-[[(diethylamino)acetyl][amino]*-N-*(2,6-dimethylphenyl)benzamide* (**4.140**) *in mice* [86]. The metabolites formed by consecutive *N*-deethylation (**4.141** and **4.142**) as well as of the parent compound are hydrolyzed at the amido bond to produce ameltolide (**4.143**).

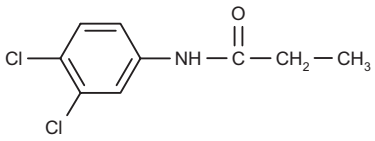
4.3.2.4. *Toxicity of Anilides*

Hydrolysis of the amide bond generally leads to inactivation of the substrate and accelerates excretion of the products. In the case of aminoacylanilides, however, such hydrolysis may represent a pathway of toxification, since it liberates *aromatic amines*, which are potentially hematotoxic, nephrotoxic, hepatotoxic, and/or carcinogenic.

The role of deacetylation in methemoglobinemia induced by acetanilide (**4.101**) and phenacetin (**4.107**) has been demonstrated. Indeed, concomitant *i.p*. administration of BNPP considerably reduced the hematotoxicity of these compounds [87]. Recent studies have shown that *N*-hydroxyphenetidine (**4.144**), a metabolite of deacetylated phenacetin, is responsible for hemolysis and methemoglobin formation [88].



4.107 **4.144**



4.145

A hydroxylamino product was also found to be the hematotoxic metabolite of the herbicide propanil (**4.145**) [89]. After administration of lidocaine (**4.128**, *Fig. 4.5*) to humans, 2,6-dimethylaniline–hemoglobin adducts were detected in blood. Again, an *N*-hydroxylated metabolite appears to be involved [90].

Acetaminophen (**4.108**) is hepatotoxic at higher doses, a toxicity explained by a cytochrome P450 dependent activation to *N*-acetyl-*p*-benzoquinonimine, which binds covalently to critical proteins (Chapt. 7 in [21]). However, the role of the hydrolytic step in acetaminophen-induced nephrotoxicity is not entirely clear. Early studies suggested a deacetylase-dependent activation of

acetaminophen [63][91][92]. Thus, pretreatment of *Fischer 344* rats with BNPP provided protection against elevation of urea concentration in blood and lessened the severity of histological lesions following acetaminophen administration. Subsequent studies in the CD-1 mouse, however, showed that BNPP or tri(2-methylphenyl) phosphate, another inhibitor of hydrolases, did not decrease the nephrotoxicity of acetaminophen. Immunochemical analysis of mouse kidney demonstrated covalent binding of acetaminophen-derived material to renal proteins [93]. Similarly, no requirement for deacetylation of acetaminophen to 4-aminophenol was found in the generation of covalently bound material in either hepatic or renal S9 fractions obtained from *Sprague–Dawley* rats [94]. At this stage, it was concluded that the nephrotoxicity of neither phenacetin nor acetaminophen is related to deacetylation.

A more recent *in vivo* study, in which blood urea concentration was used as an index of nephrotoxicity, showed that both oxidative and hydrolytic routes may contribute to acetaminophen-induced nephrotoxicity in *Sprague–Dawley* rats [95]. Indeed, pretreating rats with either 1-amino-1*H*-benzotriazole (a suicide inhibitor of cytochrome P450) or tri(2-methylphenyl) phosphate prevented an acetaminophen-induced elevation of blood urea concentrations. Furthermore, pretreatment with both inhibitors significantly decreased covalent binding in kidneys. It was suggested that the discrepancy between *in vitro* and *in vivo* results could be explained by enzymatic bioactivation of the hydrolytic product 4-aminophenol, possibly in the liver, producing nephrotoxic glutathione conjugates [95].

Deacylation may also be important in the carcinogenicity of arylamides. Phenacetin was found to exhibit higher mutagenicity in the *Salmonella* test system when incubated with hamster liver microsomes than with rat liver microsomes. The metabolism of phenacetin in microsomal fractions revealed no significant species difference in *N*-hydroxylation, but rates of deacylation were much higher in rats than in hamsters. The species difference in mutagenicity observed may, therefore, be attributed to the differences in deacylating activity between rat and hamster liver microsomes [96].

The herbicide *alachlor* (**4.146**, *Fig. 4.7*) also displayed species-dependent toxicity, since it induced nasal tumors in rats but not in mice. Its metabolic scheme in rats and mice (*Fig. 4.7*) shows that alachlor can be transformed into 2,6-diethylaniline (**4.149**) by two different pathways, one of which proceeds *via* formation of **4.147**. The other pathway implies glutathione (GSH) conjugation, followed by β-lyase-mediated liberation of the thiol, followed by *S*-methylation to produce the methylsulfide **4.148**. The two secondary amides **4.147** and **4.148** were hydrolyzed by microsomal arylamidases, but alachlor itself was not a substrate for this enzyme. The hydrolytic product 2,6-diethylaniline (**4.149**) was oxidized in nasal tissues to the electrophilic quinonimine metabolite **4.150**, which can bind covalently to proteins. Aryl-

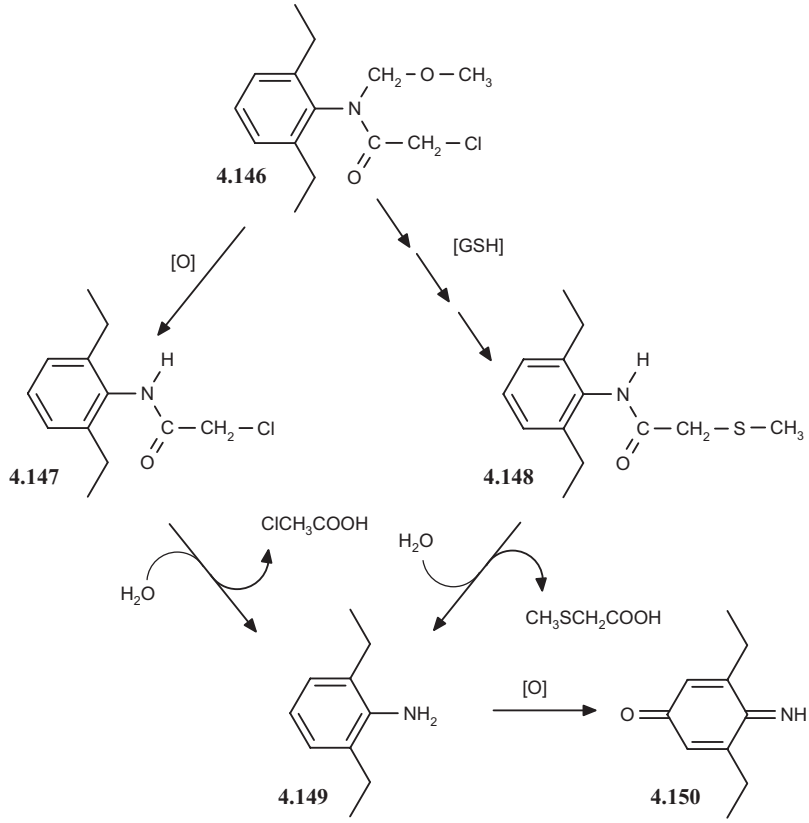
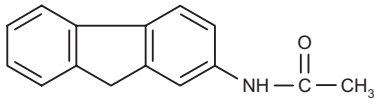


Fig. 4.7. *Metabolic activation of alachlor* (**4.146**) *to the cytotoxic quinonimine* **4.150** *in rats and mice* [97]. Only the two secondary amides **4.147** and **4.148** are hydrolyzed by microsomal arylamidases. (GSH = glutathione).

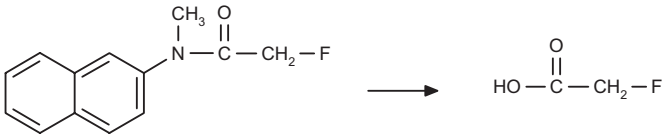
amidase activity was found to be 14- to 20-fold higher in rat than in mouse nasal tissue. Thus, the observed species differences in nasal carcinogenesis could be explained by differences in the rates of hydrolysis [97].

Deacylation also plays a role in the carcinogenicity of 2-(acetylamino)fluorene (**4.151**) in rats. Indeed, paraoxon and BNPP inhibited both the deacetylation of 2-(acetylamino)fluorene and its incorporation to DNA in rat hepatocytes [98].

The reactive mutagens formed from arylamides are *hydroxylamines* (*Fig. 4.8*) produced either by deacetylation and subsequent *N*-hydroxylation (*Pathway a*), or by *N*-hydroxylation and subsequent deacetylation (*Pathway b*). An alternative pathway to form *N*-hydroxyaminofluorene involves *transacetylation* by *N*,*O*-acyltransferase, producing *N*-acetoxyarylamine (*Pathway c*). The latter mode of activation seems to operate in human hepatocytes, since



4.151



4.152 **4.153**

organophosphates inhibited deacylation but not incorporation into DNA [99]. Thus, deacylation appears to be important for the activation of arylamides in rat hepatocytes, but may be less important in human cells.

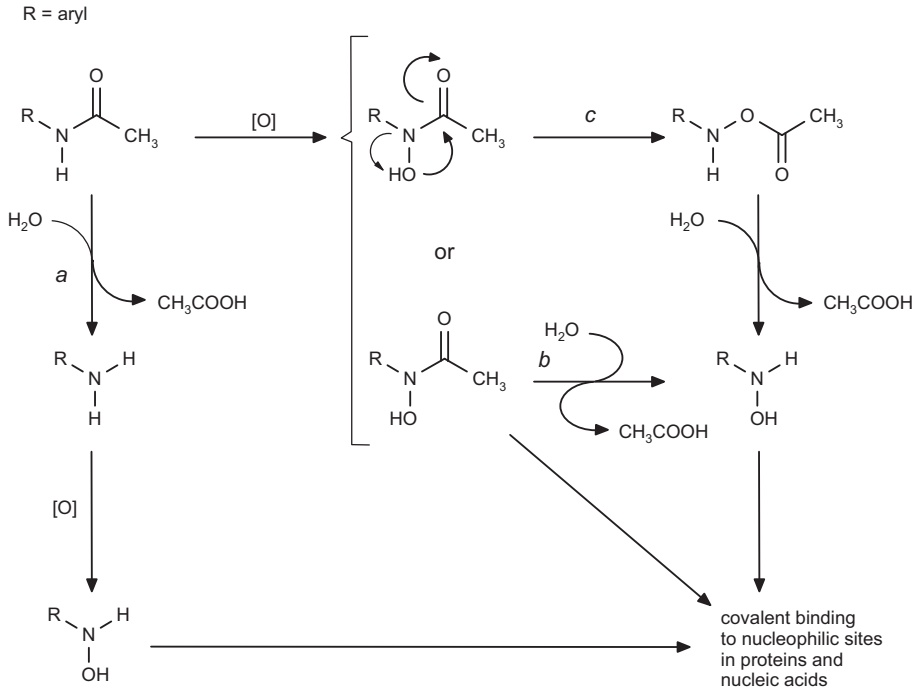


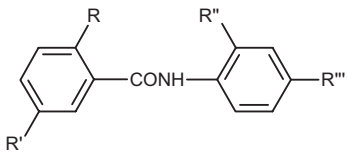
Fig. 4.8. *Formation of mutagenic* N-*hydroxyamines from arylamides. Pathway a*: *via* deacetylation and subsequent *N*-hydroxylation. *Pathway b*: *via N*-hydroxylation and subsequent deacetylation. *Pathway c*: *via N*-acetoxy arylamine produced by *N*,*O*-acyltransferases. [99]. Activation of hydroxylamines and hydroxylamides by *O*-sulfation is not shown. In all cases, the ultimate electrophile may be a nitrenium ion.

In mice, also, deacetylation is involved in the hepato-carcinogenesis induced by 2-(acetylamino)fluorene. Indeed, BNPP-inhibited DNA-adduct formation in murine liver microsomes as well as tumor initiation by *N*-hydroxy-2-(acetylamino)fluorene in infant male B6C3F_1 mice [100].

The toxicity of the acaricide 2-fluoro-*N*-methyl-*N*-(naphth-1-yl)acetamide (MNFA) (**4.152**) to mammals is related to its hydrolysis. In this case, however, toxicity was mainly due to the acid formed. Indeed, the 2-fluoroacetic acid (**4.153**) liberated by hydrolysis was further metabolized to fluorocitrate, which inhibits the tricarboxylic acid cycle [101].

4.3.3. Amide Bonds That Link Two Aromatic Systems

Having discussed amides that carry an aromatic group on either the nitrogen or the carboxy side of the amide bond (*i.e.*, anilides or benzamides, respectively), we continue our presentation with compounds in which the amide bond links two aromatic systems. The simplest structure in this class is *N*-phenylbenzamide (**4.154**). The influence of the nature and position of substitution on the rate of hydrolysis of a series of *N*-phenylbenzamides was investigated in mouse and sheep liver homogenates, with the goal of elucidating the metabolism of the anthelminthic niclosamide (**4.158**) [102].



4.154 R = R' = R" = R"' = — H

4.155 R = R' = R" = — H, R"' = — NO_2

4.156 R = — H, R' = — Cl, R" = R"' = — H

4.157 R = — OH, R' = R" = R"' = — H

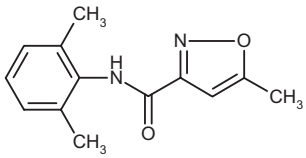
4.158 R = — OH, R' = — Cl, R" = — Cl, R"' = — NO_2

The unsubstituted *N*-phenylbenzamide showed the most rapid hydrolysis. The introduction of a 4-NO_2 group (**4.155**) in the anilino moiety reduced the rate of hydrolysis twofold. The effect of a 2-Cl substituent (**4.156**) was similar. The compound with a 2-OH group in the benzoyl moiety (**4.157**) was hydrolyzed at a rate tenfold lower than that of the unsubstituted compound.

Niclosamide (**4.158**), which carries the three substituents, was hydrolyzed by neither mammalian liver nor helminth tissue preparations. The resistance of niclosamide to enzymatic hydrolysis was explained by steric hindrance caused by the two substituents adjacent to the amide bond [102]. But, since monosubstitution also considerably reduced hydrolysis, one can postulate that H-bonding and electronic effects also contributed to the hydrolytic stability of niclosamide.

After oral administration of ameltolide (**4.143**, *Fig. 4.6*) to rats, no hydrolytic products were detected in biological fluids [103]. One could argue that the two *o*-Me groups afforded steric protection to the amide bond. However, steric hindrance may not be the main reason for the absence of metabolic cleavage in this compound, since, for lidocaine (**4.12**, *Fig. 4.5*), cleavage of the amide bond represents a major metabolic route in mammals. The apparent absence of hydrolysis in the metabolism of ameltolide may be caused by the predominance of major alternative pathways, namely *N*-acetylation and hydroxylation. Furthermore, the small fraction of unidentified polar metabolites may contain some products of hydrolysis.

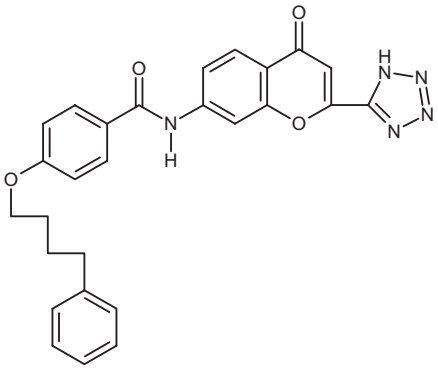
Our next example is the anticonvulsant agent *D2624* (**4.159**), which contains a 2,6-dimethylanilido moiety, and which showed marked species differences in metabolism [104]. The agent D2624 was metabolized by two major routes, namely hydroxylation of the Me group on the isoxazole ring and hydrolysis of the amide bond. In rats, half of the dose was hydrolyzed to 2,6-dimethylaniline either directly (*ca.* 50%) or indirectly (*ca.* 15%) *via* the hydroxylated metabolite. It is interesting to note that methyl hydroxylation at a site distant from the amide bond reduced the susceptibility to hydrolysis. In humans, however, most of the drug was hydroxylated and little was hydrolyzed [104]. Indeed, 2,6-dimethylaniline was detected in human plasma at very low concentrations and only at the highest doses.



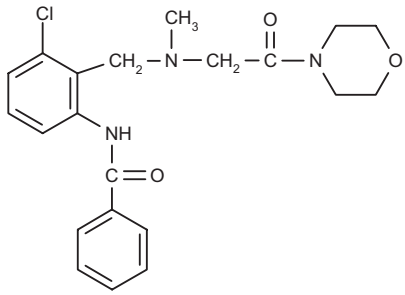
4.159

Marked species differences in hydrolytic cleavage were also observed for *pranlukast* (**4.160**), a leukotriene receptor antagonist. In rats, amide hydrolysis represented a major metabolic pathway, whereas, in humans, it was apparently absent. Investigations with purified enzymes showed that pranlukast

was hydrolyzed in rat liver by the carboxylesterase p*I* 6.2 (also called hydrolase B) but not by the p*I* 6.0 enzyme (hydrolase A) [105]. The corresponding human enzymes (carboxylesterases p*I* 5.3 and p*I* 4.5) were unable to catalyze the hydrolysis of pranlukast [106]. It has been proposed that pranlukast may serve as a model for elucidation of substrate specificities exhibited by rat and human carboxylesterases [105].



4.160



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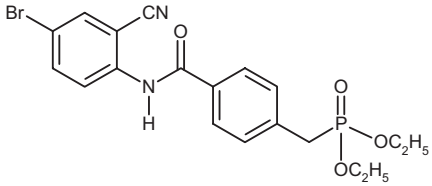
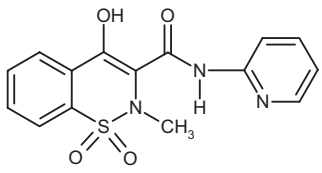


4.162

Hydrolysis of the amide bond linking its two aromatic rings represents a major metabolic pathway for the antitussive agent *fominoben* (**4.161**). In humans, *ca.* 30% of the dose was cleaved hydrolytically at the benzoylamido group [107]. Interestingly, no hydrolysis was observed for the amide bond linking the morpholino moiety to the rest of the molecule. The amide bond of diethyl 4-[(4-bromo-2-cyanophenyl)carbamoyl]benzylphosphonate (N0-1886, **4.162**), a potential antilipidemic agent, was also hydrolyzed in the rat. The resulting amine was further metabolized, whereas the acidic fragment was eliminated unchanged [108].

Piroxicam (**4.163**), a well-known anti-inflammatory agent, contains an amide bond between a benzothiazine and a pyridine ring. One of the prod-

ucts of amide hydrolysis, 3-carboxy-2-methyl-4-oxobenzothiazine 1,1-dioxide (**4.164**), was isolated from the urine of rhesus monkeys [109]. However, this metabolite was not found in the rat or the dog, perhaps because it was completely degraded by subsequent decarboxylation, ring contraction, and *N*-demethylation reactions to form *N*-methylsaccharin and saccharin [109]. In humans, the same multistep pathway seems to operate, but the metabolites produced by this route represented less than 5% of a dose. The biotransformation of the closely related isoxicam (**4.165**) also produced *N*-methyl-saccharin and saccharin but by a peroxidative route [110]. There is no evidence that other oxicams undergo amide hydrolysis [111][112].



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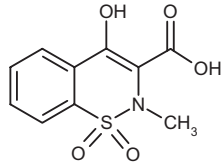

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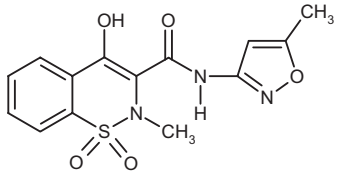
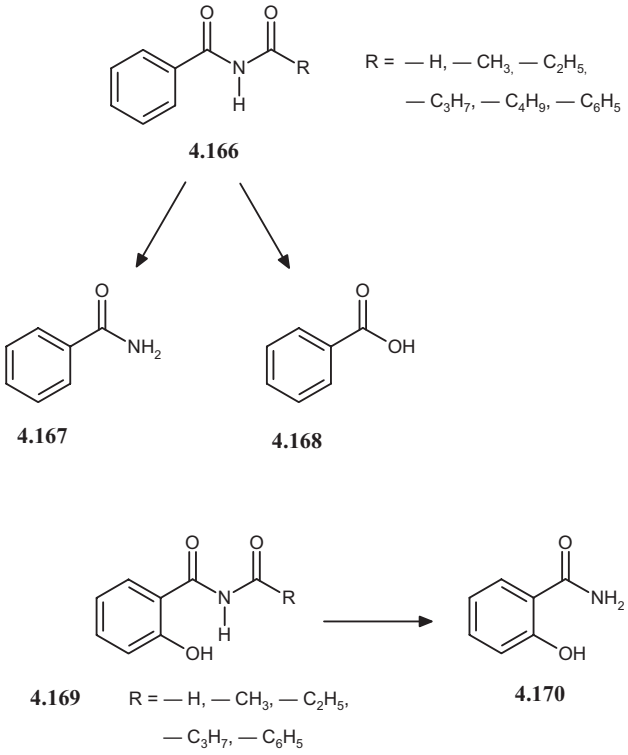


4.165

4.4. Imides

4.4.1. Linear Imides

Imides are N-*monoacylated amides*. Linear (or acyclic) imides are rarely found among drugs and agrochemicals because they are relatively unstable, being readily reduced, photolyzed, or hydrolyzed. The susceptibility of linear imides to undergo hydrolysis may be useful in the design of prodrugs of amides. For example, *Kahns* and *Bundgaard* [113] explored the potential usefulness of *N*-acylamides as prodrugs. For this, they examined the kinetics of hydrolysis and plasma-catalyzed degradation of a number of *N*-acylated benzamides and salicylamides of the general structures **4.166** and **4.169**.



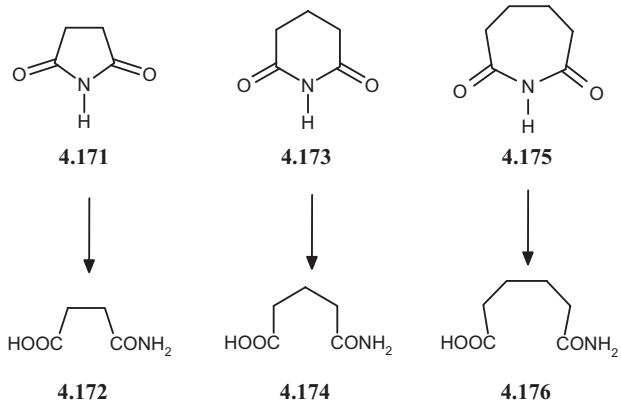
In neutral or alkaline buffer solution at 37°, the hydrolytic breakdown of *N*-formylbenzamide (**4.166**, R=H) produced only benzamide (**4.167**). In contrast, the higher homologues were hydrolyzed at the two amide bonds, with benzamide (**4.167**) and benzoic acid (**4.168**) formed in a 3:2 ratio. Plasma-catalyzed hydrolysis occurred predominantly at the distal amide bond to produce benzamide. Under these conditions, hydrolysis was very rapid for *N*-formylbenzamide (80% hydrolysis in 15 min, *i.e.*, *ca.* 500-fold faster than under abiotic conditions). The rate of enzymatic hydrolysis was also markedly influenced by the length of the *N*-acyl group, and decreased in the order H>Me>Bu>Pr>Et.

The salicylimides (**4.169**) were found to be markedly more resistant to chemical hydrolysis than **4.166**. These compounds were hydrolyzed exclusively at the distal amide bond, meaning that hydrolysis produced only salicylamide (**4.170**) and not salicylic acid. This behavior has been ascribed to steric hindrance by the 2-OH group. An intramolecular general base catalysis does not seem to be involved since, as stated, the salicylamides were less reactive than the corresponding benzamides. The rate of plasma-catalyzed hydrolysis of the *N*-acylsalicylamides was also dependent on the nature of

the *N*-acyl group, but differently than the *N*-acylbenzamides. Thus, the rate of enzymatic hydrolysis decreased in the order H>Et>Me>Pr>$PhCH_2$ [113]. Since the linear imides investigated were relatively stable in solution but readily hydrolyzed enzymatically, the authors concluded that they might be useful in prodrug design. They also pointed out a potential drawback, *i.e.*, that the site of cleavage depends on the nature of both the carboxamido and acyl moieties. This situation probably prevented further development of *N*-acylamides as prodrugs.

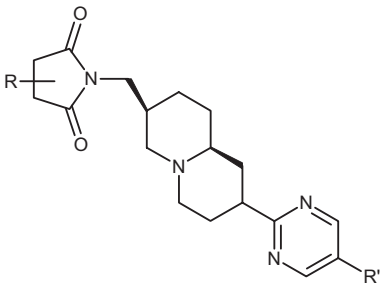
4.4.2. Cyclic Imides

In contrast to linear imides, various cyclic imides are drugs or agrochemicals. Cyclic imides undergo ring opening, not by amidases/esterases, but by *dihydropyrimidine aminohydrolase* (DHPase, EC 3.5.2.2), an enzyme of the pyrimidine degradation pathway (see *Sect. 4.5.2*). Using partially purified DHPase from rat liver, *Maguire* and *Dudley* [114] showed that the five-, six-, and seven-membered cyclic imides, namely succinimide (**4.171**), glutarimide (**4.173**), and adipimide (**4.175**) were cleaved hydrolytically to give the corresponding acid amides (**4.172**, **4.174**, and **4.176**, respectively), with adipimide being hydrolyzed much faster than its two lower homologues.



In the following paragraphs, we examine bioactive cyclic imides substituted on the ring and/or on the N-atoms, and begin with N-*substituted succinimides*. CP-93,393 (**4.177**, R=R′=H) is a selective 5-HT_1 serotonin autoreceptor agonist developed for the treatment of anxiety and depression. In the rat, hydrolysis of the succinimide bond was observed for the parent drug as well as for metabolites hydroxylated on the succinimide ring (**4.177**, R=OH,

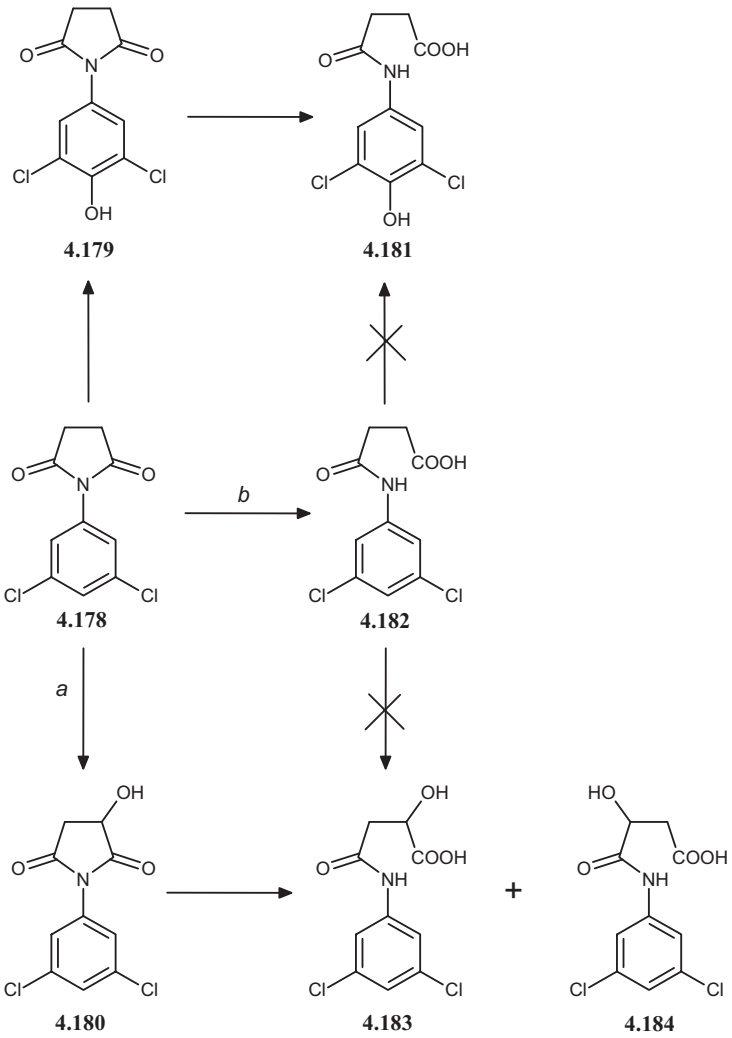
R′=H) or the pyrimidine ring (**4.177**, R=H, R′=OH) [115]. Hydrolytic cleavage of the succinimide ring also occurred in monkeys. Interestingly but unexplainably, greater amounts of the hydrolysis product of CP-93,393 were found in female than in male monkeys [116]. In humans, hydrolysis of the succinimide ring, in combination with 5-hydroxylation and/or conjugation, accounted for *ca.* 5% of the dose [117].



4.177

N-*(3,5-Dichlorophenyl)succinimide* (**4.178**), which was initially developed as an agricultural fungicide, was shown to be nephrotoxic in rats. Its metabolic pattern was investigated with the objective of determining how biotransformation may be related to toxicity [118]. The compound underwent hydroxylation at both the phenyl and succinimide rings to yield **4.179** and **4.180**, respectively. The succinimide ring in the parent compound and in the two hydroxylated metabolites could be cleaved by enzymatic or nonenzymatic hydrolysis. The cleavage product **4.181** of the phenolic metabolite **4.179** was found to accumulate in the kidney but produced no adverse renal effect when administered to rats. The cleavage of **4.180** produced **4.183** and **4.184**, which proved to be responsible for the nephrotoxicity observed in rats. The metabolites **4.181** and **4.183** could also conceivably be generated from **4.182**, but *in vitro* studies with rat liver and kidney homogenates discounted this possibility. At higher doses, the oxidative metabolism (*i.e.*, formation of **4.180** leading to **4.183** and **4.184**) became more important than hydrolysis (*i.e.*, the formation of **4.182**). This shift toward increased formation of the nephrotoxic metabolites **4.183** and **4.184** with increasing dose could explain the steep dose–toxicity relationship observed.

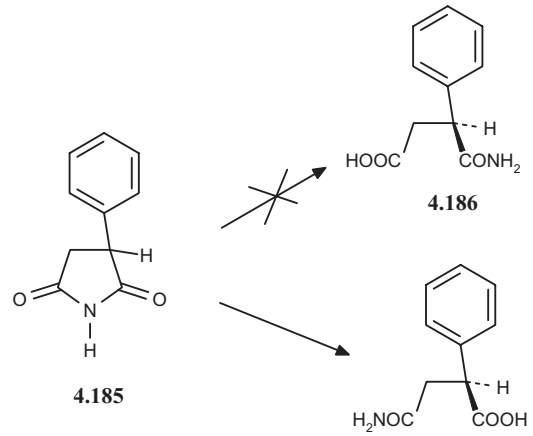
Our next examples concern *ring-substituted succinimides*. 2-Phenylsuccinimide (**4.185**), the *N*-demethylated metabolite of the antiepileptic drug phensuximide, was found to be a better substrate of DHPase than succinimide itself. Two ring-opened metabolites are conceivable, 3-phenylsuccinamic acid (**4.186**) and 2-phenylsuccinamic acid (**4.187**), but only the latter was formed enzymatically. 2-Phenylsuccinimide is chiral, and ring-opening

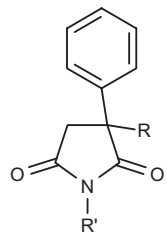


was found to be stereoselective, with only (–)-(*R*)-2-phenylsuccinamic acid (**4.187**) being formed from the racemate substrate [119].

The parent drug *phensuximide* (**4.188**) was not enzymatically cleaved, suggesting that *N*-alkylation abolishes enzymatic ring opening. Furthermore, no ring-opened metabolites of methsuximide (**4.189**) were detected. In this case, however, disubstitution of C(2) rather than *N*-alkylation prevented hydrolysis, since 2-methyl-2-phenylsuccinimide (**4.190**), its *N*-demethylated metabolite, was not a substrate of DHPase [119].

Turning to *tetrahydrophthalimide derivatives*, we find the pyrethroid insecticide tetramethrin (**4.191**). In the rat, a major metabolic pathway involves



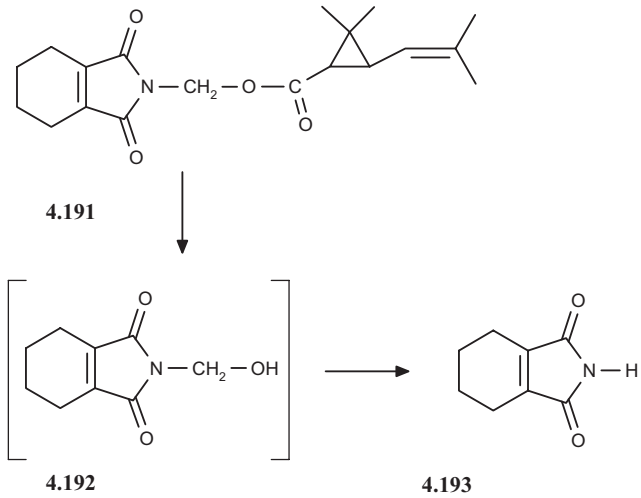


4.188 R = — H, R' = — CH_3

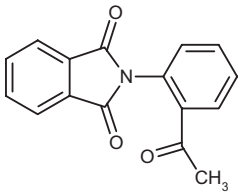
4.189 R = R' = — CH_3

4.190 R = — CH_3, R' = — H

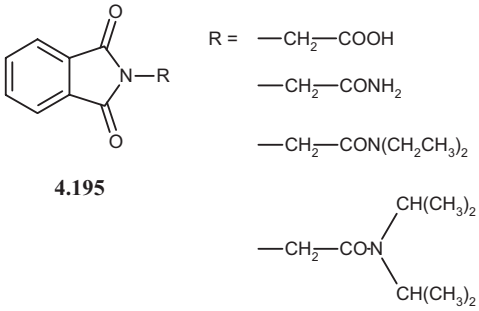
ester cleavage to produce **4.192**, an unstable carbinolamide that spontaneously loses formaldehyde to produce tetrahydrophthalimide (**4.193**). It is the latter metabolite that undergoes hydrolytic ring opening [120].



The phthalimido moiety can be found in a number of xenobiotics, but the relative importance of its hydrolysis varies widely. For example, hydrolysis was a major metabolic pathway in the rat for *N*-(2-acetylphenyl)phthalimide (**4.194**) [121].



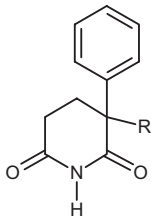
4.194



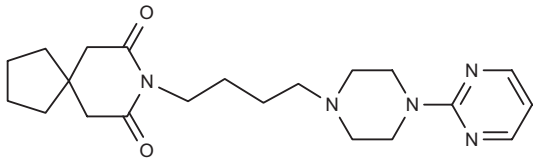
4.195

The potential of four *phthaloylglycine derivatives* (**4.195**) as antiepileptic agents was explored in rats and dogs [122]. These phthalimides were designed as potential carriers of glycine to increase the cerebral delivery of this neurotransmitter. However, phthaloylglycine (**4.195**, R=CH_2COOH) did not demonstrate any anticonvulsant activity, whereas phthaloylglycinamide (**4.195**, R=CH_2CONH_2) and its two *N*-alkylated derivatives were active. The three amides were retained for longer periods in the body due to their lower clearance and greater volume of distribution. No hydrolytic cleavage of the phthalimide ring was observed for any of the compounds. Consequently, they could not operate as chemical drug-delivery systems of glycine, but at least the three amides were directly active.

Considering now *glutarimide derivatives*, we find that 2-phenylglutarimide (**4.196**, R=H) was not a substrate for the partially purified rat liver DHPase, in contrast to glutarimide itself [114][123]. Thus, here, the introduction of the Ph group had the opposite effect than in succinimide derivatives. Similarly, no ring-opened metabolite of glutethimide (**4.196**, R=Et) was found either *in vitro* or *in vivo* [124].



4.196



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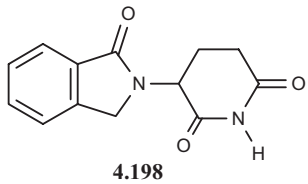
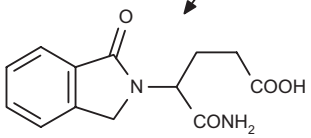
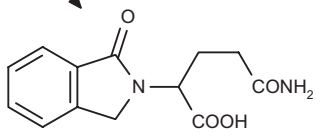


4.198



4.199



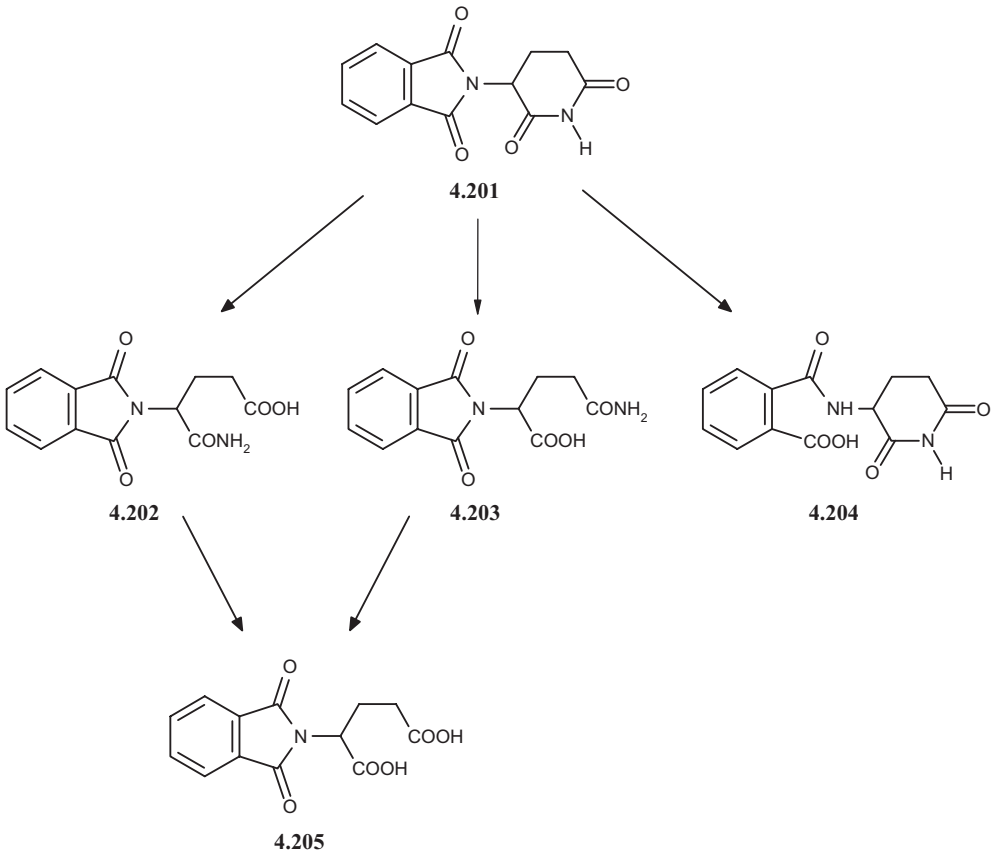
4.200

Although slow hydrolysis of the glutarimide ring occurs in *buspirone* (**4.197**), ring opening proceeds by intramolecular catalysis, as discussed in *Chapt. 11*.

One should not conclude from the above that the glutarimide ring is always resistant to hydrolysis. Indeed, its hydrolysis is a major metabolic pathway for the thalidomide analogue EM12 (**4.198**). After administration of EM 12 to marmoset monkeys, two products of hydrolysis, **4.199** and **4.200**, were found in urine. The concentrations of the two metabolites were similar after administration of the racemate. In contrast, regioselectivity (*i.e.*, different ratios of the two metabolites) was seen after separate administration of the enantiomers [125].

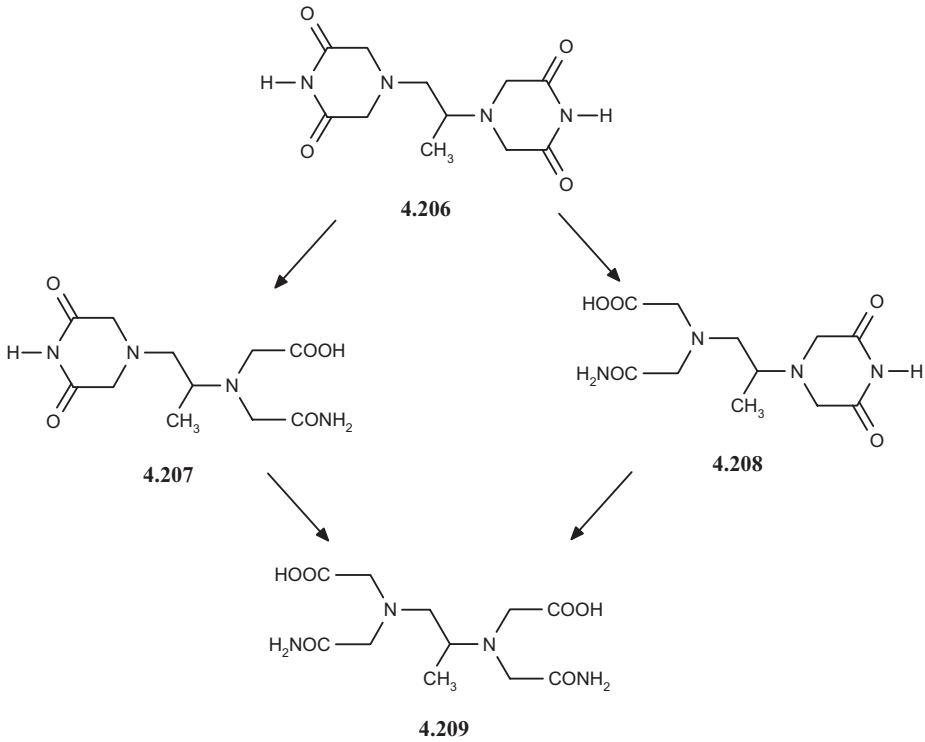
In *thalidomide* (**4.201**), both the glutarimido and phthalimido moieties undergo hydrolysis. This sedative agent proved highly teratogenic and caused the greatest drug-related tragedy in history. Recently, new clinical uses have been discovered that render thalidomide useful in the treatment of leprosy and even HIV infections [126–129]. All four amide bonds in thalidomide are susceptible to hydrolytic cleavage [130]. The main urinary metabolites in humans were shown to be 4-phthalimidoglutaramic acid (**4.202**, *ca.* 50% of a dose) and α-(2-carboxybenzamido)glutarimide (**4.204**, *ca.* 30% of a dose)

[131]. Metabolites **4.203** and **4.205** were minor. Metabolite **4.204** was the main product in rats and dogs. Toxicological investigations revealed that, of the twelve hydrolysis products of thalidomide, only the three containing the intact phthalimido moiety show teratogenic activity, namely metabolites **4.202**, **4.203**, and **4.205**) [132][133].



Our last example of a six-membered cyclic imide involves a 3,5-dioxopiperazine derivative. (+)-(*S*)-Dexrazoxane (**4.206**) has been shown to protect against doxorubicin-induced cardiotoxicity, and it acts probably by diffusing into cells and hydrolyzing to its ring-opened, metal-chelating metabolites **4.207**, **4.208**, and **4.209**. Indeed, these metabolites may chelate free iron or displace iron bound to the iron-doxorubicin complex, thus preventing iron-catalyzed oxygen radical formation [134]. Chemical hydrolysis of dexrazoxane under physiological conditions (pH 7.4, 37°) was slow, with $t_{1/2}$ values of *ca.* 8, 8, and 28 h for the formation of **4.207**, **4.208**, and **4.209**, respectively

[135]. Dexrazoxane was also hydrolyzed enzymatically in the liver and kidney by dihydropyrimidine aminohydrolase. This enzyme could hydrolyze one but not a second ring of this molecule. Levrazoxane, the enantiomer of dexrazoxane, was also hydrolyzed enzymatically by DHPase in liver homogenates, but at a rate 4.5-fold slower [136]. However, *in vivo* studies in rats dosed with razoxane (the racemic mixture of levrazoxane and dexrazoxane) revealed only a relatively small difference in elimination of the two enantiomers. This suggests that distribution and excretion reduced the impact of stereoselective biotransformation on the pharmacokinetics of these two enantiomers [137].



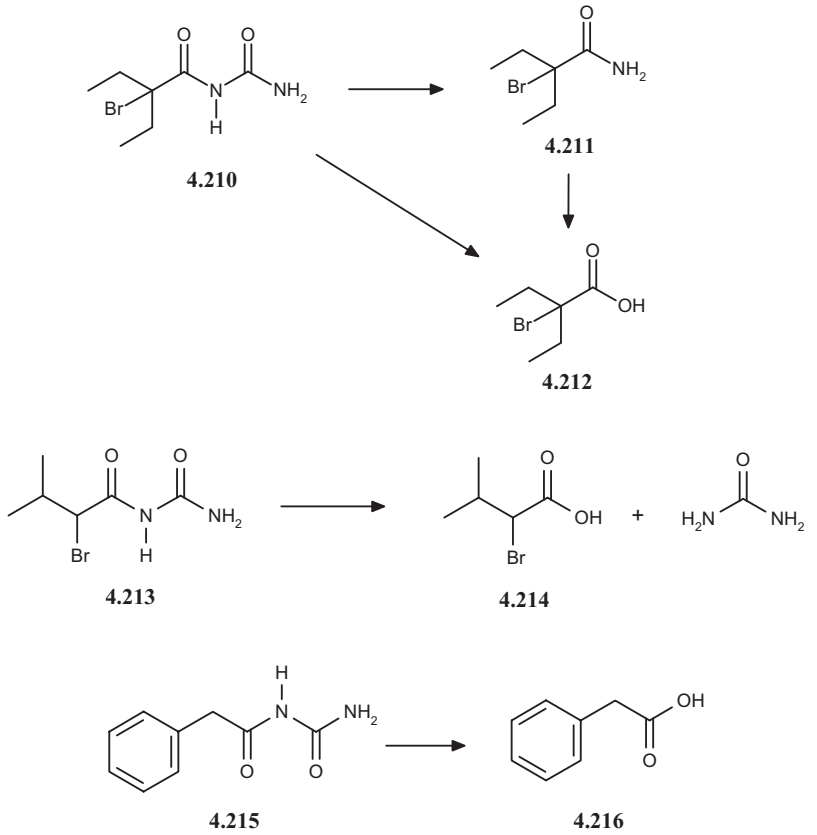
4.5. Ureides

4.5.1. Linear Ureides

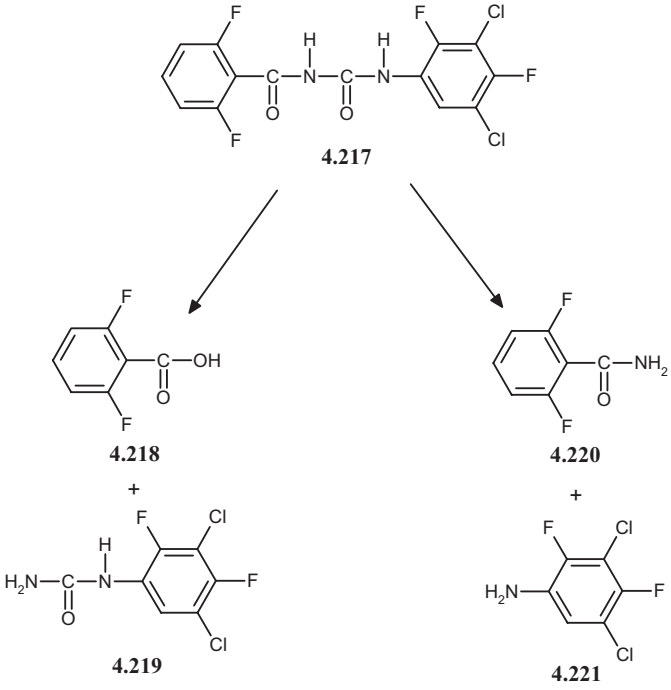
Acylated derivatives of urea are referred to as ureides. Acylation of urea with a monoacid produces acyclic ureides, whereas diacylation with malonic acid (a diacid) yields the cyclic structure of barbiturates. The acyclic ureides *carbromal* and *bromisoval*, now outdated hypnotics, can be considered ring-opened analogues of the barbiturates to be examined in the next subsection.

Hydrolytic cleavage plays an important role in the metabolic fate of both carbromal (**4.210**) and bromisoval (**4.213**). A product of hydrolysis of carbromal, 2-bromo-2-ethylbutyramide (**4.211**), was isolated from the urine of patients intoxicated with carbromal as well as from the urine of rats, mice, and dogs dosed with the drug [138]. A suspected further metabolite is 2-bromo-2-ethylbutyric acid (**4.212**). Other metabolic transformations of carbromal involve hydroxylation and debromination.

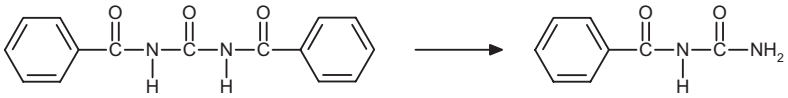
Hydrolysis and debromination are also the major routes of metabolism of bromisoval (**4.213**). Its amidase-catalyzed hydrolysis yielded bromoisovaleric acid (**4.214**) and urea. The displacement of the Br-atom is the result of glutathione conjugation. The C-atom of bromisoval bearing the Br-atom is a stereogenic center, and pronounced stereoselectivity in the metabolism of the enantiomers was observed. In isolated rat hepatocytes, (*R*)-bromisoval was both conjugated and hydrolyzed, but metabolite **4.214** was not [139]. Hydrolytic removal of urea was also observed in rodents for the anticonvulsant phenacetylurea (**4.215**), producing phenylacetic acid (**4.216**) [140].



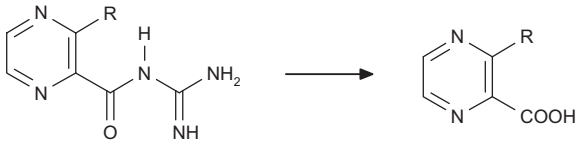
The benzoylphenylurea *teflubenzuron* (**4.217**) is an insecticide that acts by inhibiting chitin synthesis in larvae. Its urinary metabolites in rats were identified as benzoate derivatives (2,6-difluorobenzoic acid (**4.218**) and 2,6-difluorobenzamide (**4.220**)) and aniline derivatives (3,5-dichloro-2,4-difluorophenylurea (**4.219**) and 3,5-dichloro-2,4-difluoroaniline (**4.221**)) [141]. These metabolites are liberated from the parent molecule by hydrolysis of the urea bridge. The amount of urinary benzoate-type metabolites was about eight times that of the aniline-type metabolites, a discrepancy explained by the benzoate derivatives being excreted mainly as such, whereas the aniline derivatives were further metabolized.



The next example is *1,3-dibenzoylurea* (**4.222**), a diacetylated linear urea. The hydrolysis kinetics and enzymatic cleavage of 1,3-dibenzoylurea together with various other *N*-acylbenzamides were studied to assess the suitability of these compounds as prodrugs for the amido group [113]. At pH 7.4 and 37°, 1,3-dibenzoylurea was hydrolyzed quantitatively to benzoic acid and *N*-benzoylurea (**4.223**) with a $t_{1/2}$ value of 39 h. Since the hydrolysis of 1,3-dibenzoylurea was not catalyzed by human plasma, it was concluded that the acyclic diureide structure is not appropriate in prodrug design.



4.222 **4.223**



4.224 **4.225**

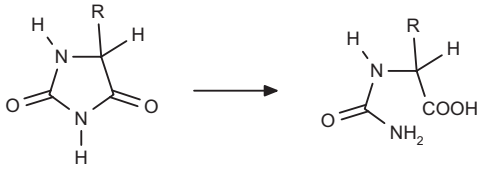
Acylated guanidines can be considered derivatives of ureides in which the carbonyl O-atom is replaced by an NH group. For this reason, we examine here *pyrazinoylguanidine* (**4.224**, R=H). Pyrazinoylguanidine inhibits urea reabsorption, and has antihypertensive and antilipidemic effects. Pyrazinoylguanidine was hydrolyzed in aqueous solution and faster in human serum. In contrast, (3-aminopyrazinoyl)guanidine (**4.224**, R=NH_2) was stable in both buffer and serum. In humans and dogs *in vivo*, pyrazinoylguanidine was rapidly metabolized to pyrazinoic acid (**4.225**, R=H), with a $t_{1/2}$ value in serum of *ca.* 1 h in both species. In dogs, introduction of the 3-NH_2 group doubled the half-life [142].

4.5.2. Cyclic Ureides

This subsection examines the hydrolytic stability of cyclic structures containing a ureido link. Schematically, ring closure can be achieved by *N*-alkylation or by *N*-acylation of the second N-atom of the ureido moiety. The former results in the formation of, *e.g.*, hydantoins and dihydropyrimidines. The latter ring closure leads to, *e.g.*, barbituric acids. Taken together, cyclic ureides can also be regarded as ring structures that contain an imido function with an adjacent N-atom. We begin our discussion with the five-membered hydantoins, to continue with six-membered structures, namely dihydropyrimidines, barbituric acids, and xanthines.

The heterocyclic ring of *hydantoins*, like that of succinimides (see *Sect. 4.4.2*), is hydrolytically cleaved by *dihydropyrimidine aminohydrolase* (DHPase, EC 3.5.2.2). Since both hydantoins and succinimides are hydrolyzed by the same enzyme, it is not surprising that structural features, such as absolute configuration, ring-substitution, and *N*-substitution, exhibit comparable influence on catalysis.

5-Monosubstituted compounds such as 5-methylhydantoin (**4.226**), 5-isopropylhydantoin (**4.227**), and 5-phenylhydantoin (**4.228**) are converted to their corresponding 2-hydantoic acids (**4.229**) when incubated with partially purified DHPase from rat liver [119]. The ring-opening reaction was stereoselective, with only the (*R*)-isomer of 5-alkylhydantoins being hydrolyzed. Interestingly, the incubation of (+)-(*S*)-5-phenylhydantoin also yielded (*R*)-2-phenylhydantoic acid. This observation can be explained by a base-catalyzed racemization of the parent drug [143].

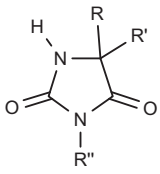


4.229

4.226 R = — CH_3

4.227 R = — $CH(CH_3)_2$

4.228 R = — C_6H_5



4.230 R = — H, R' = — C_6H_5, R'' = — CH_3

4.231 R = R' = — C_6H_5, R'' = — H

4.232 R = — C_2H_5, R' = — C_6H_5, R'' = — H

4.233 R = — C_2H_5, R' = — C_6H_5, R'' = — CH_3

N-Substitution and 5,5-disubstitution prevent ring opening as demonstrated by various examples. Ethotoin (3-ethyl-5-phenylhydantoin, **4.230**), in contrast to its *N*-deethylated metabolite, was not detectably hydrolyzed by DHPase. No ring-opened metabolite was found for phenytoin (5,5-diphenylhydantoin, **4.231**) or nirvanol (5-ethyl-5-phenylhydantoin, **4.232**), which is the *N*-demethylated metabolite of mephenytoin (**4.233**) [144].

It has been shown recently that papain exhibits hydantoinase activity. This enzyme of plant origin hydrolyzes not only 5-monosubstituted but also 5,5-disubstituted hydantoins to the corresponding *N*-carbamoylamino acids. Since chemical hydrolysis of the latter yields the corresponding amino acids, this approach may be of interest in amino acid synthesis [145].

Note that the hydantoin ring can also be cleaved after an initial oxidative step [146]. Indeed, the major metabolic route of 1-methylhydantoin (**4.234**,

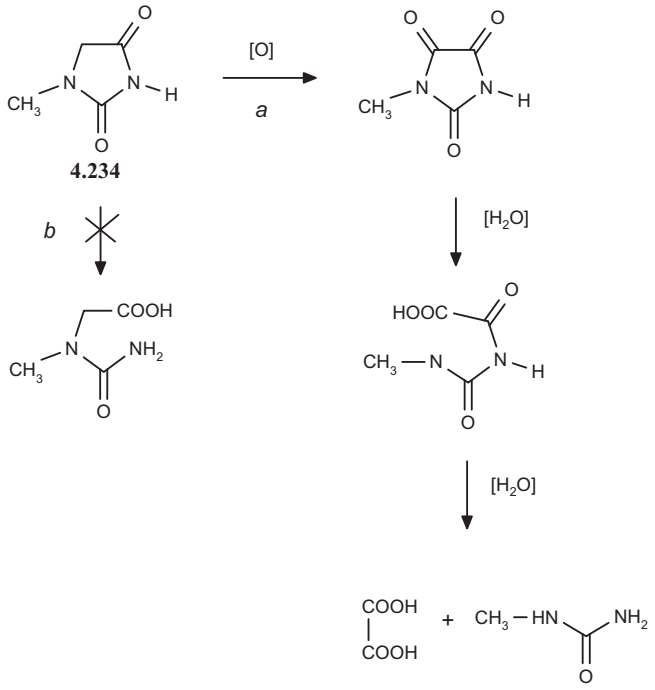


Fig. 4.9. *Mechanisms of ring opening of 1-methylhydantoin* (**4.234**). *Pathway a*: ring oxidation followed by two hydrolytic cleavage reactions [146]; *Pathway b*: has not been observed.

Fig. 4.9) in mammals involves ring oxidation followed by two hydrolytic cleavage reactions (*Fig. 4.9*, *Pathway a*). The hydrolytic product that should arise *via Pathway b* was not found.

Continuing with *six-membered rings*, we find that the mammalian degradation of uracil (**4.235**, R=H) and thymine (**4.235**, R=Me) involves several steps that are catalyzed by different enzymes (*Fig. 4.10*). In the first step, these *pyrimidine derivatives* (**4.235**) are reduced to 5,6-dihydropyrimidines by dihydrouracil dehydrogenase (EC 1.3.1.2). This is followed by a hydrolytic ring-opening catalyzed by dihydropyrimidine aminohydrolase (EC 3.5.2.2) to produce carbamoyl-β-alanine or carbamoyl-β-aminoisobutyrate. Carbamoyl-β-alanine amidohydrolase (EC 3.5.1.6) acts on carbamoyl-β-amino acids derived from uracil or thymine, removing the carbamoyl group as CO_2 and ammonia [147–149].

The antitumor agent *5-fluorouracil* (**4.236**) is rapidly metabolized to 2-fluoro-β-alanine (**4.237**) according to the sequence depicted in *Fig. 4.10* [150][151]. The degradation of 5-fluorouracil occurs in all tissues, but tumor tissues contain very small amounts of dihydropyrimidine aminohydrolase.

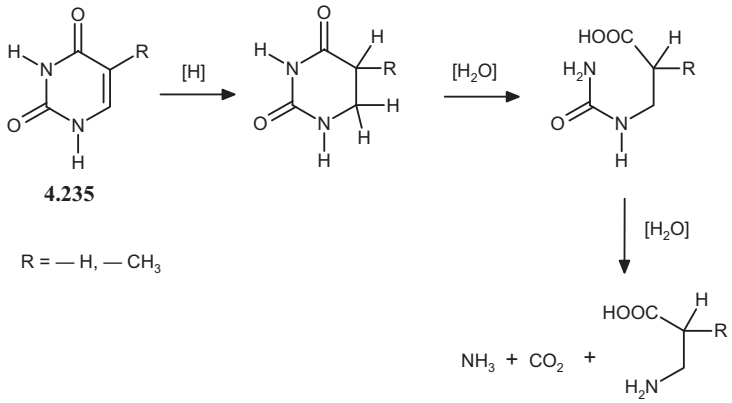
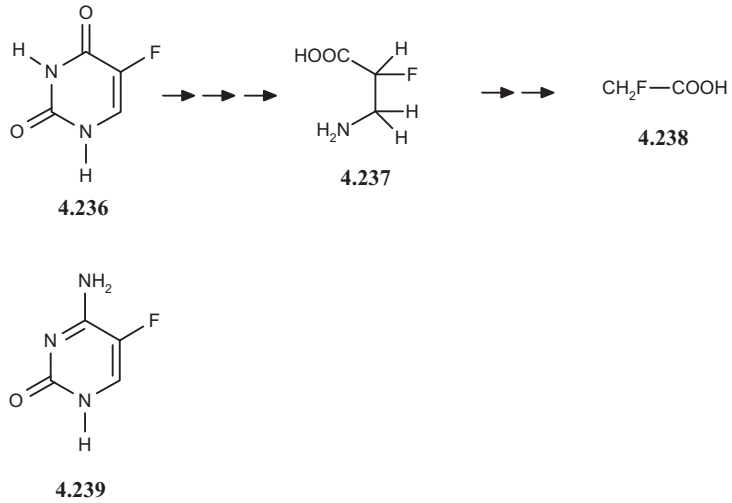


Fig. 4.10. *Mammalian degradation of pyrimidines* (**4.235**, uracil, R = H; thymine, R = Me). For details, see text.

The catalytic efficiency of this enzyme to hydrolyze 5-fluoro-5,6-dihydrouracil was found to be approximately twice that toward 5,6-dihydrouracil [152]. 2-Fluoro-β-alanine can either be eliminated *via* the bile after conjugation with bile acids, or be converted to fluoroacetate (**4.238**) [153]. The latter metabolite is transformed to fluorocitrate, a potent inhibitor of the aconitase-catalyzed conversion of citrate to isocitrate. This inhibition probably explains the clinical neurotoxicity of 5-fluorouracil [154][155].

Traces (0.1%) of 2-fluoro-β-alanine were also found in the urine of patients treated with the antifungal agent *flucytosine* (5-fluorocytosine, **4.239**) [156]. However, it appears that flucytosine, the amino analogue of 5-fluoro-



uracil, is not a substrate for dihydropyrimidine aminohydrolase. Flucytosine must be deaminated to 5-fluorouracil before the ring can be metabolically cleaved. It was believed that human cells lack this enzyme, and the very small amounts of 5-fluorouracil found in the serum of patients dosed orally with flucytosine were attributed to deamination by intestinal microflora. Recently, however, it has been shown that 5-fluorouracil is also present in patients receiving intravenous flucytosine, thus, the deamination pathway, although very minor, is also present in humans [156].

The *barbiturate ring*, obtained by condensation of malonic acid and urea, undergoes only very slow chemical hydrolysis under physiological conditions, but the rate of hydrolysis increases markedly with increasing pH and temperature. For butalbital (**4.240**, *Fig. 4.11*), two pathways of hydrolytic cleavage have been characterized, with one or both being operative depending on conditions. One route (*Fig. 4.11*, *Pathway a*) proceeds through a reversible 1,6-ring opening to the malonuric acid derivative **4.241**. The other route (*Fig. 4.11*, *Pathway b*) is *via* 1,2-cleavage to produce the diamide **4.242** after spontaneous decarboxylation of the intermediate carbamic acid. At about neutral pH, hydrolysis takes place solely *via Pathway a*; at higher pH values, *Pathway b* also plays a role [157].

Ring-opened products of *pentobarbital* (**4.243**), *amobarbital* (**4.244**), and *hexobarbital* (**4.245**) were isolated from the urine of dogs and rabbits

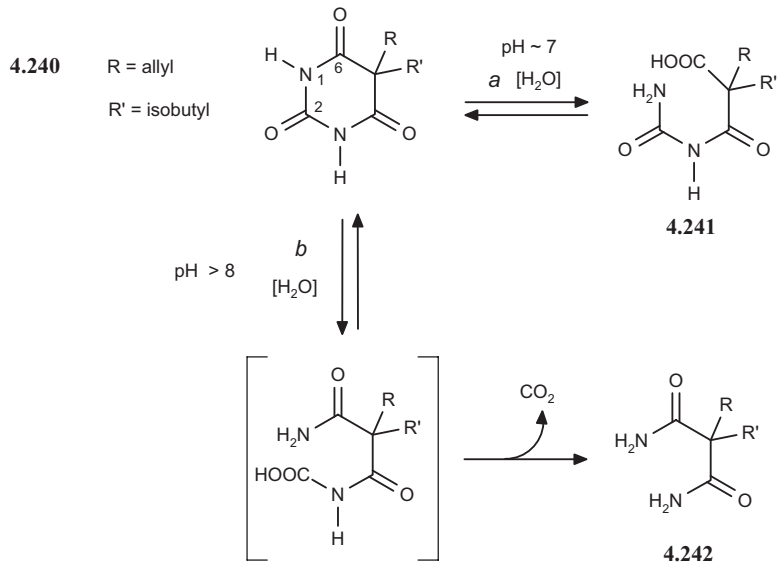
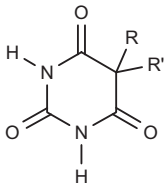


Fig. 4.11. *Chemical hydrolysis of butalbital* (**4.240**). For details, see text.

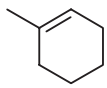
[158][159]. However, metabolic cleavage represents only a very minor pathway for barbiturates, and it has been questioned whether the ring-opened products are really metabolites or artifacts resulting from workup procedures. The most important metabolic pathway for barbiturates is oxidation of the 5-substituent.



4.243 R = — C_2H_5, R' = — $CH(CH_3)CH_2CH_2CH_3$

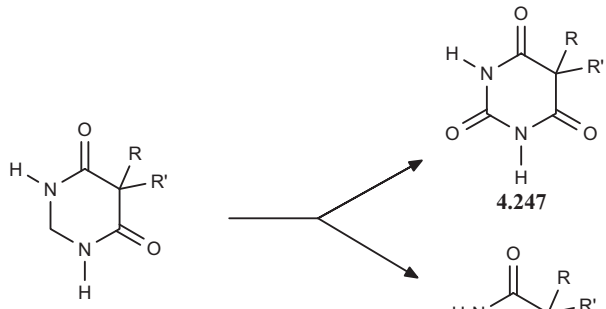
4.244 R = — C_2H_5, R' = — $CH_2CH_2CH(CH_3)_2$

4.245 R = — CH_3, R' =



The anticonvulsant agent *primidone* (**4.246**) is the 2-dihydro derivative of phenobarbital (**4.247**), which is one of its metabolites. The second major metabolite, 2-ethyl-2-phenylmalondiamide (**4.248**), is produced by a double C–N cleavage [160]. The profile of plasma levels in rats strongly suggests that 2-ethyl-2-phenylmalondiamide is not derived from the metabolite phenobarbital, but directly from primidone. Indeed, a C(2)-hydroxylated metabolite serves as an intermediate for both detected metabolites (see also Chapt. 6 in [21]). *N*-Alkyl derivatives of primidone yield a greater proportion of ring-opened metabolites, an observation explained by their higher susceptibility to oxidative metabolism at C(2) [161].

A particular mechanism of barbiturate ring opening has been observed for some barbiturates hydroxylated on the side-chain. The mechanism and relevance of this tautomeric lactam–lactone equilibrium are discussed in *Chapt. 11*.



4.246 R = — C_2H_5

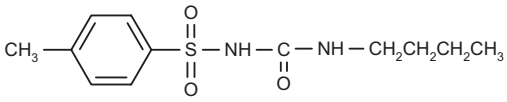
R' = — C_6H_5

4.247

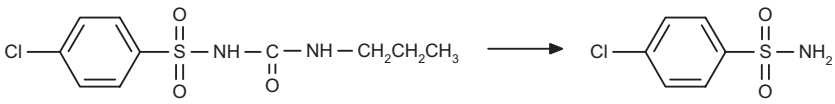
4.248

4.6. Sulfonylureas

The sulfonylurea linkage is present in many *hypoglycemic drugs*, such as tolbutamide (**4.249**). Although the metabolism of these drugs has been studied in detail, direct hydrolysis of the sulfonylurea linkage has not been observed. Only in the case of chlorpropamide (**4.250**) has some hydrolytic breakdown of the urea moiety been detected. The resulting 4-chlorobenzenesulfonamide (**4.251**), however, was not a genuine metabolite but an artifact formed from the intermediate (4-chlorophenyl)sulfonylurea by chemical hydrolysis at acidic pH during extraction [162].



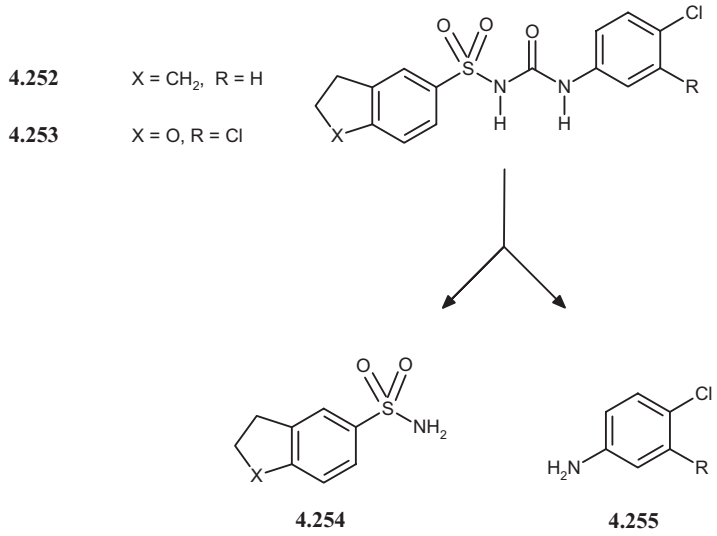
4.249



4.250 **4.251**

In contrast to the above examples, chemical and metabolic hydrolysis becomes significant for compounds substituted directly with aryl groups on both sides of the sulfonylurea linkage. Their chemical hydrolysis at neutral or slightly basic pH seems to be favored by the electronegativity of the aromatic groups, which renders the sulfonamido NH group weakly acidic. Such a *diarylsulfonylurea structure* can be found in anticancer agents and herbicides.

Thus, the diarylsulfonylurea *sulofenur* (**4.252**), a compound with antitumor activity, undergoes chemical hydrolysis at slightly acidic pH, resulting in the formation of indane-5-sulfonamide (**4.254**, X=CH_2) and 4-chloroaniline (**4.255**, R=H). 4-Chloroaniline is a minor metabolite in mice, rats, and humans, but it is of toxicological importance since it is responsible for the high levels of methemoglobin seen in animal studies and clinical trials of sulofenur [163][164]. Methemoglobin formation and associated anemia are responsible for the dose-limiting toxicity of sulofenur. In a series of sulofenur-related sulfonylureas, the size of the arylsulfonamido substituent was correlated with the extent of enzymatic hydrolysis, whereas there was no or only a very weak correlation with electronic and hydrophobic parameters [164].



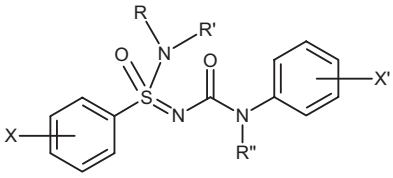
4.254 **4.255**

Recently, the disposition of the sulfonylurea oncolytic agent LY295501 (**4.253**), a more potent analogue of sulofenur, was investigated in the mouse, rat, and monkey [165]. As with sulofenur, metabolic cleavage of the sulfonylurea linkage occurred, and small amounts of 3,4-dichloroaniline (**4.255**, R = Cl), and its metabolites were found in urine. 3,4-Dichloroaniline, like 4-chloroaniline, is a potent inducer of methemoglobin formation. However, the hydrolytic pathway has only minor toxicological significance in this case, since, for poorly understood reasons, methemoglobinemia represents only a minor toxicity problem of LY295501.

A series of *sulfonimidamide analogues* (**4.256**) of oncolytic diarylsulfonylureas were evaluated for their cytotoxicity, antitumor activity, and metabolic fate [166]. The replacement of the sulfonamido O-atom with a N-atom notably reduced the acidity of this group. Therefore, these diarylsulfonimidamides, unlike sulfonylureas, do not undergo chemical hydrolysis *in vivo*. However, metabolic cleavage did occur in mice, which excreted 4-chloroaniline after being dosed with diarylsulfonimidamides [166].

The second class of compounds to be discussed are the *diarylsulfonylurea herbicides*. A good knowledge of their metabolism and toxicity is necessary, given that herbivores may consume plants contaminated with such herbicides. Thus, sulfometuron methyl (**4.257**) was metabolized in lactating goats by cleavage of the sulfonylurea bridge [167]. Significant amounts of the hydrolytic metabolites were found bound to proteins in the liver and kidneys.

The environmental impact of sulfonylurea herbicides is also of significance. Based on hydrolytic studies, *Dinelli et al.* [168] predicted the dissipation rate of four herbicides following aquifer contamination. The chemical



R = H, CH_3, acetyl

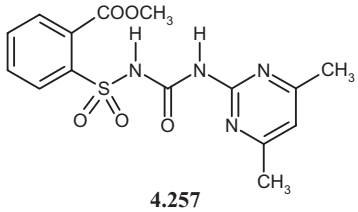
R' = H, alkyl, acyl, acetyl

R" = H, CH_3, acetyl

X = alkyl, halogen

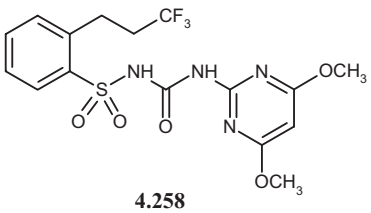
X' = alkyl, acyl, halogen

4.256



4.257

hydrolysis of these sulfonylureas involves the breakdown of the sulfonylurea bridge to give sulfonamide and the corresponding heterocyclic amine. The kinetics of their degradation in water was a function of chemical structure, temperature, and pH. Prosulfuron (**4.258**) and primasulfuron methyl (**4.259**) were sufficiently stable to enter the ground water. These compounds tend to persist due to their slow degradation at neutral and subalkaline pH. In contrast, thifensulfuron methyl (**4.260**) and rimsulfuron (**4.261**) would not create problems of aquifer pollution since they undergo rapid hydrolysis over a wide range of pH values [168].



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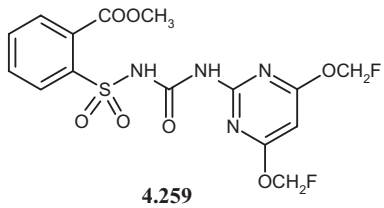

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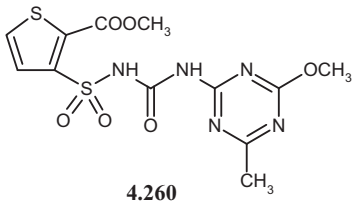

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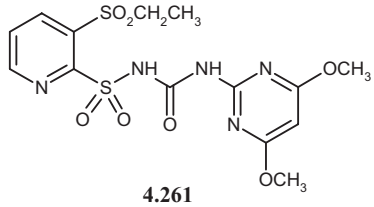


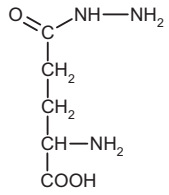
4.261

4.7. Hydrazides and Aminoacyltriazenes

Hydrazides are formed by the acylation of hydrazines, and have a C–N bond of rather low chemical stability toward hydrolysis. It is, therefore, not surprising that the cleavage of this bond represents a major metabolic pathway for most hydrazides. The reaction is catalyzed by amidases since it can be inhibited by *O*-ethyl *O*-(4-nitrophenyl) phenyl phosphothionate or bis(4-nitrophenyl) phosphate, which are classical inhibitors of this enzyme.

Both *aliphatic* and *aromatic derivatives* undergo *in vivo* hydrolysis. Thus, the presence of hydrazine ($H_2N–NH_2$) was demonstrated in the urine of rabbits following an intraperitoneal injection of glutamylhydrazine (**4.262**). After oral administration of benzoylhydrazide (**4.263**) to rats, a large proportion of the dose (50–60%) was hydrolyzed to free hydrazine and benzoic acid. The ring substitution of benzoylhydrazides may influence the rate of hydrolysis. Although 4-chlorobenzoyl- and 4-methylbenzoylhydrazide are extensively hydrolyzed like benzoylhydrazide, very little hydrolysis was observed for 4-hydroxybenzoylhydrazide, which was mainly glucuronidated at the 4-OH group [169]. This example shows that the importance of the hydrolytic pathway can be greatly influenced by competitive metabolic routes.

N-Alkylhydrazides also undergo metabolic hydrolysis. Thus, the major metabolites of the monoamine oxidase inhibitor *isocarboxazid* (**4.264**)



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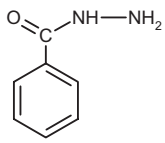

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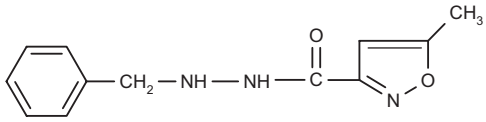

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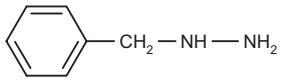

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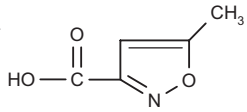
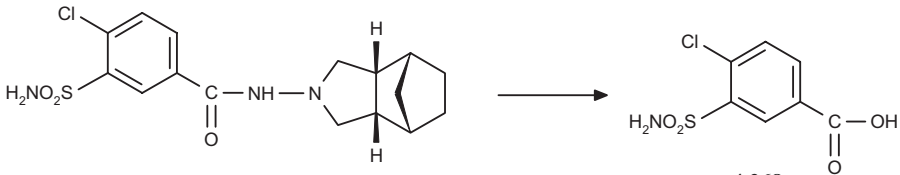


4.266

formed by rat liver and kidney homogenates were benzylhydrazine (**4.265**) and 5-methylisoxazole-3-carboxylic acid (**4.266**) [170].

The antihypertensive agent *tripamide* (**4.267**), when incubated with rat liver microsomes or partially purified microsomal arylamidase, was extensively hydrolyzed to 4-chloro-3-sulfamoylbenzoic acid (**4.268**) [171]. This metabolite seems to be produced by direct hydrolysis, since the other metabolites formed by oxidation of the cycloalkyl moiety remained unchanged when incubated with rat liver microsomes. The mechanism of hydrolysis of tripamide has not yet been fully elucidated. The inhibition of the reaction by *O*-ethyl *O*-(4-nitrophenyl) phenyl phosphothionate indicates that amidases may be involved.

The metabolic fate of isoniazid and iproniazid, two isonicotinoylhydrazides, has been extensively studied, and it has been shown that metabolic hydrolysis represents an important step in their toxification. *Isoniazid* (**4.269**) is employed as a first-line tuberculostatic drug, but prolonged therapy is associated in 1–2% of patients with significant hepatotoxicity. Isoniazid can be metabolized by either of two primary pathways, hydrolysis and direct *N*-acetylation. Isonicotinic acid (**4.271**), the product of hydrolysis, can be formed either di-



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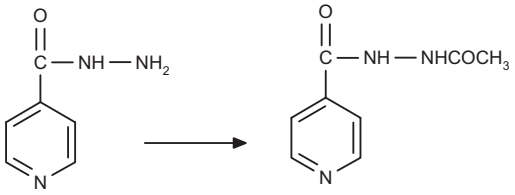
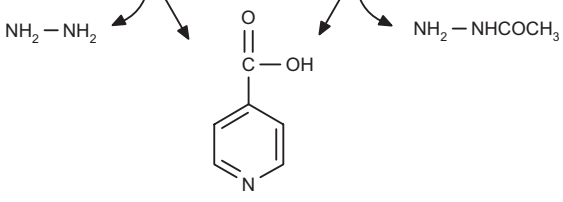


4.269 **4.270**



4.271

rectly or from the metabolite *N*-acetylisoniazid (**4.270**). In humans, most of the isonicotinic acid formed results from the hydrolysis of acetylisoniazid [172].

The mechanism of toxification of isoniazid was investigated in rats pretreated with inducers or inhibitors of microsomal enzymes or an inhibitor of acylamidases. In animals pretreated with the acylamidase inhibitor bis(4-nitrophenyl) phosphate, isoniazid and acetylisoniazid produced less liver necrosis than in control animals. The treatment had no effect on the necrosis due to acetylhydrazine [173]. In animals pretreated with inducers of microsomal cytochrome P450 such as phenobarbital, acetylisoniazid, and acetylhydrazine caused markedly increased necrosis, while pretreatment with cytochrome P450 inhibitors decreased necrosis. In contrast, the toxicity of isoniazid and hydrazine was not modified by phenobarbital pretreatment. From these observations, *Trimbell et al.* [173] concluded that the hydrolysis of acetylisoniazid is a prerequisite for hepatotoxicity, and that microsomal enzymes transform acetylhydrazine, the product of hydrolysis, to a toxic species.

Further studies with radiolabeled metabolites of isoniazid seem to have confirmed this hypothesis. It was observed that the acetyl fragment of acetylisoniazid or acetylhydrazine was significantly bound to liver proteins, whereas little binding was detected for the isonicotinoyl fragment of acetylisoniazid. Furthermore, the changes in the degree of covalent binding paralleled the changes in the extent of necrosis induced by the various pretreatments. The metabolic activation of acetylhydrazine seems to involve initial *N*-hydroxylation to ultimately yield radicals that bind covalently to cellular macromolecules [172][174][175].

According to another hypothesis, the hydrolysis of acetylisoniazid may not be the main pathway leading to hepatotoxicity. Indeed, it has been suggested that hydrazine is the metabolite predominantly responsible for isoniazid-induced hepatotoxicity [176], implicating a minor metabolic pathway, *i.e.*, direct hydrolysis to yield hydrazine and isonicotinic acid [177][178]. *Blair et al.* [179] have shown that plasma levels of free hydrazine were higher in slow than in rapid acetylators receiving isoniazid over 14 d. If hydrazine were the toxic agent, one would expect slow acetylators to be at higher risk of developing hepatic injury.

Interesting information stems from studies of the hepatotoxic effect of the concomitant administration of rifampicin, another antituberculostatic drug (and a potent inducer of cytochrome P450) often used in combination with isoniazid. Rifampicin alone is not hepatotoxic but increases significantly the incidence of hepatitis in patients simultaneously dosed with isoniazid. In human volunteers (6 slow and 8 rapid acetylators), daily administration of rifampicin increased the release of hydrazine from isoniazid [180]. In slow acetylators, the proportion of the dose metabolized to hydrazine increased

from 2.9% when isoniazid was administered alone to 6.2% when rifampicin was co-administered. In rapid acetylators, the proportions were 0.3 and 2.5%, respectively. Thus, the increased formation of hydrazine could explain the substantially higher occurrence of hepatitis in slow compared to rapid acetylators among tuberculosis patients treated daily with rifampicin and isoniazid.

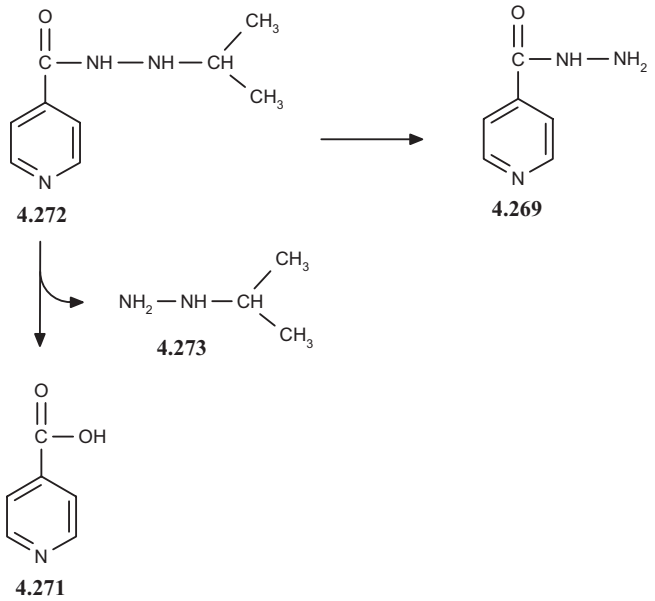
Recently, the role of hydrazine in the mechanism of isoniazid hepatotoxicity was confirmed by *Sarich et al.* [181]. Using a model of isoniazid-induced hepatotoxicity in rabbits, they found that hydrazine plasma concentrations correlated significantly with plasma argininosuccinic acid lyase, a sensitive marker of hepatic necrosis. In contrast, no correlation was found between plasma levels of isoniazid or acetylisoniazid and the markers of induced hepatic necrosis.

It is interesting to note that pretreatment with rifampicin, followed by a single dose of isoniazid, or chronic administration of a combination of rifampicin and isoniazid reaching steady-state, have opposite effects on the plasma levels of hydrazine. Thus, hydrazine plasma levels in rifampicin-pretreated rabbits were significantly lower than those in the control animals [182]. No remarkable change was detected in the plasma levels of isoniazid, acetylisoniazid, and acetylhydrazine. Similar observations were made in the rat [182]. The faster elimination of hydrazine was shown to be due to the induction of liver cytochrome P450 by rifampicin. One can speculate that the increased hydrazine levels during chronic administration of an isoniazid-rifampicin association result from an increased formation (by hydrolysis of isoniazid) of hydrazine, thus exceeding the capacity of oxidative elimination, even increased by induction.

A recent study shows that hydrolysis is not the only metabolic mechanism leading to the cleavage of the hydrazido bond in isoniazid. Indeed, the formation of isonicotinic acid from isoniazid was significantly increased in hepatocytes pretreated with classical cytochrome P450 inducers such as phenobarbital or 3-methylcholanthrene [183]. The contribution of oxidation to the formation of isonicotinic acid was found to be relatively small compared to the major route of hydrolysis by hydrolases. Other mammalian enzymatic systems may also cleave oxidatively isoniazid, namely prostaglandin synthase and myeloperoxydase in neutrophiles and monocytes [184]. Finally, the cleavage of isoniazid by a peroxidase of *Mycobacterium tuberculosis* producing a carbonyl radical seems to contribute to the pharmacological activity of the drug [185].

Iproniazid (**4.272**) was originally synthesized as a tuberculostatic drug but was found to be an antidepressant due to its inhibitory effect on monoamine oxidase. However, this compound had to be withdrawn from clinical use due to a high incidence of hepatotoxicity. The metabolism and the mechanism of toxification of iproniazid were found to be comparable to those of

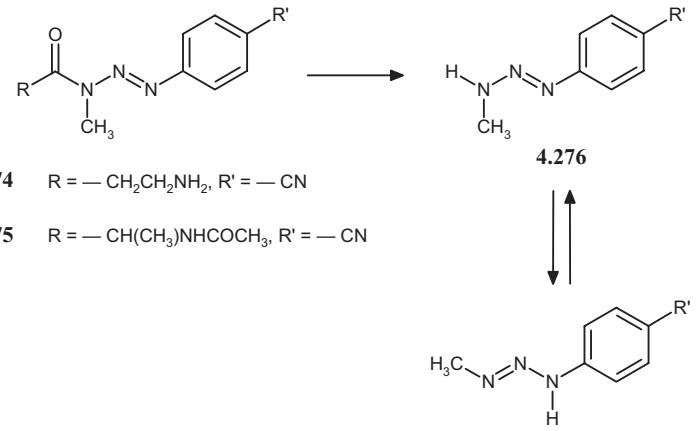
isoniazid. Thus, iproniazid hepatotoxicity was increased by inducers of cytochrome P450 and decreased by its inhibitors. Administration of the acylamidase inhibitor bis(4-nitrophenyl) phosphate prevented the hepatotoxicity caused by iproniazid but had no effect on the necrosis caused by isopropylhydrazine (**4.273**). It was, therefore, concluded that hydrolysis of iproniazid is a prerequisite metabolic step for hepatotoxicity [186]. Experiments with iproniazid labeled at the i-Pr group have indicated that this fragment binds covalently to proteins. The extent of covalent binding to macromolecules was correlated with hepatic necrosis [187].



The hepatic injury caused by iproniazid could also be due to the formation of the toxic metabolite hydrazine by *N*-dealkylation followed by hydrolysis. Indeed, *N*-dealkylation is a main route in the metabolism of iproniazid, with plasma levels of hydrazine in rabbits three- to sixfold higher than after isoniazid [188].

We have included in this Section the presentation of *aminoacyltriazenes* since they can be formally regarded as imino derivatives of hydrazide. Aminoacyltriazenes have been developed as chemically stable *triazene prodrugs* capable of enzymatic hydrolysis under physiological conditions to liberate cytotoxic monomethyltriazene antitumor agents **4.276** [189]. The aminoacyltriazene prodrugs were found to undergo hydrolysis in isotonic phosphate buffer and in human plasma. A β-alanyl derivative (**4.274**) was more stable

in phosphate buffer ($t_{1/2}$ 180 min) than in plasma ($t_{1/2}$ 53 min). An *N*-acetylated α-alanyl derivative (**4.275**) was chemically stable in phosphate buffer ($t_{1/2}$ 10 h) but liberated the cytotoxic drug in plasma with a $t_{1/2}$ value of 40 min. It seems that acylation of the α-amino group is an effective and simple way to reduce the chemical reactivity of α-aminoacyl derivatives while retaining a rapid rate of enzymatic hydrolysis.



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Chapter 5

The Hydrolysis of Lactams

Contents

5.1. Introduction

Lactams are cyclic amides that have several features in common with their ester counterparts (lactones). As with lactones, the ring opening of lactams should be regarded as *hydration* rather than hydrolysis, since lactams,

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unlike amides and esters, yield only one product or metabolite. There are other similarities between lactams and lactones. Both lactam and lactone bonds become chemically more stable with increasing ring size, and five- and six-membered rings can, in both cases, be formed spontaneously from the appropriate precursor. The difference between these ring systems lies in their reactivity: lactams are generally less reactive than lactones, in the same sense that amides are more stable than esters. The differences in the mechanisms of hydrolytic cleavage of the amide and lactam bond will be discussed in this chapter.

The greatest part of this text is devoted to the smallest lactam ring of significance in medicinal chemistry, namely the *β-lactam ring*. This four-membered ring is a well-known structural element of β-lactam antibiotics (*e.g.*, penicillins and cephalosporins). The reactivity of the β-lactam ring largely determines the chemical and pharmacological behavior of these molecules. In the following sections, we will discuss the important role that ring opening plays in the antibacterial activity of β-lactams, in their resistance to β-lactamases, in the activity of β-lactamase inhibitors, and, finally, in the resistance of β-lactams to chemical hydrolysis.

Most of this chapter (*Sect. 5.2*) focuses on the chemical reactivity of the lactam bond and its hydrolysis by bacterial enzymes (lactamases), rather than to its metabolic degradation by mammalian enzymes. This is in contradistinction to other chapters of this book, where metabolism in mammals is the focus of discussion. The reason for the attention given here to the chemical reactivity and bacterial degradation of β-lactams is that these issues have caused more pharmaceutical and clinical problems than metabolic hydrolysis. This also explains why the chemical stability of β-lactams and their resistance to β-lactamases have been the subject of countless studies, while the metabolism of these compounds has received less attention.

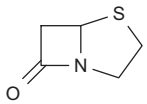
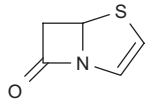
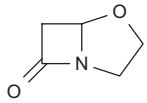
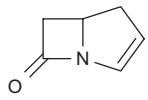
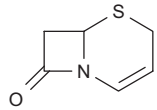
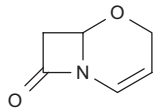
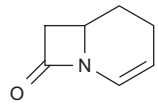
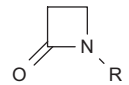
Three short sections complete this chapter. In *Sect. 5.3*, we examine the metabolism of nonantibiotic lactams of medicinal or toxicological interest. *Sect. 5.4* is concerned with complex lactams defined as containing one or more additional heteroatoms in the ring. Finally, we will discuss in *Sect. 5.5* the fate of lactams generated as metabolites by the oxidation of heterocycles containing a N-atom.

5.2. β-Lactam Antibiotics

5.2.1. Structural Diversity

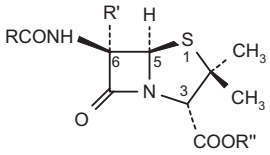
Since the discovery of penicillin, research has produced a large number of different types of derivatives with the aim to extend antibacterial activity and to improve pharmacokinetic properties [1][2].

Table 5.1. *Some Ring Systems in β-Lactam Antibiotics*

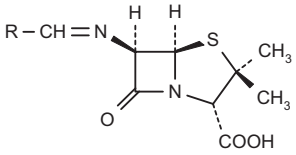
Structure	Class	Examples
	Penam	Penicillins
	Penem	SCH 29482
	Oxapenam	Clavulanic acid
	Carbapenem	Thienamycin
	Cephem	Cephalosporins
	Oxacephem	Oxacephalosporins
	Carbacephem	Carbacephalosporins
	Monocyclic β-lactams	Monobactams

The β-lactam family can be divided into several groups according to the basic skeleton (*Table 5.1*), which always contains a β-lactam ring. Except for monocyclic lactams, the β-lactam ring is fused through the N-atom and the adjacent tetrahedral C-atom to a second ring, be it five-membered or six-membered. The penams, oxapenams, cephems, and monobactams occur naturally, whereas oxacephams, carbacephams, and penems are synthetic analogues.

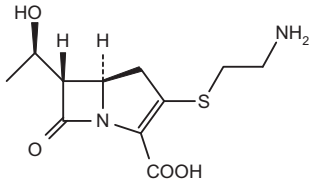
A large variety of *substituents* have been attached to these different skeletons, making the β-lactam antibiotics one of the most varied chemical classes in medicinal chemistry. In extended structure–activity studies, each position



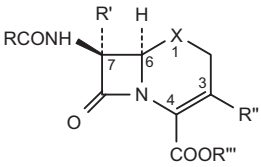
5.1



5.2



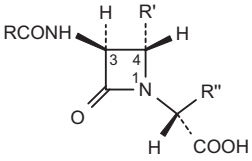
5.3



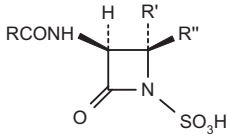
5.4 X = S

5.5 X = O

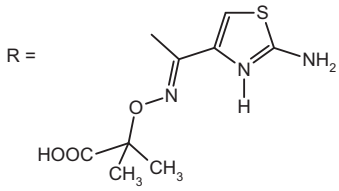
5.6 X = CH_2



5.7



5.8



R' = —CH_3

R" = —H

in penicillins and cephalosporins, with the exception of the bridgehead N-atom, has undergone alteration [3–7]. Structures **5.1**–**5.7** show the positions where modifications have resulted in clinically useful compounds. For penicillins (**5.1**), cephalosporin derivatives (**5.4**–**5.6**), and monobactams (**5.7** and **5.8**), the presence of an acylated amino group on the C-atom opposite the lactam N-atom has been found to be necessary for antimicrobial activity. Structural variations of the acylamino side chain have generated a large number of compounds with useful biological properties. However, not all β-lactam antibiotics carry an acylamino side chain. In mecillinam (**5.2**, R = hexahydroazepin-1-yl), the acylamino side chain is replaced with an amidino group. Thienamycin (**5.3**) has a 1-hydroxyethyl side chain on the carbapenem skeleton. Another common feature shared by penicillins, cephalosporins, and the monocyclic nocardins (**5.7**) is the presence of a carboxy group on the C-atom attached to the lactam N-atom. Esterification of this carboxy group with appropriated substituents has yielded prodrugs with improved bioavailability. In contrast to other β-lactam antibiotics, aztreonam (**5.8**), a monobactam, has a sulfonic acid directly attached to the β-lactam N-atom.

Stereochemical features are of great importance for antibacterial activity. The stereogenic centers at C(5) and C(6) in penicillins correspond to C(6)

and C(7) in cephalosporins. Penicillins have a third stereogenic center at the C-atom that carries the carboxy group (C(3)). The absolute configuration of natural penicillins is (3*S*,5*R*,6*R*) and that of the cephalosporins (6*R*,7*R*). The configurational orientation of the ring substituents can also be expressed with α and β notation, according to which the acylamino side chain of penicillins, cephalosporins, and monobactams is situated at the β-position, whereas the 1-hydroxyethyl side chain of thienamycin (**5.3**) is attached to the α-position. The absolute configuration of thienamycins is (5*R*,6*S*,8*S*). The presence of a 6α-MeO group in penicillins (R′ in **5.1**), or a 7α-MeO group in cephalosporins (R′ in **5.4**), increases resistance to chemical and enzymatic hydrolysis (see below). A second major locus of variation in cephalosporins is the side chain at C(3). Chemical modifications at this position, which profoundly affect chemical and biological properties, have allowed the discovery of a great number of clinically useful cephalosporins.

The large number and diversity of available β-lactams, mainly penicillins and cephalosporins, necessitate their classification. Penicillins can be classified primarily according to chemical structure. *Table 5.2* shows that there is good correspondence between chemical structure and properties. The categorization of cephalosporins into chemically similar groups is not useful because their antimicrobial spectrum is not closely correlated with chemical structure, and *classification into generations* is based on their spectrum of microbial activity (*Table 5.3*).

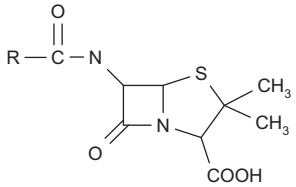
5.2.2. Reactivity of the β-Lactam Ring

The opening of the β-lactam ring by nucleophilic attack is an important determinant of the fate of β-lactam antibiotics. Indeed, their biological activity, resistance to chemical and enzymatic hydrolysis, and the occurrence of the most important side effects (*i.e.*, allergic reactions) all involve the cleavage of the lactam bond (*Fig. 5.1*).

5.2.2.1. *Antibacterial Activity*

The first chemical step in the *mechanism of action* of β-lactams is the opening of the β-lactam ring by a serine OH group of a target transpeptidase enzyme (D-alanyl-D-alanine peptidases) to form an acyl–enzyme intermediate (*Fig. 5.1*, *Pathway a*) [8]. The acylation of this membrane-bound enzyme is irreversible and interferes with the final stage of bacterial cell-wall synthesis, namely, the cross-linking reaction in peptidoglycan synthesis during which the pentaglycine bridge becomes attached to the D-alanine of a neigh-

Table 5.2. *Classification of Penicillins*



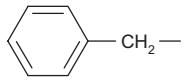
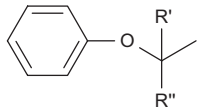
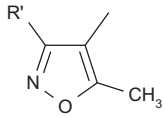
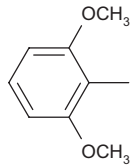
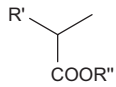
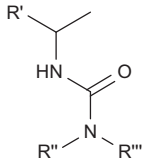
Class	R=		Stability to β-lactamases and acid hydrolysis
Benzylpenicillin and its salts	Benzyl		• unstable in acidic media • not resistant to β-lactamases
Oral penicillins	Phenoxy		• stable toward acidity • low resistance to β-lactamases
β-Lactamase-resistant penicillins	Isoxazolyl		• stable toward acidity
	2,6-Methoxy-phenyl		• stable toward acidity
Broad-spectrum penicillins	Amino		• stable toward acidity • not resistant to β-lactamases
	Carboxy		• unstable in acidic media • not resistant to β-lactamases
	Acylureido		• unstable in acidic media • not resistant to β-lactamases

Table 5.3. *Schematic Classification of Cephalosporins*

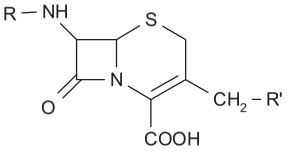
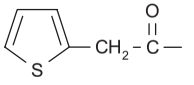
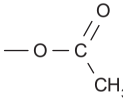
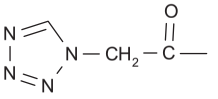
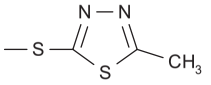
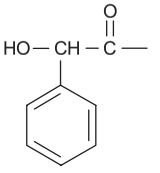
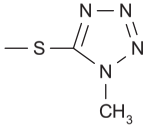
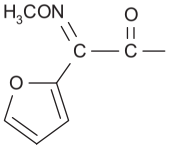
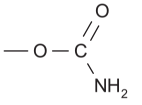
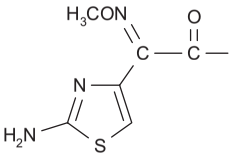
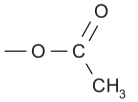
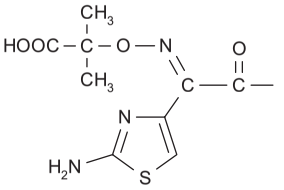
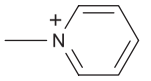
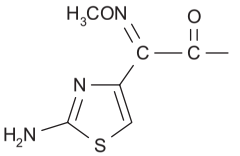
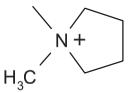
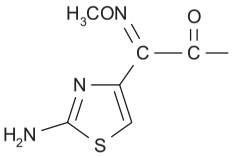
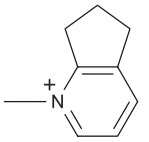
Generation	Biological activity	Examples	 R	R′
First	• Activity against *Gram*-positive bacteria similar to that of penicillins	Cefalothin		
	• Relatively narrow spectrum against *Gram*-negative species due to their lability to β-lactamases	Cefazolin		
Second	• Improved activity against *Gram*-negative bacteria due to their better lactamase stability	Cefamandole		
	• Diminished potency against *Gram*-positive bacteria compared with first-generation agents	Cefuroxime		

Table 5.3 (cont.)

Third	• Greatly improved *Gram*-negative antibacterial spectrum, excellent lactamase stability	Cefotaxime		
	• Diminished activity against *Gram*-positive organism compared with first-generation agents	Ceftazidime		
Fourth	• Activity against *Gram*-negative bacteria comparable to third-generation agents, potency against *Gram*-positive bacteria similar to first-generation agents	Cefepime		
	• Improved stability against lactamases to avoid many resistance problems	Cefpirome		

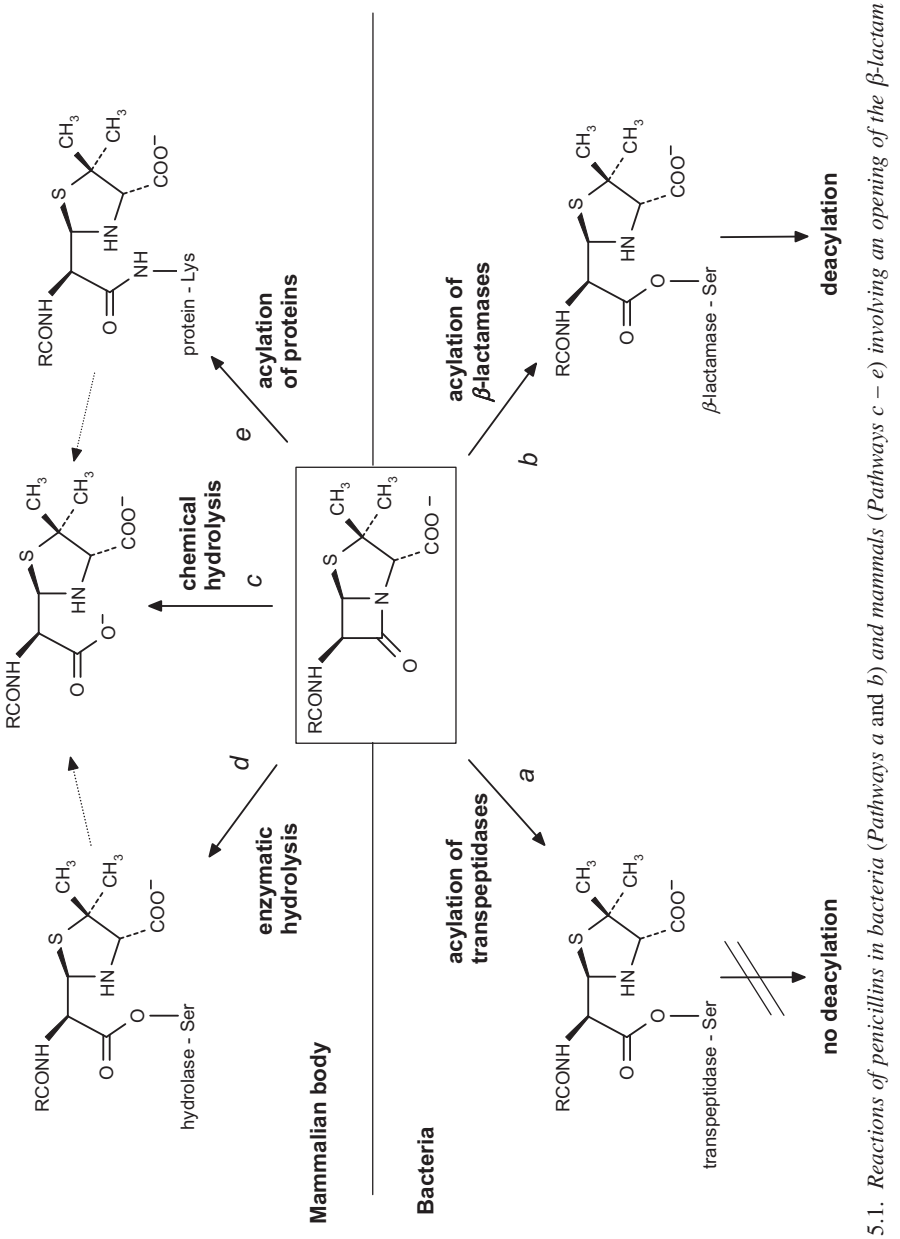


Fig. 5.1. *Reactions of penicillins in bacteria* (*Pathways a* and *b*) *and mammals* (*Pathways c – e*) *involving an opening of the β-lactam ring*

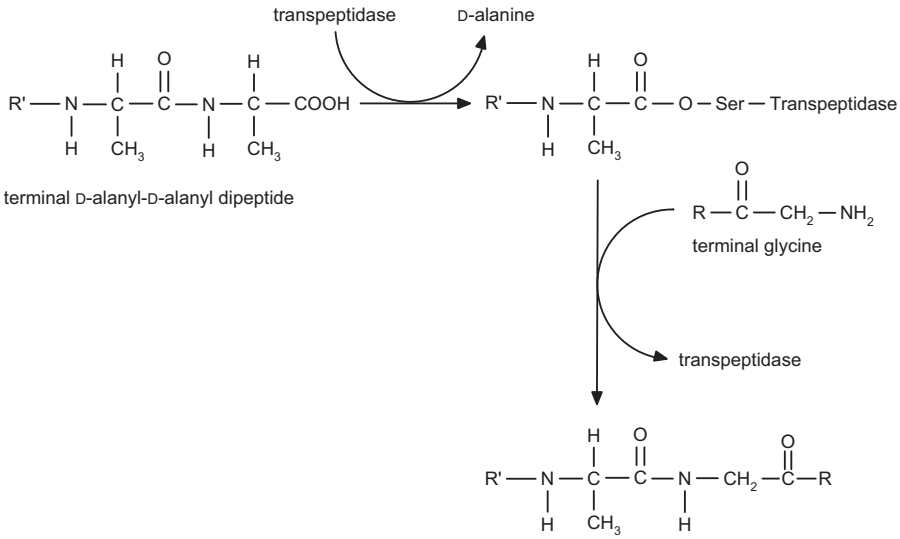


Fig. 5.2. *Mechanism of transpeptidases in the bacterial cell wall, the target of inhibitory β-lactam antibiotics* (*Fig. 5.1, Pathway a*)

boring pentapeptide chain [9][10] (*Fig. 5.2*). Other enzymes involved in peptidoglycan synthesis, in particular carboxypeptidases, can also be acylated [11]. The enzymes inactivated by β-lactam antibiotics are termed collectively '*penicillin-binding proteins*' (PBPs) [8][12–14].

5.2.2.2. *Enzymatic Hydrolysis by β-Lactamases*

β-Lactamases (EC 3.5.2.6) inactivate β-lactam antibiotics by hydrolyzing the amide bond (*Fig. 5.1*, *Pathway b*). These enzymes are the most important ones in the bacterial defense against β-lactam antibiotics [15]. On the basis of catalytic mechanism, β-lactamases can be subdivided into two major groups, namely *Zn^{2+}-containing metalloproteins* (class B), and *active-serine enzymes*, which are subdivided into classes A, C, and D based on their amino acid sequences (see *Chapt. 2*). The metallo-enzymes are produced by only a relatively small number of pathogenic strains, but represent a potential threat for the future. Indeed, they are able to hydrolyze efficiently carbapenems, which generally escape the activity of the more common serine-β-lactamases [16][17]. At present, however, most of the resistance of bacteria to β-lactam antibiotics is due to the activity of *serine-β-lactamases*. These enzymes hydrolyze the β-lactam moiety *via* an acyl–enzyme intermediate similar to that formed by transpeptidases. The difference in the catalytic pathways of the two enzymes is merely quantitative (*Fig. 5.1*, *Pathways a* and *b*).

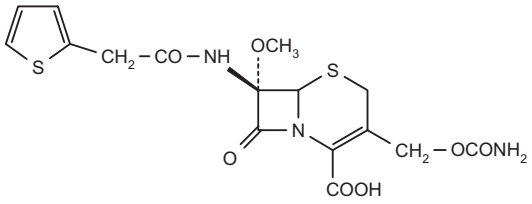
β-Lactamases have very high rates of deacylation compared to those of transpeptidases. This quantitative difference translates into very different qualitative results, since β-lactams inactivate transpeptidases but are destroyed by β-lactamases [18]. The mechanism underlying the differences in rate constant for deacylation between transpeptidases *vs.* β-lactamases remains poorly understood [19].

Two strategies have been developed to overcome the destructive effect of β-lactamases. The first is based on the development of β-lactam antibiotics that are not substrates of these enzymes. The second approach uses the association of a potent inactivator of β-lactamases with a classical β-lactam antibiotic [20][21]. The first strategy tends to evade the enzyme, while the second neutralizes it [22].

The development of β-lactam antibiotics that are *resistant to β-lactamases* began with the introduction of *steric hindrance* around the side-chain amido group [23]. Thus, penicillins carrying a 2,6-dimethoxyphenyl, 2-ethoxynaphth-1-yl, or 3,5-disubstituted isoxazol-4-yl substituent at the 6α-position (see *Table 5.2*) are resistant to hydrolysis by β-lactamases. These bulky substituents greatly reduce the affinity for β-lactamases by preventing a proper fit. In third- and fourth-generation cephalosporins, enhanced β-lactamase stability is obtained with the 7-(2-aminothiazol-4-yl)acetamido side chain bearing a (*Z*)-configured oxime residue (see *Table 5.3*) [24].

The presence of a *α-MeO group* at C(6) of penicillins or at C(7) of cephalosporins improves the resistance to β-lactamases [6]. This effect has been attributed to steric hindrance. However, substitution in the 6α- or 7α-position very often also impairs bacteriostatic activity by altering the affinity to the PBPs [3]. *Matagne et al.* [25] revealed interesting details of the interaction between β-lactamases and compounds bearing an α-MeO substituent, *e.g.*, cefoxitin (**5.9**), moxalactam (**5.10**), and temocillin (**5.11**). On the basis of crystallographic and molecular-graphics studies, they proposed that the extremely slow acylation of class-A β-lactamases is due to the displacement of the H_2O molecule that connects the Glu^{166} and Ser^{70} side chains (see *Chapt. 2*) [26–29]. Acylation becomes difficult when this H_2O molecule (which is involved in the activation of the Ser^{70} OH group by Glu^{166}) is displaced by the α-MeO group of the substrate. By contrast, class-C β-lactamases acylate cefoxitin and moxalactam with high efficiency [30]. This phenomenon can be explained by the differences in the acylation mechanisms of the two classes of lactamases. In class-C β-lactamases, the Tyr^{150} phenolic group rather than a H_2O molecule acts as a general base [31–34].

Furthermore, the 3D-location of Tyr^{150} is quite different from that of Glu^{166} in class-A β-lactamases. The observed resistance of the methoxylated cephalosporins to class-C β-lactamases is due to a slow deacylation step [35]. This inhibition is the result of the formation of an acyl–enzyme intermediate



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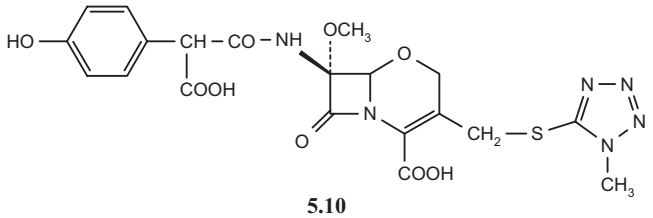

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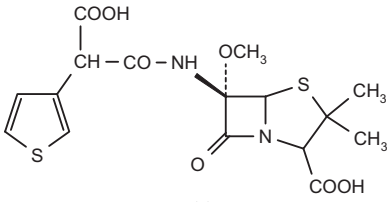


5.11

that is relatively inert to hydrolysis. The molecular mechanism of this type of inhibition will be discussed in the next subsection.

The *α-Me group* at C(4) protects the monobactam aztreonam (**5.8**) against β-lactamase attack. It has been shown that the configuration at C(3) and C(4) in the azetidinone ring is of importance: compounds with a *cis*-configuration are more rapidly inactivated by β-lactamases than the corresponding *trans*-isomer [36].

The strategy for development of β-lactamase-resistant β-lactams has some limitations. Indeed, it has often been found that the more-resistant compounds are less-efficient antibiotics. Furthermore, the natural weapons wielded by bacteria: mutation, gene transfer, and natural selection, combine to counter β-lactamase resistance. Thus, β-lactamase mutants have emerged that efficiently hydrolyze compounds that were previously considered β-lactamase-resistant [37–41]. The overproduction of enzymes – either PBPs or the original β-lactamases – as well as a decrease in the permeability of the bacterial membrane to antibiotics – are other defense strategies of the bacteria [42][43].

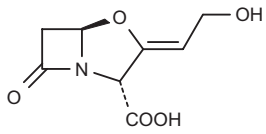
5.2.2.3. *Mechanism-Based Inactivators of β-Lactamases*

The first clinical application of the *second strategy* (to overcome bacterial resistance by neutralizing the β-lactamases) was the combination of clavulanic acid (**5.12**) and amoxicillin. The efficacy of clavulanic acid has stimulated research on other inhibitors of β-lactamases, leading to the discovery of a number of other inactivators such as sulbactam (**5.13**), 6β-bromopenicillanic acid (**5.14**), and olivanic acid (**5.15**) [44][45].

Mechanistic investigations have shown that these compounds behave as suicide inhibitors (preferably called *mechanism-based inactivators*) in the sense that they are recognized by β-lactamases as substrates, but the great stability of the acyl–enzyme intermediate blocks turnover of the enzyme [46][47]. β-Lactamase inhibitors can be divided into two classes, class I and class II: class-I inhibitors (*e.g.*, clavulanic acid (**5.12**)), in contrast to those of class II (*e.g.*, olivanic acid (**5.15**)), have a heteroatom at position 1 that can lead to ring opening at C(5). The mechanistic consequences of this difference in structure are illustrated by the general scheme in *Fig. 5.3*.

In the case of *class-II inhibitors*, the acyl–enzyme undergoes either normal deacylation (*Fig. 5.3*, *Pathway c*) or a chemical re-arrangement in which the enamine tautomerizes to an imine, generating an acyl–enzyme with a Δ^1-pyrroline structure (*Fig. 5.3*, *Pathway b*). This second acyl–enzyme intermediate hydrolyzes at a much slower rate, and the decreased turnover leads to transient inhibition of the enzyme [22][50].

The interaction of *class-I inhibitors* with β-lactamases is more complex. Subsequent to acylation, the five-membered ring is cleaved at the C–hetero-



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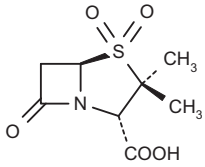

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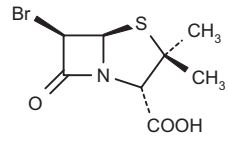

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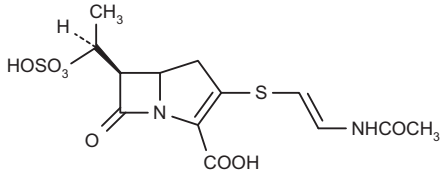

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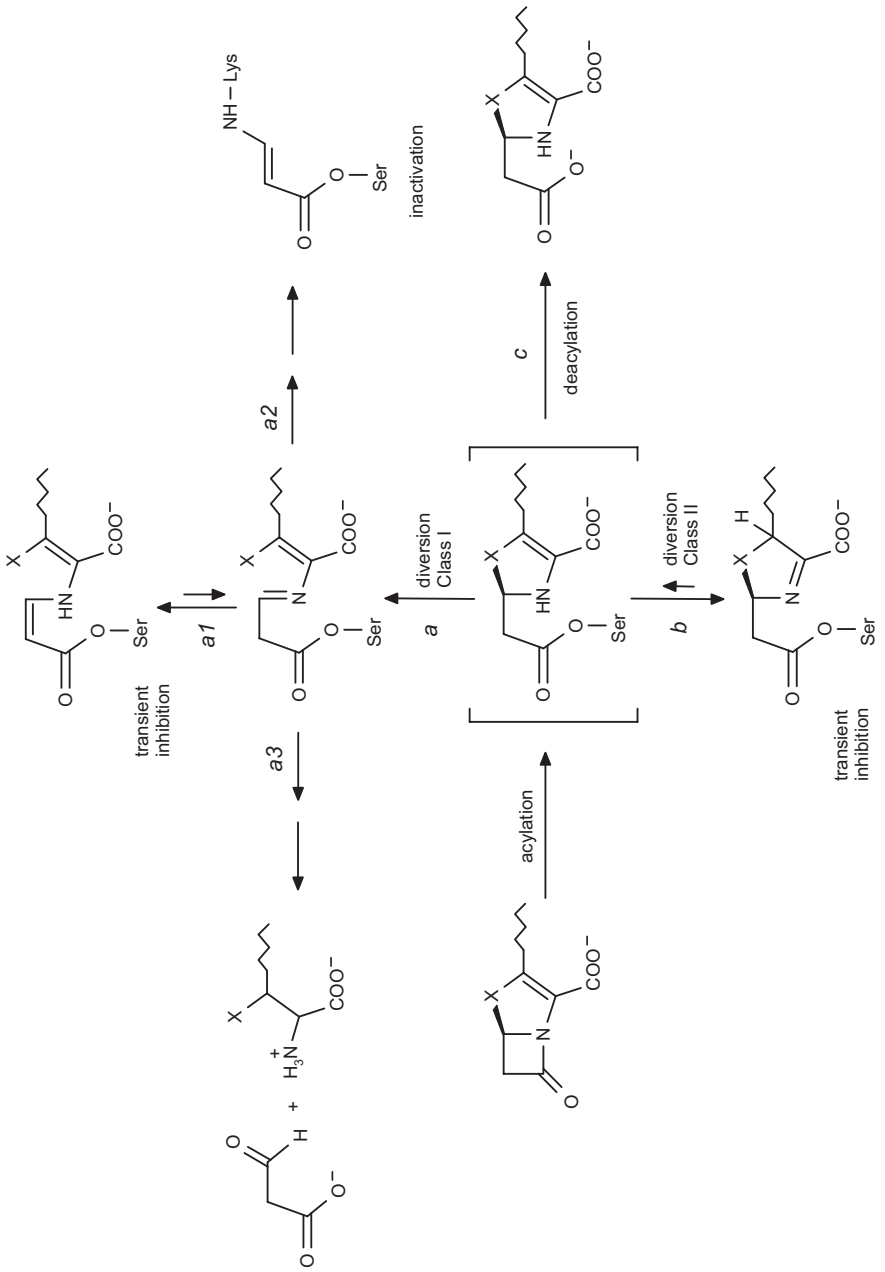
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atom bond, giving rise to a linear acyl species (*Fig. 5.3*, *Pathway a*). This intermediate can react in three ways (*Fig. 5.3*, *Pathways a1–a3*). The deacylation step liberates the imine (*Fig. 5.3*, *Pathway a3*), which is ultimately hydrolyzed to malonsemialdehyde and a penicillamine derivative. The second possible reaction of the intermediate is the tautomerization to a β-aminoacrylate by abstraction of the 6β-H-atom (*Fig. 5.3*, *Pathway a1*). The slow hydrolysis of this product leads to transient inhibition of the enzyme. Finally, the transimination of the intermediate with a lysine residue in the enzyme results in a covalently modified site, which leads to inactivation of the enzyme (*Fig. 5.3*, *Pathway a2*) [48][49][51].

Combinations of β-lactamase inhibitors with β-lactam antibiotics are very useful in the treatment of infections, since they are relatively immune to the emergence of new resistance. However, a β-lactamase resistant to inactivation by clavulanic acid has been identified [52].

Some *cephalosporins* can be both *substrates and inhibitors of β-lactamases*. The acyl–enzyme intermediate can undergo either rapid deacylation (*Fig. 5.4*, *Pathway a*) or elimination of the leaving group at the 3′-position to yield a second acyl–enzyme derivative (*Fig. 5.4*, *Pathway b*), which hydrolyzes very slowly [35][53]. Thus, cephalosporins inactivate β-lactamases by a mechanism similar to that described above for class-II inhibitors. It has been hypothesized that differences in the rate of deacylation of the acyl–enzyme intermediates derive from their different abilities to form H-bonds. A H-bond to NH in *Fig. 5.4*, *Pathway a*, may be necessary to assure a catalytically essential conformation of the enzyme, whereas the presence of a H-bond acceptor in *Fig. 5.4*, *Pathway b*, may drive the enzyme to an unproductive conformation. The *ratio between hydrolysis and elimination*, and, consequently, the relative importance of substrate and inhibitor behaviors of cephalosporins, is determined by the nature of the leaving group at C(3′). An appropriate substitution at C(3′) of cephalosporins may, therefore, increase the β-lactamase inhibitory properties and yield potentially better antibiotics [53].

◀ Fig. 5.3. *Generalized scheme for mechanism-based inactivators of β-lactamases* (*Fig. 5.1*, *Pathway b*) *detailing the different modes of action of class-I and class-II inhibitors. Pathway a*: class-I inhibitors (X = heteroatom, *e.g.*, clavulanic acid (**5.12**)); their interaction with β-lactamases involves acylation, followed by ring cleavage at the C–heteroatom bond. The resulting linear acyl species can react in three ways: *Pathway a1*: tautomerization to a β-aminoacrylate; the slow hydrolysis of this product leads to a transient inhibition of the enzyme; *Pathway a2*: transimination with a lysine residue of the enzyme, which leads to the inactivation of the enzyme; *Pathway a3*: deacylation to liberate the imine, which hydrolyzes to malonsemialdehyde and a penicillamine derivative. *Pathways b* and *c*: class-II inhibitors (X = CH_2, *e.g.*, olivanic acid (**5.15**)) can interact with β-lactamase in two ways: *Pathway b*: chemical arrangement resulting in an acyl–enzyme with a Δ^1-pyrroline structure. The slow hydrolysis of this acyl–enzyme intermediate leads to a transient inhibition of the enzyme; *Pathway c*: normal deacylation of the acyl–enzyme complex [22][48 – 51].

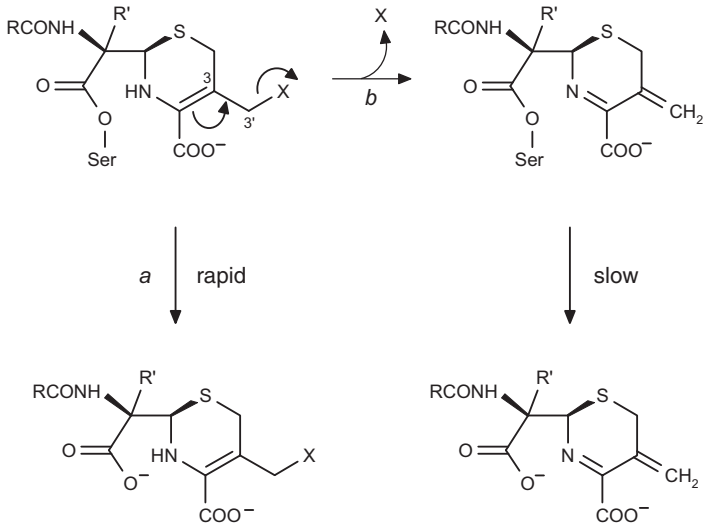
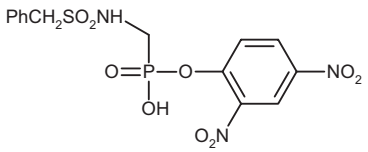


Fig. 5.4. *Inactivation of β-lactamases by cephalosporins* (*Fig. 5.1*, *Pathway b*). The mechanism of this inactivation is similar to that of class-II inhibitors (*Fig. 5.3*, *Pathway b*) and is based on the slow hydrolysis of the acyl–enzyme complex (*Pathway b*). The normal deacylation of the acyl–enzyme complex represented by *Pathway a* results in the lost of antibacterial activity of the drug. The ratio between *Pathways a* and *b* is determined by the nature of the leaving group at C(3′) [53].

Recently, a *structure–inhibition–activity relationship study* revealed that carbapenem derivatives could lead to a new class of specific inhibitors of bacterial β-lactamases [54]. Indeed, phosphonyl derivatives, **5.16**, and their cyclic analogues, **5.17** – *i.e.*, structures not based on the β-lactam nucleus – can also afford effective inhibitors of β-lactamases [55][56].

The β-lactam structure can also react with *active-serine hydrolases other than PBPs and β-lactamases*. It has been shown that appropriately substituted cephalosporins (*e.g.*, **5.18**) are potent mechanism-based inactivators of *human leukocyte elastase* (HLE, EC 3.4.21.37), a serine endopeptidase involved in the pathogenesis of pulmonary emphysema and other connective tissue diseases [57–60]. Subsequent work has demonstrated that substituted β-lactams such as **5.19** or **5.20** are more stable HLE inhibitors and have improved potencies [61–63].



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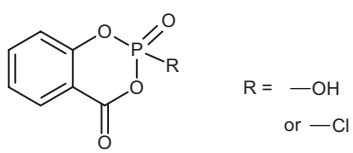

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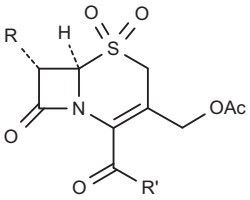

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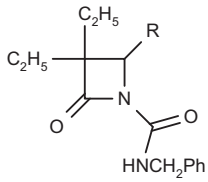

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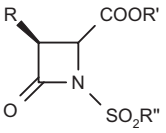

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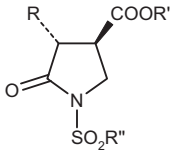

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Other potential elastase inhibitors based on the β-lactam nucleus include cephem derivatives [64], penam derivatives [65], as well as novel bicyclic β-lactams [66]. Monocyclic γ-lactams (**5.21**) with appropriated substitution might also yield useful inhibitors [67].

As already noted in *Fig. 5.1*, the ring opening of β-lactam antibiotics is not only a major event in their interaction with bacterial enzymes, but occurs also in the mammalian body. Chemical hydrolysis in body fluids, metabolism by mammalian enzymes and interactions with plasma proteins, all influence the pharmacological activity of these antibiotics. These aspects will be considered below. We begin with the discussion of the chemical reactivity of the β-lactam nucleus, which plays a key role in these events.

5.2.2.4. *Chemical Hydrolysis – Reactivity of the Nucleus*

β-Lactams are generally more reactive toward nucleophiles than are normal amides. The ease of the nucleophilic attack on the lactam carbonyl group is usually attributed to either relief of strain upon opening the ring [68], or to a reduction in the usual amide resonance due to nonplanarity of the bicyclic system [69]. However, the evidence to support unusual strain in the ring or reduced amide resonance in β-lactam antibiotics is ambiguous.

Some information concerning *ring strain* and *amide resonance* can be obtained by comparing monocyclic and bicyclic β-lactams. Monocyclic β-lactams are very resistant to alkaline and acid hydrolysis [70][71] due to *reso-*

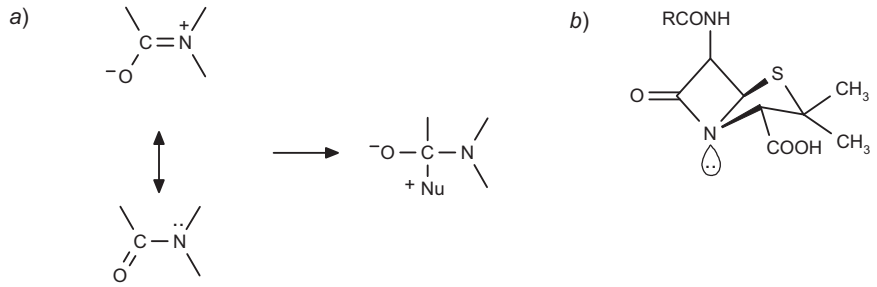


Fig. 5.5. *Factors influencing the reactivity of the β-lactam bond: a) amide resonance, and b) the nonplanar butterfly shape of penicillins*

nance stabilization of the lactam bond (*Fig. 5.5,a*). Obviously, the strain energy inherent to the ring does not contribute to the reactivity of these compounds. Because of their butterfly shape, bicyclic β-lactams have additional strain in the β-lactam ring (*Fig. 5.5,b*). Furthermore, this configuration prevents the normal planar arrangement of the amide bond, which is assumed to be necessary for delocalization of the N-atom's lone pair electrons. The higher rate of hydrolysis observed for bicyclic β-lactams seems to support these arguments. However, the magnitude of the increase is far lower than that expected for the release of strain upon opening of a four-membered ring or from a system with reduced amide resonance [72].

Page demonstrated in 1992 in a critical analysis [73] that bicyclic β-lactam antibiotics do not exhibit exceptional chemical reactivity. He concluded that neither kinetic nor ground-state effects indicate a significant degree of inhibition of amide resonance in penicillins and cephalosporins [72][74]. Indeed, in comparison to normal amides, the β-lactam N-atom does not exhibit any enhanced ability to donate its electron pair to either protons or metal ions [75][76].

Greater ring strain does not necessarily lead to more rapid alkaline hydrolysis or to better antibiotic activity [70][71]. In fact, the greater ring-strain energy in bicyclic structures does not significantly facilitate C–N cleavage [72]. Thus, comparable rates of hydrolysis of cephalosporins and penicillins catalyzed by HO^- are observed, despite the greater amount of ring strain inherent in the five-membered thiazolidine ring of penicillins than in the six-membered dihydrothiazine ring of cephalosporins [76]. Furthermore, monocyclic β-lactams with suitable electron-withdrawing substituents can be as reactive as the bicyclic systems.

An explanation for the smaller-than-expected effect of strain energy upon the rate of hydrolysis of β-lactam rings can be found upon examination of the mechanism of cleavage of the C–N bond: amides and lactams are hydro-

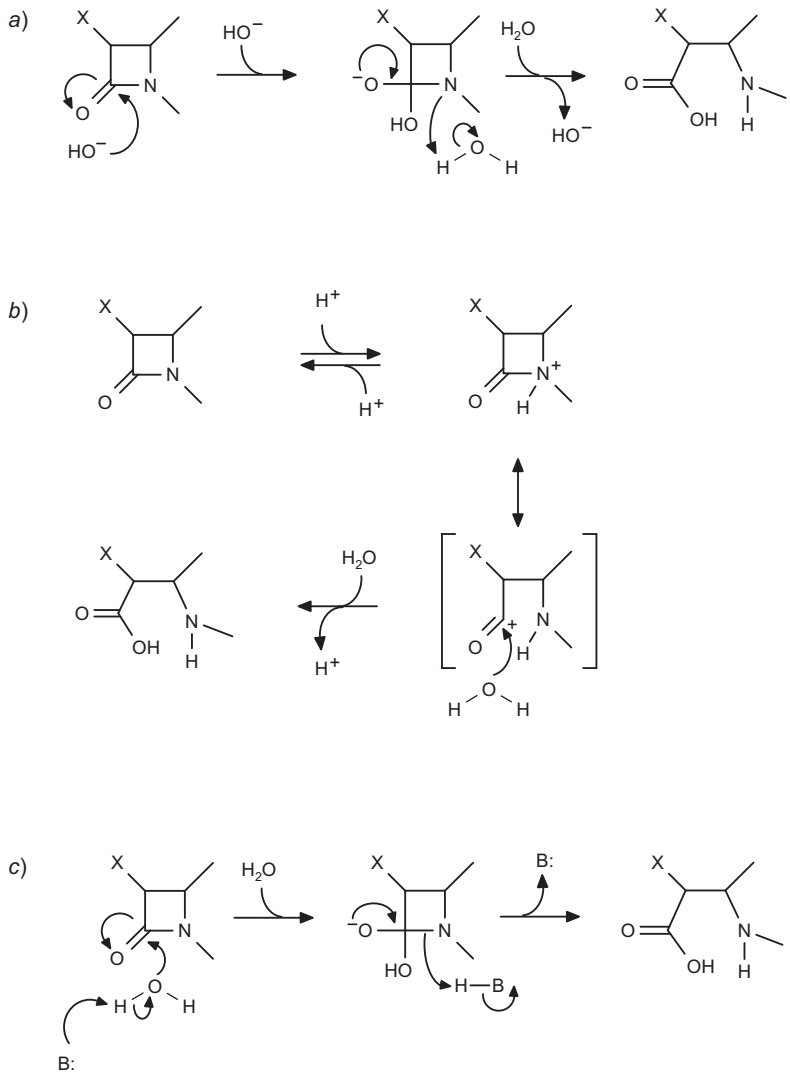


Fig. 5.6. Different mechanisms of hydrolysis of the β-lactam bond, namely a) *base-catalysis,* b) *acid-catalysis, and* c) *general base catalysis by* H_2O

lyzed by different mechanisms. For amides, the rate-limiting step is the breakdown of the tetrahedral intermediate, whereas, for β-lactams, the rate-limiting step is the formation of the tetrahedral intermediate (*Fig. 5.6,a*) [76]. Since the rate-limiting step does not involve C–N bond fission, opening the ring does not lower the activation energy in the reaction with nucleophiles. The *strain energy* arises from a combination of bond-opposition forces (*Pit-*

zer strain) and bond-angle deformation (*Baeyer* strain) [77][78]. The latter can be released during the formation of the tetrahedral intermediate, without ring opening, by conversion of the sp^2-hybridized carbonyl C-atom to sp^3. This effect may account for the *ca.* 100-fold higher rate of alkaline hydrolysis of β-lactams compared to that of acyclic amides by amines of similar basicity [74][77].

The *mechanism of acid hydrolysis* is also different in acyclic amides and β-lactams; acid catalysis of acyclic amides proceeds *via O*-protonation (see *Chapt. 4*), whereas that of β-lactams appears to be a unimolecular A1 type process, involving *N*-protonation (*Fig. 5.6,b*) [76]. *N*-Protonation is not the result of reduced amide resonance but an intrinsic property of the β-lactam structure, since bicyclic β-lactams and monocyclic β-lactams exhibit similar reactivity and behavior [76].

5.2.2.5. *Chemical Hydrolysis – Substituent Effects*

β-Lactams undergo both acid- and base-catalyzed hydrolysis [79]. The *pH* vs. *log(rate constant) profiles* of penicillins are V-shaped with rate constants at a minimum of *ca.* pH 7, whereas, for cephalosporins, these plots are U-shaped with minimum rate constants in the pH range of 3–7. The basis for this difference is that penicillins undergo no significant uncatalyzed (pH-independent) reaction, while, in contrast, for cephalosporins, a significant degree of spontaneous, pH-independent reaction between pH 3 and 7 is often observed [80].

A mechanism involving general base catalysis by H_2O at the β-lactam ring, as shown in *Fig. 5.6,c*, has been proposed. However this proposal does not explain the different susceptibilities to *spontaneous hydrolysis* of penicillins and cephalosporins. *Llinás et al.* [81] suggested, in explaining the observation of a pH-independent hydrolysis in cephalosporins but not in penicillins, that neighboring-group participation by the amide side chain would facilitate general base catalyzed attack of H_2O. The H_2O molecule encounters little steric hindrance in the case of cephalosporins because the dihydrothiazine ring fused to the β-lactam is more planar and does not carry substituents that can interact with the side chain at C(7). In the case of penicillins, the thiazolidine ring fused to the β-lactam gives rise to a butterfly-shaped molecule, which places the geminal Me groups at C(2) very close to the amido group at C(6). This molecular geometry generates steric hindrance for the H_2O molecule involved in the catalytic process.

In the following paragraphs, we will discuss the substituent effects that influence the rate of hydrolysis of the β-lactam bond in acidic and alkaline solutions.

Substituent effects profoundly influence the reactivity of the β-lactam cycle. A number of representative examples of the effect of structural features on chemical reactivity are assembled in *Table 5.4* and discussed here.

We begin the discussion with one of the major sites of structural variation, namely at *C(6) in penicillins* and at *C(7) in cephalosporins* (*Table 5.4,A*). Electron-withdrawing substituents at C(6) in penicillins and at C(7) in cephalosporins increase the rate of base-catalyzed hydrolysis [76]. The same substituent effect has been observed for monobactams [93][94].

The rate of acid-catalyzed degradation of the penicillins also depends largely on the nature of their acylamido side chain. Structure–activity-relationship studies undertaken for the rational design of orally active penicillins have shown that the stability in gastric juice increases with the sum of *Taft*'s inductive substituent constants (σ_1 values) of the 6-amino side chain [95].

The acid degradation of penicillins is *ca.* 100-fold faster than expected from the σ value of the RCONH group [76]. This increased activity has been attributed to neighboring-group participation by the acylamido side chain. *Proctor et al.* [76] have proposed the mechanism shown in *Fig. 5.7* for this intramolecular interaction. The acylium ion, **5.22**, formed by ring opening can be trapped either by water to give penicilloic acid (**5.24**, *Fig. 5.7*, *Pathway b*) or by the intramolecular amido group to give the protonated oxazolylthiazolidine (**5.23**, *Fig. 5.7*, *Pathway a*). This intermediate is the precursor of various degradation products such as penicilloic acid (**5.24**), penicillenic acid (**5.25**), and penillic acid (**5.26**). Oral penicillins are more stable to acid because their phenoxy substituent decreases the nucleophilicity of the carbonyl C-atom in the side chain, which, in turn, reduces the formation of the oxazolylthiazolidine intermediate.

Electron-withdrawing substituents at C(7) in cephalosporins decrease the rate of acid hydrolysis just as substituents at C(6) in penicillins do. However, in terms of acid-catalyzed hydrolysis cephalosporins are much less reactive than penicillins, even when the substituents on the side chains are similar [76], because cephalosporins do not show neighboring-group participation. The absence of neighboring-group participation in cephalosporins can be explained by the lower basicity of the N-atom of the dihydrothiazine ring compared to that of the thiazolidine ring of penicillins.

Now we turn to a discussion of the influence of *α-substitution at C(6) or C(7)* on the chemical reactivity of the lactam ring (*Table 5.4,B*). This substitution has been introduced mainly to improve lactamase stability (see *Sect. 5.2.2.2*). The insertion of an additional α-substituent at C(6) or C(7) of penicillins or cephalosporins, respectively, has a relatively small effect on the rate of base hydrolysis [82][83]. 6α-Methoxypenicillin is hydrolyzed at a rate that is approximately half that observed for the unsubstituted parent penicillin. This decrease is due mainly to unfavorable steric interaction between the

Table 5.4. *Influences of Some Structural Features of β-Lactams on Their Rate of Chemical Hydrolysis*

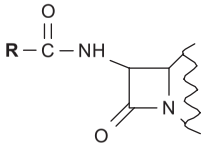
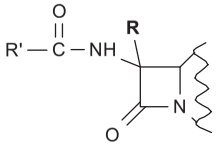
Class	Structure	Structural features	Effects	Rate of base-catalyzed hydrolysis	Rate of acid-catalyzed hydrolysis	Refs.
A		Electron-withdrawing substituents • at C(6) for *penicillins* • at C(7) for *cephalosporins*	• Inductive effect (σ) • Neighboring group participation by the acylamido side chain	• Increased by purely inductive effect • Reactivity of *cephalosporins* similar to that of *penicillins*	• Decreased in *penicillins* due to neighboring-group participation (see *Fig. 5.7*); higher rates than predicted from σ • No neighboring-group participation in *cephalosporins*	[76]
B		• *Penicillins*: C(6α)-substitution • *Cephalosporins*: C(7α))-substitution	• Inductive and steric effects	• Steric effect of R = MeO $\Rightarrow$ approx. twofold decrease • Steric effect of R = Me $\Rightarrow$ *ca.* 10-fold decrease	• Decreased	[73][82][83]

Table 5.4 (cont.)

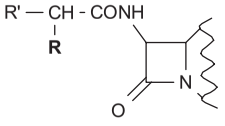
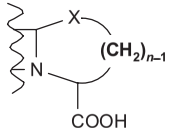
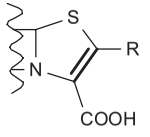
Class	Structure	Structural features	Effects	Rate of base-catalyzed hydrolysis	Rate of acid-catalyzed hydrolysis	Refs.
C		• Substitution at C(α) in the side chain	• Inductive and steric effects • R = NH_2: intramolecular catalysis	• Increased by electron-withdrawing substituents • Increased by R = NH_2 in *cephalosporins* and *monobactams* (see *Fig. 5.8*)	• No significant contribution in *cephalosporins* • Decreased in *penicillins* by electron-withdrawing and large substituents (R = NH_2 ⇒ electron attraction by positively charged amino group)	[84 – 86]
D		• Ring size • *Penicillins*: four-membered ring • *Cephalosporins*: five-membered ring	• Strain energy • Pyramidalization of lactam N-atom	• Similar for *penicillins* and *cephalosporins*	• *Cephalosporins* are *ca.* 10^4 fold less reactive than *penicillins*	[76]
E		• Incorporation of a double bond in the thiazolidine ring of a penam to give the corresponding *penem*	• Conjugation of double bond with N-atom electrons decreases the basicity of the leaving group	• Increased *ca.* 25-fold	• Lower than that of *penicillins*, higher than that of *cephalosporins*	[73][87]

Table 5.4 (cont.)

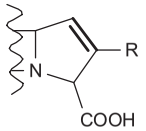
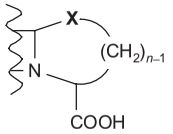
Class	Structure	Structural features	Effects	Rate of base-catalyzed hydrolysis	Rate of acid-catalyzed hydrolysis	Refs.
F		• Conversion of Δ^2-carbapenem to *Δ^1-carbapenem*	• Decrease in the basicity of the leaving group	• Increased *ca.* 25-fold		[73][88]
G		• *Penicillins*: replacement of thiazoline S-atom with CH_2 (*carbapenam*)	• C–S–C Angle smaller, C–S bond length longer ⇒ more ring strain, but leaving group is more basic	• Increased *ca.* 3-fold		[73][82][89]
		• Replacement of S- with O-atom (*oxapenams*)	• Inductive effect	• Increased *ca.* 5-fold		
		• *Cephalosporins*: replacement of dihydrothiazine S-atom with CH_2 (*carbacephalo-sporins*)		• Decreased *ca.* 30-fold		
		• Replacement of S- with O-atom (*oxacephalosporins*)		• Increased *ca.* 6-fold		

Table 5.4 (cont.)

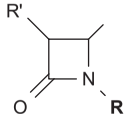
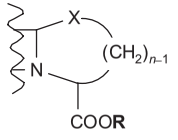
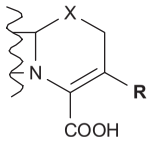
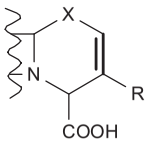
Class	Structure	Structural features	Effects	Rate of base-catalyzed hydrolysis	Rate of acid-catalyzed hydrolysis	Refs.
H		• Substitution of lactam N-atom	• Basicity of leaving group amine, electron-with-drawing substituents in amino moiety	• Increases with basicity • *Monobactams* are *ca.* 100 times less reactive than *penicillins* with leaving groups of similar basicity		[76][90]
I		• *Penicillins*: Esterification of the C(3) carboxy group	• Electron-with-drawing effect makes β-lactam carbonyl C-atom more electrophilic, and intramolecular acylamido participation (see *Fig. 5.9*)	• Increased *ca.* 16-fold		[91]
		• *Cephalosporins*: Ester or lactone at the C(4) carboxy group	• β-Lactam carbonyl C-atom more electrophilic, no intramolecular acylamido participation	• Increased up to 130-fold		

Table 5.4 (cont.)

Class	Structure	Structural features	Effects	Rate of base-catalyzed hydrolysis	Rate of acid-catalyzed hydrolysis	Refs.
J		• Substituent at C(3)	• Inductive effect	• Increases with increasing σ_1		[92]
K		• Δ^3-*Cephem* to Δ^2-*cephem*	• β-Lactam N-atom becomes planar	• Increased 2- to 3-fold		[72]

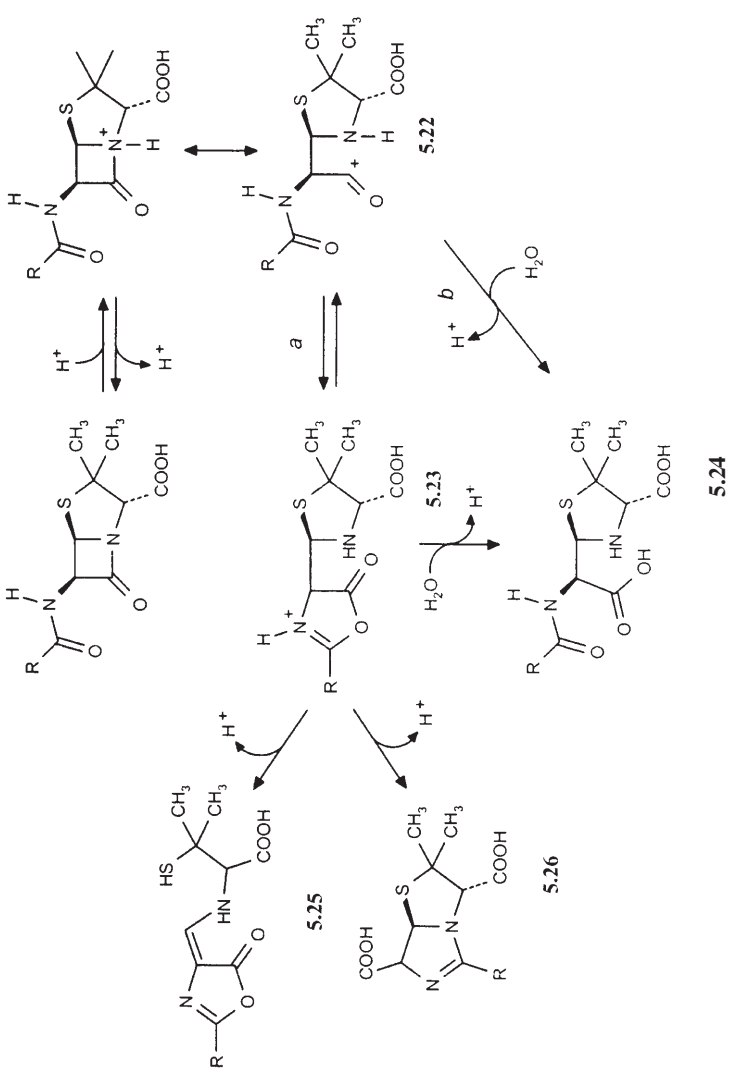
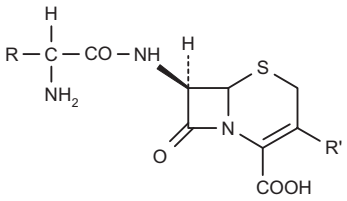
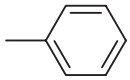
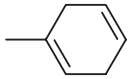
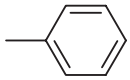


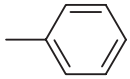
Fig. 5.7. *The acid-catalyzed hydrolysis of penicillins involves first the formation of an acylium ion* (**5.22**), *which, by reacting with* H_2O, *yields penicilloic acids* **5.24** (*Pathway b*). The participation of a neighboring 6-acylamido group increases the rate of hydrolysis. During this intramolecular reaction (*Pathway a*), oxazolylthiazolidines (**5.23**) are formed and then give rise to the degradation products penicilloic acids **5.24**, penicillenic acids **5.25**, and penillic acids **5.26** [76].



5.27 R =  R' = —CH_3

5.28 R =  R' = —CH_3

5.29 R =  R' = —CH_2OCOCH_3

5.30 R =  R' = —Cl

substituent and the tetrahedral intermediate, since we would expect the polar effects of the substituent to accelerate base hydrolysis. Steric effects are also responsible for the 10-fold reduction in the rate of hydrolysis of penicillins and cephalosporins caused by the introduction of a 6α- or 7α-Me group [82][83].

A second way to vary the structure of the acylamino side chain (at position *6β of penicillins* and *7β of cephalosporins*) is the introduction of a substituent at C(α) (*Table 5.4,C*). This effect is best exemplified by introduction of an amino group, which plays a particular role in the chemical stability of β-lactam antibiotics. At pH 8, the degradation rates of α-(2-amino-2-phenylacetyl)cephalosporins (such as cephalexin (**5.27**), cephradine (**5.28**), or cephaloglycin (**5.29**)) were *ca.* 10 to 20 times faster than those of the corresponding cephalosporins lacking an α-amino side chain. The increase in reactivity has been attributed to *intramolecular attack by the α-amino group* of the β-lactam (*Fig. 5.8,a*). Isolation of the corresponding piperazine-2,5-dione product (**5.31**) confirms this hypothesis [84a][86][96]. *Cefaclor* (**5.30**) also undergoes intramolecular aminolysis by the 7-NH_2 group at 37° in neutral solution [97].

This reaction occurs not only in bicyclic lactams, but also in monobactams. Indeed, intramolecular nucleophilic amino attack has also been observed in an arylglycine-substituted monobactam (*Fig. 5.8,b*) [84b]. However, *ampicillin* (see below, **5.43**, *Fig. 5.14*), which also carries an α-amino side chain in the 6β-position, does not exhibit such an enhanced rate. This difference in reactivity has been attributed to the steric hindrance of both the geminal Me groups and H–C(3), which impedes the attack by the α-amino group at the β-face [84a]. In contrast, penicillins with an amino substituent in the 6β-acylamido side chain show an intermolecular reaction resulting in the formation of oligomers (see *Sect. 5.2.5*).

Llinás et al. [81] examined how *intramolecular aminolysis* is influenced by configuration at C(6) in penicillins and at C(7) in cephalosporins. In con-

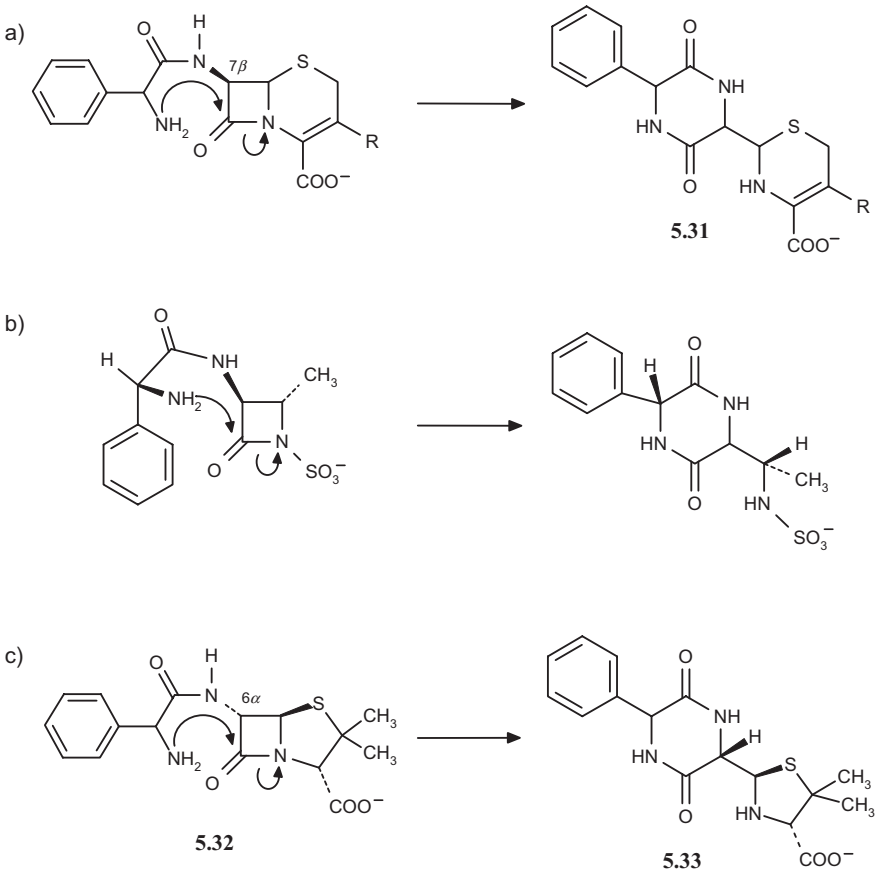


Fig. 5.8. *An α-amino group in the acylamido side chain of β-lactams increases the rate of degradation in alkaline media by favoring an intramolecular nucleophilic reaction yielding piperazine-2,5-dione products* (*e.g.*, **5.31** and **5.33**). *a*) α-(2-Amino-2-phenylacetyl)cephalosporins; *b*) arylglycine-substituted monobactams; *c*) 6α-epiampicillin (**5.32**).

trast to ampicillin and other penicillins, 6α-epiampicillin (**5.32**, *Fig. 5.8,c*) underwent pH-independent aminolysis in neutral and basic media to form the piperazine-2,5-dione **5.33**. It is likely that the 6α-position of the side chain allows intramolecular attack to take place from the more favorable α-direction (*Fig. 5.8,c*); calculations support this interpretation.

In acidic solution, the α-amino group in the side chain is protonated and becomes electron-withdrawing, thus enhancing the acid stability of the lactam ring. In contrast, the presence of the electron-donating carboxy group reduces markedly the stability in acidic solution. Substitution at the α-position of the side chain creates an additional stereogenic center, thus, the resulting

epimers potentially have different pharmacokinetic and pharmacodynamic properties [98]. However, a complete study of the chemical stability of the (*R*)- and (*S*)-epimers of moxalactam (**5.10**) in aqueous solution showed no differences in the rate constant for degradation or epimerization [99].

The differences in the rates of alkaline hydrolysis of β-lactams have been shown to depend primarily on the basicity of the amino leaving group [76]. The rate of alkaline hydrolysis increases as the pK_a of the leaving group increases. Several structural features affecting the pK_a of the leaving amino group are summarized in *Table 5.4*, the details of which follow.

In cephalosporins, the reactivity of the β-lactam ring is mainly due to the reduced basicity of the leaving amino group, a reduction that results from enamine resonance (*Table 5.4,D*). The introduction of a double bond in the thiazoline ring of a penam to give the corresponding Δ^2-penem system (*Table 5.4,E*) increases the rate of hydrolysis within the order of magnitude expected from the decrease in basicity of the amino leaving group [76]. A decrease in basicity also explains why Δ^2-carbapenems are more reactive than Δ^1-carbapenems (*Table 5.4,F*) [88]. Carbapenams hydrolyze at lower rates than penams because the replacement of the S-atom by CH_2 increases the basicity of the amino group (*Table 5.4,G*). The introduction of a 1β-Me group (see later, 1β-methylcarbapenems, *e.g.*, meropenem (**5.47**)) improved stability not only against chemical hydrolysis but also against metabolic degradation (see *Sect. 5.2.7*) compared to 1β-unsubstituted carbapenems [100].

The rate of hydrolysis in monocyclic β-lactams can also be predicted from the basicity of the amino leaving group (*Table 5.4,H*) [90].

Esterification of the carboxy group at C(3) in penicillins and at C(4) in cephalosporins has been undertaken to produce prodrugs with improved absorption characteristics (*Table 5.4,I*). However, simple aliphatic and aromatic esters have higher rates of alkaline hydrolysis of the β-lactam ring than the corresponding acids [101]. The reason for this phenomenon is that the ester group is more electron-withdrawing than the carboxylate group, and, consequently, renders the β-lactam carbonyl C-atom more susceptible to nucleophilic attack [72]. Cephalosporin esters also isomerize under basic conditions [102]. In penicillins, esterification induces neighboring-group participation by the acylamido side chain at C(6) (*Fig. 5.9*, *Pathway b*), which results in formation of an oxazol-5(4*H*)-one intermediate (**5.34**, *Fig. 5.9*) [91]. For comparison purposes, hydrolysis without neighboring-group participation is shown as in *Fig. 5.9*, *Pathway a*. To obtain useful prodrugs, the accelerating effect of simple esters on the hydrolytic cleavage of the β-lactam bond must be avoided. This can be achieved by introducing double-ester types such as (acyloxy)alkyl esters. Indeed, since the ester bond of these compounds is rapidly hydrolyzed in plasma (which removes the electron-

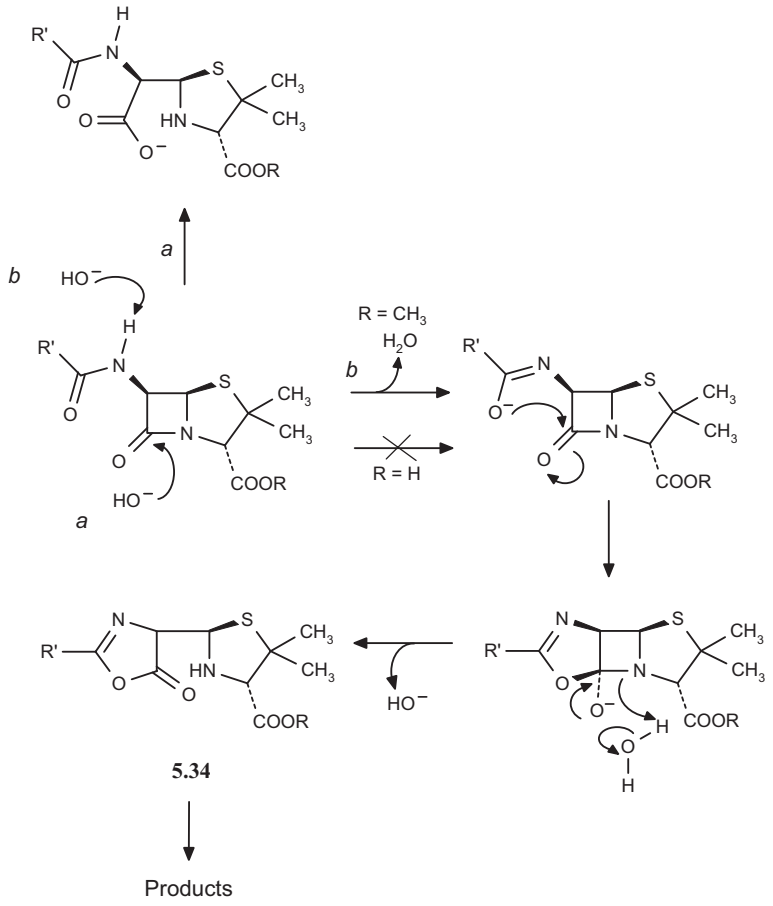


Fig. 5.9. *Esterification of the OH group at C(3) enhances alkaline hydrolysis of penicillins by inducing neighboring-group participation of the acylamido side chain at C(6)* (*Pathway b*), *which implies the formation of oxazol-5(4*H*)-one intermediate* **5.34**. *Pathway a* shows hydrolysis without neighboring-group participation [91].

withdrawing ability), the ester substituent cannot influence the stability of the β-lactam bond [103].

Another important site of structural variation in cephalosporins is C(3) (*Table 5.4.J*). Electron-withdrawing substituents at C(3) such as a Cl-atom or a MeO group increase base-catalyzed hydrolysis of cephalosporins by both resonance and inductive effects [92]. For cephalosporins carrying 3-methylene-linked substituents with leaving group ability (*e.g.*, acetate, thiol, or pyridine), it has been postulated that a concerted expulsion of the substituent facilitates the nucleophilic attack on the β-lactam carbonyl group [104][105]. However, there are also arguments for a stepwise process in which the ex-

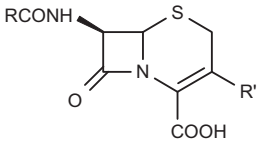
pulsion of the substituent occurs after nucleophilic attack. In this case, only the inductive effect of the substituent at C(3′) affects the rate of β-lactam C–N bond fission [106].

The change from a Δ^3-cephem to a Δ^2-cephem system increases the rate of alkaline hydrolysis two- to three-fold (*Table 5.4.K*) [72].

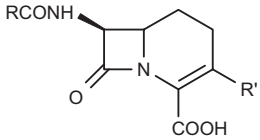
5.2.3. Relationship between Chemical Hydrolysis and Antibacterial Activity

Ring-opening, followed by nucleophilic attack, occurs as part of the mechanism of action of β-lactams (*i.e.*, acylation of bacterial transpeptidases) as well as during their alkaline hydrolysis. In the active site of transpeptidases, the serine OH group (*Fig. 5.1*, *Pathway a*) acts as the nucleophilic agent, a role played in aqueous solution by the HO^- ion (*Fig. 5.6,a*). Based on the apparent similarity between the mechanisms of enzyme acylation and alkaline hydrolysis, it has been suggested that the rate of base hydrolysis of β-lactams is correlated with their antibacterial activity [92]. In other words, it has been assumed that the chemically more labile β-lactam drugs should have greater antibacterial activity [84a]. Such qualitative correlations between chemical reactivity and antimicrobial activity have been found only within narrow series of β-lactams having a given ring system with substituent variations only at specific positions [82][107]. For example, antibacterial activity is related to the rate of alkaline hydrolysis in separate series of cephalosporins with different substituents at C(3) and C(3′) [80][108][109].

Despite these observations, neither correlation nor trend was observed when the rates of β-lactam hydrolysis in different classes of β-lactam antibiotics were compared with antimicrobial activity [72][104]. Thus, a comparison of matched pairs of cephalosporins **5.35** and their 1-carba-1-dethiacephalosporin analogues **5.36** did not reveal any relationship between microbial activity and aqueous stability [89]. The antimicrobial activities of the pairs of compounds were similar across a broad range of bacteria, whereas all the 1-carba-1-dethiacephalosporins were remarkably more stable (8- to 50-fold) than their cephalosporin counterparts. Similar observations were made for two series of monobactams. Oxamazines **5.37** showed considerable antibacterial activity, whereas their corresponding sulfur analogues (thiamazines **5.38**) were inactive. Yet, rates of β-lactam ring opening were shown to be comparable in both series [104]. The differences in antibacterial activity observed could be explained with the help of X-ray structure determinations, molecular graphics, and quantum-mechanical calculations. Indeed, these methods have revealed that thiamazines, due to their bond lengths and an-



5.35



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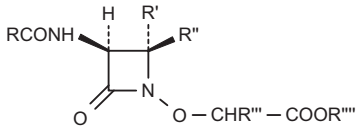

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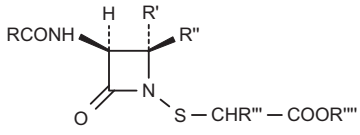


5.38

gles, would not fit as well as oxamazines within the active site of the target bacterial transpeptidase [104].

It appears that qualitative correlations between antibacterial activity and rate constants of HO^- ion catalyzed hydrolysis are fortuitous since many factors other than transpeptidase acylation contribute to antimicrobial activity. These other contributing factors include permeation of the outer membrane of the bacterial cell wall, resistance to β-lactamase, the fit in the active site of the enzyme, stability of the acylated enzyme, and, last but not least, *in vivo* pharmacokinetic behavior.

5.2.4. Stereoselectivity of Hydrolysis

β-Lactam antibiotics have several stereogenic centers, the configurations of which are critical for antibacterial activity. In the following section, some examples that illustrate stereochemical aspects of the hydrolytic degradation of β-lactams are presented.

Penicillins hydrolyze to *penicilloic acids*, which retain the same (5*R*,6*R*)-configuration (*Fig. 5.10*). In the subsequent step, however, penicilloic acids slowly epimerize in aqueous solution to their (5*S*,6*R*)-isomers [110]. The mechanism of *C(5)-epimerization* involves opening of the thiazolidine ring by C–S bond fission, followed by reclosure with inversion of configuration at C(5) (*Fig. 5.10*) [111].

In *cephalosporins*, the C(6) position corresponds to C(5) in penicillins or penicilloic acids. During the degradation of cephalosporins, epimerization at C(6) is generally not observed. However, there are exceptions to this rule. An investigation of the degradation kinetics of cefdinir (**5.39a**, *Fig. 5.11*) and its C(7)-epimer (**5.39b**) in aqueous solution showed that, after β-lactam ring

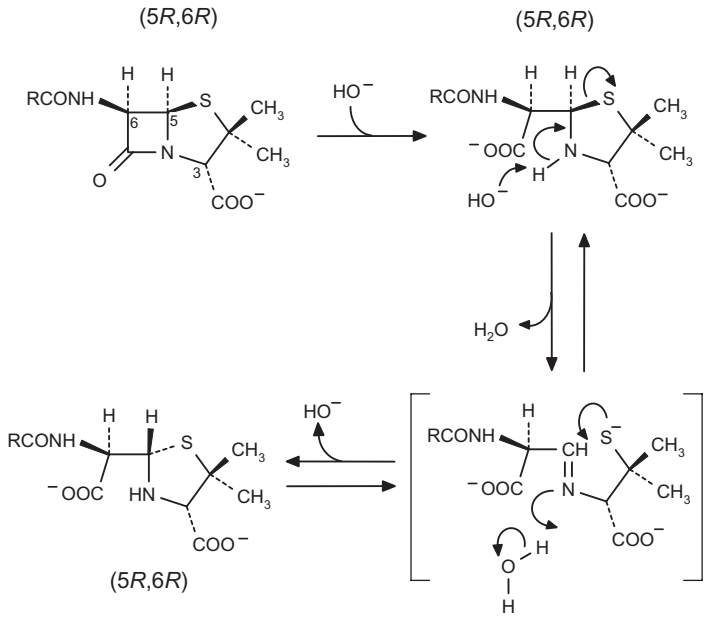
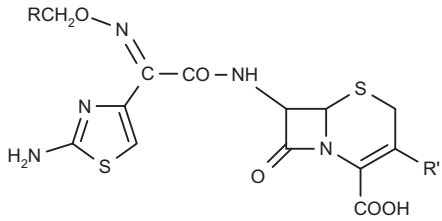


Fig. 5.10. *Base-catalyzed epimerization at C(5) in penicilloic acids involves opening the thiazolidine ring by C–S bond fission, followed by reclosure with inversion of configuration at C(5)* [111]



5.40 R = —COOH R' = —CH=CH$_2$

5.41 R = —H R' = —CH$_2$OCOCH$_3$

opening, a *C(6) epimerization* took place [112]. The resulting products underwent lactonization to produce diastereoisomeric pairs of γ-lactones (*Fig. 5.11*). Interestingly, such compounds were not found in the hydrolysis of cefixime (**5.40**), which has a very similar structure.

The γ-lactone degradation products of cefdinir were found to be also subject to C(6)-epimerization in neutral to basic solutions. The mechanism proposed for this epimerization involves deprotonation of the enamine N-atom,

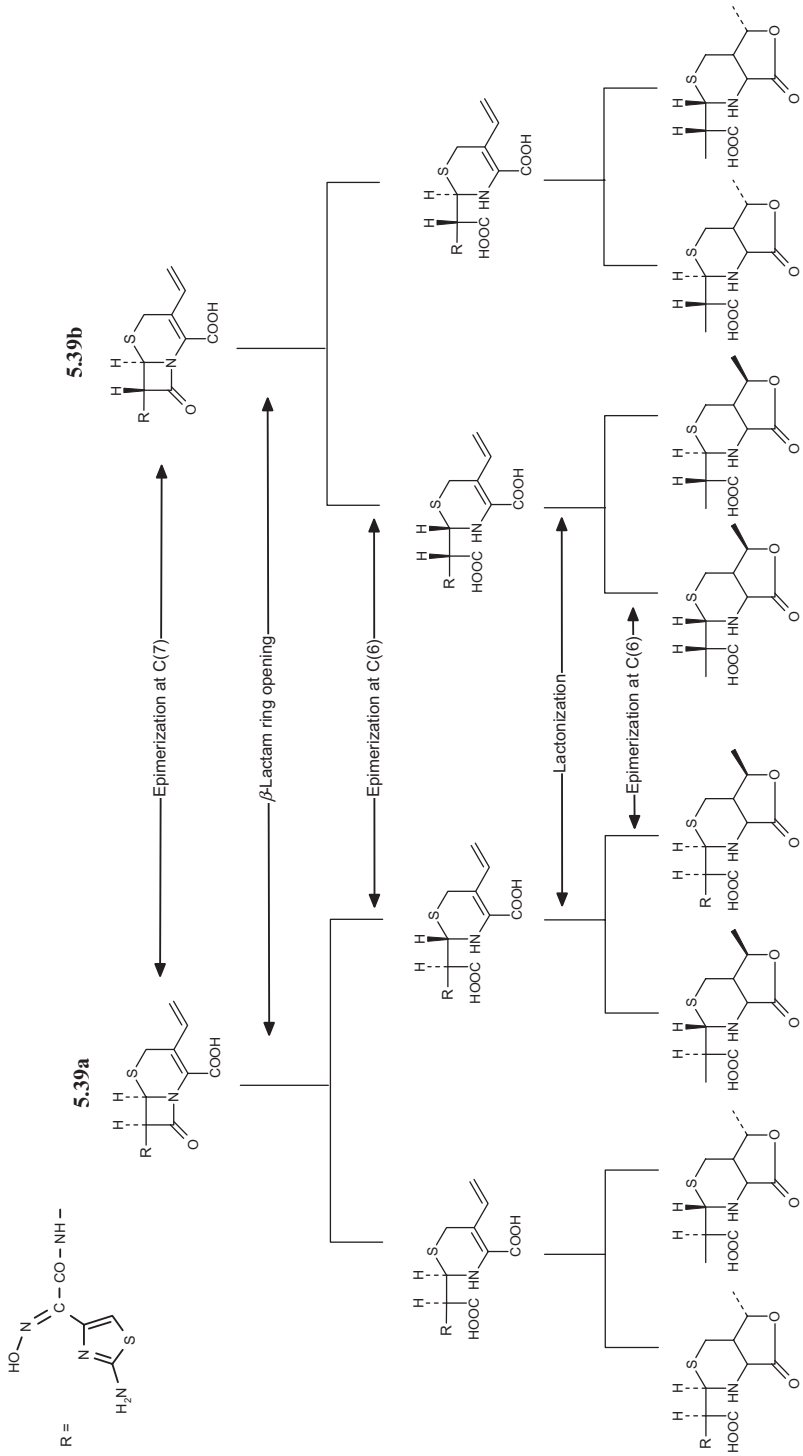


Fig. 5.11. *Family tree of the degradation products of cefdinir* (**5.39a**) *and its 7-epimer* (**5.39b**) [112]

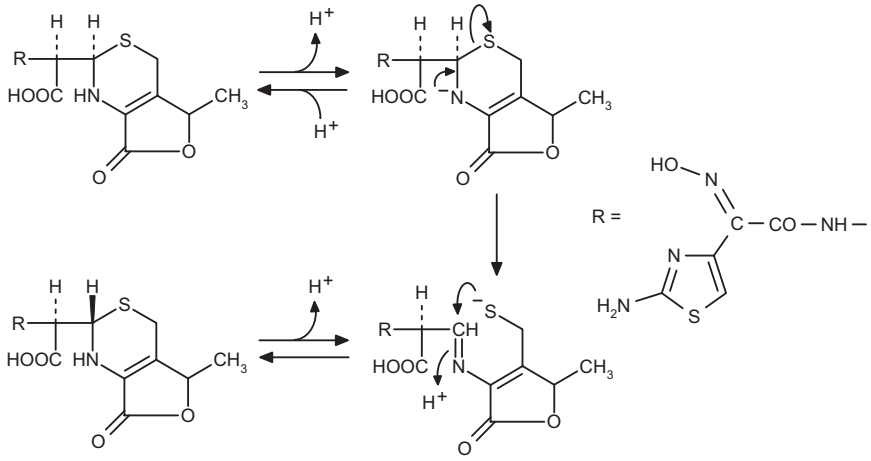


Fig. 5.12. *C(6)-Epimerization of the γ-lactone degradation products of cefdinir and its 7-epimer* (**5.39a** and **b**, *Fig. 5.11*). The mechanism involves deprotonation of the enamine N-atom, fission of the dihydrothiazine ring at the C–S bond, followed by reclosure with inversion of configuration at C(6) [112].

fission of the dihydrothiazine ring at the C–S bond, followed by reclosure with inversion of configuration at C(6) (*Fig. 5.12*) [112]. The electronegativity of the enamine N-atom seems to play an important role in favoring C(6)-epimerization.

Cephalosporins such as cefixime (**5.40**) and cefotaxime (**5.41**) undergo *epimerization at C(7)* under alkaline conditions without preliminary β-lactam ring opening. The epimerization is believed to begin with the removal of the acidic H-atom at C(7). The acidity of this proton derives from the resonance effect of the neighboring carbonyl group (*Fig. 5.13*) enhanced by the presence of electron-withdrawing substituents at C(3′) [113][114].

Hetacillin (**5.42**, *Fig. 5.14*), a prodrug developed to increase the aqueous stability and oral absorption of ampicillin, was found to be the only penicillin showing *epimerization at C(6)* (*Fig. 5.14, Pathway a*). This reaction is analogous to the C(7)-epimerization described above for cephalosporins. The configurational change is initiated by removal of the H-atom at C(6) by a HO^- ion, leading to the formation of a planar carbanion. In aqueous solution at high pH, epimerization rather than conversion to ampicillin (**5.43**, *Fig. 5.14*, *Pathway b*) is the major route of degradation of hetacillin [115].

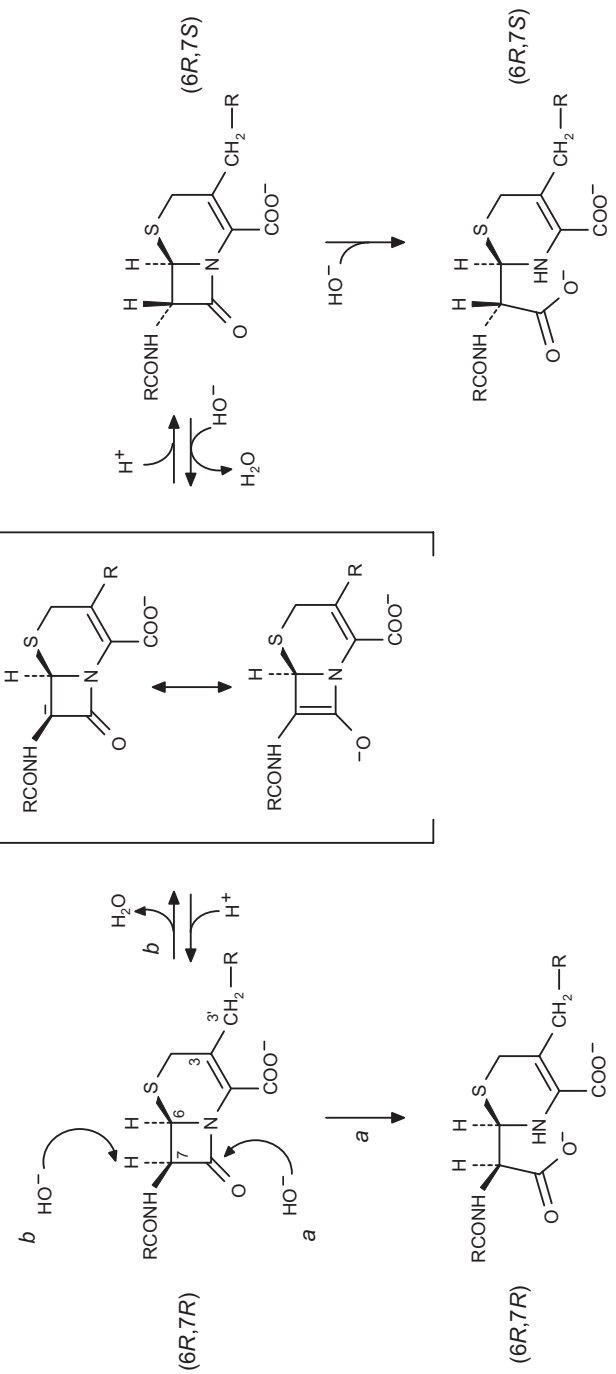


Fig. 5.13. *Base-catalyzed epimerization at C(7) in cephalosporins involves the removal of the acidic H-atom at C(7) (Pathway b).* Electron-withdrawing substituents at C(3′) enhance the acidity of the C(7) proton and, thus, favor epimerization compared to hydrolysis *(Pathway a)* [113][114].

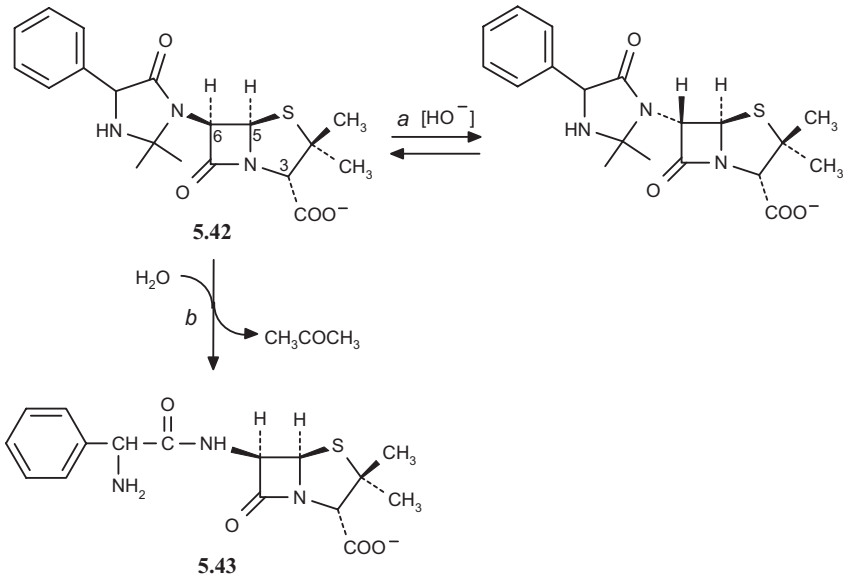


Fig. 5.14. *Degradation of the ampicillin prodrug hetacillin* (**5.42**). In an alkaline medium, *Pathway a* (epimerization at C(6)) is more important than *Pathway b* (hydrolysis producing ampicillin (**5.43**)) [115][116].

5.2.5. Intermolecular Reactions

Some β-lactam antibiotics carry nucleophilic substituents that can react with the lactam bond of a neighboring molecule. Such intermolecular reactions occur with *α-aminopenicillins* such as ampicillin, producing *polymeric degradation products* in aqueous solution (*Fig. 5.15*). The formation of these oligomers involves attack by the α-amino group of one molecule on the β-lactam ring of a second molecule. The degree and rate of polymerization depends on pH, the initial concentration of the compound, and the basicity of its α-amino group [117][118].

Cephalosporins are mostly degraded *via* intramolecular aminolysis (see above, *Fig. 5.8*). However, high drug concentrations may also favor degradation *via* oligomer formation [119]. In the case of loracarbef (**5.44**, *Fig. 5.16*), which is not subject to intramolecular aminolysis, the formation of dimeric structures becomes predominant under moderately acidic conditions [120].

Intermolecular reactions also play an important role in the stability of carbapenems. *Thienamycin* (**5.45**), the first carbapenem discovered, is destroyed very rapidly in aqueous solution; its chemical instability is due to intermolecular aminolysis of the β-lactam induced by the NH_2 group in the C(2) side

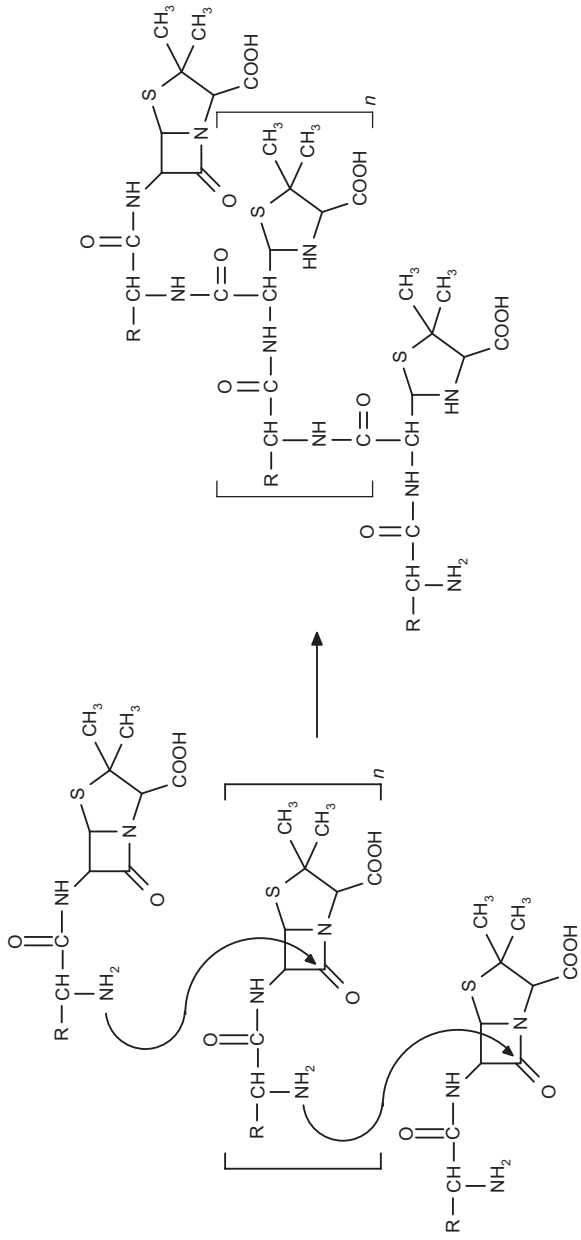


Fig. 5.15. *The oligomer formation of α-aminopenicillins involves the intermolecular attack by the α-amino group on the β-lactam ring* [117]

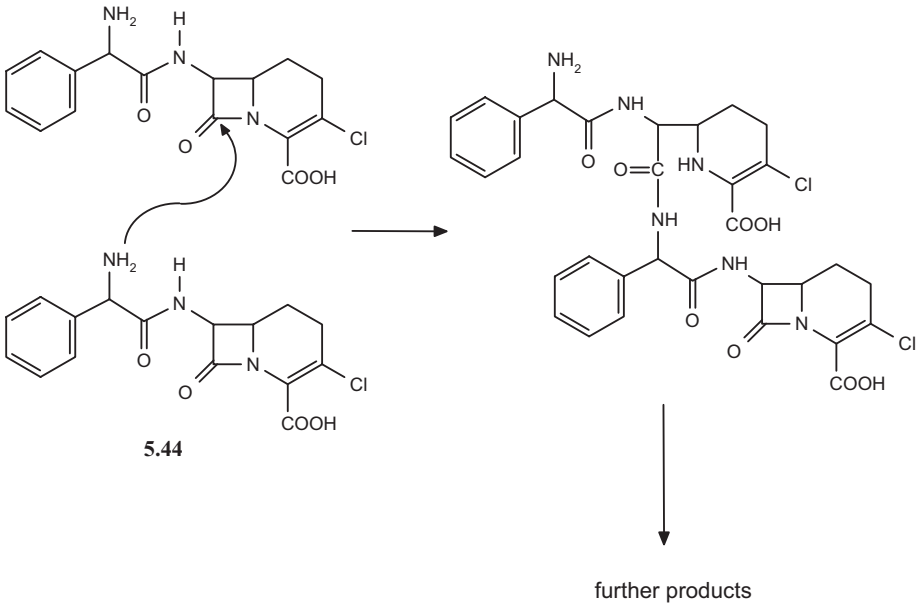
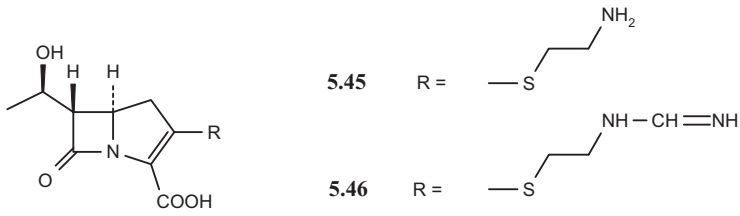


Fig. 5.16. *Dimerization of loracarbef* (**5.44**) [120]



chain [121]. To effectively stabilize the carbapenem nucleus, several derivatives (*e.g.*, imipenem and meropenem) were designed that minimize intermolecular aminolysis through a decrease in basicity and nucleophilicity of the NH_2 group.

Imipenem (**5.46**) has not completely fulfilled such expectations [122]. Indeed, this compound is unstable in both acidic and alkaline media. In weakly acidic solutions, imipenem undergoes complex oligomerization, a reaction initiated by the intermolecular attack of the carboxy group on the β-lactam (*Fig. 5.17*) and yields, finally, a diketopiperazine compound. In weakly alkaline solution, an intermolecular reaction between the β-lactam and (iminomethyl)amino group was observed (*Fig. 5.18*). This reaction proceeds *via* an unstable dimer that breaks down to thienamycin (**5.45**) and a β-lactam ring-opened compound bearing a *N*-formyl group [123].

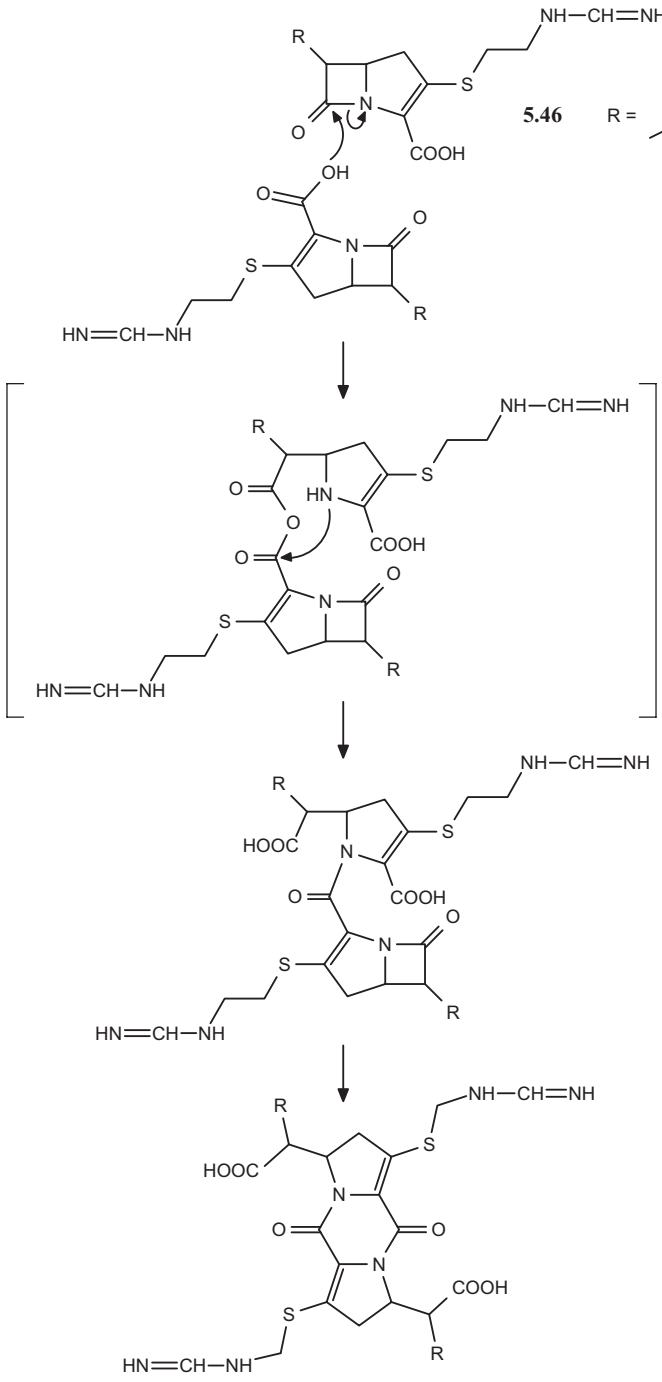


Fig. 5.17. *Acid-catalyzed oligomerization of imipenem* (**5.46**) *starting with an intermolecular attack by the carboxy group on the β-lactam bond* [123]

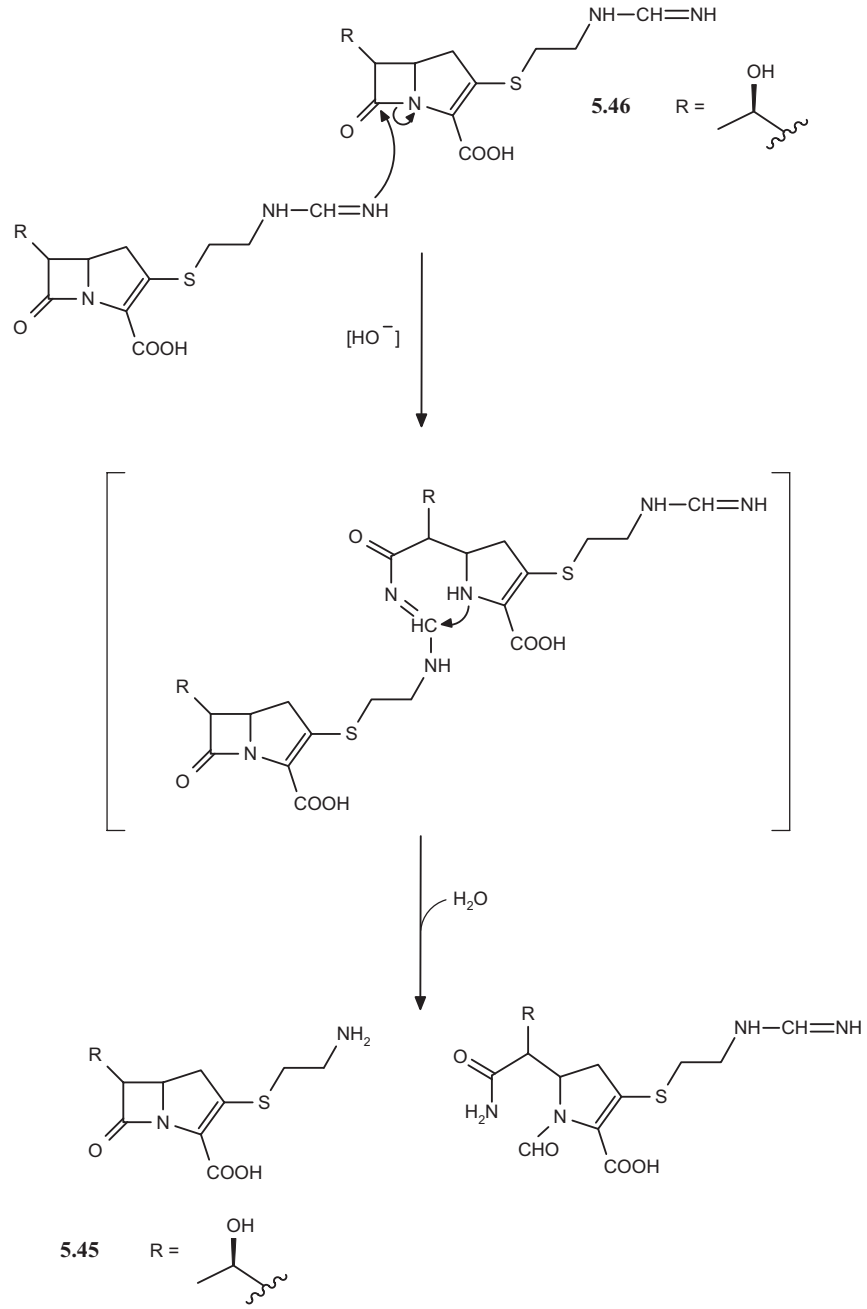
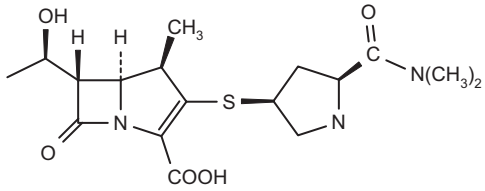


Fig. 5.18. *Base-catalyzed decomposition of imipenem* (**5.46**) *initiated by an intermolecular reaction between the β-lactam and* (*iminomethyl*)*amino group.* The reaction generates one molecule of thienamycin (**5.45**) [123].



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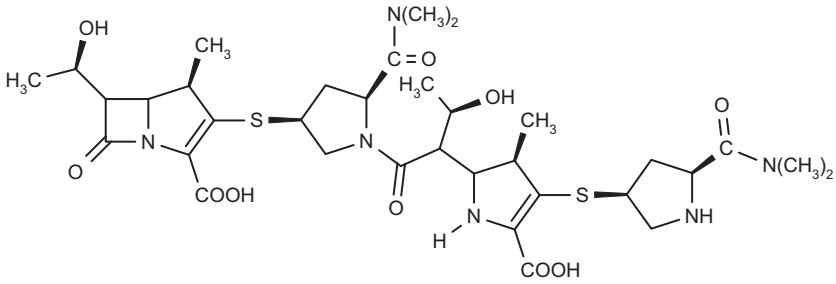
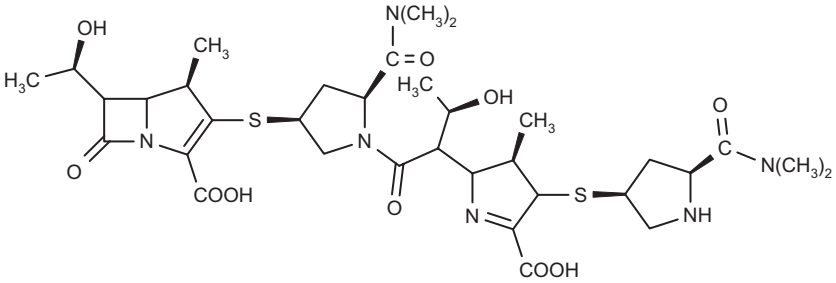


5.48a



5.48b

Efforts to produce more-stable compounds have yielded *meropenem* (**5.47**), which, although superior to other carbapenems, is less-stable than penicillins or cephalosporins. This lack of stability is confirmed by the formation of breakdown products identified as the dimers (**5.48a** and **b**) resulting from intermolecular aminolysis of the β-lactam ring [100].

Interestingly, the formation of dimers and trimers by intermolecular aminolysis has also been observed in concentrated solution (>1%) of the monobactam aztreonam (**5.8**) [124].

5.2.6. External Factors Influencing Stability

The stability of the β-lactam ring is strongly influenced by the molecular factors discussed above and, obviously, also by external factors such as biological fluids or pharmaceutical matrices. Some of these factors are discussed below.

5.2.6.1. *Buffer-Catalyzed Hydrolysis*

The presence of buffers often enhances the rate of hydrolysis of β-lactam antibiotics, *i.e.*, the rate constant of degradation increases linearly with an increase in buffer concentration at constant pH [86][125–130]. Acetate, borate, citrate, and, especially, phosphate buffers have a strong catalytic effect, whereas carbonate buffers are not, or are barely, catalytic. The catalytic effect of buffers on the β-lactam hydrolysis can in most cases be explained by *general acid* and *general base catalysis*. An investigation of the hydrolysis of benzylpenicillin by oxygen bases revealed that the mechanism of catalysis depends on the strength of the base involved [72]. Weak bases such as acetate probably act as a general base catalyst. The reaction with strong bases, however, proceeds by *nucleophile-catalyzed hydrolysis*. Thus, the catalytic effect of phosphate buffer on the degradation of penicillin and ampicillin in neutral aqueous solution has been shown to be *via* a nucleophilic reaction mechanism. This mechanism involves the reaction of the monohydrogen phosphate anion with the β-lactam group to produce an ester intermediate (*Fig. 5.19*) [131]. Similarly, the formation of an ester intermediate is responsible for the increased degradation of penicillins and cephalosporins observed in the presence of polyhydroxy alcohols (sorbitol, mannitol, and glycerol) and various carbohydrates (glucose, fructose, and dextrans) [132].

5.2.6.2. *Metal Ion Catalyzed Hydrolysis*

Transition metal ions cause a dramatic increase in the rate of hydrolysis of β-lactam antibiotics [75][133][134]. For example, *copper(II)* and *zinc(II)* ions increase the rate of alkaline hydrolysis *ca.* 10^8-fold and 10^4-fold, respectively [76]. It has been suggested that the metal ion coordinates with both the carboxylate group and the β-lactam N-atom of *penicillins* (**A**, *Fig. 5.20*). This complex stabilizes the tetrahedral intermediate and, thus, facilitates cleavage of the C–N bond catalyzed by the HO^- ion [74][75]. Such a model appears applicable also to clavulanic acid, imipenem, and monobactams, but it re-

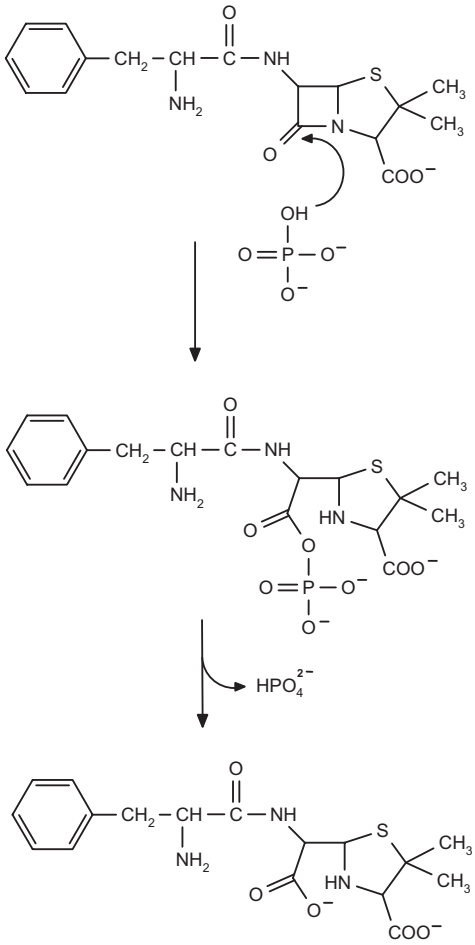


Fig. 5.19. *Nucleophilic phosphate-catalyzed degradation of penicillins involving the formation of a penicilloyl phosphate intermediate* [131]

mains hypothetical [123][135]. Based on kinetic studies, *Hay et al.* [136] proposed that Cu^{II} binds to the deprotonated N-atom of the amide side chain. In this case, β-lactam hydrolysis of benzylpenicillin involves intramolecular attack on the carbonyl group by a Cu-coordinated hydroxide species (**B**, *Fig. 5.20*).

In *cephalosporins*, the lactam N-atom has a lower basicity due to enamine conjugation and a geometry that is less favorable for binding metal ions. Nevertheless, cephalosporins bind Cu^{II} ions ten times more tightly than penicillins, suggesting that the metal ion coordination site is different from that of the penicillins. Indeed, molecular models of cephalosporins seem to

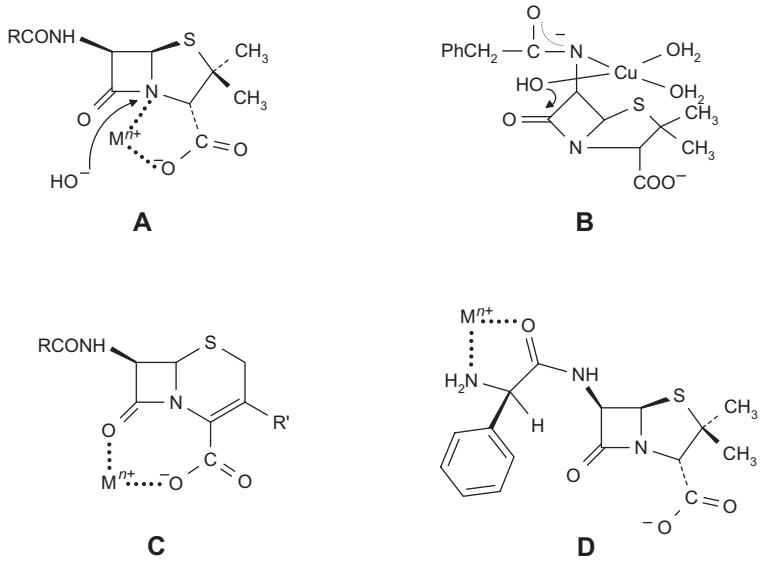


Fig. 5.20. *Modes of coordination of transition metal ions with β-lactam antibiotics.* Complex **A**: In penicillins, the metal ion coordinates with the carboxylate group and the β-lactam N-atom. This complex stabilizes the tetrahedral intermediate and facilitates the attack of HO^- ions from the bulk solution. Complex **B**: In benzylpenicillin Cu^{II} binds to the deprotonated N-atom of the amide side chain. The hydrolysis involves an intramolecular attack by a Cu-coordinated HO^- species on the carbonyl group. Complex **C**: In cephalosporins, coordination of the metal ion is by the carbonyl O-atom and the carboxylate group. Because the transition state is less stabilized than in **A**, the acceleration factor of metal ions for the hydrolysis of cephalosporins is lower than for penicillins. Complex **D**: β-Lactams with a basic side chain bind the metal ion to the carbonyl and the amino group in their side chain. This binding mode does not stabilize the tetrahedral transition complex and, therefore, does not affect the rate of hydrolysis.

indicate that coordination occurs between the carbonyl O-atom and the carboxylate group (**C**, *Fig. 5.20*). As a result, metal-catalyzed hydrolysis of cephalosporins is characterized by a transition state of lower stability and a lower acceleration factor than for penicillins [75].

Another chelate structure exists for *β-lactams bearing a basic side chain*. In ampicillin and cephalexin, *e.g.*, the metal ion is bound by the carbonyl and amino groups (**D**, *Fig. 5.20*) [137]. When so attached, the metal ion does not appear to stabilize the tetrahedral transition-state complex, and, indeed, Cu^{II} ions did not significantly affect the hydrolysis of cefaclor [125].

The way in which β-lactams interact with metal ions is obviously important to their stability in biological fluids and pharmaceutical preparations. Studies with metal ions have also been undertaken to understand how zinc-dependent β-lactamases hydrolyze β-lactams (see *Chapt. 3*) [135][138][139].

5.2.6.3. *Micelle-Catalyzed Hydrolysis*

Surface-active agents used as adjuvants in pharmaceutical preparations to improve drug dissolution may affect the stability of β-lactams. Thus, the presence of micelles of cetyl(trimethyl)ammonium bromide (CTAB) enhanced up to 50-fold the rate of alkaline hydrolysis of penicillins [140]. In the case of cephalosporins, micelle-promoted catalysis of the intramolecular degradation process (see *Sect. 5.2.2*) was also observed [85][141]. It has been proposed that the negatively charged penicillins and cephalosporins are attracted by the cationic micelles. This attraction increases substrate concentration in the micellar phase, in turn accelerating the rate of HO^- ion attack. Ion exchange at the micellar surface and electrostatic stabilization of the transition state may also contribute to the increased rate [142][143].

The failure of anionic micelles to promote HO^--catalyzed hydrolysis can be attributed to electrostatic repulsion between β-lactams and micelles. The same explanation holds for the inhibition of acid-catalyzed degradation by cationic micelles [140][144].

5.2.6.4. *Interactions with Serum Albumin*

Penicillins and cephalosporins bind irreversibly to serum albumin. It has been shown that drug–protein conjugates result from the aminolysis of the β-lactam bond by the ε-amino group of lysine residues in the protein (*Fig. 5.1*, *Pathway e*). The bound penicilloyl group appears to be the major antigenic determinant of penicillin allergy [145–148].

The metabolic fate of the benzylpenicillin–human serum albumin conjugate was studied in rats [149]. The conjugate was taken up by the liver, where it underwent enzymatic cleavage to form benzylpenicilloic acid. Thus, the benzylpenicilloic acid excreted in urine may be formed either by direct hydrolysis of the β-lactam ring, or by catabolism of protein conjugates formed *in vivo*.

5.2.7. Metabolism in Mammals

The previous sections have covered both degradation by microbial enzymes (β-lactamases) and chemical hydrolysis. We now turn to considerations of the mammalian metabolism of β-lactams involving the opening of the β-lactam ring.

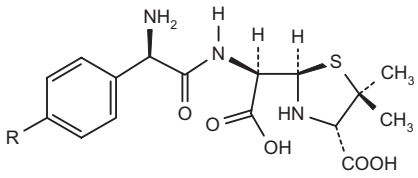
Only a limited number of papers describe the isolation and identification of mammalian metabolites of penicillins and cephalosporins, which is sur-

prising when one considers the extensive literature devoted to the chemical and enzymatic stability of these antibiotics (see above). In the past, the identification of penicillin and cephalosporin metabolites was not a priority, probably because of the low toxicity of these drugs and because a major portion of a dose is usually excreted unchanged.

In metabolic studies of β-lactam antibiotics, there is a major difficulty in distinguishing between enzymatic and chemical reactions. A review of relevant studies shows the importance of the analytical methodology to differentiate the products of *metabolic and chemical hydrolysis*.

High-performance liquid chromatography (HPLC) is often used to detect penicillin metabolites in biological samples [98][150]. The (5*S*)- and (5*R*)-epimers of penicilloic acids (**5.49a** and **b**) as well as piperazine-2,5-diones **5.50** were identified in biofluids and are regarded as metabolites of ampicillin and amoxycillin [110][151]. However, the degradation of penicillins may continue during analysis, making it difficult to decide which of the detected compounds are true metabolites and which are simply degradation products.

Mass spectrometry coupled to liquid chromatography (LS/MS) eliminates the need for derivatization and, thus, circumvents one of the perturbing factors. Using an LS/MS method, *Suwanrumpha* and *Freas* [152] identified the above-mentioned metabolites of ampicillin (**5.49** and **5.50**) in human



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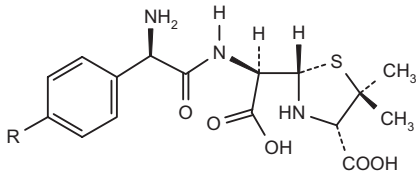
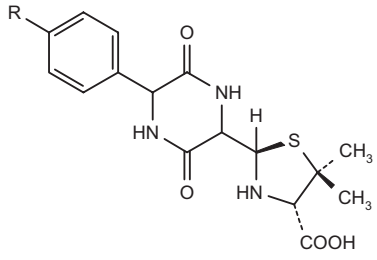


5.49b



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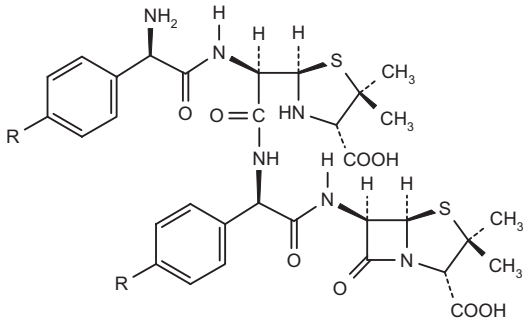
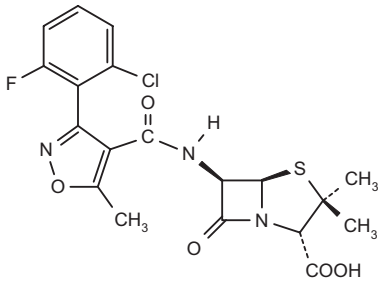


5.51

urine and showed the presence of both the (5*S*)- and (5*R*)-epimers of the penicilloic acid derived from ampicillin. It was speculated that only the (5*R*)-epimer is eliminated from the body, and that the (5*S*)-epimer detected in urine samples is an artefact.

Nuclear magnetic resonance (NMR) spectroscopy of untreated biological fluids has been used successfully in metabolic studies of penicillins. *Connor et al.* [153] used this method to investigate the metabolism and urinary excretion of ampicillin and amoxycillin in humans and rats. In addition to the metabolites **5.49** and **5.50**, they detected a dimer of amoxycillin (**5.51**) in rat urine.

In ^{1}H- and ^{19}F-NMR spectroscopy studies of the metabolites of flucloxacillin (**5.52**) in rat urine, the presence of both (5*R*)- and (5*S*)-flucloxacillin penicilloic acids was demonstrated [154]. During the first collection period, the concentration of the (*R*)-epimer largely exceeded that of the (*S*)-epimer. The (*R*)/(*S*) ratio then decreased progressively until an excess of (*S*)-epimer was reached. These findings are in agreement with the hypothesis that the (*S*)-epimer is formed by epimerization in urine (see above) [152].



5.52

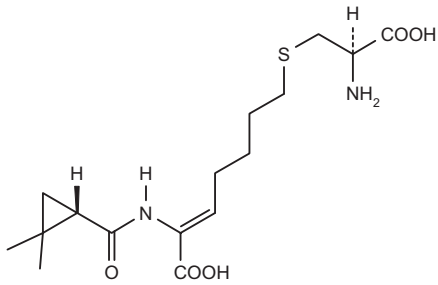
Even when hydrolysis and epimerization can be avoided during sample preparation and handling, it is not possible to conclude definitively whether the compounds found in plasma and urine are true metabolites or simply degradation products. Indeed, chemical degradation can also occur within the body since urine and plasma contain a wide variety of potential catalysts, including metal ions, phosphate ions, proteins, and sugars (see *Sect. 5.2.6*). Whereas the existence of mammalian enzymes that act on penicillins and cephalosporins is considered possible [155], no such mammalian enzyme appears to have been identified to date.

In contrast to penicillins and cephalosporins, for which it is unclear whether metabolism takes place, there is proof that penems and carbapenems are metabolized in mammals. Indeed, penems and carbapenems, although re-

sistant to microbial β-lactamases, undergo significant renal metabolism [156][157]. The responsible enzyme has been identified as the renal tubular brush border *dihydropeptidase I* (DHP-I, EC 3.4.13.11) [158][159]. Penicillins, cephalosporins, and monobactams have been shown to be insensitive to DHP-I [160].

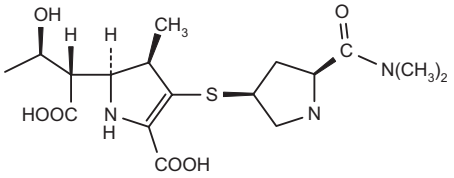
Chemical modifications have been carried out in the *carbapenem* series with a view to reduce the extent of metabolic degradation. The first compound in this series was thienamycin (**5.45**), which was found to undergo extensive metabolism in rodents and monkeys [121][156]: the degree of metabolic degradation, together with its chemical instability, have prevented marketing of this compound.

The first carbapenem released for clinical use was *imipenem* (**5.46**), a compound with relatively high resistance to microbial β-lactamases. The addition of the (iminomethyl)amino side chain renders imipenem chemically more stable than thienamycin. But, like thienamycin, imipenem is also easily hydrolyzed by renal dehydropeptidase I, producing a mixture of β-lactam ring-opened 1-pyrrolidine epimers at C(3) [161]. The renal metabolism of imipenem can be minimized by co-administration of cilastatin (**5.53**), a competitive inhibitor of DHP-I [156][162].

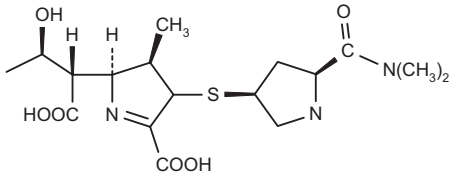


5.53

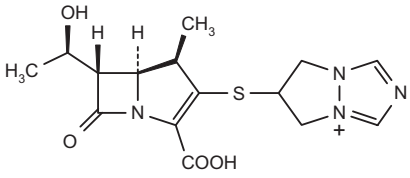
Further improvement of chemical and biological stability has been obtained by the introduction of a 1β-Me substituent in the carbapenem structure [163]. Thus, meropenem (**5.47**) is remarkably resistant to human renal dehydropeptidase I and does not require co-administration of a DHP-I inhibitor for clinical use. After intravenous administration to humans, *ca.* 70% of a dose was recovered in urine unchanged. Ring-opened forms of the compound, *i.e.*, a mixture of the two isomers of meropenoic acid (**5.54a** and **5.54b**), accounted for the remainder of the dose [100][164]. Substituents other than the 1β-Me group may also enhance resistance to DHP-I. Thus, both the 1β-Me group and the triazolium ring appear to be responsible for the enhanced resistance of LJC 10,627 (**5.55**) to metabolic degradation [165].



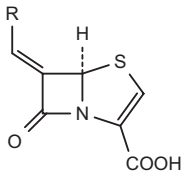
5.54a



5.54b



5.55



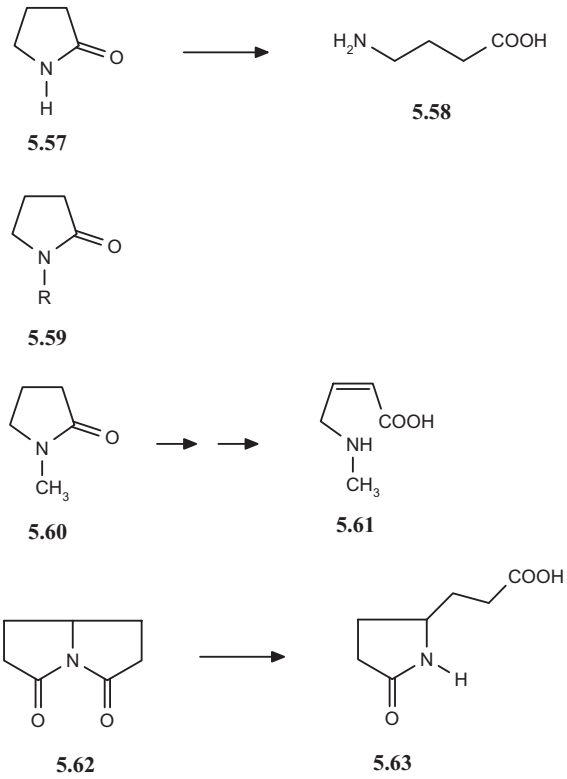
5.56

Like carbapenems, several *penems* have also been found to be susceptible to renal dehydropeptidase degradation [165–167]. 6-Substituted methylidene-penems **5.56** are very potent broad-spectrum inhibitors of bacterial β-lactamase, with the inhibitory activity residing predominantly in the (5*R*)-enantiomers. As a rule, the (5*S*)-enantiomers are less stable than the (5*R*)-enantiomers toward DHP-I [168].

5.3. Simple Lactam Derivatives

This section is devoted to the metabolic fate of lactam rings with five or more members but no additional heteroatoms, a group of structures we categorize as simple lactams.

The simplest *five-membered lactam* is *pyrrolidin-2-one* (**5.57**), the cyclic form of γ-aminobutyric acid (GABA (**5.58**)), an important inhibitory neurotransmitter in the central nervous system (CNS). Systemically administered GABA does not produce pharmacological effects since it does not cross the blood–brain barrier. In contrast, the more lipophilic pyrrolidin-2-one readily penetrates the CNS. Further, brain slices hydrolyze pyrrolidin-2-one, leading to the suggestion that it might serve as a prodrug of GABA. *Callery et al.* [169] detected labeled GABA in mouse brain after intravenous administration of deuterium-labeled pyrrolidin-2-one. However, the increase in GABA concentration in the brain was less than 1%. This small increase may be rationalized by the slow rate of hydrolysis of pyrrolidin-2-one and the rapid turnover of GABA in the brain.

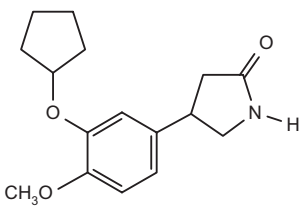


5.57 **5.58** **5.59** **5.60** **5.61** **5.62** **5.63**

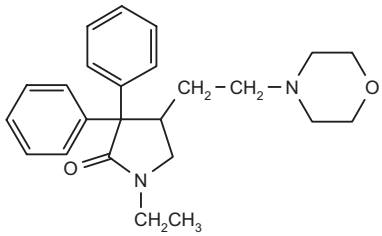
More recently, *Nakamura et al.* [170] again took up the concept of GABA prodrugs based on pyrrolidin-2-one. They tested the lipophilic derivatives 1-dodecanoylpyrrolidin-2-one (**5.59**, R = dodecanoyl) and 1-dodecylpyrrolidin-2-one (**5.59**, R = dodecyl). The former was degraded to GABA in mouse liver and in brain homogenates, whereas the latter was stable. However, neither derivative increased GABA levels in mouse brain after intraperitoneal administration.

Little is known on the metabolic fate of *1-methylpyrrolidin-2-one* (**5.60**), an industrial solvent also useful as a solubilizing agent and a penetration enhancer in topical formulations. A preliminary investigation of the disposition and metabolism of labeled 1-methylpyrrolidin-2-one in the rat showed that the compound is excreted mainly in urine [171]. Three urinary metabolites were detected, the major of which (*ca.* 75% of the dose) was 4-(methylamino)but-2-enoic acid (**5.61**). This unsaturated product may likely have been formed by H_2O elimination from a hydroxylated metabolite.

Rolziracetam (**5.62**), a nootropic agent, has a structure containing two fused lactam rings. This compound is stable in buffer solutions, but is rapidly metabolized in laboratory animals, yielding exclusively the ring-opened



5.64



5.65

metabolite **5.63** [172]. The remaining intact pyrrolidin-2-one ring appeared to be resistant to hydrolysis.

Substituted pyrrolidin-2-ones are generally resistant to metabolic hydrolysis as seen in the cases of rolipram and doxapram. Metabolic studies of the antidepressant *rolipram* (**5.64**) in several mammalian species, including humans, did not reveal any cleavage of the pyrrolidinone ring [174]. Similarly, no metabolite with an opened pyrrolidin-2-one ring was found for *doxapram* (**5.65**), a respiratory stimulant [174].

We will now consider *supidimide* (**5.66**) as an example of a molecule containing a *six-membered lactam ring*. The piperidin-2-one ring of this potential sedative underwent slow chemical hydrolysis in buffer solution to yield **5.67**, but was resistant to metabolic hydrolysis. The other amide bond was stable [175]. Supidimide was primarily metabolized by oxidation of the piperidin-2-one ring to yield a glutarimide ring, which then was hydrolyzed as described in *Sect. 4.4*.

5.66 **5.67**

Caprolactam (**5.68**, *Fig. 5.21*) has a *seven-membered lactam ring* and is a major industrial compound in the production of *Nylon*™, its polymer. This compound shows only moderate levels of toxicity in mice and rats when administered orally. The hydrolysis product 6-aminohexanoic acid (**5.69**) was a minor metabolite in rats [176]. Hydroxylation in the γ-position to yield **5.70** without preliminary hydrolysis of the lactam linkage has been shown to be the major metabolic pathway. This metabolite hydrolyzes in urine to produce 6-amino-4-hydroxyhexanoic acid (**5.71**), which is in equilibrium with the corresponding lactone (**5.72**).

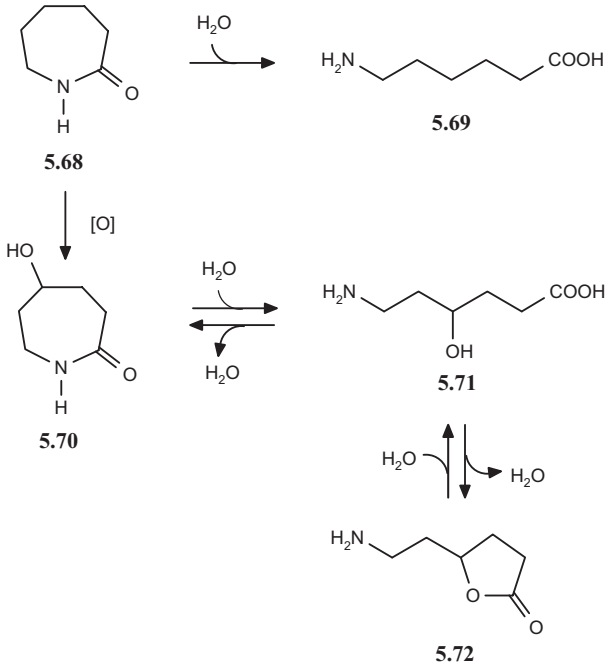
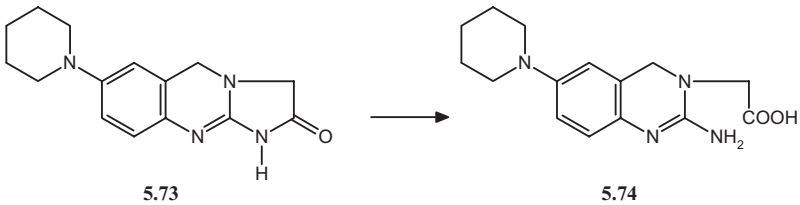


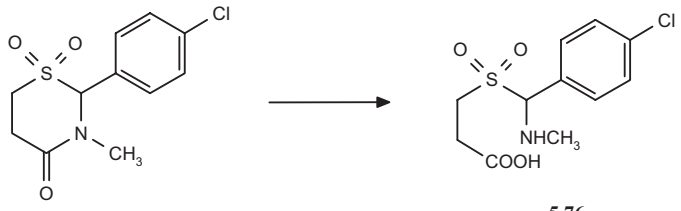
Fig. 5.21. *Metabolism of caprolactam* (**5.68**) *in rats.* Hydrolysis to 6-aminohexanoic acid (**5.69**) represents a minor pathway. Hydroxylation in the γ-position to yield **5.70** is the major metabolic pathway. Metabolite **5.70** hydrolyzes in urine to produce 6-amino-4-hydroxyhexanoic acid (**5.71**), which is in equilibrium with the corresponding lactone **5.72** [176].

5.4. Complex Lactam Derivatives

We now examine the metabolic fate of lactam bonds located in rings containing an additional heteroatom, designated here as *complex lactams*. Our first example is *DN-9893* (**5.73**) a platelet-aggregation inhibitor [177]. Its ring-opened metabolite **5.74** was detected in rat urine after intravenous administration of DN-9893. However, insufficient evidence exists to determine whether hydrolysis of the lactam ring was enzymatic or nonenzymatic.

The main human plasma metabolite of *chlormezanone* (**5.75**), a muscle relaxant, was found to be the ring-opened compound **5.76**. Since hydrolysis



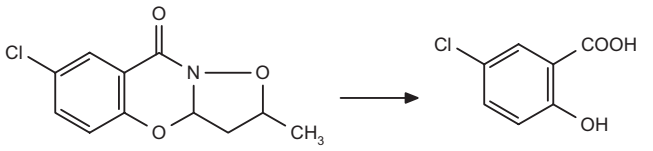


5.75 **5.76**

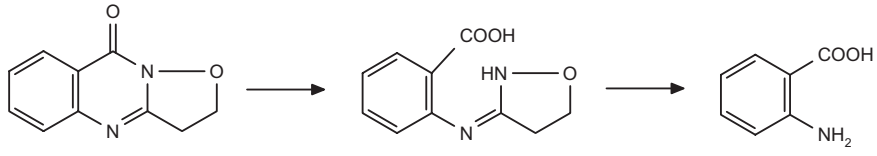
of the amide bond in the six-membered heterocyclic ring also occurs readily in buffers, it can be assumed that a nonenzymatic reaction contributes at least in part to the formation of this metabolite [178]. The ring-opened metabolite **5.76** is structurally related to the muscle relaxant baclofen (see below, **5.93**, *Fig. 5.25*). This similarity suggests that the ring-opened metabolite **5.76** might be pharmacologically active.

No intact drug was found in human plasma after oral administration of *seclazone* (**5.77**), an anti-inflammatory agent. During absorption, the compound undergoes extensive first-pass metabolism, yielding 5-chlorosalicylic acid (**5.78**) [179]. The sequence of metabolic cleavage reactions has been established for an analogue of seclazone, 2,3-dihydro-9*H*-isoxazolo[3,2-*b*]quinazolin-9-one (*W-2429*, **5.79**). Hydrolysis of the pyrimidone ring yields 3-[(2-carboxyphenyl)imino]isoxazolidine (**5.80**), which is converted in turn to anthranilic acid (**5.81**) [180].

Hydrolytic cleavage of a seven-membered ring occurs in the metabolism of chlordiazepoxide (**5.82**, *Fig. 5.22,a*) and other *benzodiazepines* (see also *Sect. 11.9*). The 'lactam ring opened' metabolite **5.83** was detected in humans and dogs and is believed to be generated by hydrolysis of the intermediate lactam [181][182]. However, the diazepine ring can be split by other mech-



5.77 **5.78**



5.79 **5.80** **5.81**

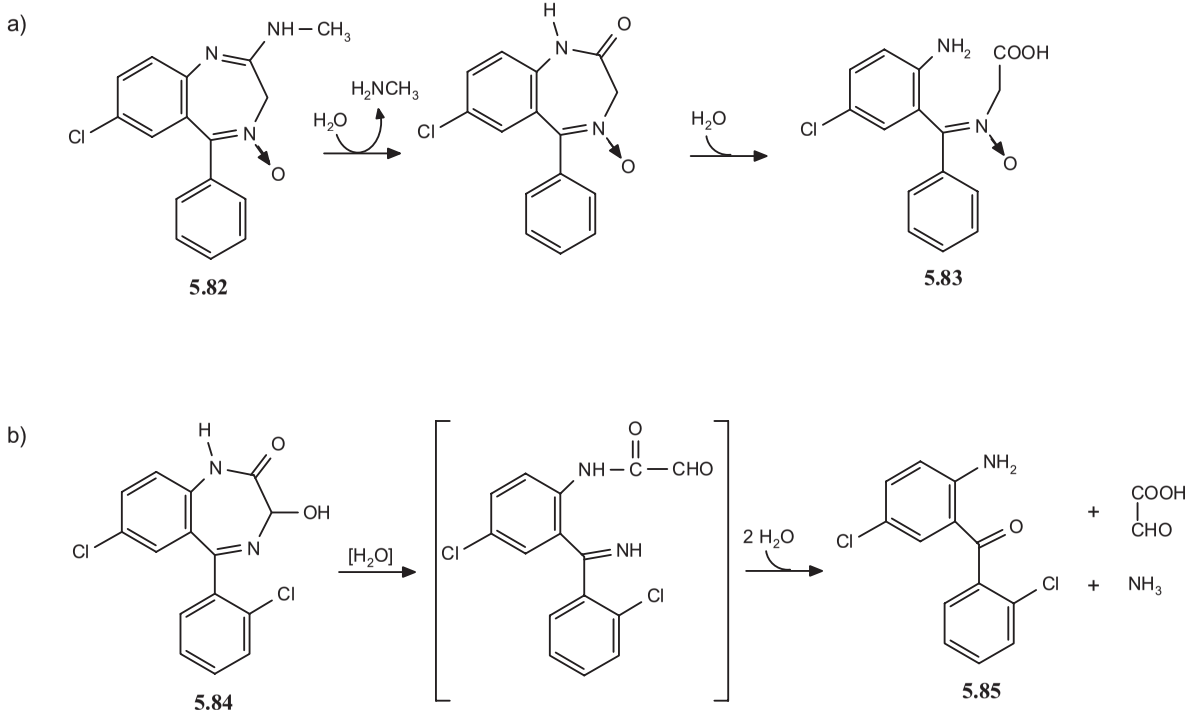


Fig. 5.22. *Two mechanisms for the cleavage of the diazepine ring. a*) In chlordiazepoxide (**5.82**), the ring-opened metabolite **5.83** is generated by hydrolysis of the intermediate lactam [181][182]. *b*) In oxazepam (**5.84**), ring opening occurs *via* an imino aldehyde produced by hydrolysis of the carbinolamine bond. The benzophenone derivative **5.85** is produced *via* hydration of the C=N bond to form the keto group and by hydrolytic cleavage of the amide bond [183].

anisms. Thus, ring opening in *oxazepam* (**5.84**, *Fig. 5.22,b*) occurs *via* an imino aldehyde produced by hydrolysis of the carbinolamine bond. Benzophenone derivatives such as metabolite **5.85** are produced *via* hydration of the C=N bond to form the keto group (*Sect. 11.6*) and by hydrolytic cleavage of the amide bond [183].

5.5. Lactam Metabolites

5.5.1. Formation of Lactam Metabolites

Azaheterocycles can undergo oxidation at endocyclic α-C-atoms, forming metabolites with a lactam structure. In some cases, ring-opened metabolites (*i.e.*, ω-amino acids) can also be detected. One might expect that these metabolites are the result of the hydrolytic cleavage of the lactam bond. However, comprehensive studies have revealed another mechanism for ring-cleavage (*Fig. 5.23*). A detailed discussion of this mechanism can be found elsewhere (Chapt. 6 in [184]). Briefly, the first step is an α-hydroxylation by cytochrome P450 (*Fig. 5.23*, *Reaction a*). The resulting unstable carbinolamine is in equilibrium with an open-chain amino aldehyde. Two metabolic reactions can follow. In the first, aldehyde oxidase (EC 1.2.3.1) converts the carbinolamine to the lactam derivative (*Fig. 5.23*, *Reaction b*). In the second alternative, aldehyde dehydrogenase transforms the ring-opened tautomer into the ω-amino acid derivative (*Fig. 5.23*, *Reaction c*).

The metabolism of most five-membered azaheterocycles such as *pyrrolidines* can be described by the scheme given in *Fig. 5.23*. Thus, nicotine (**5.86**, *Fig. 5.24*), tremorine (**5.89**, *Fig. 5.24*), a pharmacological reagent used to produce experimental parkinsonism, and the antidepressant *prolintane* (**5.90**, *Fig. 5.24*) are metabolized to both lactam and ω-amino acid derivatives [185–187].

In contrast to these examples, the antibacterial agent piromidic acid (**5.91**, *Fig. 5.24*) is not converted to a lactam derivative but yields only the ω-amino acid derivative [188]. Steric features might underlie this metabolic difference. Bulky groups in close proximity to the N-atom seem to favor the formation of the amino acid metabolite, whereas substrates with an unhindered N-atom seem to favor the formation of a lactam metabolite.

Based on the idea that pyrrolidines can be metabolized selectively to γ-aminobutyric acid derivatives, *Wall* and *Baker* [189] developed a '*retro*-metabolic' approach with 3-(4-chlorophenyl)pyrrolidine (**5.92**, *Fig. 5.25*) as a prodrug for the GABA-ergic agent baclofen (**5.93**, *Fig. 5.25*). *In vitro* studies showed that metabolism of **5.92** yields a pair of isomeric lactam metabolites (**5.94**, **5.96**) and a pair of isomeric amino acid metabolites (**5.93**, **5.95**). The formation of the metabolites resulting from the oxidation

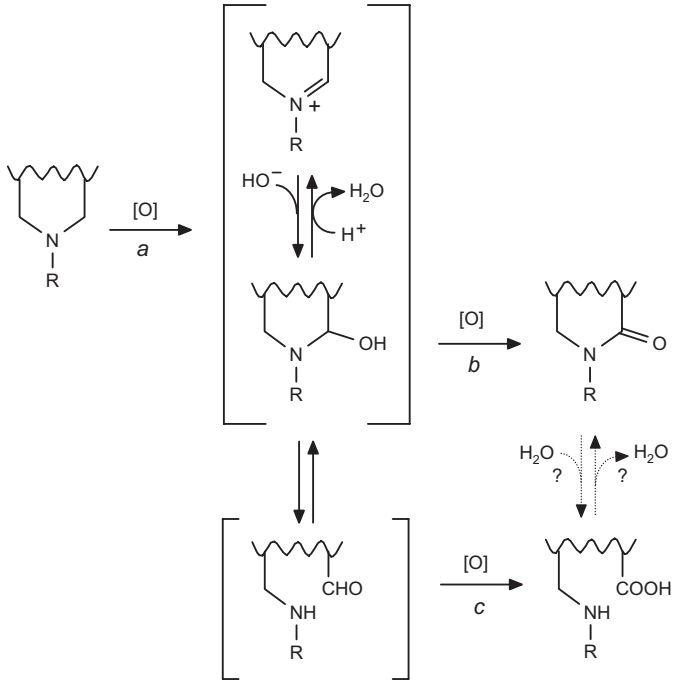


Fig. 5.23. *Mechanism of oxidative opening of azaheterocycles.* Hydroxylation at the α-position (*Reaction a*) yields an unstable carbinolamine, which is in equilibrium with an open-chain amino aldehyde. The carbinolamine can be converted by aldehyde oxidase to a lactam derivative (*Reaction b*), while the open-chain amino aldehyde can be converted by aldehyde dehydrogenase to a ω-amino acid derivative (*Reaction c*).

of the sterically less hindered 5-position (*i.e.*, **5.93**, **5.94**) was greater than that resulting from oxidation of the 2-position (*i.e.*, **5.95**, **5.96**). Furthermore, the lactam metabolites were formed *ca.* 100-fold faster than the amino acid metabolites in rat liver homogenates, while, in brain homogenates, the lactam and amino acid pathways were approximately equivalent. Since the lactam metabolites are not or are very slowly hydrolyzed (see below), the use of **5.92** as a GABA-ergic prodrug is seriously impeded since a significant portion of the dose is lost to nonproductive formation of lactams in the liver.

5.5.2. Hydrolysis of Lactam Metabolites

What about the susceptibility of lactam metabolites to metabolic hydrolysis to yield the corresponding ω-amino acid metabolite? Little is

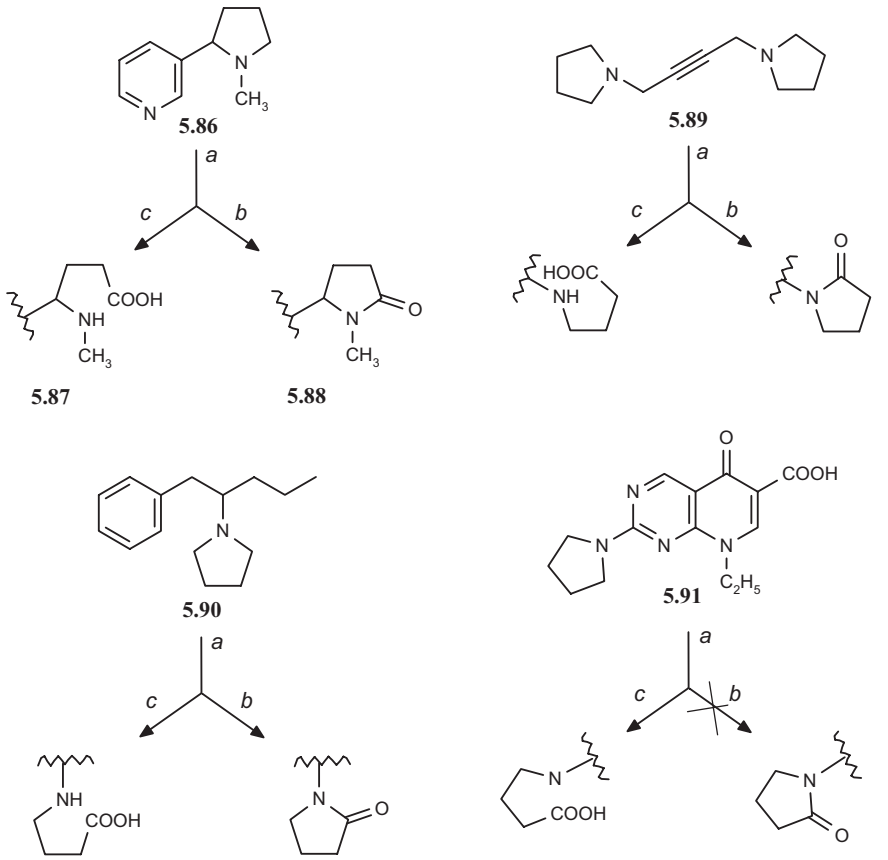


Fig. 5.24. *Metabolism of the pyrrolidine ring according to the mechanism in* Fig. 5.23. Nicotine (**5.86**), tremorine (**5.89**), and prolintane (**5.90**) are metabolized to both lactam and ω-amino acid derivatives [185–187]. Due to steric hindrance next to the N-atom, piromidic acid (**5.91**) yields only the amino acid derivative [188].

known about the hydrolytic stability of the lactam metabolites in the examples given above. This may be due in part to the difficulty in separating and detecting the resulting amino acid metabolites, and in assessing the relative contributions of the direct and indirect pathways in their formation. Valuable information has been obtained from metabolic studies of cotinine (**5.88**, *Fig. 5.24*). This nicotine metabolite is relatively stable and has, therefore, been used to indirectly determine plasma nicotine levels in smokers since its half-life is considerably longer than that of nicotine in humans. The principal metabolites of cotinine (5- and 3-hydroxycotinine, cotinine 1-*N*-oxide, and 4-oxo-4-(pyrid-3-yl)-*N*-methylbutyramide) are

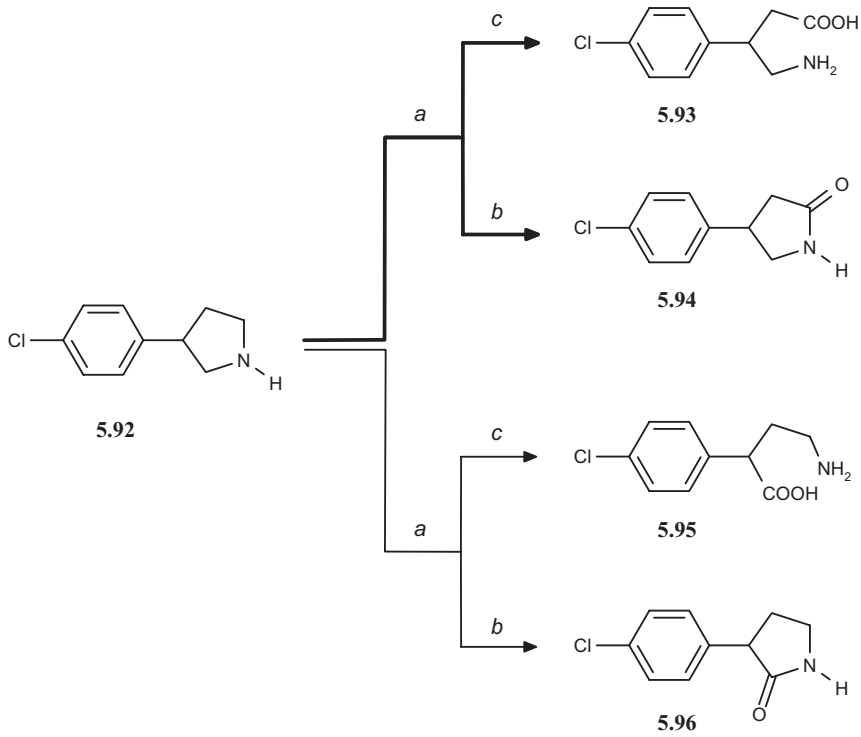


Fig. 5.25. *Metabolism of 3-(4-chlorophenyl)pyrrolidine* (**5.92**) *according to* Reactions a – c *in* Fig. 5.23. The formation of the metabolites resulting from the oxidation of the sterically less-hindered 5-position (**5.93**, **5.94**) is greater than that resulting from oxidation of the 2-position (**5.95**, **5.96**) [189].

formed by oxidative reactions, and no hydrolysis of its lactam ring has been reported.

One may, therefore, assume that the lactam metabolites formed are rather resistant to hydrolytic degradation and do not contribute significantly to the formation of the ω-amino acid derivatives. This is in line with the observation that substituted pyrrolidin-2-ones are generally resistant to metabolic hydrolysis (see *Sect. 5.3*).

Six-membered azaheterocycles (*i.e.*, *piperidino derivatives*) show a metabolic pattern similar to that of pyrrolidine compounds. Thus, the antiemetic drug diphenidol (**5.97**, *Fig. 5.26*) and *DN-9893* (**5.73**) are both metabolized to their ω-amino acid and lactam derivatives [177]. In contrast, no lactam metabolite was detected for phencyclidine (**5.98**, *Fig. 5.26*), a potent analgesic also widely abused [190]. One may assume that, like for piromidic acid (**5.91**, *Fig. 5.24*), steric hindrance at the N-atom is unfavorable for the formation of lactam metabolites.

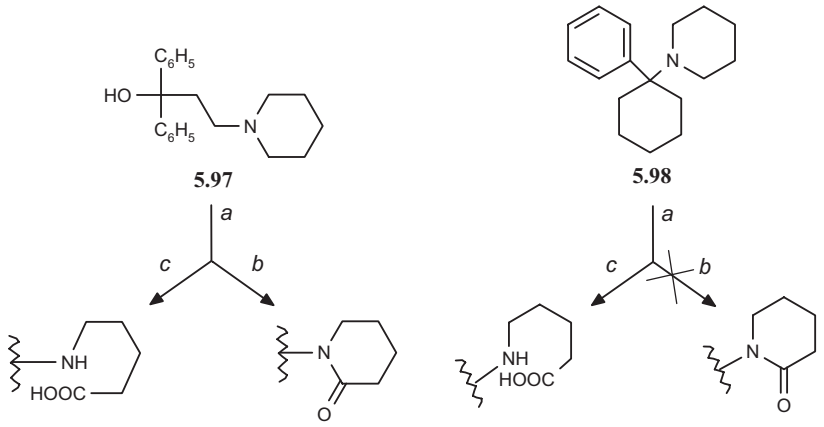
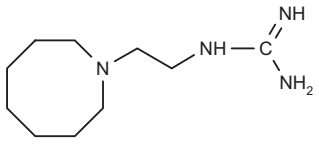


Fig. 5.26. *Metabolism of the piperidine ring according to the mechanism in* Fig. 5.23. Diphenidol (**5.97**) and DN-9893 (**5.73**) yield both amino acid and lactam metabolites [177]. Phencyclidine (**5.98**) yields only the amino acid derivative; steric hindrance at the N-atom appears to impede formation of the lactam metabolite [190].



5.99

The azocane derivative *guanethidine* (**5.99**), an antihypertensive agent, is largely converted to the *ω*-amino acid metabolite without formation of a lactam metabolite [191], a reactivity that is difficult to explain.

To conclude this section, we present two azaheterocycles that contain two heteroatoms and undergo metabolic ring-opening reactions. *Morpholino derivatives*, such as the antidepressant viloxazine (**5.100**, *Fig. 5.27*) and the analgesic CERM 1841 (**5.101**) yield morpholinone and ring-opened metabolites [192][193]. In these early studies, the morpholinone metabolites were considered precursors of the ring-opened metabolites. Subsequently, it has been shown for the antidepressant indeloxazine (**5.102**) that the morpholinone metabolite is not an intermediate in the formation of the ring-opened metabolites [194]. It is, therefore, reasonable to assume that ring opening occurs directly in analogy to pyrrolidine and piperidine derivatives (see above).

The *imidazoline ring* has also medicinal significance. The oral hypoglycemic agent midaglizole (**5.103**) undergoes little metabolism in humans and is excreted mainly unchanged. In dogs and rats, however, cleavage of the 2-imidazoline ring represents one of the principal metabolic routes [195]. The metabolic pattern of the closely related iminoimidazolidine derivative cloni-

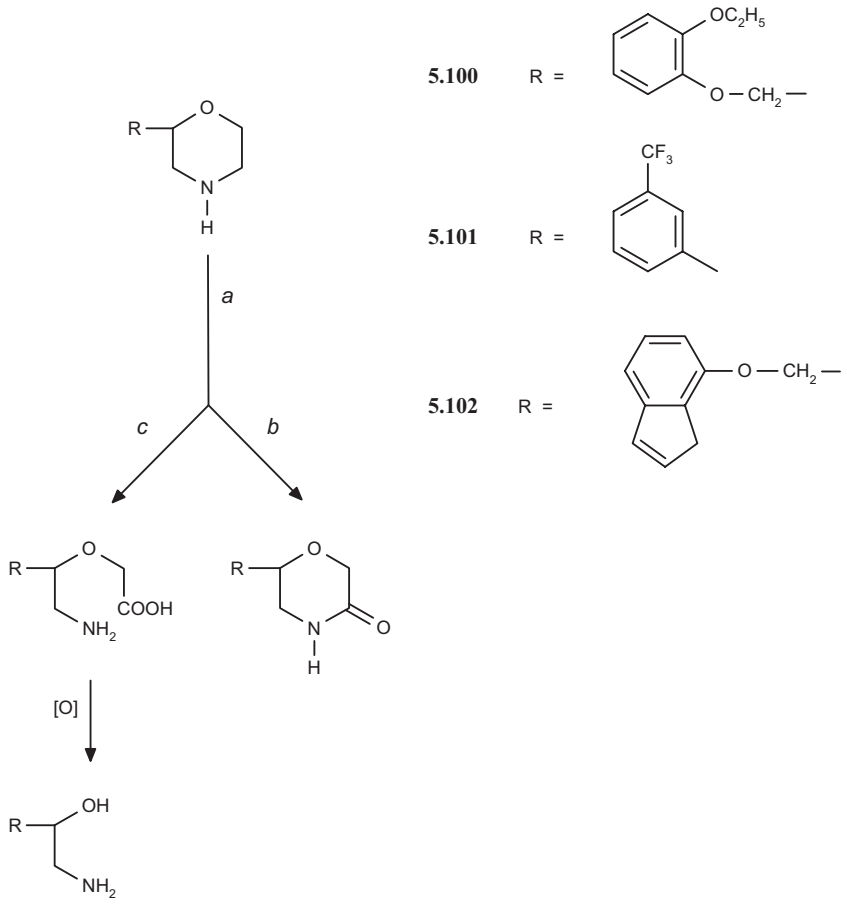
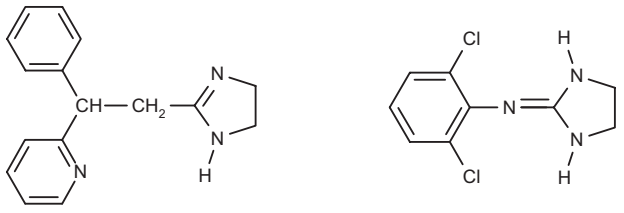


Fig. 5.27. *Metabolism of the morpholino moiety in viloxazine* (**5.100**), *CERM 1841* (**5.101**), *and indeloxazine* (**5.102**) *according to the mechanism in* Fig. 5.23 [194]



5.103 **5.104**

dine (**5.104**), an antihypertensive agent, is very similar [196]. It is reasonable to assume that cleavage of the imidazoline ring occurs, like for other azaheterocycles, *via* formation of a carbinolamine intermediate according to *Fig. 5.23*.

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Chapter 6

The Hydrolysis of Peptides

Contents

Hydrolysis in Drug and Prodrug Metabolism. Bernard Testa, Joachim M. Mayer

6.1. Introduction

In *Chapt. 4*, we have examined the hydrolysis of a variety of amido groups occurring in drugs and other xenobiotics. As we saw in *Chapt. 5*, cyclic amides (*i.e.*, lactams) constitute a class of special interest in medicinal chemistry. The present chapter is dedicated to another class of amides of even greater medicinal and biochemical interest, namely that of peptides.

6.1.1. Some Definitions

As defined by the *IUPAC-IUB Commission on Biochemical Nomenclature*, '*a peptide is any compound produced by amide formation between a carboxy group of one amino acid and an amino group of another*'. *Peptide* is, thus, a general term applicable to a chain of amino acids of any length, and this is the meaning used here. According to common usage, peptides made of 2 to 9 amino acid residues are called *oligopeptides*, peptides containing from 10 to *ca.* 50 residues are *polypeptides*, whereas *proteins* are often defined as having more than *ca.* 50 residues. Note, however, that some biochemists prefer to set the borderline between oligopeptides and polypeptides up to *ca.* 20 residues, and that between polypeptides and proteins at *ca.* 100 residues. In fact, the trend is now to consider as a protein any polypeptide that can exhibit a stable tertiary structure with at least two different elements of secondary structure (*e.g.*, α-helix, β-strand; see next *Sect.*).

The *Commission on Biochemical Nomenclature* (*CBN*) of the *International Union of Pure and Applied Chemistry* (*IUPAC*) and of the *International Union of Biochemistry and Molecular Biology* (*IUBMB*) has issued many recommendations and rules on peptides and amino acids. These recommendations and rules concern nomenclature, notations, abbreviations, symbols, *etc.* (see www.chem.qmul.ac.uk/iupac/AminoAcid/ and www.chem.qmul.ac.uk/iubmb/).

Twenty amino acids are constituents of the peptide chains found in nature. These are generally referred to as the '*common amino acids*' (*Table 6.1*). Less-common amino acids are listed in *Table 6.2*, including selenocysteine, often called the '21st natural amino acid'. Although of little relevance here, we note that the '22nd amino acid', pyrrolysine, was discovered in 2002 in the genetic code of certain Archea and eubacteria [1].

The amide bonds in peptides are usually called *peptide bonds*, and they are formed between C(1) of one amino acid and N(2) of another (sometimes called *eupeptide bonds*). Peptides may also include compounds linked by other amide bonds (sometimes called *isopeptide bonds*).

6.1.2. Chemical Background

When considered in isolation, peptide bonds are in many ways analogous to standard amide bonds. They are *kinetically stable*, in the sense that hydrolysis under physiological conditions of temperature and pH is very slow, with $t_{1/2}$ values that can be counted in years. However, they are *thermodynamically unstable* in the sense that the equilibrium constant favors hydrolysis over condensation by a factor of 10^3 to 10^4.

The characteristic properties of peptides result from the presence of a chain of several or many amide bonds. A first problem is that of *numbering*, and here *Fig. 6.1* taken from the *IUPAC-IUB* rules may help. A second and major aspect of the structure of peptides is their *conformational behavior*. Three torsion angles exist in the backbone (*Fig. 6.2*). The dihedral angle ω (omega) describes rotation about C–N, ϕ (phi) describes rotation about N–C^{α}, and ψ (psi) describes rotation about C^{α}–C. *Fig. 6.2* represents a peptide in a fully extended conformation where these angles have a value of 180°.

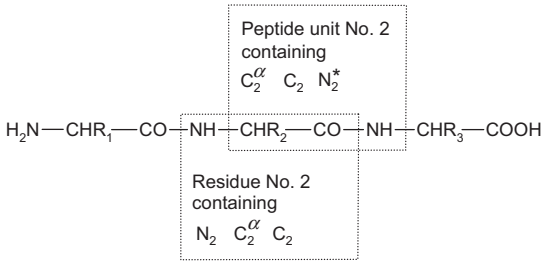


Fig. 6.1. IUPAC-IUB Commission on Biochemical Nomenclature *rules defining residues and peptide units in peptides.* In the example shown, residue No. 2 contains the backbone atoms N_2, C_2^{α}, and C_2. Peptide unit No. 2 contains the backbone atoms C_2^{α}, C_2, and N_2^{*}. R_1, R_2, and R_3 are the side chains of the amino acid residues (www.chem.qmul.ac.uk/iupac/AminoAcid/).

Table 6.1. *The Twenty Common Amino Acids*[a])

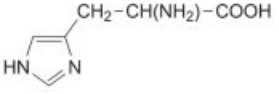
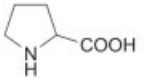
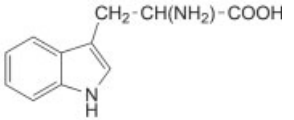
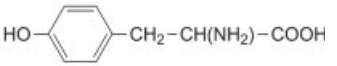
Trivial name[b])	Abbreviation[c])		Systematic name[e])	Formula
	Three letter	One letter[d])		
Alanine	Ala	A	2-Aminopropanoic acid	$CH_3-CH(NH_2)-COOH$
Arginine	Arg	R	2-Amino-5-guanidinopentanoic acid	$H_2N-C(=NH)-NH-(CH_2)_3-CH(NH_2)-COOH$
Asparagine	Asn[f])	N[g])	2-Amino-3-carbamoylpropanoic acid	$H_2N-CO-CH_2-CH(NH_2)-COOH$
Aspartic acid	Asp[f])	D[g])	2-Aminobutanedioic acid	$HOOC-CH_2-CH(NH_2)-COOH$
Cysteine	Cys	C	2-Amino-3-mercaptopropanoic acid	$HS-CH_2-CH(NH_2)-COOH$
Glutamic acid	Glu[h])	E[i])	2-Aminopentanedioic acid	$HOOC-(CH_2)_2-CH(NH_2)-COOH$
Glutamine	Gln[h])	Q[i])	2-Amino-4-carbamoylbutanoic acid	$H_2N-CO-(CH_2)_2-CH(NH_2)-COOH$
Glycine	Gly	G	Aminoethanoic acid	H_2N-CH_2-COOH
Histidine	His	H	2-Amino-3-(1*H*-imidazol-4-yl)propanoic acid	
Isoleucine	Ile	I	2-Amino-3-methylpentanoic acid[j])	$CH_3CH_2-CH(CH_3)-CH(NH_2)-COOH$
Leucine	Leu	L	2-Amino-4-methylpentanoic acid	$(CH_3)_2CH-CH_2-CH(NH_2)-COOH$
Lysine	Lys	K	2,6-Diaminohexanoic acid	$H_2N-(CH_2)_4-CH(NH_2)-COOH$
Methionine	Met	M	2-Amino-4-(methylthio)butanoic acid	$CH_3-S-(CH_2)_2-CH(NH_2)-COOH$
Phenylalanine	Phe	F	2-Amino-3-phenylpropanoic acid	$C_6H_5-CH_2-CH(NH_2)-COOH$

Table 6.1 (cont.)

Trivial name[b])	Abbreviation[c])		Systematic name[e])	Formula
	Three letter	One letter[d])		
Proline	Pro	P	Pyrrolidine-2-carboxylic acid	
Serine	Ser	S	2-Amino-3-hydroxypropanoic acid	$HO-CH_2-CH(NH_2)-COOH$
Threonine	Thr	T	2-Amino-3-hydroxybutanoic acid[k])	$CH_3-CH(OH)-CH(NH_2)-COOH$
Tryptophan	Trp	W	2-Amino-3-(1*H*-indol-3-yl)propanoic acid	
Tyrosine	Tyr	Y	2-Amino-3-(4-hydroxyphenyl)propanoic acid	
Valine	Val	V	2-Amino-3-methyl-butanoic acid	$(CH_3)_2CH-CH(NH_2)-COOH$
Unspecified amino acid	Xaa	X		

[a]) See www.chem.qmul.ac.uk/iupac/AminoAcid/. [b]) The trivial name refers to the L- or D-, or DL-amino acid. [c]) The three-letter symbols refer to L-amino acids, unless otherwise indicated by the D-prefix, *e.g.*, D-Ala. All natural amino acids except the achiral glycine, and a few produced by microorganisms, have the L-configuration. In all cases except cysteine, the L-configuration is described by (*S*). [d]) The use of one-letter symbols should be restricted to reporting of long sequences. [e]) The fully systematic forms ethanoic, propanoic, butanoic, and pentanoic may alternatively be called acetic, propionic, butyric, and valeric, respectively. Similarly, butanedioic = succinic, 3-carbamoylpropanoic = succinamic, pentanedioic = glutaric, and 4-carbamoylbutanoic = glutaramic. [f]) The symbol Asx denotes Asp or Asn. [g]) B denotes D or N. [h]) Glx denotes Glu or Gln; also used for any substance, such as 4-carboxyglutamic acid (Gla) or 5-oxoproline (= pyroglutamic acid, Glp) that yields glutamic acid upon acid hydrolysis of peptides. [i]) Z denotes E or Q. [j]) The absolute configuration of L-isoleucine is (2*S*,3*S*). [k]) The absolute configuration of L-threonine is (2*S*,3*R*).

Table 6.2. *Some Less-Common Peptide Constituents*[a])

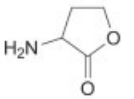
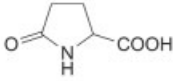
Trivial name	Three-letter symbol	Systematic name[b])	Formula
β-Alanine	βAla	3-Aminopropanoic acid	$H_2N-(CH_2)_2-COOH$
2-Aminobutyric acid	Abu	2-Aminobutanoic acid	$CH_3CH_2-CH(NH_2)-COOH$
2-Aminovaleric acid (Norvaline[c]))	Ape (Nva[c]))	2-Aminopentanoic acid	$CH_3-(CH_2)_2-CH(NH_2)-COOH$
2-Aminohexanoic acid (Norleucine[c]))	Ahx (Nle[c]))		$CH_3-(CH_2)_3-CH(NH_2)-COOH$
2-Aminoadipic acid	Aad	2-Aminohexanedioic acid	$HOOC-(CH_2)_3-CH(NH_2)-COOH$
3-Aminoadipic acid	βAad	3-Aminohexanedioic acid	$HOOC-(CH_2)_2-CH(NH_2)-CH_2-COOH$
2-Aminopimelic acid	Apm	2-Aminoheptanedioic acid	$HOOC-(CH_2)_4-CH(NH_2)-COOH$
Citrulline	Cit	2-Amino-5-ureidovaleric acid	$H_2N-CO-NH-(CH_2)_3-CH(NH_2)-COOH$
2,3-Diaminopropionic acid	A_2pr or Dpr	2,3-Diaminopropanoic acid	$H_2N-CH_2-CH(NH_2)-COOH$
2,4-Diaminobutyric acid	A_2bu or Dab	2,4-Diaminobutanoic acid	$H_2N-(CH_2)_2-CH(NH_2)-COOH$
2,6-Diaminopimelic acid	A_2pm or Dpm	2,6-Diaminoheptanedioic acid	$HOOC-CH(NH_2)-(CH_2)_3-CH(NH_2)-COOH$
Homocysteine	Hcy	2-Amino-4-mercaptobutanoic acid	$HS-(CH_2)_2-CH(NH_2)-COOH$
Homoserine	Hse	2-Amino-4-hydroxybutanoic acid	$HO-(CH_2)_2-CH(NH_2)-COOH$
Homoserine lactone[d])	Hse > or Hsl or Hse		
Ornithine	Orn	2,5-Diaminopentanoic acid	$H_2N-(CH_2)_3-CH(NH_2)-COOH$

Table 6.2 (cont.)

Trivial name	Three-letter symbol	Systematic name[b])	Formula
Pyroglutamic acid (5-oxoproline)[e])	Glp or <Glu or 	5-Oxopyrrolidine-2-carboxylic acid	
Sarcosine	Sar	*N*-Methylglycine	$CH_3-NH-CH_2-COOH$
Selenocysteine	Sec (U[f]))		$HSe-CH_2-CH(NH_2)-COOH$

[a]) See www.chem.qmul.ac.uk/iupac/AminoAcid/. [b]) See *Footnote e* in *Table 6.1*. [c]) This name and the corresponding three-letter symbol are not recommended. [d]) Homoserine lactone can form only one peptide bond, namely with its amino group. Its only position in a peptide is therefore the C-terminus. [e]) Pyroglutamic acid can form only one peptide bond, namely with its carboxy group. Its only position in a peptide is therefore the N-terminus. [f]) One-letter symbol.

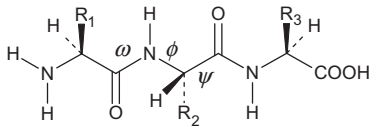


Fig. 6.2. *Torsion angles in peptides, namely* ω (omega) *describing rotation about C–N,* ϕ (phi) *describing rotation about N–*C^{α}*, and* ψ (psi) *describing rotation about* C^{α}*–C bond* (www.chem.qmul.ac.uk/iupac/AminoAcid/). The prototypic peptide shown here is in a fully extended conformation where all three torsional angles are equal to 180°.

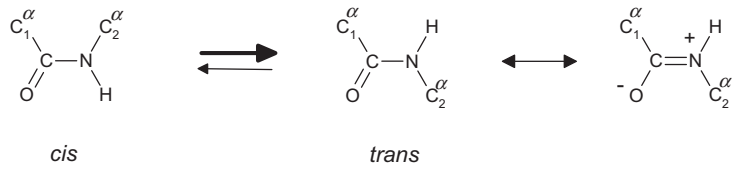


Fig. 6.3. *Conformational equilibrium about* ω*, showing its strong preference* (*ca.* 90 kJ mol^{-1}) *for the* trans*-conformation, as stabilized by its mesomeric form with C=N bond character*

The torsion angle ω, which is common to peptides and nonpeptidic amides, always prefers a planar over a nonplanar conformation due to the partial double-bond character of the amide bond (*Fig. 6.3*, right). Thus, a peptide bond resembles an amide bond in conformational and electronic terms [2][3]. However, peptides differ from amides in that both the carbonyl C-atom and the amido N-atom are nearly always bound to an sp^3-hybridized C-atom. As a result, the *trans*-conformer (ω=180°) is consistently preferred over the *cis*-conformer, the energy difference usually being *ca.* 90 kJ mol^{-1} (*Fig. 6.3*). The relationship between the partial double-bond character of the amide bond (*Fig. 6.3*, right) and hydrolysis will be considered in *Sect. 6.3*.

In contrast to the practically frozen angle ω, the angles ϕ and ψ have some flexibility and allow the peptides to exist in a variety of prefered conformations. Thus, one allowed region in ϕ *vs.* ψ plots (*Ramachandran* plots) is defined by ϕ=−60° to −150° and ψ=90° to 180°. This region comprises dihedral angles characteristic of antiparallel-chain pleated β-sheets ($\phi\approx$−139°, $\psi\approx$135°) and parallel-chain pleated sheets ($\phi\approx$−119°, $\psi\approx$113°). Another region (ϕ=−60° to −150° and ψ=−40° to −60°) contains the dihedral angles characteristic of a right-handed α-helix ($\phi\approx$−57°, $\psi\approx$−47°). Left-handed α-helices are characterized by $\phi\approx$57° and $\psi\approx$47°. These major elements of the *secondary structure* of peptides are among the factors that influence the hydrolysis (chemical and enzymatic) of peptide bonds.

In addition to the backbone angles discussed above, the conformational hyperspace of peptides also includes the dihedral angles of the residue side

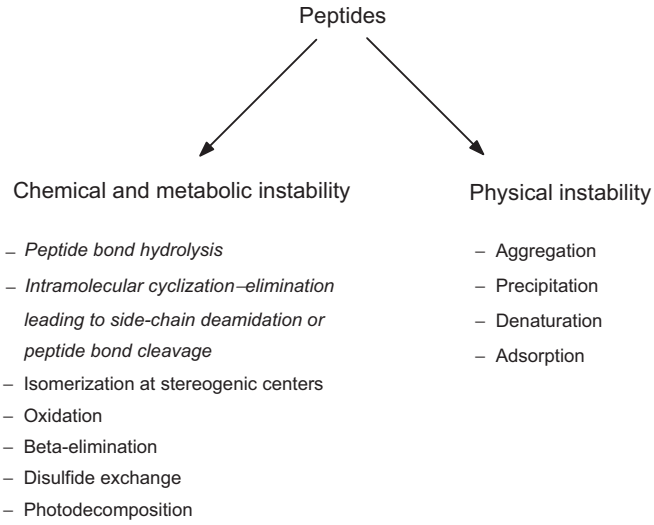


Fig. 6.4. *Chemical, metabolic, and physical processes of instability in pharmaceutical peptides* (modified from [9]). The italicized reactions are the focus of this chapter.

chains. These are the χ (chi) angles, written with adequate subscripts and superscripts to define the χ angle of each single bond (www.chem.qmul.ac.uk/iupac/AminoAcid/). The conformational hyperspace of the side chains, together with their physicochemical properties, plays an essential role in the folding of polypeptide chains and in processes of intermolecular recognition [4][5].

Peptides can undergo a variety of *degradation* reactions (*Fig. 6.4*) [6–9]. Pathways of physical degradation include aggregation, precipitation, and adsorption. Denaturation, *i.e.*, an often-irreversible alteration of the tertiary structure of a peptide, is also considered a type of physical degradation. These physical reactions fall outside the scope of this work.

Chemical reactions of degradation include hydrolysis, deamidation, isomerization (racemization and epimerization), oxygenation, β-elimination, disulfide exchange, and photodecomposition. The reactions of relevance in the context of this book are *deamidation* and *peptide bond hydrolysis*. The latter reaction can be nonenzymatic or enzymatic. Of particular interest are intramolecular reactions of cyclization–elimination, which can result in peptide bond cleavage or side-chain deamidation. As will be seen, a few general rules exist to predict which peptide bonds are chemically less-stable. Thus, peptide bonds formed by the amino group of serine or threonine have been found to be less stable than many other amide bonds. The same is true for some peptide bonds involving aspartic acid. Also, the Asp–Pro bond is more susceptible than others to acid-catalyzed cleavage.

Oligopeptides, polypeptides, and proteins are of great relevance and interest in medicinal chemistry. This is true of both endogenous peptides (*e.g.*, neuroactive and hormonal peptides, functional proteins) and exogenous compounds (*e.g.*, peptide analogues and peptidomimetics) [10–13]. However, the development of bioactive peptides is often hindered by undesirable pharmaceutical (*e.g.*, low water solubility, poor chemical stability), pharmacokinetic (*e.g.*, low membrane permeability, fast enzymatic degradation), and pharmacodynamic properties (*e.g.*, low selectivity, short duration of action) [7][14]. This is a major contemporary issue in drug research, and attempts by a variety of strategies to solve these problems have been made:

- Derivatization to protect endogenous peptides or create prodrugs thereof.
- Design and development of unnatural peptides based on D-amino acids, β-amino acids, artificial amino acids, *etc.*
- Protective derivatization of such unnatural peptides.
- Design and development of pseudopeptides and peptidomimetics.

The present chapter focuses on specific aspects of these challenges, namely *peptide bond hydrolysis* (chemical and enzymatic) and *intramolecular reactions of cyclization–elimination* (*Fig. 6.4*). This will be achieved by considering, in turn: *a*) the enzymatic hydrolysis of prodrugs containing a peptide pro-moiety (*Sect. 6.2*), *b*) the chemical hydrolysis of peptides (*Sect. 6.3*), *c*) the enzymatic hydrolysis of peptides containing only common amino acids (*Sect. 6.4*), *d*) the hydrolysis of peptides containing nonproteinogenic amino acids (*Sect. 6.5*), and, finally, *e*) the hydrolysis of peptoids, pseudopeptides and peptidomimetics (*Sect. 6.6*).

6.2. Prodrugs That Contain a Peptide Pro-Moiety

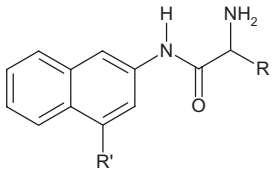
A number of clinically useful drugs have been derivatized with a peptide pro-moiety with the view to improve pharmaceutical properties (*e.g.*, solubility) and/or pharmacokinetic behavior (*e.g.*, absorption or targeted delivery). In such cases, and in contrast to the peptides and derivatives and analogues discussed in *Sect. 6.3 – 6.6*, the *peptide unit is not part of the pharmacophore*.

In the present section, we examine pro-moieties consisting of one to several residues, with the pro-moiety always *attached to the drug by an amide bond*. In other words, the peptide pro-moiety is attached *via* either the NH_2 or COOH terminal group to a COOH or NH_2 group, respectively, of the drug. The case of prodrugs linked by a carboxylic acid ester bond to an amino acyl pro-moiety will be presented in *Chapt. 8*. In the prodrugs discussed here, there may exist competitive hydrolytic reactions between the linking amido group and one or more peptide bonds in the pro-moiety. It will, therefore, be

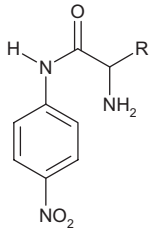
useful to begin our presentation with prodrugs in which the pro-moiety consists of a single amino acid. Many such examples may be found in the literature [15].

6.2.1. Prodrugs for Improved Solubility and Absorption

Preliminary information useful in prodrug design has been obtained with amino acids attached to model aromatic amines. Thus, N-*(naphthalen-2-yl) amides of amino acids* (**6.1**, R = side chain of amino acid, R′ = H) proved to be of interest as test compounds to monitor peptidase activity such as aminopeptidase M (membrane alanyl aminopeptidase, microsomal aminopeptidase, EC 3.4.11.2) [16][17]. In the presence of purified rabbit kidney aminopeptidase M or human cerebrospinal fluid (CSF) aminopeptidase activity, the rate of hydrolysis decreased in the order Ala->Leu->Arg->Glu-2-naphthylamide. Ala-2-naphthylamide, in particular, proved to be a good test compound, as its rate of hydrolysis was influenced by experimental conditions (preparation, inhibitors, *etc.*), as was the hydrolysis of a number of low-molecular-weight opioid peptides and circulating vasoactive peptides.



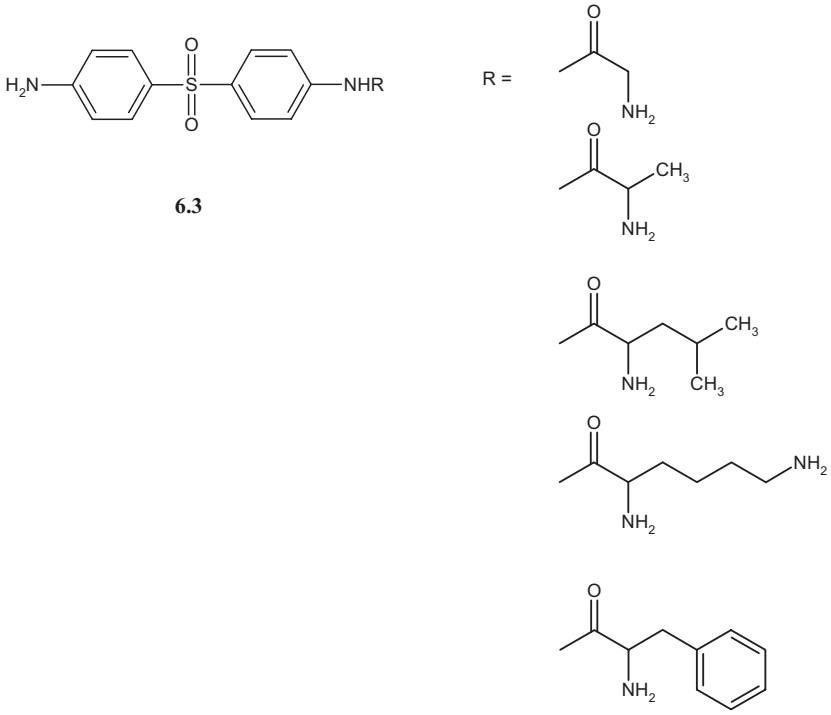
6.1



6.2

Similarly, the 4-methoxy-2-naphthylamides of Leu, Ala, Arg, and Glu (**6.1**, R = side chain of amino acid, R′ = MeO) were used to assess the type and activity of aminopeptidase in homogenates of conjunctival, nasal, buccal, duodenal, ileal, rectal, and vaginal tissues from rabbits. This systematic comparison afforded a better understanding of the role of the aminopeptidase barrier in peptide absorption from oral *vs.* non-oral routes [18]. In a comparable manner, the γ-glutamyltranspeptidase and dipeptidase activities were investigated in mammary tissue with the 4-nitroanilides of Leu, Met, Lys, Glu, and Asp (**6.2**, R = side chain of amino acid) [19].

Amides are of interest as prodrugs given their greater chemical stability but overall comparable enzymatic susceptibility relative to esters. In *Chapt. 4*, we began discussing amide prodrugs. The presentation continues in this chapter with prodrugs having an amino acid or a peptide as pro-moiety.



6.3

Amino acid pro-moieties may be of interest in preparing *water-soluble prodrugs* of active aromatic amines for parenteral administration. Indeed, the positive charge of the α-amino group affords water solubility, the amide bond affords chemical stability, and hydrolytic bioactivation occurs readily when the prodrug is a good substrate for one or more hydrolases. This is aptly illustrated by amino acid amides of *dapsone* (**6.3**), a major antileprosy drug [20]. The prodrugs investigated fulfilled the prerequisites of being stable in aqueous solution but rapidly hydrolyzed by peptidases. In aqueous solution at pH 4, the projected shelf-lives ($t_{90\%}$) were greater than 2 y, the rates of hydrolysis decreasing in the order Gly>Ala>Phe>Lys>Leu, suggesting that steric hindrance influences hydrolysis.

After intravenous administration to rabbits, the L-amino acid derivatives of dapsone were rapidly ($t_{1/2}$<2 min) and quantitatively converted to dapsone. The corresponding D-amino acid derivatives were also quantitatively converted to dapsone, but the $t_{1/2}$ values ranged from 30 to 60 min. In human blood, the $t_{1/2}$ values for hydrolysis of the L-amino acid prodrugs ranged from 1.7 to 20 min (Leu<Phe<Lys<Gly<Ala). All derivatives were substrates of leucine peptidase, with decreasing specificity constants (k_{cat}/K_m) Leu≈Ala>Phe>Lys≈Gly. Interestingly, only L-Lys-dapsone was a substrate of trypsin, whereas only L-Phe-dapsone was a substrate of chymotrypsin.

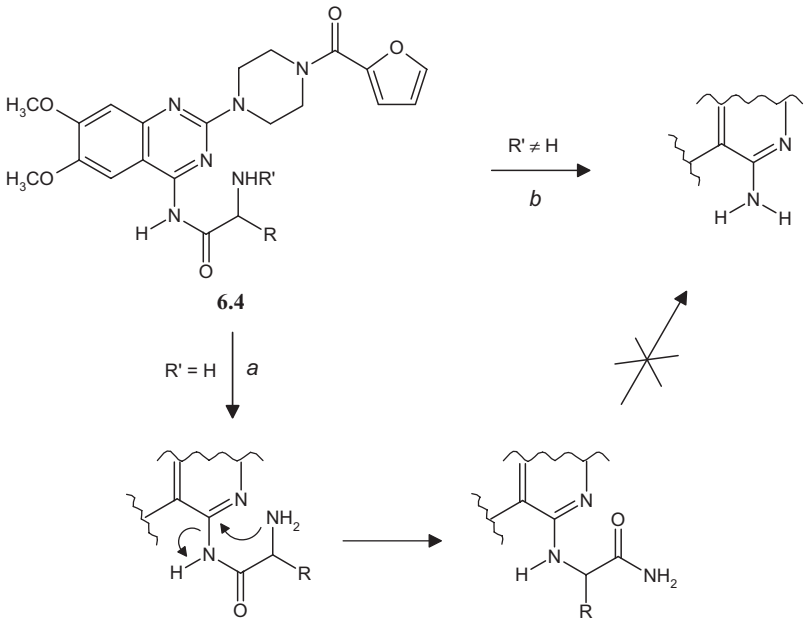
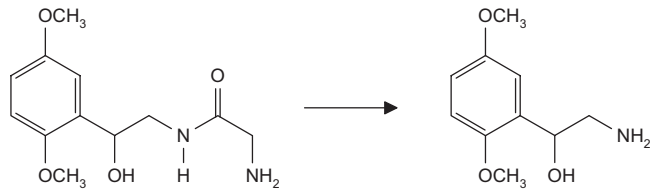


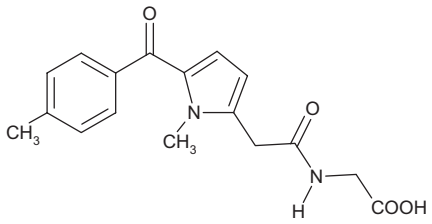
Fig. 6.5. *Competitive reactions of rearrangement* (*Pathway a*) *and hydrolysis* (*Pathway b*) *in amino acid prodrugs of prazosin* [21]

A counter-example is provided by amino acid derivatives of the α_1-antagonist *prazosin*. These compounds (**6.4**, *Fig. 6.5*) were prepared as potential prodrugs to increase the water solubility of the parent compound and to target peptidases for *in vivo* hydrolytic activation [21]. However, the amino acid derivatives examined (Ala, Pro, and Lys) degraded rapidly ($t_{1/2} \approx$ 10–50 min) in aqueous solution at neutral pH to a rearrangement product that was not prazosin, but was produced by the intramolecular process shown in *Fig. 6.5* (*Pathway a*). This particular reactivity was attributed to the nature of the aromatic heterocycle, which favors intramolecular attack by the α-amino group. After end-capping the free α-amino group (*e.g.*, by *N*-acetylation), the primary route of chemical degradation became hydrolysis of the amide bond to give prazosin (*Fig. 6.5*, *Pathway b*), but the reaction was too rapid at neutral pH to allow stable solutions to be prepared. This increased lability compared to amino acid amides of other aromatic amines was again attributed to the electronic nature of the aromatic heterocycle, and it illustrates that unexpected problems can never be excluded in prodrug design.

Prodrugs with a single amino acid as the pro-moiety have also been developed to improve *oral absorption*, a major aspect of pharmacokinetic optimization [22][23]. This is illustrated by *midodrine* (**6.5**), a long-acting α-adrenergic stimulating agent [24]. This compound is, in fact, a prodrug of ST



6.5 **6.6**



6.7

1059 (**6.6**), with glycine as the pro-moiety. Careful pharmacological studies in a variety of animal species showed that midodrine had a markedly longer duration of action than ST 1059 and showed good efficacy after oral administration. It was, therefore, concluded that the pressor agent ST 1059 was liberated enzymatically from midodrine, a well-absorbed 'transport form'.

The *glycine amide of tolmetin* (**6.7**) functions as a prodrug and was shown to be orally more potent than tolmetin as an inhibitor of adjuvant arthritis in the female *Lewis* rat [25]. The superiority of tolmetin glycine amide relative to tolmetin in these tests was demonstrated by inhibition of paw swelling and reduction of degenerative bone changes in an animal model of human chronic rheumatoid arthritis. These properties were not evident after oral administration of equimolar mixtures of tolmetin sodium and glycine. Pharmacokinetic analyses revealed that tolmetin glycine amide was completely absorbed orally and hydrolyzed to tolmetin in the adjuvant arthritic rat. The combined effects of oral absorption, distribution, and hydrolysis of tolmetin glycine amide produced lower plasma peak levels of tolmetin than an equivalent dose of tolmetin sodium, but plasma concentrations were sustained for a longer period and contributed to an apparent increase in potency. Furthermore, tolmetin glycine amide had a decreased propensity to cause gastrointestinal irritation compared to tolmetin sodium. Several additional amino acid amides of tolmetin were similar to the glycine amide in exhibiting increased potency and reduced gastrointestinal toxicity in comparison to equimolar doses of tolmetin sodium.

Interestingly, the favorable results obtained with tolmetin glycine amide were not shared by indomethacin glycine amide [25].

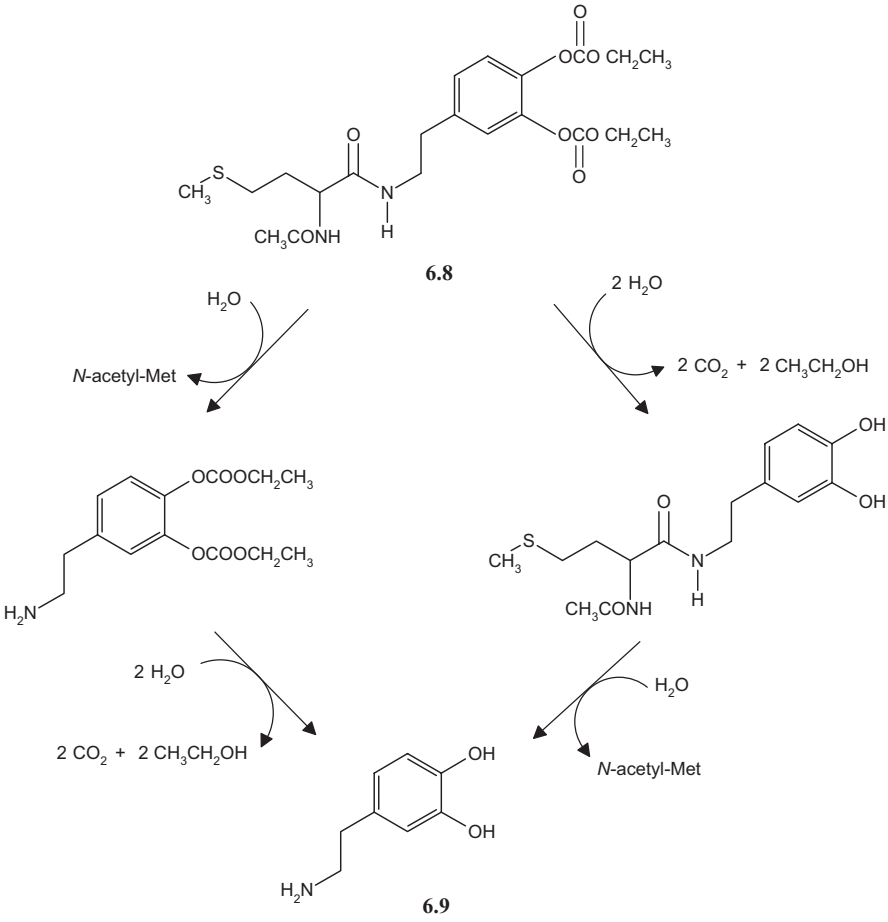


Fig. 6.6. *Pathways of hydrolytic bioactivation of docarpamine* (**6.8**) *to liberate dopamine* (**6.9**)

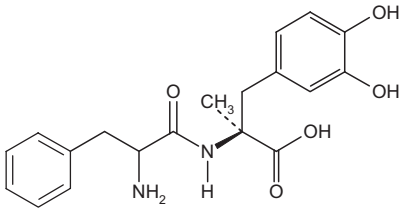
Docarpamine (**6.8**, *Fig. 6.6*) was developed as a potential *orally active prodrug* of dopamine (**6.9**, *Fig. 6.6*) to be used in the treatment of renal insufficiency, congestive heart failure, and some types of shock [26][27]. Indeed, dopamine itself is active only by intravenous infusion since it undergoes extensive inactivation by first-pass metabolism. Following oral administration to rats and dogs, the main pathway of metabolism in the small intestine was hydrolysis of the two ethoxycarbonyl pro-moieties esterifying the catechol group. Amide hydrolysis to liberate the *N*-acetylmethionine promoiety was a minor pathway. In the liver, ester hydrolysis was, again, a major pathway, but so was hydrolysis of the amide linkage (*Fig. 6.6*). Clinical studies have confirmed the safety and efficacy of docarpamine in patients undergoing cardiac surgery [28].

6.2.2. Prodrugs for Targeted Transport

6.2.2.1. *Targeting of Intestinal Transporters*

There have been sustained efforts in recent years to use the carrier systems of the brush-border membrane of intestinal mucosa to increase absorption of orally administered drugs [29][30]. One system of particular interest is the *intestinal peptide carrier* (*hPEPT1*) whose physiological function is the absorption of di- and tripeptides and whose xenobiotic substrates include β-lactam antibiotics, renin inhibitors, and angiotensin-converting enzyme (ACE) inhibitors [31].

A series of *peptide prodrugs of* L-α-*methyldopa* were prepared and shown to exhibit high affinity for the peptide carrier system [32]. In an *in situ* intestinal perfusion model, the prodrugs Phe-L-α-methyldopa (**6.10**) and L-α-methyldopa-Phe (**6.11**) showed permeabilities that were 10- and 20-times higher, respectively, than that of L-α-methyldopa. The other derivatives examined (Gly- and Pro-L-α-methyldopa, L-α-methyldopa-Pro) also had better permeabilities. These and other results indicate that the peptide transport system has a relatively low substrate specificity and can indeed be targeted by peptide prodrugs to improve absorption [33].



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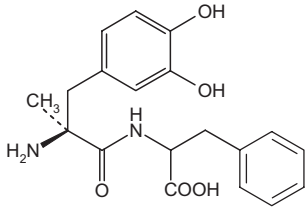

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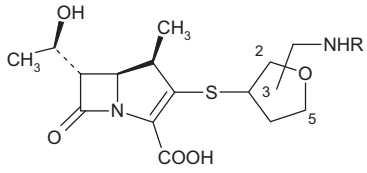
Increased permeability is just one prerequisite in the development of useful peptide prodrugs. Another condition is that efficient bioactivation must follow absorption. *Mucosal cell enzymes* able to hydrolyze peptides include exopeptidases such as aminopeptidases and carboxypeptidases, endopeptidases, and dipeptidases such as cytosolic nonspecific dipeptidase (EC 3.4.13.18), Pro-X dipeptidase (prolinase, EC 3.4.13.4), and X-Pro dipeptidase (prolidase, EC 3.4.13.9). For example, L-α-methyldopa-Pro was shown to be a good substrate for both the peptide transporter and prolidase. This dual affinity is not shared by all dipeptide derivatives, and, indeed, dipeptides that lack an N-terminal α-amino group are substrates for the peptide transporter but not for prolidase [29][33][34].

A recent example of targeting of a peptide-transport system is found in *carbapenem* antibiotics [35]. A series of (aminomethyl)tetrahydrofuranyl-1β-methylcarbapenems (**6.12**, R=H, aminomethyl side chain at C(2) or C(5)) with excellent *in vitro* broad-spectrum antibacterial activity exhibited only modest efficacy against acute lethal infections in mice following oral administration. In an effort to improve oral efficacy, a *peptide-mediated transport strategy* was investigated with a variety of amino acids substituted at the amino group. The peptidic prodrugs containing an L-amino acid demonstrated improved efficacy after oral administration, and their absorption and hydrolysis was demonstrated by high levels of the parent drug in plasma following oral administration of the prodrugs. The Ala-, Val-, Ile-, and Phe-substituted prodrugs demonstrated three- to tenfold increased efficacy by oral administration against acute lethal infections in mice. Median effective doses of <1 mg/kg against infections caused by *S. aureus*, *E. coli*, *Enterobacter cloacae*, or penicillin-susceptible *Streptococcus pneumoniae* were obtained after the administration of single oral doses. In sharp contrast to these results, the D-forms were consistently less active than the parent drug. These results suggest an active absorption process with marked stereoselectivity for the prodrugs prepared from an L-amino acid. This hypothesis is further supported by the finding that the parent drugs demonstrated greater efficacy than the prodrugs by subcutaneous administration.

The oral absorption of an entirely different class of drugs, namely *bisphosphonates*, has also been improved by a peptide prodrug strategy [36]. The drugs pamidronate (**6.13**, $n=2$) and alendronate (**6.13**, $n=3$) were derivatized with the Pro-Phe-dipeptidyl unit to yield the prodrugs Pro-Phe-pamidronate (**6.14**, $n=2$) and Pro-Phe-alendronate (**6.14**, $n=3$), the ob-



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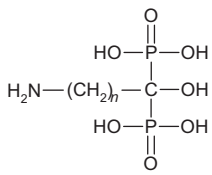

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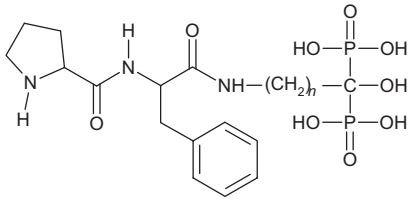


6.14

jective being to target carrier systems in the intestine, particularly the intestinal peptide carrier system (hPEPT1), as well as cytosolic peptidases. *In situ* single-pass perfusion studies revealed competitive inhibition of transport by Pro-Phe, suggesting peptide carrier-mediated transport. Prodrug transport in the Caco-2 cell line was significantly better than that of the parent drugs, and the prodrugs exhibited high affinity for the intestinal tissue. Oral administration of the dipeptidyl prodrugs in rats produced a threefold increase in drug absorption, and the bioavailability of Pro-Phe-alendronate was two to three times higher than that of the parent drug. The results indicate that the oral absorption of bisphosphonates can indeed be improved by a peptidyl prodrug strategy targeting hPEPT1 and other transporters.

6.2.2.2. *Targeting of Cell-Specific Transporters*

The tissue- or cell-selective delivery of drugs (*i.e.*, so-called 'magic bullets') remains a major challenge in medicinal chemistry and biopharmacy. A number of prodrug strategies have been explored with variable success, some of which are presented in the following pages and elsewhere in this book. Such strategies rely on *selection*, *i.e.*, the targeting of a device or process able to select by filtering or screening the agent. In the absence of selection, a drug will reach and permeate normal and target cells equally (*Fig. 6.7,a*), or a prodrug will deliver comparable concentrations of an active agent to normal and target cells (*Fig. 6.7,b*). The selection devices examined here and below are cell-specific transporters, constitutionally or pathologically expressed peptidases (*Sect. 6.2.3.1* and *6.2.3.2*), and artificially imported peptidases (*Sect. 6.2.3.3*).

The principle of targeting a cell-specific transporter to achieve selective delivery is schematically represented in *Fig. 6.8* and exemplified by a study in which the objective was selective delivery of *5-fluorouracil* (5FU) to pathological microorganisms. Because microorganisms possess specialized transport systems for the uptake of peptides, the drug was derivatized into antifungal and antibacterial prodrugs that target *microbial peptide carrier systems* and peptidases (*Fig. 6.9*) [37]. The prodrugs prepared were α-substituted glycine dipeptides, and, more specifically, the two epimers of L-Ala-2-(5-fluorouracil-1-yl)-glycine (**6.15**, *Fig. 6.9*). After permease-mediated entry into microbial cells, enzymatic hydrolysis generated an unstable α-(5FU)-glycine (**6.16**), which spontaneously decomposed to release 5FU (**6.17**). In antifungal tests, the L,L-epimer proved as active as 5FU itself, whereas the L,D-epimer was inactive. This finding is compatible with the known stereospecificity of the peptide carriers for L,L-dipeptides.

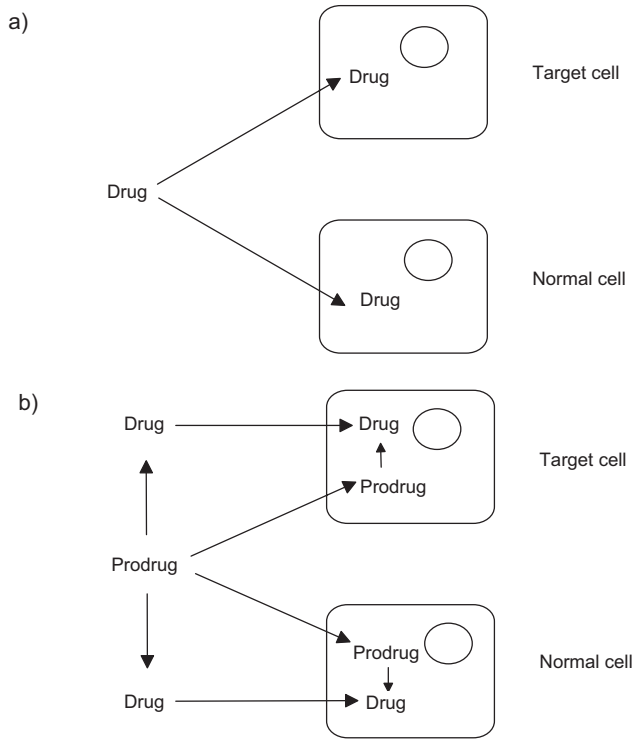


Fig. 6.7. *Schematic representation of nonselective drug delivery in the absence of a selection process. a*) Nonselective delivery of a drug. *b*) Nonselective activation of a prodrug.

Targeting of a tissue-specific transport system has been carried out on the *kidney*. This organ contains several transport systems for anionic compounds, particularly for glutathione and γ-glutamyl compounds. To take advantage of these transport systems to achieve selective renal vasodilation, the hydralazine-like vasodilator *5-butyl-2-hydrazinopyridine* (CGP 18137, **6.18**) was derivatized to its *N*-acetyl-γ-glutamyl prodrug (CGP 22979, **6.19**) [38]. In rats, the active drug was, indeed, found to accumulate selectively in the renal tissue following administration of the prodrug. A variety of experiments confirmed that the enzyme γ-glutamyl transpeptidase (see below) was not involved in the renal accumulation of CGP 18137. Active transport of the prodrug into the tubular cells, followed by intracellular hydrolysis by various enzymes, accounted for the observed renal selectivity.

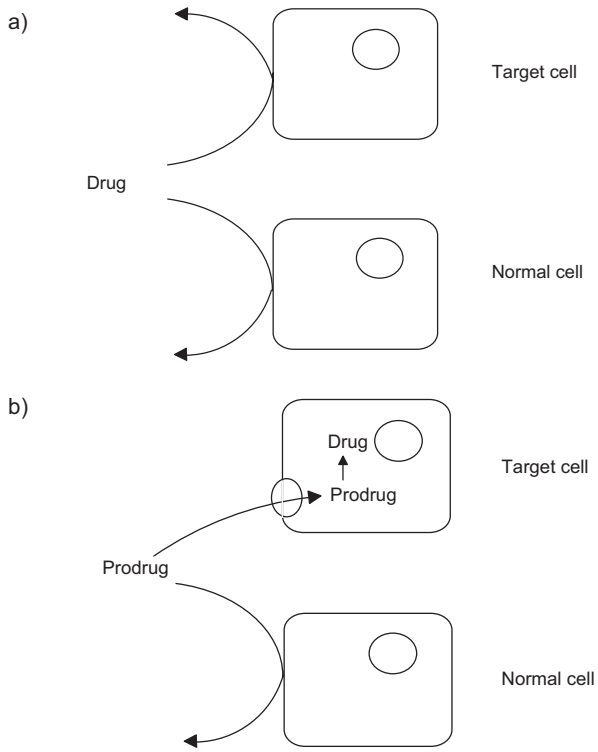
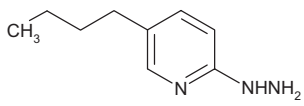


Fig. 6.8. *Schematic representation of a selective delivery obtained by targeting a cell-specific transporter. a*) The drug itself does not permeate passively into cells and is not a substrate of the transporter. *b*) The prodrug does not permeate passively into cells but is a substrate of the transporter. An additional condition of success is for the prodrug to undergo intracellular activation.



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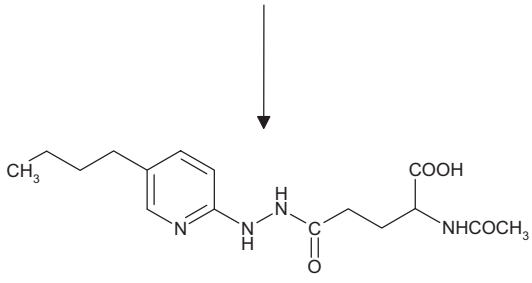

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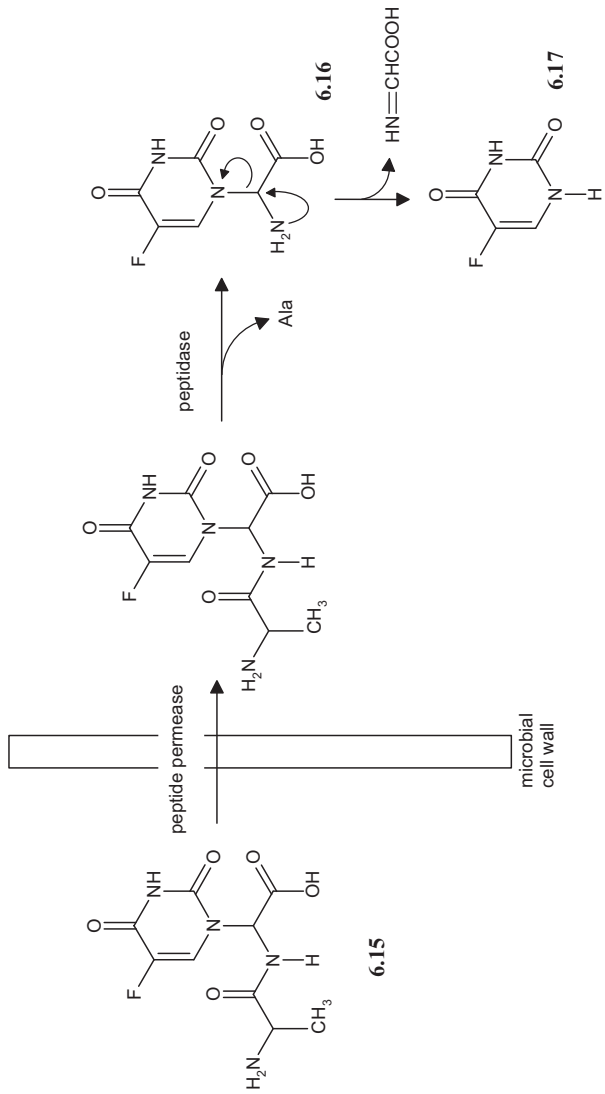


Fig. 6.9. *Rationale for the design of peptidic prodrugs* (**6.15**) *of 5-fluorouracil* (**6.17**) *targeting microbial peptides permeases and peptidases* [37]

6.2.3. Prodrugs for Targeted Metabolism

The targeting of peptidases that are expressed at particularly high levels in a given tissue or organ is a viable strategy used to achieve site-selective activation of peptide-linked prodrugs and, hence, site-selective drug delivery. A scheme depicting this strategy is shown in *Fig. 6.10*. As compared to the drug itself (*Fig. 6.10,a*), a prodrug targeted for a peptidase specifically present in and/or around target cells will undergo selective activation (*Fig. 6.10,b* and *c*). As discussed below, the peptidase may be *expressed constitutionally*, *overexpressed in pathologies*, or even *imported artificially*.

6.2.3.1. *Targeting of Peptidases Expressed Constitutionally*

Here, we use intestine-selective and kidney-selective drug delivery to illustrate the targeting of constitutionally expressed peptidases.

5-Aminosalicylic acid (5-ASA, **6.23**, *Fig. 6.11*) is a drug used in the treatment of inflammatory bowel diseases. In an attempt to improve the *intestinal delivery* of this drug, the following peptide-linked prodrugs were prepared and examined: 5-[*N*-(L-aspartyl)amino]salicylic acid (**6.21**, $n=1$), 5-[*N*-(L-glutamyl)amino]salicylic acid (**6.21**, $n=2$), [*N*-(5-amino-2-hydroxyphenyl)-L-prolyl]-L-leucine (**6.22**), ({5-[*N*-(L-aspartyl)amino]-2-hydroxyphenyl}-L-prolyl)-L-leucine (**6.20**, $n=1$), and ({5-[*N*-(L-glutamyl)amino]-2-hydroxyphenyl}-L-prolyl)-L-leucine (**6.20**, $n=2$) [39]. Selective cleavage by intestinal brush border enzymes was indeed observed (*Fig. 6.11*), in that aminopeptidase A hydrolyzed the aspartic or glutamic acid residue in **6.20** ($n=1, 2$) and **6.21** ($n=1, 2$), whereas carboxypeptidases hydrolyzed the Pro-Leu moiety in **6.20** ($n=1, 2$) and **6.22**. These results proved promising.

The targeting of peptide-linked prodrugs to the *kidney* has also been actively investigated by taking advantage of the constitutionally high renal concentrations of such enzymes as γ-glutamyl transpeptidase (EC 2.3.2.2) [15]. Other renal enzymes, such as cysteine conjugate β-lyase (EC 4.4.1.13), may be targeted by cysteine conjugates, but these fall outside the scope of this book [40]. In the example of compound **6.19** discussed above (*i.e.*, the *N*-acetyl-γ-glutamyl prodrug of 5-butyl-2-hydrazinopyridine), hydrolysis by γ-glutamyl transpeptidase did not occur since *N*-acetylated γ-glutamyl amides cannot be hydrolyzed by this enzyme.

The interplay of kidney-selective transport and/or kidney-selective activation is well illustrated by the prodrug *γ-glutamyl sulfamethoxazole* (**6.24**, R=H) and some *N*-acyl-γ-glutamyl derivatives (**6.24**, R=acyl) [41]. γ-Glu-sulfamethoxazole, indeed, released sulfamethoxazole at high rates in kidney homogenates, whereas other organs showed low or negligible activity. Its

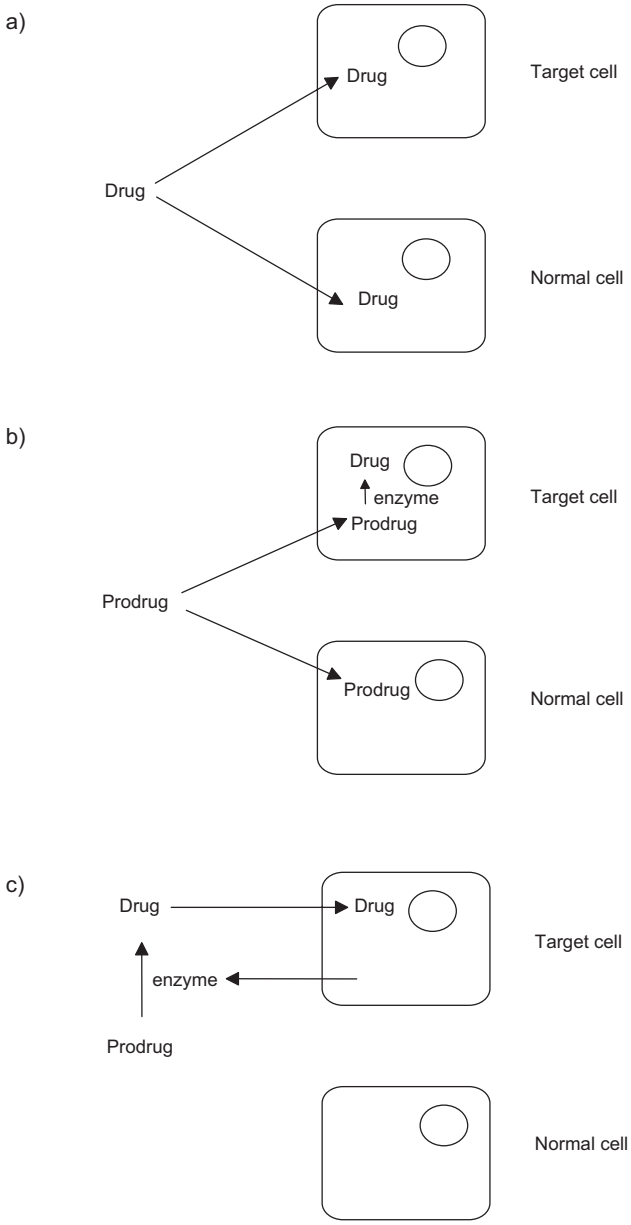


Fig. 6.10. *Schematic representation of a selective delivery obtained by targeting a cell-specific peptidase*. *a*) The drug itself permeates passively into all cells, and no selectivity is achieved. *b*) The target cells contain a specific enzyme, which activates the prodrug *in situ*. *c*) The target cells produce a specific enzyme, which activates the prodrug in their vicinity. Note that cases *b* and *c* are not mutually exclusive and may, in fact, occur simultaneously.

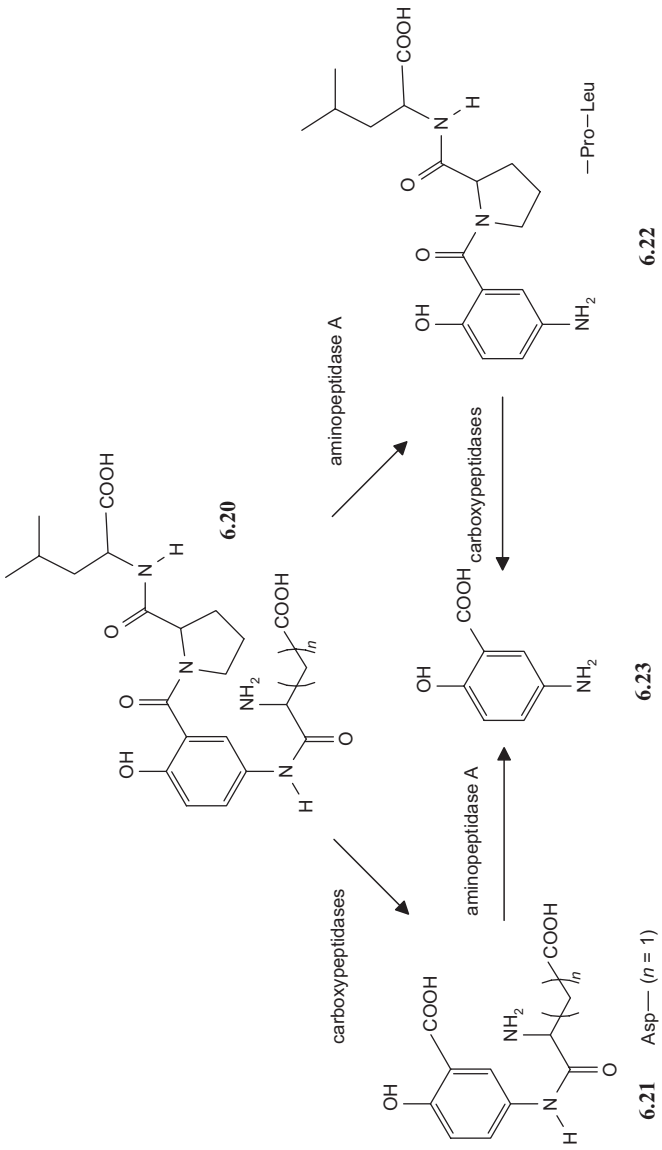
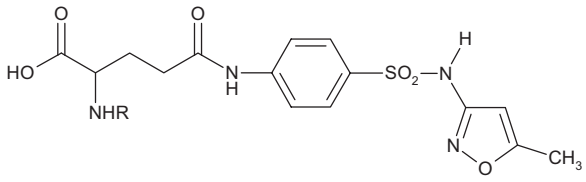


Fig. 6.11. *Peptide prodrugs* (**6.20**, **6.21**, and **6.22**) *for the intestine-selective delivery of 5-aminosalicylic acid* (**6.23**). The prodrugs undergo selective activation by intestinal brush border enzymes, namely aminopeptidase A and/or carboxypeptidases [39].

enantiomer γ-D-Glu-sulfamethoxazole was a modest substrate in kidney homogenates, and various *N*-acyl-Glu-sulfamethoxazoles were poor substrates. These *N*-acyl derivatives were even poorer substrates when purified γ-glutamyl transpeptidase was added to the organ homogenates, indicating that the *N*-acyl-γ-Glu derivatives had to be deacylated by acylases prior to being hydrolyzed by γ-glutamyl transpeptidase. Following *i.p.* administration to mice, both sulfamethoxazole and γ-Glu-sulfamethoxazole led to a somewhat selective and similar accumulation of the drug in kidneys. In contrast, a markedly higher and more selective accumulation of the drug was seen after *i.p.* administration of *N*-acyl-γ-Glu-sulfamethoxazoles.



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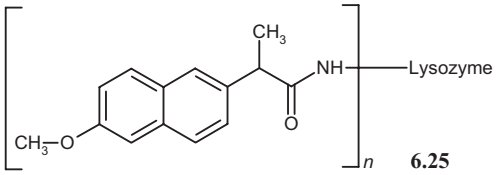
This example suggests that a more-selective kidney delivery might perhaps be obtained by targeting selective transport systems rather than γ-glutamyl transpeptidase. In chemical terms, *N*-acyl-γ-Glu prodrugs might be expected to achieve a more selective kidney delivery than γ-Glu prodrugs. However, it would be premature to generalize from the examples presented above, since the substrate specificity of γ-glutamyl transpeptidase is only partly understood. Thus, the γ-Glu-γ-Glu prodrug of a diuretic xanthine analogue was shown to be a much better substrate of γ-glutamyl transpeptidase than the corresponding γ-Glu prodrug [42]. Both prodrugs achieved significant diuresis *in vivo*. Interestingly, the *N*-acetyl-γ-Glu prodrug of this xanthine analogue was not *N*-deacetylated and, thus, remained inactive.

Renal delivery has also been attempted by making use of the capacity of the proximal tubule cells to accumulate *low-molecular-weight proteins* (LMWPs) and to degrade them to their amino acid constituents. In an exploratory study, egg-white lysozyme (a LMWP) was linked covalently to drugs or model compounds bearing a carboxy group [43]. This protein was chosen because of its low molecular weight (14400) and the presence of seven free amino groups available for drug derivatization. When incubated with rat kidney lysosomes, the naproxen–lysozyme conjugate (**6.25**, $n \approx 0.5$) liberated naproxen-Lys but no free naproxen. In other words, the protein was, indeed, degraded, but the amide bond between naproxen and the ε-amino group of a



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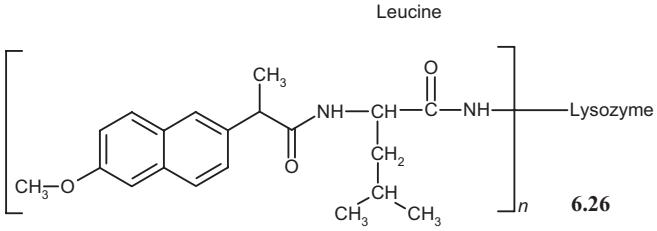

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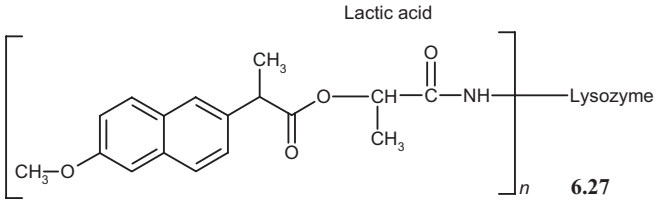


6.27

lysine residue proved stable. Other carboxylic acids such as benzoic acid and indomethacin behaved identically. Only in the case of carboxylic acids with a peptidic structure (*e.g.*, enalaprilate) was the linking bond (a peptide bond in this case) hydrolyzed to liberate the drug.

To circumvent this problem, various oligopeptide spacers were examined, *e.g.*, naproxen-Leu-lysozyme (**6.26**, $n \approx 0.3$), but the product released by lysosomes was in all cases naproxen linked to the first amino acid residue. Thus, for nonsteroidal anti-inflammatory drugs and other similar agents, a peptide spacer concept was no guarantee of rapid and effective release of the parent drug from a drug–lysozyme conjugate. Final success, *i.e.*, drug release, was achieved with conjugates having a hydroxy acid as a spacer, *e.g.*, naproxen-lactic acid-lysozyme (**6.27**, $n \approx 0.6$). In such conjugates, the spacer was attached to the drug by an ester bond, and to the LMWP by an amide bond. The critical reaction of hydrolysis was, therefore, that of an ester bond, not that of an amide link. Interestingly, these ester LMWP conjugates were found to be adequately stable in the bloodstream, from which they were re-

moved by glomerular filtration and not by hydrolysis. It was concluded that LMWPs may indeed be of use as carriers for specific renal delivery of drugs, since renal cortex homogenates and lysosomes are able to degrade the protein and generate the parent drug from drug–spacer–LMWP conjugates.

6.2.3.2. *Targeting of Peptidases Overexpressed in Pathologies*

Two cases have been selected here to illustrate the targeting of pathologically overexpressed enzymes, namely peptidases overexpressed in infected wounds and in tumors.

Exudates from *infected wounds* (*Staphyloccocus aureus*) contain elevated levels of *thrombin*. This enzymatic activity has, therefore, been used as a target for local activation of prodrugs of antibiotics such as gentamycin [44]. The device for drug delivery consisted in gentamycin coupled to a poly(vinyl alcohol) hydrogel by a thrombin-sensitive peptide linker (**6.28**, *Fig. 6.12*). Thrombin cleaved the linker at the Arg–Gly bond to release Gly-Phe-Pro-Ala-Gly-Gly-gentamycin. The latter was then digested by aminopeptidases present in serum and wounds (*Fig. 6.12*) to liberate gentamycin. This activation sequence was demonstrated when the conjugate **6.28** was incubated with *S. aureus* infected wound fluid, with thrombin and leucine aminopeptidase, or with human plasma and Ca^{2+}. The bactericidal activity of the conjugate was also demonstrated *in vivo* in infected wounds in rats.

Tumor cells express many hydrolytic enzymes, particularly peptidases, some of which are partially specific for certain tumor types, *e.g.*, plasmin, plasminogen activator protease, and cathepsins. A number of prodrug strategies have been developed for the tumor-selective delivery of cytotoxic drugs [45–47], as illustrated below with a few representative examples.

Many tumor cells contain overexpressed levels of membrane-bound plasminogen activators and, thus, produce elevated levels of *plasmin* (EC 3.4.21.7) in the milieu of the tumor. This has led to the idea of developing plasmin-activated anticancer prodrugs. Two such drugs, phenylenediamine mustard and acivicin (**6.29** and **6.30**, respectively, R=H), were derivatized to the D-valylleucyllysyl prodrugs (**6.29** and **6.30**, R=D-Val-Leu-Lys-, respectively) [48]. These prodrugs showed several-fold-increased cytotoxicity toward chicken embryo fibroblasts transformed by *Rous* sarcoma virus to produce higher levels of plasminogen activator. A number of experiments demonstrated that this selectivity was, indeed, due to plasmin-catalyzed activation. Thus, the epimeric prodrug analogue **6.29** (R=D-Val-Leu-D-Lys-) was inactive. Furthermore, the prodrug of acivicin (**6.30**, R=D-Val-Leu-Lys-) did not display selective toxicity for the transformed cells when these were cultured in plasminogen-free medium.

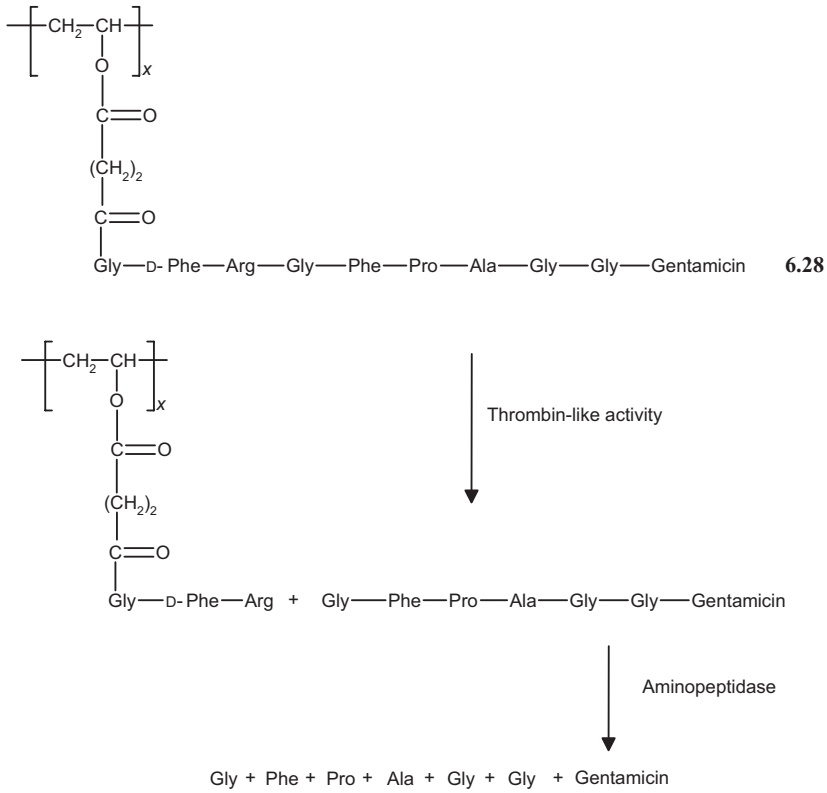
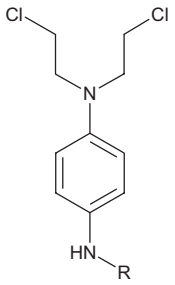


Fig. 6.12. *Two-step activation of a poly(vinyl alcohol)–linker–gentamycin device for wound-selective delivery of the antibiotic* [44]. The overexpressed enzyme thrombin is the activity targeted by the thrombin-sensitive peptide linker.

The prodrugs **6.29** and **6.30** (R=D-Val-Leu-Lys- in both cases) were also tested *in vivo* for antitumor activity. However, the results were disappointing in that no improved therapeutic index was seen when drug and prodrug were compared in mice bearing intraperitoneal B16 melanoma or early M5076 ovarian carcinoma. Permeation problems and degradation at extratumor sites were some of the factors invoked as plausible causes of *in vivo* failure.

Plasmin is not the only protease whose levels are increased at some tumor sites. Other examples include prolidase, aminopeptidase N, cathepsins, and prostate-specific antigen [47][49]. The targeting of some of these proteases is aptly illustrated with prodrugs of *doxorubicin* (DOX, **6.31**, R=H). Doxorubicin is, indeed, a highly active cytotoxic agent used with marked efficacy in breast and small-cell lung cancers. However, cumulative cardiotoxicity severely limits its use. There is, thus, an obvious rationale for the development of tumor-targeted prodrugs of doxorubicin.



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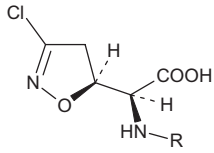

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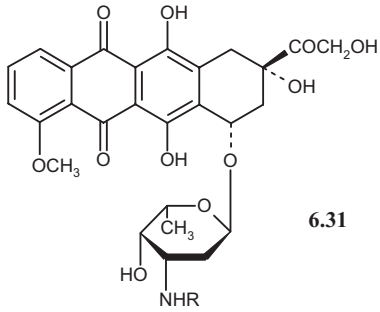

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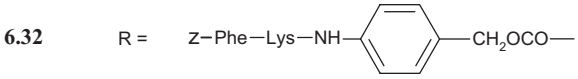
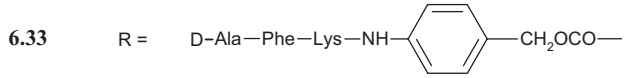




N-L-*Leucyl-doxorubicin* (Leu-DOX, **6.31**, R=Leu) appears to be a particularly promising investigational prodrug. This agent was compared with doxorubicin in human tumor xenografts growing subcutaneously in athymic nude mice. The panel of xenografts represented three different tumor types. Both compounds were administered at their maximum tolerated doses of 8 and 28 mg/kg for DOX and Leu-DOX, respectively. Leu-DOX showed antitumor activity in 10 of the 16 tumors (two of five breast, five of seven small-cell, and three of four non-small-cell lung carcinomas). In comparison, DOX was active in 7 of the 16 tumors (one breast, four small-cell lung, and two lung adenocarcinoma xenografts). In all the DOX-sensitive lung tumors,

Leu-DOX showed a higher efficacy than the parent compound. Given these results and that phase-I clinical trials with Leu-DOX had already been performed, phase-II clinical evaluation of Leu-DOX in patients with breast and lung cancer was recommended [50].

Further investigations revealed that the superior antitumor efficacy of Leu-DOX was, indeed, due to site-selective activation and delivery of doxorubicin. At equitoxic intravenous doses of DOX and Leu-DOX (8 and 28 mg/kg, respectively, see above), the plasma levels of DOX attained were comparable, whereas differences were seen in tumor tissue concentrations [51]. The enzymes involved in the tumor-selective activation of Leu-DOX were not identified, although possible candidates include the cathepsin family of proteases [47].

There are, indeed, systematic investigations to use doxorubicin prodrugs to target *cathepsin B* (EC 3.4.22.1), a member of a large family of lysosomal cysteine peptidases that function as components of the intracellular protein-degradation system. Increased cathepsin B levels and redistribution of the enzyme are well-documented in human and animal tumors [52]. Thus, two protected dipeptidyl derivatives of doxorubicin (**6.31**, R=Z-Phe-Lys- and R=Z-Val-Cit; Z=(benzyloxy)carbonyl) failed to be hydrolyzed by purified cathepsin B from bovine spleen [53]. However, introduction of the self-eliminating (4-aminobenzyloxy)carbonyl (PABC) spacer between the DOX and peptidyl moieties yielded prodrugs that were good substrates for the enzyme while remaining resistant to hydrolysis in human plasma. A particularly promising compound was doxorubicin derivative **6.32**. Mechanistic investigations revealed that activation occurred in two steps. First, cathepsin B catalyzed hydrolysis of the amide bond linking the peptide to the spacer. The PABC–DOX bond was then cleaved spontaneously in protic solvents. Other bulky cytotoxic agents such as paclitaxel were similarly derivatized to prodrugs that were activated by cathepsin B and rat liver lysosomes but not by plasma hydrolases [54].

Interestingly, a closely related prodrug of doxorubicin that has the same spacer (*i.e.*, **6.33**) was found to be a good substrate for *plasmin* while being stable in buffer and blood serum [55]. A marked selectivity was seen against a plasmin-generating MCF-7 breast cancer cell line.

The serine protease, *prostate-specific antigen* (PSA, semenogelase, EC 3.4.21.77), is expressed at high levels and in an androgen-dependent manner in prostate cancer cells and prostate cancer metastases, and is also a serological marker for prostate cancer growth. There is, thus, a clear rationale to involve this enzyme in prodrug activation. A seven amino acid peptide derivative (**6.31**, R=Ac-His-Ser-Ser-Lys-Leu-Gln-) was not hydrolyzed by PSA [55]. In contrast, the eight amino acid analogue (**6.31**, R=Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-; Mu=morpholinocarbonyl) was hydrolyzed to

Leu-DOX by PSA. Primary cultures of PC-82 human prostate cancer cells and LNCaP human prostate cancer cells produced enzymatically active PSA in sufficient amounts to activate the doxorubicin prodrug to a cytotoxic form *in vitro*. That 70 nM of the prodrug killed 50% of the PSA-producing LNCaP cells, whereas doses as high as 1 μM had no cytotoxic effect on PSA-nonproducing TSU human prostate cancer cells *in vitro*, demonstrated the specificity of the cytotoxic response [56]. The activity of **6.31** (R = Mu-His-Ser-Ser-Lys-Leu-Gln-Leu-) was further demonstrated in nude mice bearing androgen-dependent, PSA-producing PC-82 human prostate cancer xenografts [57].

6.2.3.3. *Targeting of Peptidases Imported Artificially*

The strategies for selective tumor delivery discussed above have also inspired researchers to introduce *exogenous enzymes* in tumor cells or tissues. In other words, the objective here is *a*) in a first step, to deliver an exogenous enzyme to the tumor, and *b*) in a second step, to administer a prodrug that will be specifically activated by this artificially imported enzyme. The two biotechnological protocols that currently generate considerable interest are *antibody-directed enzyme prodrug therapy* (*ADEPT*) and *gene-directed enzyme prodrug therapy* (*GDEPT*) [46][58][59].

In ADEPT, an exogenous enzyme is coupled to a monoclonal antibody (mAb) targeted to tumor cells. This enzyme–mAb conjugate is administered and allowed sufficient time to localize on the tumor cells and be cleared from circulation. In a second step, a prodrug is administered that, being a selective substrate of the exogenous enzyme, will be selectively activated at the tumor site. A schematic representation of this strategy is shown in *Fig. 6.13*, with the enzyme–mAb conjugate binding selectively to tumor cells and activating the prodrug near their surface.

We note that ADEPT is in fact a more elaborate form of antigen-targeted therapy. In this case, the active drug is coupled to an antibody raised against the tumor cells. Activation occurs by cleavage of the drug–antibody conjugate after it has localized on target cells [46]. Two relevant examples of ADEPT in which carboxypeptidases are used are presented below. Other peptidases investigated in ADEP projects include aminopeptidases, β-lactamase, and penicillin amidases. Examples concerning other enzymes will be discussed in *Chapt. 8* and *11*.

Carboxypeptidase G2 (CPG2, glutamate carboxypeptidase, EC 3.4.17.11) is a bacterial enzyme not produced in mammalian cells. It has been used with promising success to target cytotoxic alkylating agents to tumor cells [60][61]. In a series of studies, a CPG2 monoclonal antibody con-

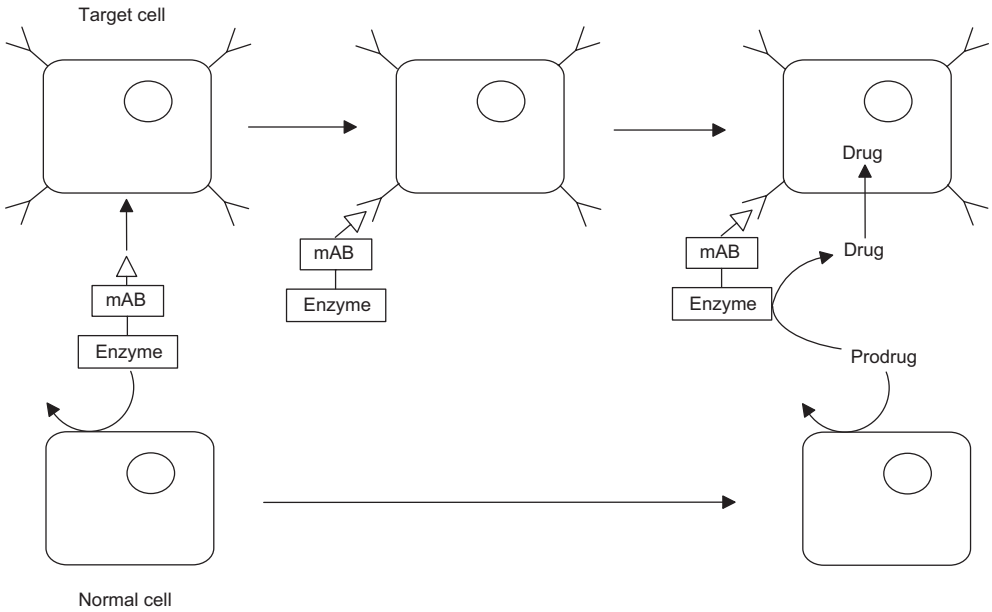
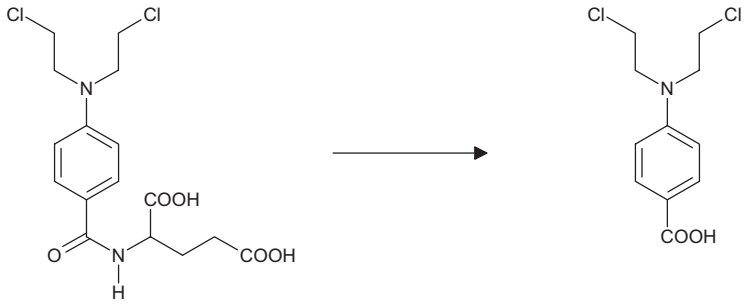


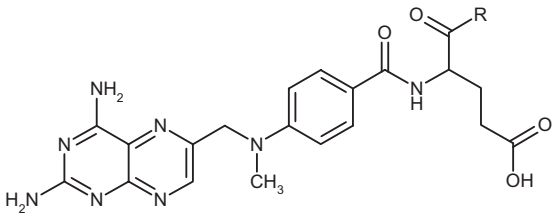
Fig. 6.13. *Schematic representation of a selective delivery obtained by antibody-directed enzyme-prodrug therapy* (ADEPT). An exogenous enzyme is coupled to a monoclonal antibody (mAb) targeted for tumor cells. In a second step, a prodrug is administered, which, as a selective substrate of the exogenous enzyme, will be selectively activated at the tumor site.

jugate was targeted to human choriocarcinoma and human colorectal cell lines. The prodrugs investigated, *N*-{4-[bis(2-chloroethyl)amino]benzoyl}-L-glutamic acid (**6.34**) and two analogues, were designed as selective substrates of CPG2. And, indeed, the two tumor cell lines labeled with the CPG2–mAb conjugate effectively activated **6.34** to the anticancer agent 4-[bis(2-chloroethyl)amino]benzoic acid (**6.35**), eliciting a strong cytotoxic response. Promising results were obtained in athymic mice with transplanted choriocarcinoma or colorectal xenografts. In a recent clinical study, an ADEPT based on prodrug **6.34** met conditions for effective antitumor therapy and gave evidence of tumor response in colorectal cancer [60].

The application of an exogenous enzyme such as carboxypeptidase G2 is certainly an attractive idea, but it presents the danger of eliciting an immune response by the patient against the enzyme. This is one of the reasons why another approach, the use of artificial mutants of human carboxypeptidases (hCPA), has been explored [62]. The drug used was *methotrexate* (**6.36**, R=H), an important agent in the treatment of solid malignancies, but one whose lack of cell selectivity is responsible for very serious toxicity to bone marrow and the gastrointestinal tract.



6.34 **6.35**



6.36

A number of prodrugs of methotrexate were prepared by linking an amino acid to the α-carboxy group of its glutamyl moiety (**6.36**, R= NH–CHR′–COOH). Such prodrugs are not substrates of the cellular folate carrier and cannot be internalized by the cells. As shown in *Table 6.3*, adding the phenylalanine pro-moiety yielded a prodrug that was a good substrate for wild-type human hCPA1 (EC 3.4.17.1) and hCPA2 (EC 3.4.17.15). In contrast, adding a negatively charged aspartic or glutamic acid pro-moiety gave poor substrates. Molecular modeling of the enzymes suggested that bulky phenylalanine- and tyrosine-based prodrugs of methotrexate would be poor substrates for hCPA1 and hCPA2. Indeed, and with a few exceptions such as the naphthylalanine derivative, prodrugs containing a bulky pro-moiety were not or hardly hydrolyzed by hCPA1 and hCPA2 (*Table 6.3*), and they were stable *in vivo* in mice.

Furthermore, the same molecular-modeling studies indicated that these bulky prodrugs would be good substrates of mutant hCPA1 and hCPA2 having an enlarged substrate binding pocket obtained by site-directed mutagenesis of Thr^{268} or Ala^{268}, respectively. The experimental results confirmed this prediction, as shown in *Table 6.3*. Thus, the 3-cyclobutyl-Phe prodrug proved

Table 6.3. *Substrate Selectivity of Human Carboxypeptidases A1 and A2* (hCPA1 and hCPA2) *and Artificial Mutants toward Methotrexate Prodrugs for Use in ADEPT* [62]

R in **6.36**	k_{cat}/K_m [M^{-1} s^{-1}]				
	hCPA1	hCPA2	hCPA1-T286G	hCPA1-T286A	hCPA2-A286G
Phe	440,000	90,000	7,350,000	2,250,000	41,000
Glu	170	0	120	n.d.[a])	n.d.
Asp	80	0	60	n.d.	76
naphthyl-Ala	1,400	1,400,000	1,400,000	640,000	5,500,000
3-cyclobutyl-Phe	0	260	1,800,000	750	2,100
2-cyclopentyl-Tyr	275	910	41,000	n.d.	260,000
3-cyclobutyl-Tyr	0	0	280,000	450	5,200
3-(*t*-Bu)-Tyr	0	0	110,000	n.d.	9,000

[a]) n.d. = not determined.

an excellent substrate for hCPA1-T286G but a very poor substrate for hCPA1 and hCPA2. Based on these promising results, the cytotoxic efficiency of hCPA1-T286G was tested in an *in vitro* ADEPT model. Here, hCPA1-T286G was chemically conjugated to ING-1, an antibody that binds to the tumor antigen Ep-Cam, or to Campath-1H, an antibody that binds to the T and B cell antigen CDw52. These conjugates were then incubated with HT-29 human colon adenocarcinoma cells (which express Ep-Cam but not Campath-1H), followed by incubation of the cells with some of the *in vivo* stable prodrugs. As expected, the ING-1–hCPA1-T286G conjugate produced excellent activation of the methotrexate prodrugs to kill HT-29 cells as efficiently as methotrexate itself. In contrast and, again, as expected, the enzyme–Campath-1H conjugate did not bind to the HT-29 cells and was without effect on the prodrugs.

In GDEPT, the gene encoding an exogenous enzyme is transferred to the tumor cells in which it is to be expressed. Two gene vectors are available, namely viral vectors (virus-directed enzyme prodrug therapy) and non-viral vectors composed of chemical gene delivery agents [46][59][63]. In a second step, a prodrug that is selectively activated by the exogenous enzyme expressed by the tumor cells is administered, as schematized in *Fig. 6.14*.

To cite an example, this strategy was also used to activate methotrexate-Phe (**6.36**, R=Phe) and other methotrexate-α-peptides in the vicinity of tumor cells [64]. Carboxypeptidase A is normally synthesized as a zymogen that is inactive without proteolytic removal of its propeptide end by trypsin. To adapt this system to GDEPT, a mutant form of the enzyme (CPA_{ST3}) was

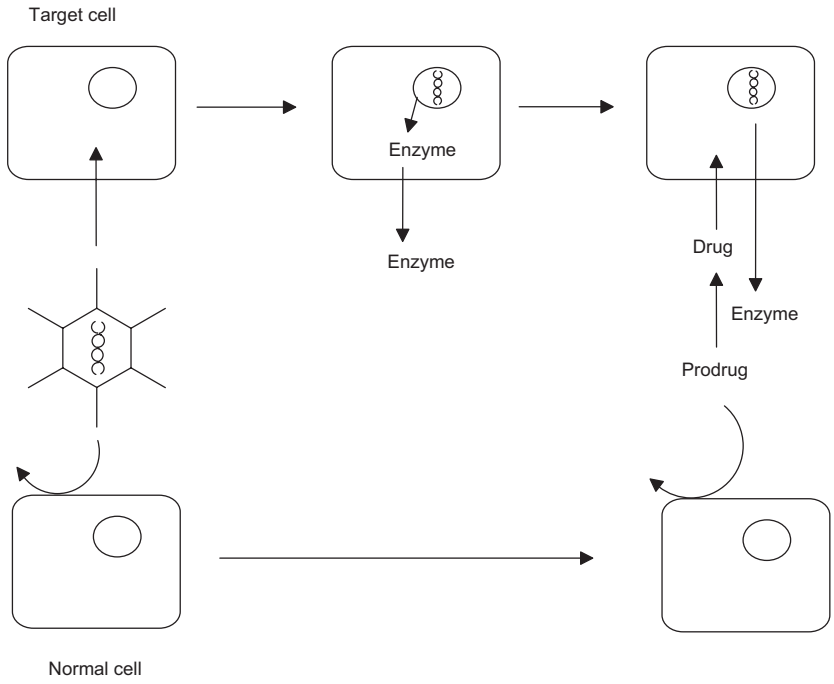


Fig. 6.14. *Schematic representation of selective delivery obtained by gene-directed enzyme-prodrug therapy* (GDEPT). The gene encoding an exogenous enzyme is transferred to tumor cells, where it is to be expressed. In a second step, a prodrug is administered that is selectively activated at the tumor site by the exogenous enzyme expressed by the tumor cells.

engineered. This mutant did not require trypsin-dependent zymogen cleavage but was activated by ubiquitously expressed intracellular propeptidases. All evidence indicated that mature CPA_{ST3} was structurally and functionally similar to the trypsin-activated, wild-type enzyme. Furthermore, tumor cells expressing CPA_{ST3} were sensitized to the methotrexate prodrugs in a dose- and time-dependent manner.

To limit diffusion of CPA_{ST3}, a cell-surface-localized form was generated by constructing a fusion protein between CPA_{ST3} and the phosphatidylinositol linkage domain from decay accelerating factor (DAF). After retroviral transduction, both CPA_{ST3} and $CPA_{ST3\text{-}DAF}$ exhibited a potent bystander effect, even when <10% of the cells were transduced, because extracellular production of methotrexate sensitized both transduced and nontransduced cells.

The examples given above may not be the ones ultimately cleared for cancer therapy. They were selected here simply to illustrate the principles of the ADEPT and GDEPT strategies relevant to our context.

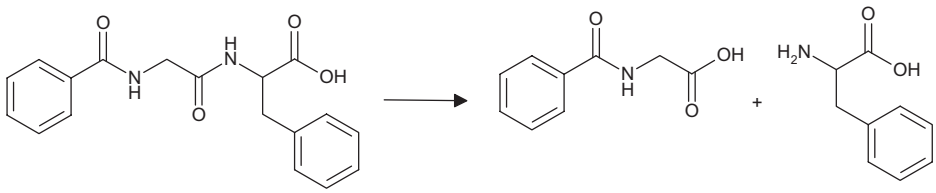
6.3. Chemical Hydrolysis of Peptides

The chemical degradation of peptides can involve a variety of reactions as schematized in *Fig. 6.4*. Before focusing on enzymatic reactions of hydrolysis, it is opportune here to discuss the chemical (*i.e.*, nonenzymatic) reactions of hydrolysis occurring in peptides. Nonhydrolytic reactions (isomerization at stereogenic centers, oxidation, β-elimination, *etc.*) and physical phenomena fall outside the scope of this work. As will be seen, nonenzymatic reactions of hydrolysis are usually but not always slow under normal conditions of temperature and medium. As such, they raise pharmaceutical problems mainly of storage that may affect the shelf-life of the peptides, but seldom pose pharmacokinetic or biological problems that could affect the *in vivo* half-life of exogenous and endogenous peptides. The major exceptions to this rule are degradation reactions occurring at asparagine residues (*Sect. 6.3.4.*), whose rate can be fast enough to be biologically significant by hastening the *in vivo* breakdown of endogenous peptides.

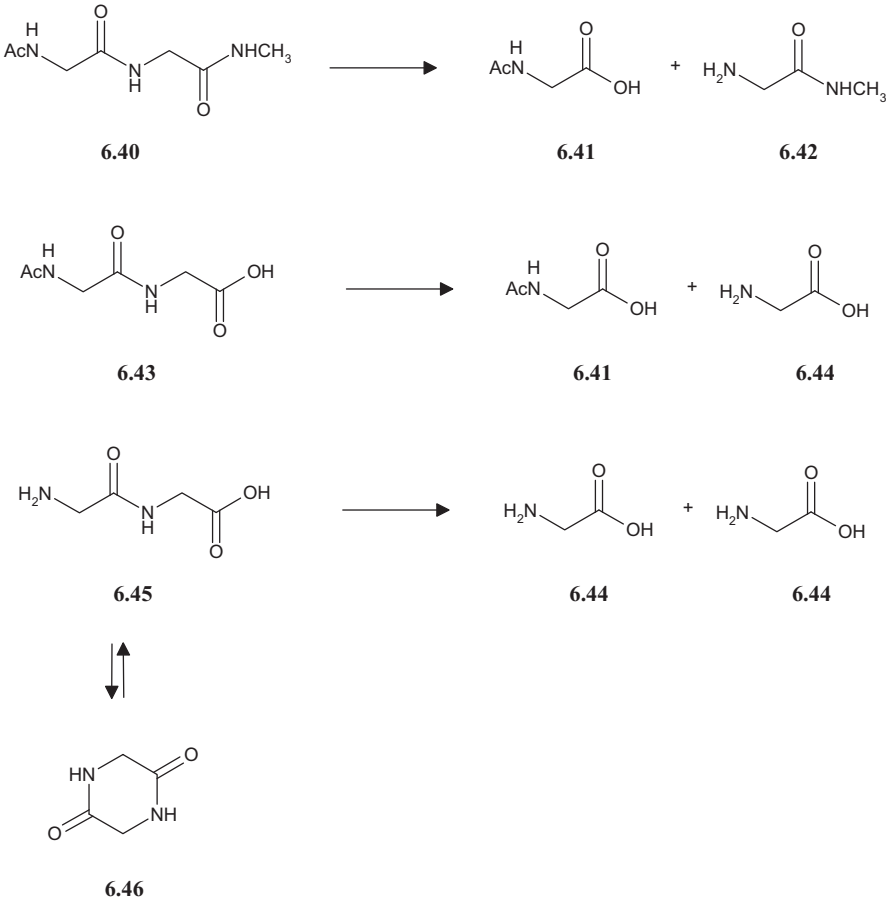
6.3.1. Chemical Hydrolysis of the Peptide Bond

The evidence examined here pertains to hydrolysis of the peptide bond and of proteins in water under various conditions of pH and temperature. Selective hydrolysis by artificial or biomimetic catalysts is not discussed (*e.g.*, [65][66]).

The *intrinsic inertness* of the peptide bond is demonstrated by a study of the chemical hydrolysis of N-*benzoyl-Gly-Phe* (hippurylphenylalanine, **6.37**) [67], a reference substrate for carboxypeptidase A (EC 3.4.17.1). In pH 9 borate buffer at 25°, the first-order rate constant for hydrolysis of the peptide bond (k_{chem}) was 1.3×10^{-10} s^{-1}, corresponding to a $t_{1/2}$ value of 168 y. This is a very slow reaction indeed, confirming the intrinsic stability of the peptide bond. Because the analytical method used was based on monitoring the released phenylalanine, no information is available on the competitive hydrolysis of the amide bond to liberate benzoic acid.



6.37 **6.38** **6.39**



The kinetic constants for the carboxypeptidase A catalyzed hydrolysis at pH 9 and 25° were $k_{cat}=61\ s^{-1}$, $K_m=0.29$ mM. In other words, the enzyme afforded a rate enhancement of 11 orders of magnitude ($k_{cat}/k_{chem}=4.7\times10^{11}$), and a catalytic proficiency of 15 orders of magnitude ((k_{cat}/K_m)/$k_{chem}=1.6\times10^{15}\ M^{-1}$).

Comparable results were obtained by investigating the kinetics of non-enzymatic hydrolysis of acetylglycineglycine *N*-methylamide (**6.40**), acetylglycineglycine (**6.43**), and glycineglycine (**6.45**) [68]. Hydrolyses were carried out at pH values of *ca.* 6.8 and at high temperatures, and then extrapolated to 25°. Thus, *Ac-Gly-Gly* N-*methylamide* (**6.40**) was hydrolyzed to acetylglycine (**6.41**) and glycine *N*-methylamide (**6.42**) with a first-order rate constant at 25° of $3.6\times10^{-11}\ s^{-1}$ ($E_a=23.5$ kcal mol^{-1}), which corresponds to a $t_{1/2}$ value of *ca.* 600 y. A similar result was obtained for the hydrolysis of *Ac-Gly-Gly* (**6.43**) to yield acetylglycine (**6.41**) and glycine (**6.44**) ($k=$

4.4×10^{-11} s^{-1}, E_a=25.0 kcal mol^{-1}, $t_{1/2}$ *ca.* 500 y). A comparison with enzymatic parameters (carboxypeptidase B and angiotensin-converting enzyme) again revealed rate enhancements of 10 to 13 orders of magnitude, and catalytic proficiencies of 13–17 orders of magnitude.

The case of *Gly-Gly* (**6.45**) is more complex, because the presence of an unprotected N-terminus, plus the lack of steric hindrance at the C^{α}-atoms, resulted in a reversible cyclization to form diketopiperazine (Gly=Gly, **6.46**; $k_1 + k_{-1} \approx 2\times10^{-8}$ s^{-1}, $t_{1/2}$ *ca.* 1 y). The cyclization reaction was competitive with hydrolysis, forming two molecules of glycine (**6.44**; k=6.3 $\times10^{-11}$ s^{-1}, E_a=23.0 kcal mol^{-1}, $t_{1/2}$ *ca.* 350 y). The problem of diketopiperazine formation at the N-terminus of peptides will be discussed again in the next section. This example, together with the previous one, confirms the very high stability of peptides bonds under physiological conditions of pH and temperature. As discussed in the next section, such a statement is no longer valid in the presence of a few side-chain residues able to react intramolecularly.

The above examples demonstrate the behavior of peptide bonds at neutral pH. Information is also available on the pH–rate profile of hydrolysis of peptide bonds, as exemplified by N-*(phenylacetyl)glycyl*-D-*valine* (**6.47**), an acyclic penicillin G analogue [69]. As a preliminary observation, we note that this compound contains a single stereogenic center, meaning that results obtained with its enantiomer *N*-(phenylacetyl)-Gly-Val would have been identical under the achiral conditions of the study.

The two hydrolytic reactions of *N*-(phenylacetyl)glycyl-D-valine (**6.47**) yield Gly-D-Val (**6.48**) and D-Val (**6.49**), respectively, as peptide products. The pH–rate profiles of the reactions at 37° (*Table 6.4*) were practically iden-

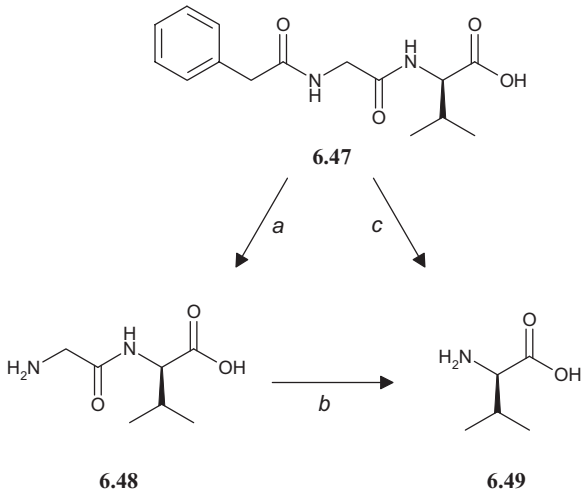


Table 6.4. *First-Order* (in s^{-1}) *and Second-Order* (in M^{-1} s^{-1}) *Rate Constants for the Hydrolysis of* N-*(Phenylacetyl)glycyl*-D-*valine* (**6.47**) *at 37°* [69]

	pH	*Reaction a*[a])	*Reaction c*[b])	*Reaction b*[c])
log k [s^{-1}]	0	–5.6	–6.0	n.d.[d])
	1	–7.0	–6.5	n.d.
	3	–8.7	–8.9	n.d.
	5	–10.3	–9.8	n.d.
	7	–10.3	–10.6	n.d.
	9	–9.4	–9.6	n.d.
	11	–8.8	–9.0	n.d.
	13	–7.6	–7.2	n.d.
	14	–5.5	–5.6	n.d.
log k_{H_2O} [s^{-1}]		–10.0	–10.1	n.d.
log k_{H^+} [M^{-1} s^{-1}]		–5.8	–5.8	–7.0
log k_{HO^-} [M^{-1} s^{-1}]		–6.0	–5.9	–5.7

[a]) Rate constants for the hydrolysis of the phenylacetyl–Gly bond in **6.47** to yield phenylacetic acid and Gly-D-Val (**6.48**). [b]) Rate constants for the hydrolysis of the Gly–D-Val bond in **6.47** to yield *N*-phenylacetyl-Gly and D-Val (**6.49**). [c]) Rate constants for the hydrolysis of Gly-D-Val (**6.48**) to yield Gly and D-Val (**6.49**). [d]) n.d. = Not determinable.

tical, with a maximum of stability in the region of pH 5–9 ($t_{1/2}$ *ca.* 220 y). Below pH 5 and above pH 9, the rates of reaction increased linearly to become four orders of magnitude larger at pH 0 and 14 ($t_{1/2}$ *ca.* 8 d).

The individual contributions of the H_2O, H^+, and HO^- catalysts to the mechanism of the reaction were further evaluated by means of the kinetics parameters (*Table 6.4*). At neutral pH, *Reactions a* and *c* were both dominated by k_{H_2O}. The second-order rate constants k_{H^+} and k_{HO^-} were identical, indicating similar efficiencies of the H^+ and HO^- catalysts. Interestingly, the second-order rate constants for the hydrolysis of Gly-D-Val (**6.48**) to yield Gly and D-Val (**6.49**) (*Reaction b*) could also be calculated (*Table 6.4*). The similarity to the corresponding rate constants of *Reactions a* and *c* suggests that the rate of peptide bond hydrolysis is not particularly sensitive to substitution at or protonation of the flanking amino and carboxy groups [69].

The great stability of unactivated peptide bonds has implications for the methods of hydrolysis used in peptide sequencing (*i.e.*, partial hydrolysis) or in determining amino acid composition (*i.e.*, complete hydrolysis). The ability of partial hydrolysis to produce smaller peptides containing overlapping sequences was already used in 1945–1953 by *Sanger* and co-workers [70] to establish the sequence of insulin. This *peptide sequencing* technique is based on the observation that acid hydrolysis of peptide bonds does not occur randomly. Bonds on the amino side of serine and threonine residues are the most labile; other rather labile bonds are those formed by aspartic acid and glycine residues (see next section). In contrast, Val–Ile and Ile–Ile bonds are extreme-

ly difficult to hydrolyze. Nevertheless, the specificity of peptide bond cleavage by acid hydrolysis is rather low, which leads to complex peptide mixtures that are difficult to analyze. This represents a major limitation of the technique. However, this problem has been overcome by application of modern mass-spectrometry (MS) techniques. For example, peptides incubated 3–30 min in 1–6N HCl at 100–110° are partially hydrolyzed to shorter peptides, which may then be sequenced *via* analysis by sophisticated MS techniques such as high-resolution plasma-desorption MS [71].

Much more drastic methods must be applied when the goal is to determine a peptide's *amino acid composition*, but partial racemization and partial loss of sensitive amino acids (*e.g.*, Ser, Thr, Trp) are difficult to avoid entirely. Also, under conditions that produce complete hydrolysis, asparagine and glutamine residues generally undergo deamidation, as do amidated C-termini. Various methods have been investigated in an extensive study, showing that conventional thermal hydrolysis in 6N HCl or 4M methanesulfonic acid solution at 110° for 24 h yields good composition data [72]. Comparable results were obtained when thermal energy is replaced with microwave radiation energy, albeit with slightly more racemization and loss of hydrolysis-sentitive residues.

Why should some peptide bonds be more sensitive to hydrolysis than others? As discussed in the next section, the *side chains of certain residues* (*e.g.*, Asp, Glu, Asn, and Gln) are able to undergo intramolecular reaction with the adjacent residue, leading to a variety of peptide bond fission and rearrangement reactions. Certain residues when located at the N- or C-terminus also exhibit a particular degree of reactivity.

A more general factor involved in chemical hydrolysis, one that remains to be fully validated, is a *decrease in the partial double-bond character of some peptide bonds* (see *Fig. 6.3*). Based on X-ray data, the C–N bond has an average length of 147 pm, with limits of 137 and 157 pm. The corresponding values for the C=N bond are 128 pm, with limits of 120 and 137 pm. Thus, the measured or calculated length of a peptide bond may give an indication of its partial double-bond character. A survey of crystallographic data suggests that the bond length of most delocalized peptide bonds in proteins are 134 and 136 pm. Non-delocalized peptide bonds range from 140–148 pm, without correlation to dihedral angle [73].

Such a difference in partial double-bond character has implications for the mechanism, and, hence, the reaction rate, of acid-catalyzed hydrolysis (*Fig. 6.15*). In delocalized peptide bonds (*Fig. 6.15,a*), protonation involves the carbonyl O-atom with its partial negative charge. In non-delocalized peptide bonds (*Fig. 6.15,b*), protonation involves the N-atom, which is rendered more basic by the lack of delocalization [73].

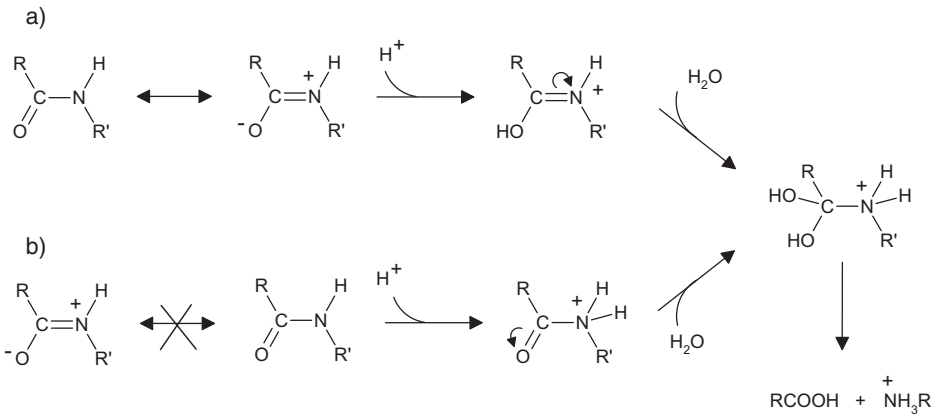


Fig. 6.15. *Simplified mechanisms of the acid-catalyzed hydrolysis of* a) *delocalized peptide bonds and* b) *non-delocalized peptide bonds*

6.3.2. Peptide Sites That Exhibit Particular Reactivity toward Hydrolysis

A limited number of sites and residues are recognized as having particular reactivity toward chemical hydrolysis, or to undergo reactions of nucleophilic substitution often accompanied by hydration or dehydration. Examples of the most significant of these reactions are discussed in the present and following two sections. In this section, we examine reactions at the *C-terminus* (hydrolysis of the $CONH_2$ group), at the *N-terminus* (cleavage of pyroglutamyl peptide bonds, formation of diketopiperazines), and at *certain residues* (peptide bonds involving Ser, side-chain hydrolysis of Arg to Cit and Orn). A few reactions occurring in the solid state are also mentioned. In *Sect. 6.3.3*, cleavage at *aspartic acid peptide bonds* is presented, whereas *Sect. 6.3.4* will be devoted to reactions of deamidation of and cleavage at *asparagine and glutamine residues*. We recall that, while, as a rule, the nonenzymatic reactions discussed in *Sect. 6.3* may pose problems during manufacturing and storage of peptide drugs, with the exception of asparagine residues, these reactions are usually too slow to affect the half-lives of peptides in the body. They are, thus, of pharmaceutical but seldom of pharmacokinetic or biological relevance. A compilation of the reactions and examples discussed is given in *Table 6.5*.

6.3.2.1. *Hydrolysis of $CONH_2$ Groups at the C-Terminus*

Numerous examples document the hydrolysis of terminal $CONH_2$ groups. Thus, the degradation products of *luteinizing hormone releasing*

Table 6.5. *Peptide Sites of Particular Reactivity toward Chemical Hydrolysis*. See text for references and further details.

Site	Reaction(s)	Examples discussed in text
C-Terminal $CONH_2$	Hydrolysis to COOH	Luteinizing hormone releasing hormone (*Fig. 6.16*)
		[Arg^6,D-Trp^{7,9},MePhe^8]substance P-(6–11)-hexapeptide (**6.52**, *Fig. 6.17*) and model tripeptides
		Klerval (*Fig. 6.18*)
		RS-26306 (*Fig. 6.19*)
		Pramlintide (*Fig. 6.20*)
		Thyrotropin-releasing hormone (*Fig. 6.21*)
N-Terminal Glp	Ring opening of Glp and cleavage of Glp peptide bonds	Luteinizing hormone releasing hormone (*Fig. 6.16*)
		Glp-Phe-Leu-Phe-Arg-Pro-Arg, Glp-Leu-Gly-Pro, Glp-His-Pro, and Glp-His
		Thyrotropin-releasing hormone (*Fig. 6.21*)
The two N-terminal residues	Loss of the two N-terminal residues *via* a diketopiperazine	Glycineglycine *N*-methylamide (**6.56**)
		Luteinizing hormone releasing hormone (Fig. 6.16)
		Arg-Trp-Phe (**6.50**, R = OH) and Phe-Trp-Arg (**6.51**, R = OH) (*Fig. 6.17*)
		Thyrotropin-releasing hormone (*Fig. 6.21*)
Ser–Xaa or Thr–Xaa bonds	Hydrolysis of peptide bonds involving a Ser or Thr residue	Luteinizing hormone releasing hormone (*Fig. 6.16*)
		RS-26306 (*Fig. 6.19*)
		Calcitonin (*Fig. 6.22*)
		Cyclosporin A (*Fig. 6.23*)
Arg	Side-chain hydrolysis to Orn and/or Cit	[Arg^6,D-Trp^{7,9},MePhe^8]substance P-(6–11)-hexapeptide (**6.52**, in *Fig. 6.17*) and model tripeptides

Table 6.5 (cont.)

Site	Reaction(s)	Examples discussed in text
Asp residues	Degradation at Asp residues (*Pathways a*, *b* and/or *c* in *Fig. 6.27*)	Arg-Gly-Asp-Phe (**6.70**)
		Val-Tyr-Pro-Asp-Gly-Ala, Val-Tyr-Pro-Asp-Ser-Ala, Val-Tyr-Pro-Asp-Val-Ala, and Val-Tyr-Gly-Asp-Gly-Ala (*Fig. 6.28*)
		Klerval (*Fig. 6.18*)
		Human parathyroid hormone
		Secretin
		Human basic fibroblast growth factor
Asn and Gln residues	Degradation at Asn and Gln residues (*Pathways d*, *e* and/or *f* in *Fig. 6.29*)	Val-Tyr-Pro-Asn-Gly-Ala (*Fig. 6.30*)
		N-Pivaloyl-Gly-Asn-Sar-Gly-*tert*-butylamide (**6.75**)
		Glucagon
		Calcitonin (*Fig. 6.22*)
		Xaa-Gln dipeptides
		Ribonuclease A
		Val-Xaa-Asn-Ser-Val and Val-Ser-Asn-Xaa-Val
		Pramlintide (*Fig. 6.20*)
		Human parathyroid hormone
		Human tissue plasminogen activator
		Human growth hormone (hGH)
		Insulin (*Fig. 6.32*)

hormone (LHRH, gonadorelin-releasing hormone (GnRH), Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2; *Fig. 6.16*) were determined in aqueous solution at neutral pH at various temperatures [74]. One of the predominant routes of degradation was hydrolysis of the terminal $CONH_2$ group (*Fig. 6.16*, *Reaction a*) to yield the terminal free acid form. Other predominant reactions will be considered later in this section. Kinetics studies over a range of pH and temperature values showed that LHRH is maximally stable at pH 4–6. Thus, the first-order rate constants at 25° were *ca.* 8×10^{-10} s^{-1} at pH 5 and *ca.* 2×10^{-9} s^{-1} at pH 7.4, corresponding to $t_{1/2}$ values of *ca.* 27 and 11 y, respectively [75]. Physical phenomena such as peptide aggrega-

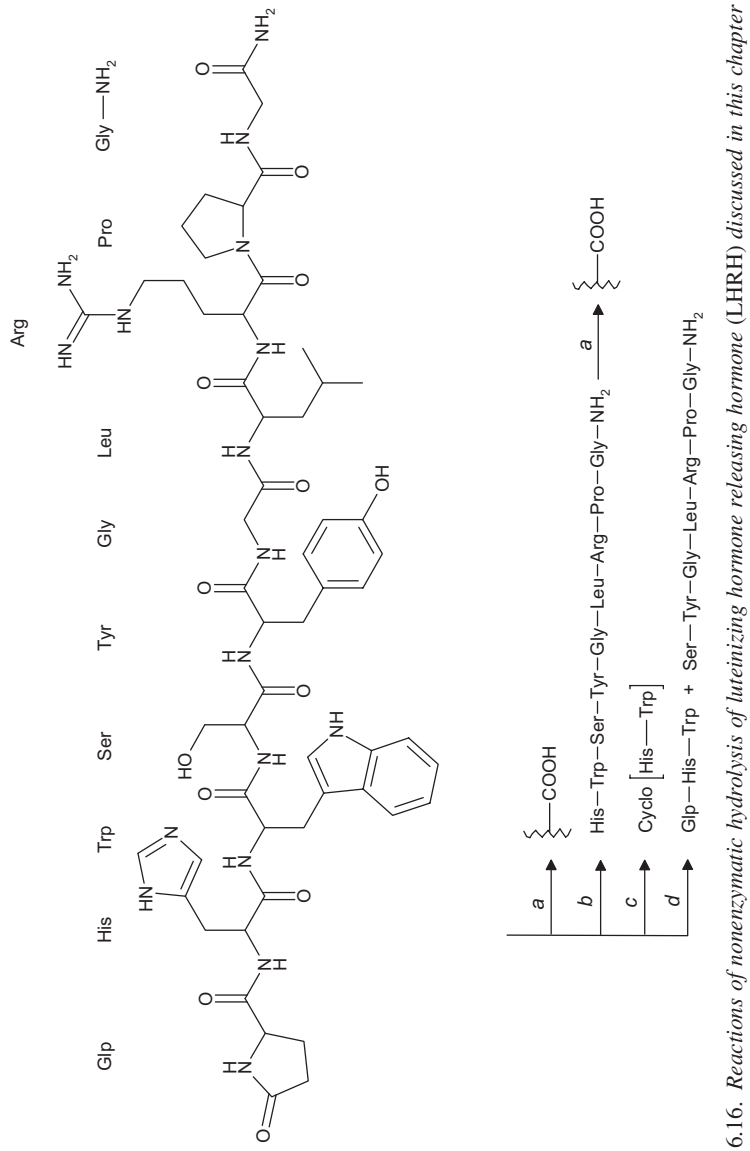


Fig. 6.16. *Reactions of nonenzymatic hydrolysis of luteinizing hormone releasing hormone* (LHRH) *discussed in this chapter* [73]

tion appear to be a greater cause for pharmaceutical concern than chemical stability.

The antagonist *[Arg^{6},D-Trp^{7,9},MePhe^{8}]substance P-(6–11)-hexapeptide* (**6.52**, *Fig. 6.17*), which has been investigated as a potential antitumor agent, has been found to undergo some chemical degradation. The tripeptides Arg-Trp-Phe-NH_2 (**6.50**, R=NH_2), Arg-Trp-Phe (**6.50**, R=OH), Phe-Trp-Arg-NH_2 (**6.51**, R=NH_2), and Phe-Trp-Arg (**6.51**, R=OH) were, therefore, examined to obtain more insight into the breakdown processes [76a]. A study of the kinetics of degradation as well as identification of degradation products in alkaline and acidic media was undertaken. The amidated forms were less stable than the carboxylic acid forms. Indeed, Arg-Trp-Phe-NH_2 and Phe-Trp-Arg-NH_2 exhibited maximal stability at pH 4–5 ($k=2\times10^{-6}$ s^{-1} at 80°, $t_{1/2}$ 4 d) and a $t_{1/2}$ value at pH 7.4 and 80° of *ca.* 1.6 d. The corresponding results for Arg-Trp-Phe and Phe-Trp-Arg were a maximum of stability at pH 5–7 ($k=1\times10^{-6}$ s^{-1} at 80°, $t_{1/2}$ 8 d) and a $t_{1/2}$ value at pH 7.4 and 80° of *ca.* 5 d.

The tripeptides in *Fig. 6.17* underwent a few breakdown reactions (N-terminus elimination, Orn formation, peptide bond hydrolysis), some of which will be considered later in this section. Of relevance here was that, of the two amidated tripeptides, the amide at the C-terminus underwent deamidation predominantly (*Fig. 6.17*, *Reaction a*), which, perhaps, explains the somewhat lesser stability compared to the free carboxylic acid forms. While the hexapeptide (**6.52**, *Fig. 6.17*) followed a different pattern of decomposition [76b], deamidation was also a predominant hydrolytic reaction at all pH values. Thus, the procedure to extrapolate results from small model peptides to larger medicinal peptides appears to be an uncertain one, since small modifications in structure can cause large differences in reactivity.

Another example is found with *klerval* (*Fig. 6.18*), a compound developed as a platelet anti-aggregant and inhibitor of thrombus formation [77]. This compound, an (*N*-acyl-*N*-ethylglycyl)aspartyl-cyclohexylalanine amide, hence, a derivatized peptide containing an artificial amino acid (according to the definitions given in *Sect. 6.4* and *6.5*), demonstrates that the reactions of chemical hydrolysis discussed in *Sect. 6.3* are not restricted to peptides that contain only proteinogenic amino acids. This compound was relatively stable in the pH range of 5–8 ($t_{1/2}$ 16 d at 80°), where C-terminal deamidation was minimal or not detectable. In contrast, klerval was markedly less stable below pH 5 (proton-catalyzed hydrolysis) and above pH 8 (base-catalyzed hydrolysis), with deamidation becoming the predominant reaction at pH 1 and 9 (*Fig. 6.18*, *Reaction a*). Other breakdown reactions, which occur on either side of the aspartic acid residue, will be discussed in *Sect. 6.3.3*.

The luteinizing hormone releasing hormone antagonist *RS-26306* (*Fig. 6.19*) is another example of a peptide analogue that undergoes hydrolysis of its terminal $CONH_2$ group. This decapeptide contains five synthetic amino

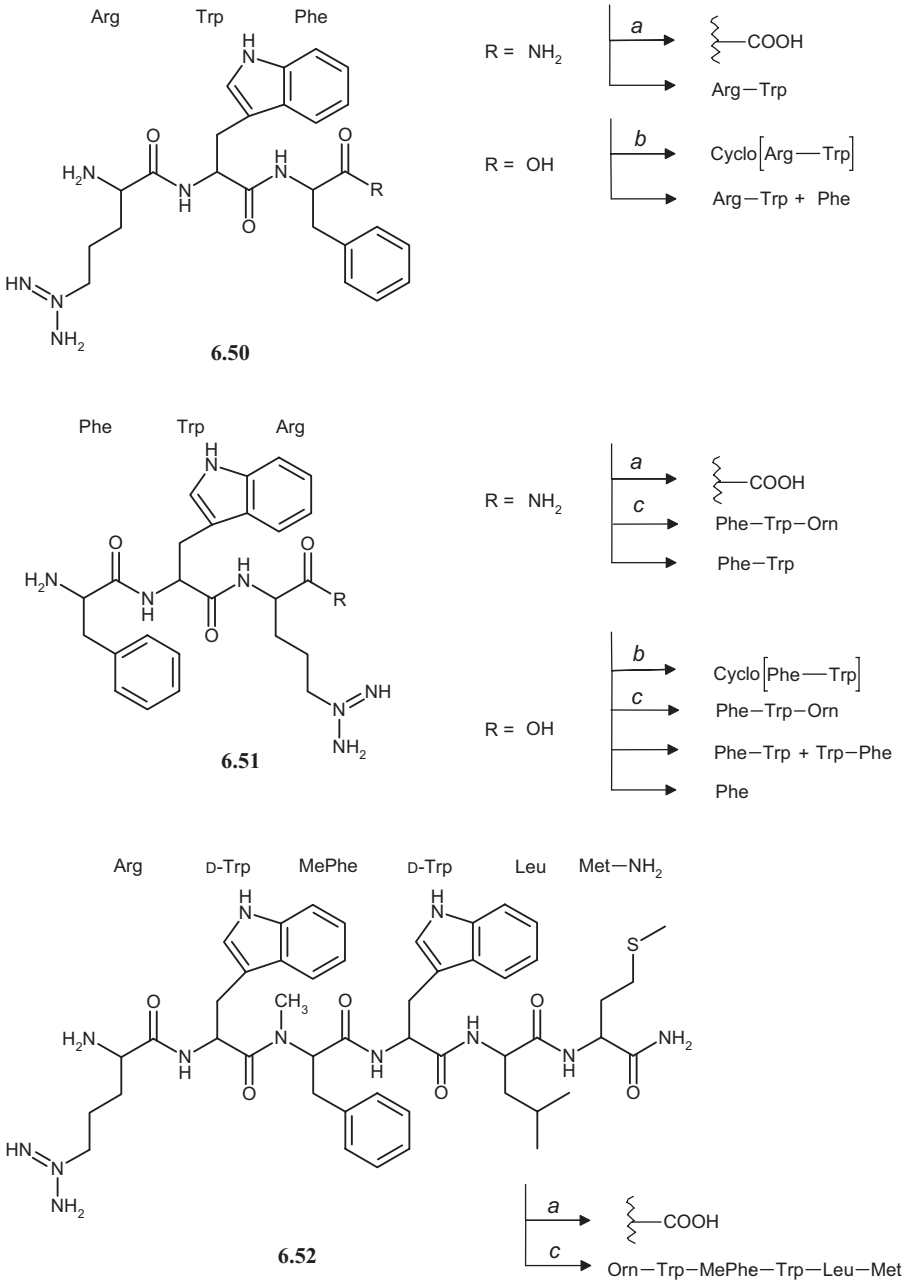


Fig. 6.17. *Reactions of nonenzymatic hydrolysis of model tripeptides* (**6.50** and **6.51**) *and of the antagonist [Arg*^{6}*,*D*-Trp*^{7,9}*,MePhe*^{8}*]substance P-(6–11)-hexapeptide* (**6.52**) *discussed in this chapter* [75]

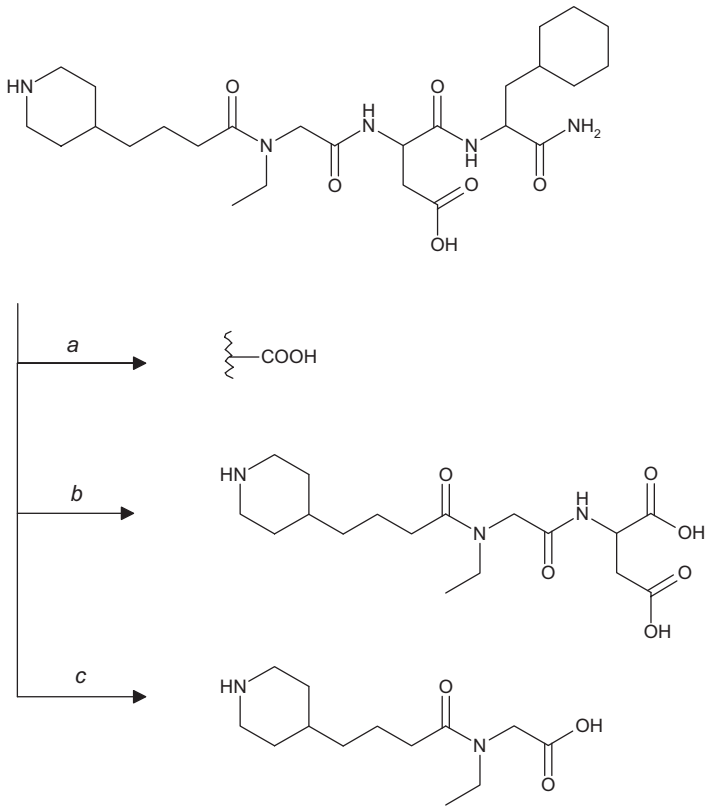


Fig. 6.18. *Reactions of nonenzymatic hydrolysis of klerval discussed in this chapter* [77]

acids (naphthylalanine, 4-chlorophenylalanine, (pyridin-3-yl)alanine, and two *N*,*N*-diethylhomoarginines), and five of its residues are in the D-configuration. Its N- and C-termini are protected by acetylation and amidation, respectively. In buffered aqueous solution, the compound was most stable in the pH range of 4–6, with a $t_{1/2}$ value at 80° and 25° of *ca.* 130 d and 100 y, respectively [78]. Deamidation of the terminal $CONH_2$ group (*Fig. 6.19*, *Reaction a*) is the major reaction at pH<4. Other reactions were intramolecular serine-catalyzed peptide hydrolysis (to be discussed below), and isomerization at C^{α}, which predominates at pH>6. Interestingly, the pH profile of the reaction of deamidation was found to be slowest at pH 5, and to increase regularly and almost symmetrically in the lower and higher pH ranges.

To close this section, we briefly mention that reactions of deamidation are not restricted to oligopeptides, as might be deduced from the above examples. For example, *pramlintide* (*Fig. 6.20*), a synthetic analogue of human amylin, is a hormone that acts as a partner to insulin in controlling blood glu-

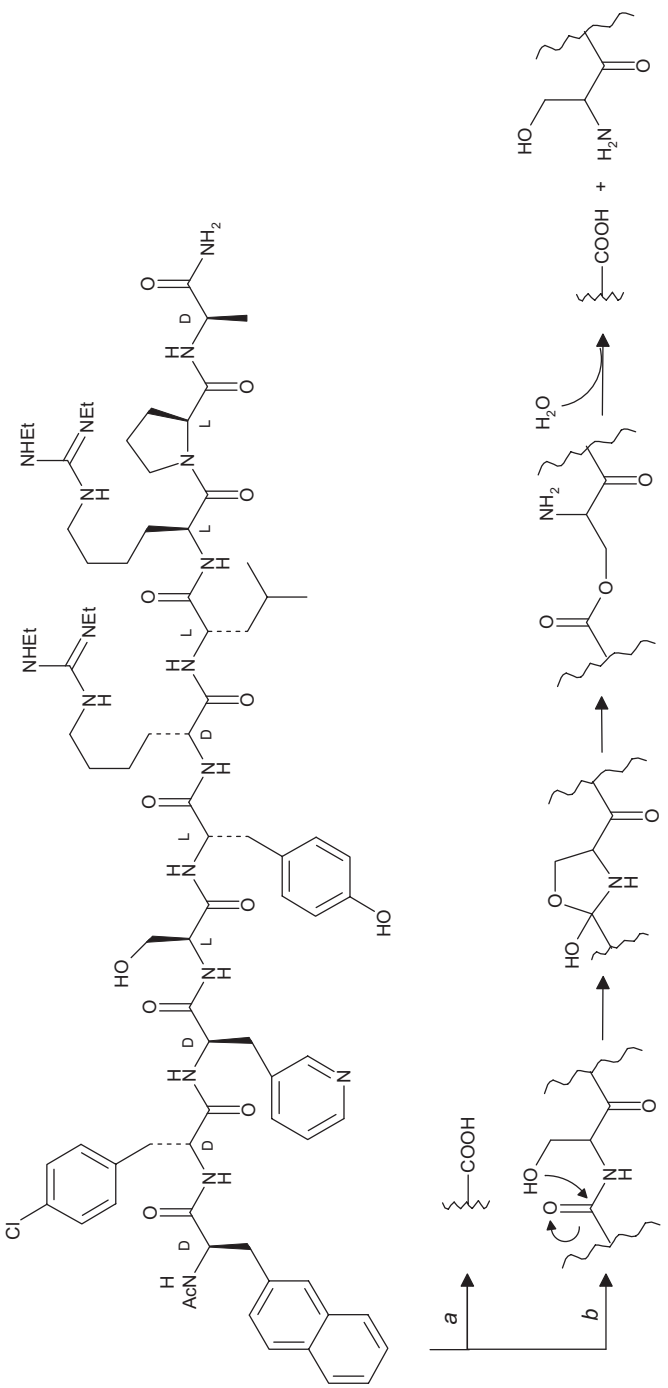


Fig. 6.19. *Reactions of nonenzymatic hydrolysis of RS-26306 discussed in this chapter* [78]

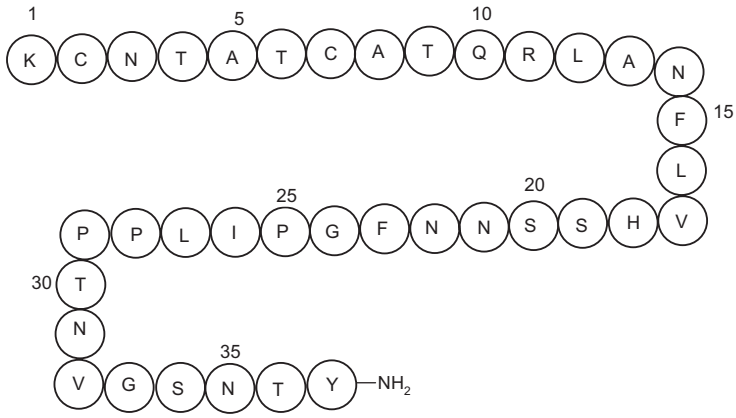


Fig. 6.20. *The chemical structure of pramlintide* [79]

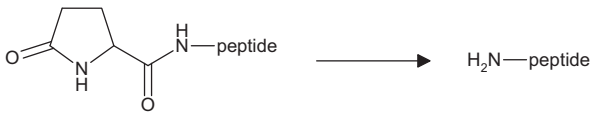
cose. Indeed, the product of C-terminus deamidation, [Tyr^{37}-OH]-pramlintide, has been identified among other breakdown products in pharmaceutical solutions at pH 4 heated at 40° for 45 d [79].

6.3.2.2. *Hydrolysis of and at Pyroglutamyl Residues*

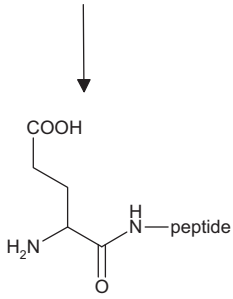
A Glp residue (**6.53**) located at the N-terminus has been found to be labile to acid hydrolysis. Two reactions can occur, namely *opening of the pyrrolidone ring* to produce the analogous Glu peptide (**6.54**), or *cleavage of the Glp peptide bond* to yield the shortened peptide (**6.55**).

Reactions of this nature have been noted in a number of *model peptides* related to bioactive peptides, namely Glp-Phe-Leu-Phe-Arg-Pro-Arg, Glp-Leu-Gly-Pro, Glp-His-Pro, and Glp-His [80]. When incubated in 1N HCl at 60° for 6 h, these peptides all underwent ring opening (*ca.* 40%) and Glp peptide bond cleavage (10–20%). The two reactions were clearly competitive and not sequential, and, indeed, the Glu peptide (**6.54**) products underwent little or no Glu peptide bond cleavage. Interestingly, decreasing temperature and increasing HCl concentration reduced the relative importance of ring opening, such that, in 36% HCl at 0°, this reaction became negligible [81].

The identity of the amino acid residue neighboring the Glp moiety also influences ring opening and cleavage reactions [82]. In tetrapeptides of general structure Glp-Xaa-Ala-Phe, the $t_{1/2}$ values for total hydrolysis in 1N HCl at 60° ranged from 1.8 to 4.4 h and increased in the series Thr<Se<Gly <Asp<Tyr<Ala<Glu=His<Lys<Arg=Ile<Pro. The ratio of pyrrolidone ring opening to Glp peptide bond cleavage also varied markedly, the ex-



6.53 **6.55**



6.54

tremes being 1.4 for Xaa=Ser and 0.4 for Xaa=Ile. These results suggest that a sterically hindered and bulky side chain including a positive or negative charge brought about resistance of the analogue to acid hydrolysis. The inductive effect of the OH groups of serine and threonine may explain the higher susceptibility of the Glp–Ser and Glp–Thr bonds to acid hydrolysis.

An example of direct biological relevance is afforded by *thyrotropin-releasing hormone* (TRH) (*Fig. 6.21*). Incubations in 1N HCl at 60° afforded a number of products, most of which are shown in *Fig. 6.21* [83]. After 6 h, the total of products hydrolyzed at the terminal $CONH_2$ group (*Reactions a*) was *ca.* 45% of the incubated amount. Hydrolytic ring opening (*Reactions b*) and Glp peptide bond cleavage (*Reaction c*) were also predominant reactions (*ca.* 70 and 25%, respectively). Again, increasing acid concentration and decreasing temperature increased markedly the relative importance of Glp peptide bond cleavage.

In LHRH, cleavage of the Glp peptide bond was a major reaction (*Fig. 6.16*, *Reaction b*) [74].

6.3.2.3. *Formation of Diketopiperazines*

Diketopiperazine formation is a reaction that occurs specifically at the N-terminus of peptides and leads to cleavage of the first two residues. *Glycylglycine* N-*methylamide* (**6.56**) has proven to be particularly useful as a model compound in investigations of the mechanism and kinetics of the reaction as compared to the reactivity of analogues **6.40**, **6.43**, and **6.45** discussed in *Sect.*

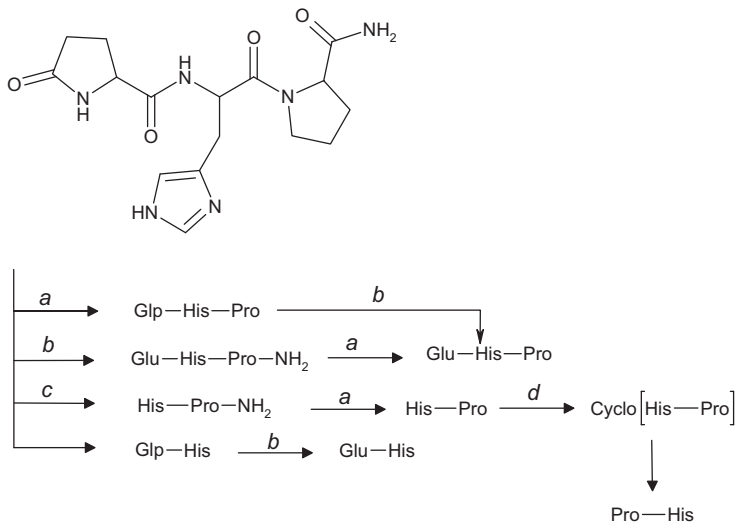
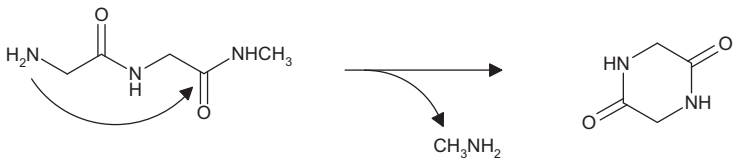


Fig. 6.21. *Reactions of nonenzymatic hydrolysis of thyrotropin-releasing hormone* (TRH) *discussed in this chapter* [83]

6.3.1 [68]. In this study, at neutral pH, Gly-Gly-NHMe underwent a single reaction, namely the formation of diketopiperazine (cyclo(Gly-Gly), **6.46**) with loss of $MeNH_2$. This nucleophilic reaction of cyclization–elimination necessitates a free amino group at the N-terminus, as shown by the failure of Ac-Gly-Gly-NHMe (**6.40**) to form a diketopiperazine. With Gly-Gly-NHMe, the reaction proceeded quite rapidly ($k=8 \times 10^{-11}$ s^{-1}, $E_a=17.2$ kcal mol^{-1}, $t_{1/2}$ *ca.* 100 d), being three orders of magnitude faster than cleavage of the Gly–Gly bond in Ac-Gly-Gly-NHMe (**6.40**), Ac-Gly-Gly (**6.43**), or Gly-Gly (**6.45**).



6.56 **6.46**

While we are not aware of any systematic investigation of the conditions and structural factors that influence diketopiperazine formation, the literature contains a number of examples of such reactions. Thus, cyclo(His-Trp), a degradation products of LHRH (*Fig. 6.16*, *Reaction c*), is a secondary product formed after cleavage of the Glp–His bond. After an incubation of 90 d at neutral pH, cyclo(His-Trp), represented 4 and 10% of the breakdown products obtained at 37° and 50°, respectively [74].

Diketopiperazine formation was also examined in the peptides **6.50**, **6.51**, and **6.52** shown in *Fig. 6.17* [76a]. Clear evidence for the formation of a diketopiperazine product was obtained only for Arg-Trp-Phe (**6.50**, R=OH). In this case, the product was cyclo(Arg-Trp) (*Reaction b*). The diketopiperazine formed from Phe-Trp-Arg (**6.51**, R=OH) was not seen directly, but the presence of Trp-Phe together with Phe-Trp afforded indirect evidence. Interestingly, diketopiperazine formation occurred during acid-catalyzed degradation but not under basic conditions, and, as explained, was restricted to the two deamidated tripeptides.

Diketopiperazine formation occurred to a marked extent during the degradation of *thyrotropin-releasing hormone* in 1N HCl at 60° (*Fig. 6.21*, *Reaction d*) [83]. After 6 h, cyclo(His-Pro) accounted for *ca.* 15% of the initial amount incubated.

The examples found in the literature concern mostly oligopeptides, with the reactive N-terminal amino acid being Gly, His, or Arg. Interestingly, the former is particularly flexible, whereas His and Arg contain a basic side chain. Whether these are fortuitous observations or have general value remains to be seen.

6.3.2.4. *Hydrolysis of Serine Peptide Bonds*

In a number of peptides, a serine residue has been found to be a site of chemical peptide bond cleavage. This was well-illustrated in a careful study of the degradation of the RS-26306 (*Fig. 6.19*) [78]. The rate constant for cleavage of the peptide bond between D-(pyridin-3-yl)alanine and L-serine ($k \approx 4 \times 10^{-8}$ s^{-1} at 80°, $t_{1/2} \approx 200$ d) was pH-independent in the investigated pH range of 3–8. The mechanism deduced was an *intramolecular nucleophilic addition* of the serine OH group to the neighboring peptide bond to form an intermediate oxazolidine, which rearranged to an acylated serine intermediate (*i.e.*, an ester) in the rate-determining step (*Fig. 6.19*, *Reaction b*).

A similar reaction was observed during degradation of LHRH itself. Here, cleavage of the Trp^3–Ser^4 bond was evidenced by production of the tripeptide and heptapeptide (*Fig. 6.16*, *Reaction d*). After 90 d at neutral pH and 50°, this reaction accounted for *ca.* 10% of the degradation observed [74]. Similar reactions were observed with various analogues of LHRH.

Calcitonin, a peptide containing 32 amino acids in a single chain characterized by a disulfide bridge between Cys^1 and Cys^7 (*Fig. 6.22*), offers an example and three counter-examples of hydrolysis at serine residues. Its rate of chemical degradation, like that of other peptides, is highly pH-dependent: at 70°, the minimum rate was observed at pH 3.3 ($k = 7 \times 0^{-6}$ s^{-1}, $t_{1/2}$ 1 d) [84]. From an activation energy of 15.4 kcal mol^{-1} at pH 6, it can be calculated

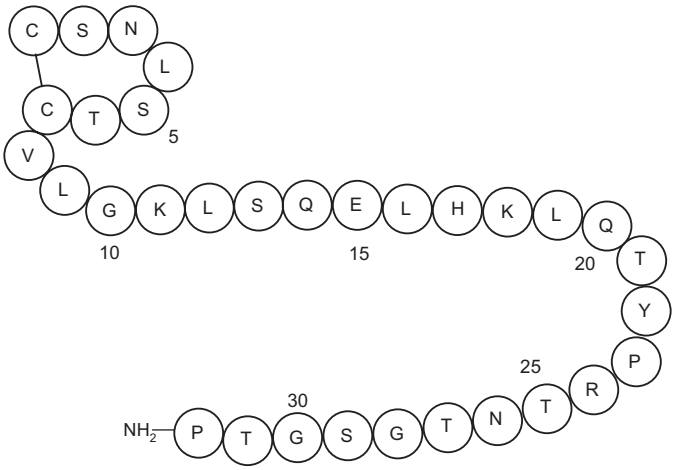
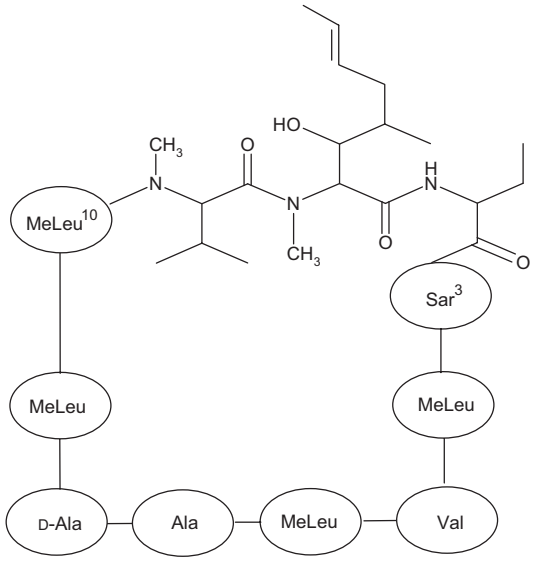


Fig. 6.22. *The structure of salmon calcitonin* (sCT)

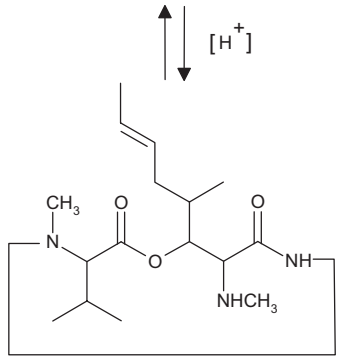
that, at this pH and at 25°, $k = 1.1 \times 10^{-6}$ s^{-1} and $t_{1/2}$ *ca.* 7 d. Two relevant hydrolytic reactions dominate the degradation of calcitonin, namely deamidation of glutamine residues (to be discussed later) and hydrolysis of the Cys^1–Ser^2 peptide bond [85]. The cleavage of this bond occurred with unexpected efficiency compared to cleavage of other peptides. Also important is the observation that the peptide bonds adjacent to each of the three other serine residues in the molecule (Ser^5, Ser^{13}, and Ser^{29}) were stable under the conditions of the study.

This example illustrates that the reactivity of peptide bonds involving serine residues is not due solely to the presence of serine: other, as yet poorly understood factors must also play a role. *Threonine residues* also show this particular type of reactivity [9]. Here, we examine the case of *cyclosporin A* (CsA), a cyclic undecapeptide that contains some unusual amino acids (**6.57**, *Fig. 6.23*), whose major breakdown reaction is hydrolytic cleavage at a threonine analogue. Position 1 is occupied by 4-[(*E*)-but-2-enyl]-*N*,4-dimethylthreonine, whose OH group reacts as a nucleophile at the carbonyl C-atom of the adjacent *N*-methylvaline (position 11) [86].

The reaction is acid-catalyzed and yields isocyclosporin A (iso-CsA, **6.58**, *Fig. 6.23*) as the major product. At 50°, the kinetics of the first-order reaction were $k = 7 \times 10^{-6}$ s^{-1} ($t_{1/2}$ *ca.* 1.1 d) at pH 1, and $k = 1.7 \times 10^{-8}$ s^{-1} ($t_{1/2}$ *ca.* 1.2 y) at pH 4. Iso-CsA (*i.e.*, the *O*-peptide) had a much greater chemical stability than CsA (*i.e.*, the *N*-peptide) under acidic conditions, in contrast to other findings where the opposite was true. Interestingly, *O*-acetyl-CsA did not yield iso-CsA and exhibited a much greater stability than CsA, consistent with the nucleophilic mechanism mentioned above.



6.57



6.58

Fig. 6.23. *Simplified representation of cyclosporin A* (**6.57**) *and its breakdown product isocyclosporin A* (**6.58**)

6.3.2.5. *Other Reactions in Solution and in the Solid State*

Some studies document *hydrolysis of an arginine residue* to ornithine and/or citrulline [87]. The reactions are insufficiently understood, but an attractive hypothesis is that they involve the hydrolysis of the imine bond of the guanidino group (see also *Sect. 11.6*).

As shown in *Fig. 6.24*, the guanidino group of arginine (**6.59**) can exist in two tautomeric forms. According to the hypothetical mechanism present-

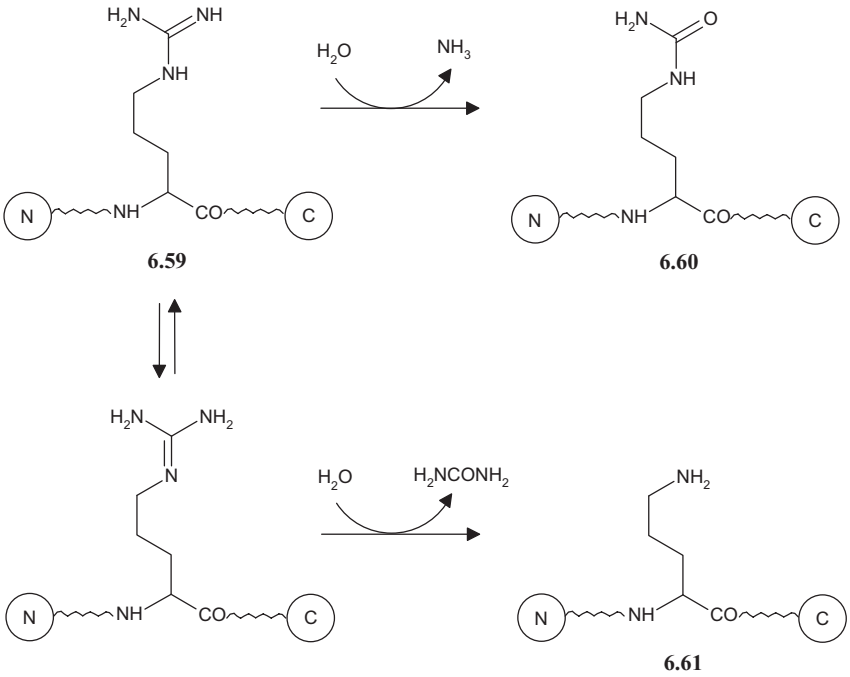


Fig. 6.24. *Hypothetical mechanism for the hydrolysis of an arginine residue* (**6.59**) *to an ornithine* (**6.60**) *or a citrulline* (**6.61**) *residue*

ed in *Fig. 6.24*, hydrolysis of one tautomer would yield an ornithine residue (**6.60**), whereas hydrolysis of the other would yield citrulline (**6.61**).

Examples of this reaction are provided by the hexapeptide [Arg^{6},D-Trp^{7,9},MePhe^{8}]substance P-(6–11) (**6.52**, *Fig. 6.17*) and the two model tripeptides Phe-Trp-Arg-NH_2 and Phe-Trp-Arg (**6.51**, R=NH_2 and OH, respectively) [76b]. Under alkaline conditions, all three compounds formed the ornithine analogue as an important product (*Fig. 6.17*, *Reactions c*), irrespective of whether arginine was at the N-terminus (**6.52**) or the C-terminus (**6.51**). In contrast, the model tripeptides Arg-Trp-Phe-NH_2 and Arg-Trp-Phe (**6.50**, R=NH_2 and OH, respectively), which also have arginine at the N-terminus, did not yield ornithine. This discrepancy was ascribed to steric differences, as the tripeptides contain L-Trp and the hexapeptide contains D-Trp.

Besides the specific reactions discussed in the previous and subsequent sections, degradation studies often reveal that peptide bond cleavage can occur at *unexpected sites* (see, *e.g.*, the unlabeled reactions in *Figs. 6.17* and *6.21*). Future investigations will certainly expand our understanding of lability factors in peptides.

We now turn briefly to the problem of *peptide stability in the solid state* [8][88]. First, we note that most – if not all – reactions discussed in the previous and subsequent sections can also occur in the solid state, although the kinetics and mechanisms of the reactions can be quite different from those observed in solution. Moisture content, the presence of excipients that act as catalysts, and surface phenomena are all factors whose roles are all-but-impossible to predict. As a result, each formulation poses a new challenge to pharmaceutical scientists. As a rule, solution data cannot be used to predict the shelf-life of solid formulations, and extrapolating from one solid formulation to another can be misleading.

In addition to the reactions discussed in the present section, specific reactions caused by the presence of some excipients may occur in solids. A detailed overview would be beyond the scope of this work, and we will simply present here two *non-redox breakdown reactions that involve reducing sugars* (*i.e.*, sugars that contain an aldehyde function). The two reactions, however, are of a non-redox nature in that the aldehyde group does not act as a reducing reagent.

The first reaction of interest is a rather general one known as the *Maillard* reaction (*Fig. 6.25,a*). The first step is a condensation between the carbonyl group of a reducing sugar and an amino group. Since the reaction involves the loss of a H_2O molecule, it is facilitated in media of low H_2O content. Lysine residues are usually the most reactive sites in a peptide, given their side-chain amino group. The side chain of arginine can also react, and there is evidence that asparagine and glutamine residues may also be reactive [8]. The intermediate imine formed by condensation (*Reaction a*) is unstable and tautomerizes to an enol (*Reaction b*), which, in turn, undergoes an *Amadori* rearrangement (*Reaction c*) to a ketone. The *Maillard* reaction is of particular significance to the food industry, since it causes browning of food and changes in the chemical and physiological properties of proteins.

Human relaxin, a hormone that plays an important role in biological responses of reproductive tissues during pregnancy, is another important example of a peptide that undergoes the *Maillard* reaction [89]. In lyophilized formulations that contained glucose and were stored at 40° for up to two weeks, the *Maillard* reaction was found to occur at three or, more probably, four basic side chains, namely Lys^{A9}, Lys^{B9}, Arg^{B17}, and probably Lys^{A17} or Arg^{B13} (*Fig. 6.26*). After 2 weeks at 40°, almost 50% of the initial amount of relaxin had reacted with glucose (including a reaction of a serine residue, discussed below). In contrast, *ca.* 90% of the relaxin remained when glucose was replaced with a nonreducing sugar such as mannitol or trehalose.

As part of the same study, relaxin was also found to react with glucose in an unexpected manner to form *des-Ser^{B29} peptides*. The reaction did not

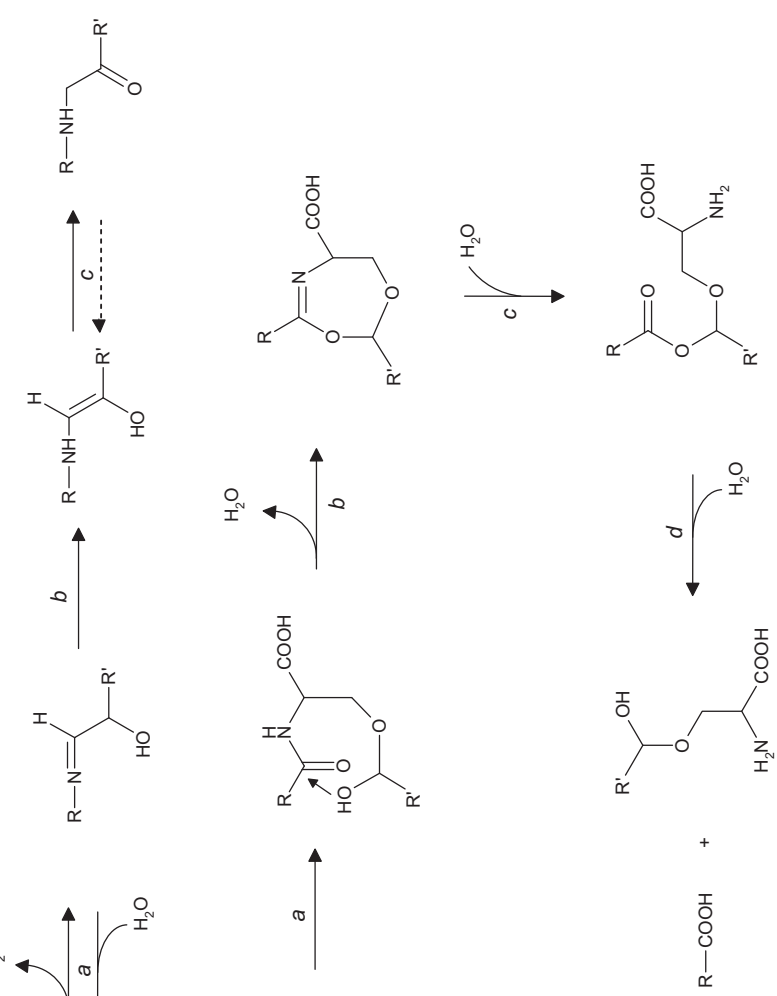


Fig. 6.25. *Simplified mechanism of two degradation reactions between peptides and reducing sugars occurring in solids. a) Maillard* reaction between a side-chain amino (or amido) group showing the formation of an imine (*Reaction a*), followed by tautomerization to an enol (*Reaction b*) and ultimately to a ketone (*Reaction c*). *Reaction c* is known as the *Amadori* rearrangement (modified from [8]). *b)* Postulated mechanism of the reaction between a reducing sugar and a C-terminal serine. The postulated nucleophilic addition yields an hemiacetal (*Reaction a*) and is followed by cyclization (intramolecular condensation; *Reaction b*). Two subsequent hydrolytic steps (*Reactions c* and *d*) yield a serine–sugar conjugate and the des-Ser-peptide (modified from [89]).

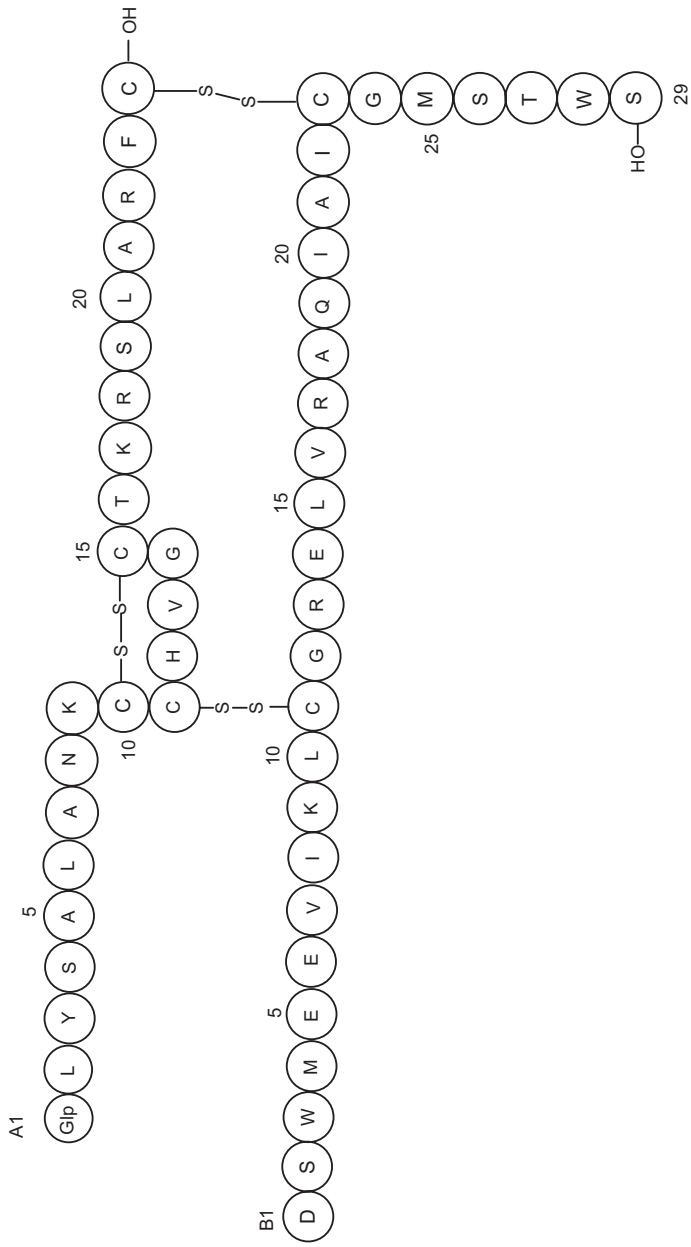


Fig. 6.26. *The primary structure of human relaxin, showing chain A* (24 amino acids), *which contains an internal disulfide bridge and is joined to chain B* (29 amino acids) *by two disulfide bridges.* The two C-termini are indicated with an OH group. Sites of *Maillard* reaction are Lys^{A9}, Lys^{B9}, Arg^{B17}, and probably Lys^{A17} or Arg^{B13}. Ser^{B29} also reacted with glucose resulting in cleavage of the Trp^{28}–Ser^{29} bond (modified from [89]).

occur when glucose was replaced with mannitol or trehalose. As postulated, the reaction probably involves nucleophilic addition of glucose to Ser^{B29} to form a hemiacetal (*Fig. 6.25,b*, *Reaction a*). This is followed by an intramolecular condensation (*Reaction b*). The resulting heterocyclic intermediate then undergoes two hydrolytic steps (*Reactions c* and *d*) to yield a serine–sugar conjugate and the des-Ser peptides [89]. After 2 weeks at 40°, loss of Ser^{B29} involved *ca.* 20–35% of the initial relaxin, depending on moisture content but not on glucose content (1 or 2%). The reaction was significantly less in solutions that contained 10 or 20% glucose.

6.3.3. Reactivity of Aspartic Acid Residues

6.3.3.1. *Reaction Mechanisms*

It is a common observation that a peptide bond formed by an aspartic acid residue is cleaved in dilute acids at a rate at least 100-fold faster than other peptide bonds. Three reaction mechanisms are now known to account for this specific reactivity [9][90–95].

Indeed, the *α-aspartyl peptide* (**6.62**) can break down as shown in *Fig. 6.27*. The first mechanism shown (*Pathway a*) begins with a nucleophilic attack of the C-neighboring N-atom (*i.e.*, the amido N-atom of the $n + 1$ residue) on the carbonyl C-atom of the Asp side chain. The resulting product is a *succinimidyl peptide* also known as an *aspartoyl peptide* (**6.63**). The modified residue (aspartic succinimide) is sometimes abbreviated as *Asu* in peptide sequences. The succinimide intermediate can then be hydrolyzed according to two competitive reactions, the preferred one forming an *iso-aspartyl peptide* (**6.64**), also known as a *β-aspartyl peptide*. In addition, the succinimidyl peptide can hydrolyze back to the α-aspartyl peptide (**6.65**). But, since the stereogenic center in the succinimide ring is configurationally labile, the α-aspartyl peptide shown (**6.65**) must be a mixture of two epimers (*i.e.*, the L- and the D-α-aspartyl peptide).

The second mechanism (*Pathway b*) begins with a nucleophilic attack internal to the aspartic acid residue, whose free side-chain carboxy group reacts with its C(1) atom, resulting in *cleavage of the Asp–Xaa bond*. The entire C-terminal peptide attached to aspartic acid is, thus, released, together with N-terminal peptide containing aspartic anhydride (**6.66**). The cyclic anhydride is highly reactive and hydrolyzes to the N-terminal peptide ending with a C-terminal aspartic acid (**6.67**). A somewhat different mechanism with the same outcome, namely nucleophilic attack at the same (but protonated) carbonyl group by a H_2O molecule, and activation of the latter by the aspartic acid side chain (general acid catalyzed hydrolysis), is also possible and will be discussed below in relation to the Asp–Gly bond.

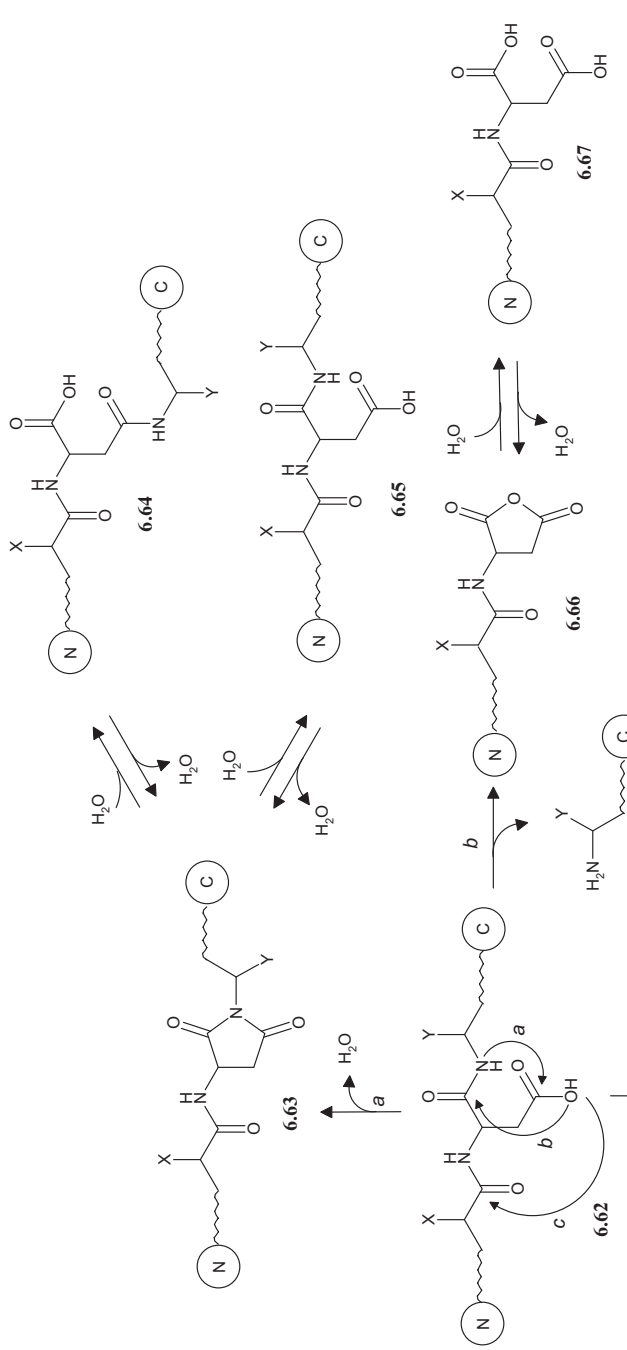
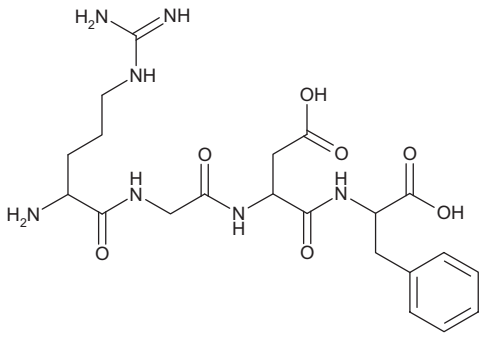


Fig. 6.27. *Simplified representation of the reaction mechanisms by which aspartic acid residues can facilitate the hydrolytic cleavage of Asp–Xaa or Xaa–Asp peptide bonds. Pathway a* begins as a nucleophilic attack of the C-flanking N-atom (*i.e.*, the amide N-atom of the $n+1$ residue) at the carbonyl C-atom of the Asp side chain. *Pathway b*, which cleaves the Asp–Xaa bond, begins as a nucleophilic attack internal to the Asp residue. *Pathway c*, which cleaves the Xaa–Asp bond, is analogous to *Pathway b*, except that the attack is at the carbonyl C-atom of the N-flanking residue (*i.e.*, the carbonyl of the $n-1$ residue).

The third mechanism (*Fig. 6.27*, *Pathway c*) involves *cleavage of the Xaa–Asp bond*. It begins as a nucleophilic reaction that resembles *Pathway b*, except that the attack is on the carbonyl of the N-flanking residue. The resulting tetrahedral intermediate (**6.68**) breaks down by cleavage of the $C(sp^3)$–N bond and hydrolysis of the ester bond. The end products of the reaction are the N-terminus fragment ending with Xaa and the C-terminus fragment beginning with Asp (**6.69**).

The relative importance of the three pathways depends on *internal and external factors*. External factors such as pH will be discussed in the pages to follow, as will internal factors such as the nature of the adjacent residue. There is, however, one internal factor that can be deduced from *Fig. 6.27*, namely that *Pathway b* is priviledged relative to *Pathway c* since the formation of a five-membered ring is entropically more favorable than that of a six-membered ring [93][96].



6.70

An example of a substrate that is subject to these three pathways is provided by model peptide *Arg-Gly-Asp-Phe* (**6.70**) [91]. The overall rate of degradation of this peptide decreased by approximately one order of magnitude with increasing pH from 2 to 7, remained practically constant in the pH range of 7–10, and increased slightly above pH 10. At pH 3 and 50°, only two reactions occurred, namely *i*) formation of a succinimide partly followed by that of the iso-aspartyl peptide (*Fig. 6.27*, *Pathway a*), and *ii*) cleavage of the Asp^3–Phe^4 bond (*Fig. 6.27*, *Pathway b*). After 800 h, each of the two pathways accounted for *ca.* 45% of the initial amount of substrate.

Under mildly acidic (pH 4–5) and neutral (pH 6–8) conditions, isomerization to the iso-aspartyl peptide predominated over peptide bond hydrolysis. However, an additional pathway was observed under these conditions,

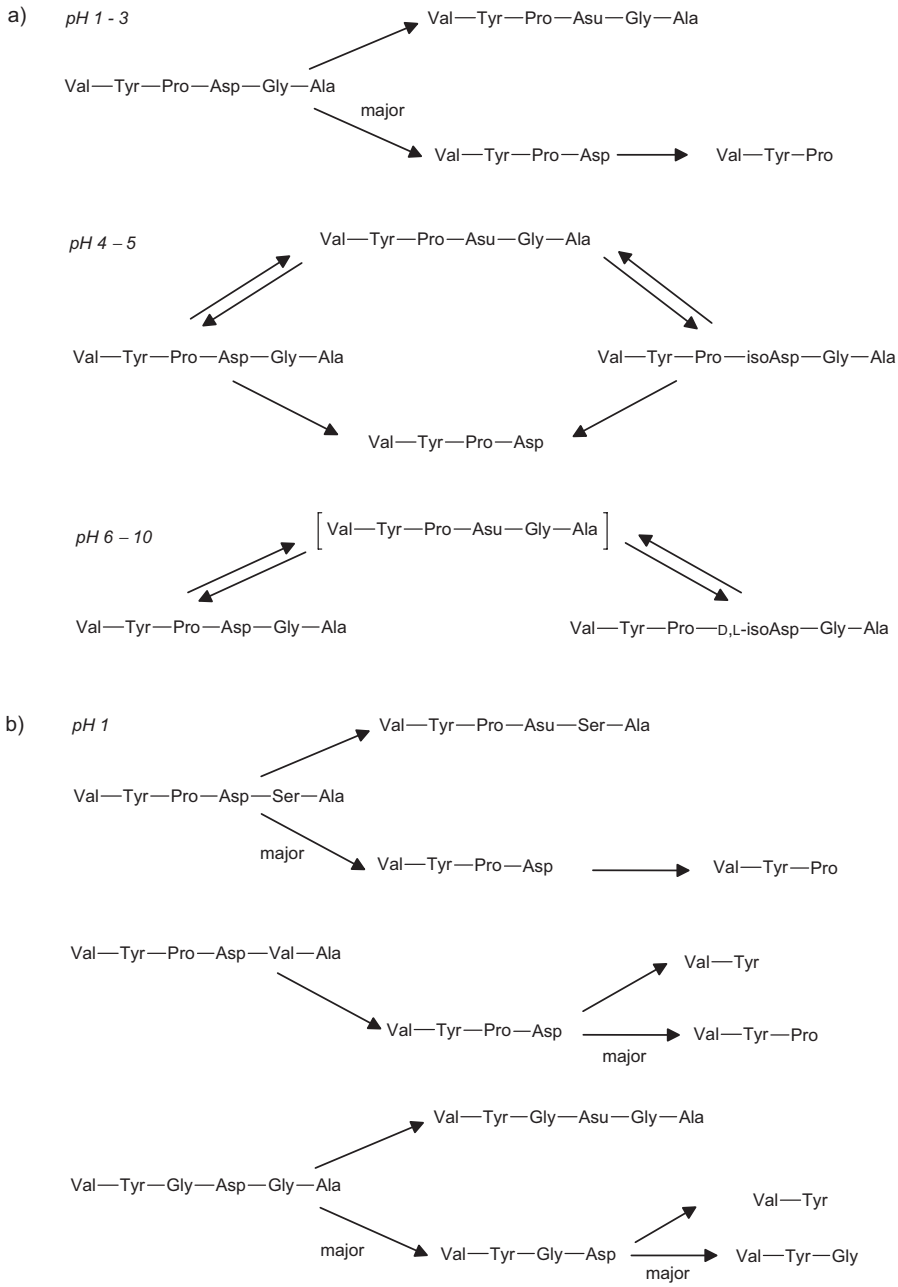


Fig. 6.28. *Breakdown reactions of Val-Tyr-Xaa*[3]*-Asp-Xaa*[5]*-Ala as a function of pH* (Asu = aspartic succinimide) [93][96]. *a*) Breakdown reactions of Val-Tyr-Pro-Asp-Gly-Ala in pH ranges 1–3, 4–5, and 6–10. *b*) Breakdown reactions of Val-Tyr-Pro-Asp-Ser-Ala, Val-Tyr-Pro-Asp-Val-Ala, and Val-Tyr-Gly-Asp-Gly-Ala at pH 1. *c*) Breakdown reactions of the same three peptides at pH 10.

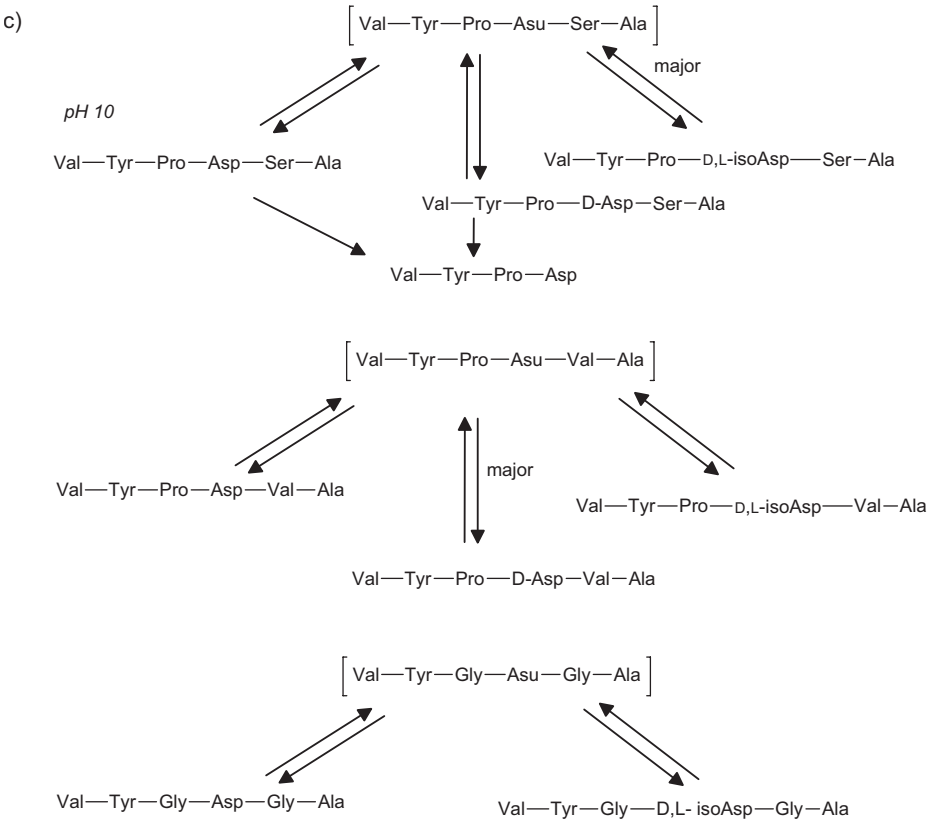


Fig. 6.28 (cont.)

namely cleavage of the Gly^2–Asp^3 bond (*Fig. 6.27*, *Pathway c*). That it occurred at all can be rationalized by the minimal steric hindrance and flexibility inherent to the Gly^2 residue.

Further insights into the influence of pH on the reactivity at aspartic acid residues are provided by a study of the *model peptide Val-Tyr-Pro-Asp-Gly-Ala* (*Fig. 6.28,a*) [93]. At pH 1 and 37°, the $t_{1/2}$ value for degradation was *ca.* 450 h, with cleavage of the Asp–Gly bond predominating approximately fourfold over formation of the succinimidyl hexapeptide. At pH 4 and 37°, the $t_{1/2}$ value was *ca.* 260 h due to the rapid formation of the succinimidyl hexapeptide, which was slowly replaced by the iso-aspartyl hexapeptide. Cleavage of the Asp–Gly bond was a minor route. At pH 10 and 37°, the $t_{1/2}$ value was *ca.* 1700 h, and the iso-aspartyl hexapeptide was the only breakdown product seen. In *Sect. 6.3.3.2*, we will compare this peptide with three analogues to evaluate the influence of flanking residues.

A medicinal example is provided by *klerval* (*Fig. 6.18*). The aspartic acid residue in this tripeptide analogue is also a site of chemical instability. At pH 1, cleavage of the Asp–Xaa bond (*Fig. 6.18*, *Reaction b*) was second in importance after C-terminal deamidation (see *Sect. 6.3.2.1*), and cleavage of the Xaa–Asp bond (*Fig. 6.18*, *Reaction c*) was third. At pH 4, cleavage of the Asp–Xaa bond was the major reaction and was accompanied by the formation of the succinimide and the iso-aspartyl peptide; cleavage of the Xaa–Asp bond was minor. At pH 7, the major products were the L-iso-Asp and D-iso-Asp peptides, together with minor amounts of the D-Asp peptide.

The pharmaceutical significance of the role of aspartic acid residues is also aptly illustrated by *recombinant human parathyroid hormone* (rhPTH) [97]. This hormone, a peptide of 84 residues, is secreted by the parathyroid gland and acts as a homeostatic regulator of the level of calcium in blood. Its maximal stability in the examined range of pH 2–10 was at pH 5. The two pathways of breakdown were *i*) cleavage at aspartic acid residues, and *ii*) deamidation, rearrangement, and cleavage at asparagine residues, which predominates above pH 5 (see *Sect. 6.3.4*). Below pH 5, cleavage at aspartic acid residues was the only detectable pathway. The polypeptide contains five aspartates, listed here together with the flanking residue, namely Gln^{29}-Asp^{30}-Val^{31}, Arg^{44}-Asp^{45}-Ala^{46}, Glu^{55}-Asp^{56}-Asn^{57}, Ala^{70}-Asp^{71}-Lys^{72}, and Ala^{73}-Asp^{74}-Val^{75}. Cleavage occurred at all five aspartate residues, and always on the C-terminal side (*i.e.*, the Asp–Xaa bond). Interestingly, none of the flanking residues is known to facilitate Asp–Xaa or even Xaa–Asp cleavage, in contrast to the facilitating residues discussed in *Sect. 6.3.3.2*. This may at least partly explain why all five reactions appear to make comparable contributions to the breakdown of rhPTH.

These and a number of other examples point to the following pH ranges at which *Pathways a–c* can be seen (see *Fig. 6.27*):

- Formation of *succinimidyl peptides* (**6.63**, *Pathway a*): pH *ca.* 2–6;
- Formation of *iso-aspartyl peptides* (**6.64**, *Pathway a*): pH *ca.* 5–10;
- Formation of D-*Asp* and D-*iso-Asp peptides* (**6.64** and **6.65**, *Pathway a*): pH >6–7;
- Cleavage of *Asp–Xaa bonds* (**6.67**, *Pathway b*): pH<6;
- Cleavage of *Xaa–Asp bonds* (**6.69**, *Pathway c*): pH<6.

All the evidence presented in this section concerns aspartic acid residues, and one may wonder whether *glutamic acid residues* display similar reactivity. The answer is clearly that they do not, in particular for entropy reasons. In fact, replacement of a reactive aspartic acid residue by a glutamic acid residue can greatly increase the chemical stability of a peptide. This is exemplified by *human epidermal growth factor* (hEGF), an important promoter of

cell division. Long-term storage of hEGF resulted in conversion to a new species that had only 20% of the biological activity of native hEGF [98]. The evidence suggested that Asp^{11} had been converted to an iso-aspartyl residue, and, indeed, replacement of Asp^{11} with glutamic acid resulted in a fully active EGF analogue that did not form detectable amounts of the corresponding rearrangement product.

6.3.3.2. *Structural Determinants*

The major structural factors that influence the reactivity of aspartic acid residues are *i*) *conformational aspects* of the peptide, particularly the local *flexibility* of the peptide chain as dictated by primary, secondary, and tertiary structure, and *ii*) the amino acid sequence (*i.e.*, *the nature of the adjacent residues*). Most of the available evidence concerns the influence of adjacent residues, as discussed in this section.

Before doing so, we briefly examine the *influence of conformation and flexibility*. Indeed, formation of succinimide is limited in proteins due to conformational constraints, such that the optimal value of the ϕ and ψ angles (*Sect. 6.1.2*) around the aspartic acid and asparagine residues should be +120° and –120°, respectively [99]. These constraints often interfere with the reactivity of aspartic acid residues in proteins, but they can be alleviated to some extent by local backbone flexibility when it allows the reacting groups to approach each other and, so, favors the intramolecular reactions depicted in *Fig. 6.27*. When compared to the same sequence in more-flexible random coils, elements of well-formed secondary structure, especially α-helices and β-turns, markedly reduce the rate of succinimide formation and other intramolecular reactions [90][100].

The influence of *backbone flexibility* was seen, for example, when the stability of the linear tetrapeptide **6.70** was compared to that of a cyclic hexapeptide derivative of the same sequence [91]. Indeed, the cyclic peptide was approximately one order of magnitude more stable than the linear peptide in the pH range of 2–7. The results at higher pH values were inconclusive since the stability of the cyclic peptide decreased dramatically due to the degradation of a disulfide bond absent in the linear peptide.

As to the *influence of adjacent residues*, four amino acids are known to increase the reactivity of aspartic acid. These are glycine, proline, histidine, and serine, as explained and exemplified below [101]. To the best of our knowledge, most of the available evidence concerns the facilitating effects of glycine and proline.

The facilitating effect of *glycine* is ascribed to the local flexibility induced by the absence of a side chain. This implies that the cleavage of

Gly–Asp bonds (*Fig. 6.27*, *Pathway c*) as well as rearrangement and cleavage at Asp-Gly sites (*Fig. 6.27*, *Pathways a* and *b*) will be facilitated, although, as explained, Asp-Gly sites are more reactive than Gly-Asp sites.

Proline is also a labilizing residue in that acid-catalyzed cleavage is facilitated at Asp–Pro bonds. This is assumed to be due to the greater basicity of the secondary amino group of proline, which causes enhanced protonation of the leaving group during hydrolysis (*Fig. 6.27*, *Pathway b*) [87][94]. Thus, Asp–Pro bonds are 8–20-fold more labile at pH 2 than other Asp–Xaa or Xaa–Asp bonds [102]. Such a mechanism is obviously not valid for Pro–Asp bonds. Peptides for which Asp–Pro cleavage was a major, or the major, degradation reaction under acidic conditions include recombinant *human nerve growth factor* [103], recombinant *human macrophage colony-stimulating factor* [104], and recombinant *human interleukin 11* [105].

Histidine flanking an aspartic acid residue has been found to increase the rate of succinimide formation up to tenfold (*Fig. 6.27*, *Pathway a*) [96][100]. This effect may be due to the ability of the histidine residue to facilitate succinimide formation by protonating the OH leaving group of the aspartic acid side.

Serine flanking an aspartic acid residue on the carboxylate side also facilitates cleavage of the Asp–Ser bond under alkaline conditions. Two mechanisms have been postulated, namely *i*) H-bonding between the serine OH group and the α-carbonyl group of aspartic acid, which increases polarization of this carbonyl group and facilitates attack by the side-chain COOH group (*Fig. 6.27*, *Pathway b* facilitated by intramolecular general acid catalysis), and *ii*) independently of the presence of Asp, *N*,*O*-acyl migration as depicted in *Fig. 6.19*, *Reaction b* [96]. Such a facilitating effect has also been seen with *cysteine* in place of serine [92].

The influence of N- and C-side flanking residues on the reactivity of aspartic acid residues was investigated with model hexapeptides of the general structure *Val-Tyr-Xaa*^{3}*-Asp-Xaa*^{5}*-Ala*. One such compound was investigated in great detail and has been discussed in *Sect. 6.3.3.1* [93]. Here, we compare its degradation with that of three analogues, namely Val-Tyr-Pro-Asp-Ser-Ala, Val-Tyr-Pro-Asp-Val-Ala, and Val-Tyr-Gly-Asp-Gly-Ala [96].

At pH 1 and 70°, the four hexapeptides had comparable $t_{1/2}$ values for the degradation reaction (10–25 h). And, as shown in *Fig. 6.28,a* and *b*, cleavage of the Asp–Xaa bond was either the predominant primary reaction (Xaa = Gly or Ser, with the minor reaction being succinimide formation), or the only detectable one (Xaa = Val). Cleavage of the Xaa–Asp bond occurred not in the hexapeptide but in the four Val-Tyr-Xaa-Asp tetrapeptides as a secondary reaction. Taken globally, these results suggest that, in strongly acidic media, the catalytic effect of H_3O^+ greatly predominates over the labilizing influence of the residues flanking aspartic acid on either side.

At pH 10 and 70°, the hexapeptides had $t_{1/2}$ values for degradation of *ca.* 30–40 h, with the exception of Val-Tyr-Pro-Asp-Val-Ala, the stability of which was much greater ($t_{1/2}$ *ca.* 800 h). Here, the hexapeptides containing Asp-Gly or Asp-Ser were clearly much more reactive than the Asp-Val hexapeptide. Interestingly, the D,L-iso-aspartic hexapeptide was the only product formed from the Asp-Gly hexapeptides, and it was the major product from the Asp-Ser hexapeptide (*Fig. 6.28,a* and *c*). Formation of the D-Asp hexapeptide was observed for the Asp-Ser hexapeptide, and it was the major one for the Asp-Val hexapeptide, presumably because base-catalyzed epimerization had ample time to occur given the very slow rate of other breakdown reaction.

We now examine the degradation of two selected bioactive peptides containing aspartic acid residues flanked by activating residues.

Secretin a basic linear peptide comprised of 27 amino acids, whose primary function is the stimulation of exocrine pancreatic secretion. The medicinal use of this peptide is rendered difficult by a rapid loss of activity during storage due to degradation. Secretin was found to be most stable in pH 7 solution, less stable in acidic solution, and least stable in alkaline solution [95]. The two reactive sites were characterized as Asp^{3}-Gly^{4} and Asp^{15}-Ser^{16}. Cleavage at both sites was observed in strongly and weakly acidic solutions. In addition, the Asu^{3} peptide was the major product in weakly acidic solutions, but it was rapidly hydrolyzed in neutral and alkaline solutions to yield the iso-Asp^{3} peptide.

Human basic fibroblast growth factor (bFGF) is a globular protein containing a single chain of 155 residues. Potentially reactive sites include Asp^{15}-Gly^{16}, Asp^{28}-Pro^{29}, Asp^{36}-Gly^{37}, Asp^{46}-Gly^{47}, Asp^{50}-Gly^{51}, Asp^{57}-Gly^{58}, and Asp^{88}-Gly^{89}. However, only two sites proved reactive at pH 5, namely Asp^{15}-Gly^{16} and Asp^{28}-Pro^{29}. Cleavage occurred at both sites. In addition, the major degradation product at this pH was a succinimide at Asp^{15} (>10% after 3 months at 25°). The succinimide was no longer detectable after storage at pH 6.5, having been converted to iso-Asp [94].

6.3.4. Reactivity of Asparagine and Glutamine Residues

6.3.4.1. *Introduction*

Asparagine residues (and glutamine residues, see below) are sites of particular instability in peptides. As will be exemplified below, rates of degradation at asparagine residues are markedly faster (tenfold and even much more) than at aspartic acid residues. As reported, the $t_{1/2}$ values for the internal *asparagine* in a large number of pentapeptides ranged from 6 to 507 d under

physiological conditions of pH and temperature, while those of internal *Gln* ranged from 96 to 3409 d [6]. Degradation at asparagine residues can, thus, be the major cause of nonoxidative damage to proteins, with consequences that go well beyond synthesis and storage, since its occurrence *in vivo* may affect long-lived proteins. This may result in loss of function, trigger proteolytic breakdown, and increase immunogenicity, among other effects [90].

It has been suggested that this post-translational protein modification may be a necessary biochemical process, serving as a 'biological clock' to facilitate the rapid turnover of biologically important proteins [106]. Such modifications have been observed for triosephosphate isomerase, serine hydroxymethyltransferase, and ribonuclease A [107–111]. It is also conceivable that the reaction plays an important role in the biogenesis of many human disorders that are associated with aging. For example, the α-, β-, and γ-crystallins, proteins located in the lens, cannot be regenerated and must remain practically intact during the entire lifetime of an individual. As a result, these have evolved to be, perhaps, the most stable proteins in the human body. Increased degradation at Asn^{143} has been observed in human cataractous lenses [112].

6.3.4.2. *Reaction Mechanisms*

The degradation pathways engendered by the presence of *asparagine* residues are *i*) deamidation to Asp peptides, *ii*) formation of intermediate cyclic imides known as succinimides, followed by epimerization and ring opening to iso-Asp peptides, and *iii*) backbone cleavage by formation of a succinimide (*Fig. 6.29*) [6][8][9][87][113][114]. Because all these pathways lead to deamidated products, degradation at asparagine residues is sometimes globally referred to as *deamidation*. We will discuss these pathways before examining the influence of structural factors.

The simplest degradation displayed by asparagine and glutamine is *direct hydrolytic deamidation* of the side-chain carboxamido group (*Fig. 6.29*, *Pathway d*). Such a reaction, however, is seen only at low pH values, and its biological significance appears negligible. Its product is the Asp peptide (**6.62**) whose further reactions have been presented in *Fig. 6.27*.

The most important degradation mechanism of asparagine and glutamine residues is formation of an intermediate *succinimidyl peptide* (**6.63**) without direct backbone cleavage (*Fig. 6.29*, *Pathway e*). The reaction, which occurs only in neutral and alkaline media, begins with a nucleophilic attack of the C-neighboring N-atom at the carbonyl C-atom of the Asn side chain (slow step). The succinimide ring epimerizes easily and opens by hydrolysis (fast step), as shown in *Fig. 6.27*, to yield the *iso-aspartyl peptide* (**6.64**) and the *aspartyl peptide* (**6.65**) in a ratio of 3:1.

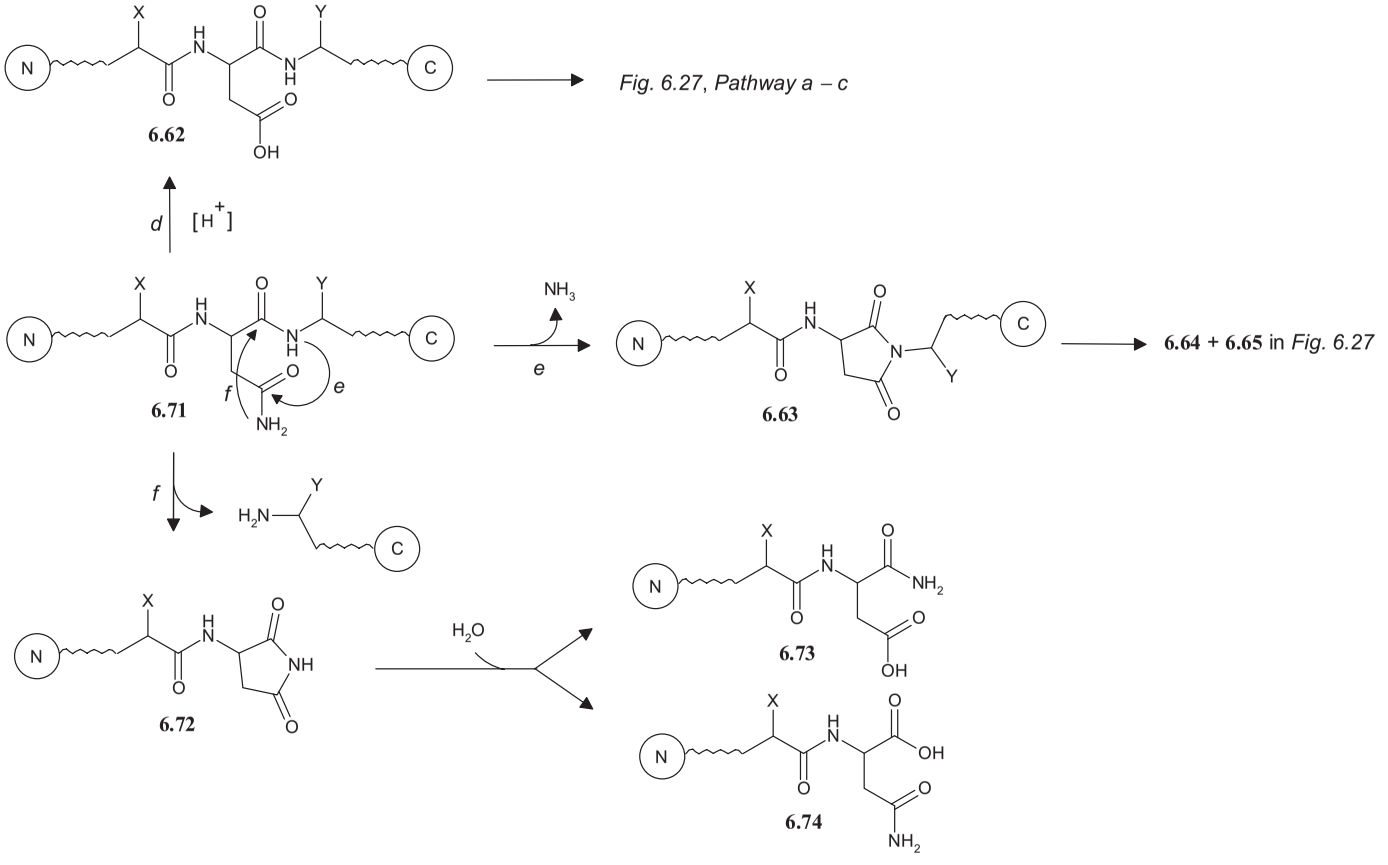


Fig. 6.29. *Simplified representation of the degradation reactions at asparagine residues. Pathway d* is the acid-catalyzed, direct hydrolysis of the Asn peptide (**6.71**) into the corresponding Asp peptide (**6.62**); the degradation reactions of the latter have been depicted in *Fig. 6.27. Pathway e* begins as a nucleophilic attack of the C-flanking N-atom at the carbonyl C-atom of the Asn side chain, producing a succinimide (**6.63**), further reactions of which have been depicted in *Fig. 6.27. Pathway f*, which cleaves the Asn–Xaa bond, begins as a nucleophilic attack internal to the Asn residue to produce a C-terminal succinimide (**6.72**).

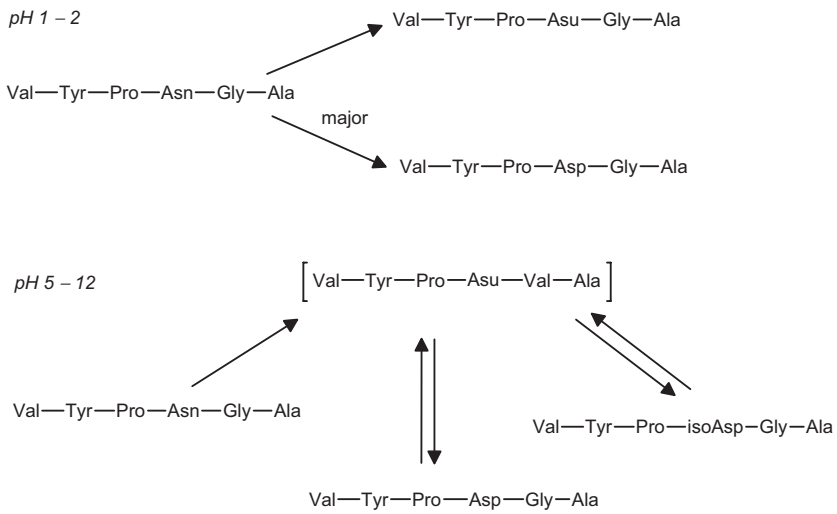


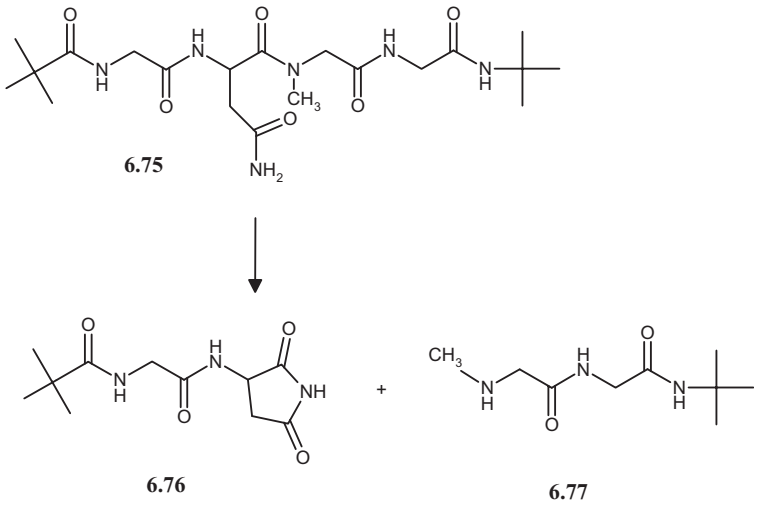
Fig. 6.30. *Breakdown reactions of Val-Tyr-Pro-Asn-Gly-Ala as a function of pH* (Asu = aspartic succinimide) [130]

In a study of the model hexapeptide *Val-Tyr-Pro-Asn-Gly-Ala* (*Fig. 6.30*) [115], maximal stability was seen at pH 4 ($k \approx 2 \times 10^{-8}$ s^{-1}, $t_{1/2}$ *ca.* 400 d, 37°). At pH 2 and 37°, two products were found ($k \approx 6 \times 10^{-8}$ s^{-1}, $t_{1/2}$ *ca.* 130 d). The very minor product was the succinimidyl hexapeptide (stable at this pH), and the Asp hexapeptide (*Fig. 6.29*, *Pathway d*), which accounted for most of the degradation.

In the pH range of 5–12, the degradation of Val-Tyr-Pro-Asn-Gly-Ala showed a completely different pattern, the only products seen being the Asp and the iso-Asp hexapeptide. Both originated from the succinimidyl hexapeptide, which was not stable enough in this pH range to be detected. The first-order rate constant k at 37°, pH 7.4, 0.1M phosphate buffer, was *ca.* 8×10^{-6} s^{-1} ($t_{1/2}$ *ca.* 24 h). This is a highly significant result since it highlights the biological and pharmacokinetic relevance of asparagine degradation.

The last reaction in *Fig. 6.29* (*Pathway f*) is also a cyclization–elimination, but, in contrast to *Pathway e*, the entire C-terminal fragment attached to asparagine is released, in analogy to *Pathway b* in *Fig. 6.27*. The other product of the reaction is the N-fragment having a *C-terminal succinimide* (**6.72**). The latter product can hydrolyze to the two fragments **6.73** and **6.74**. Such a pathway is seldom observed and seems to require special structural conditions. Indeed, when the C-side neighbor of asparagine is a *N*-alkyl amino acid (*e.g.*, sarcosine or proline) or has a bulky side chain, attack of Asn according to *Pathway e* is precluded, and the alternative route becomes *Pathway f*.

Such a reaction was seen in the degradation of N-*pivaloyl-Gly-Asn-Sar-Gly-(*tert-*butyl)amide* (**6.75**) [116], leading to the formation of the two fragments **6.76** and **6.77**, the former undergoing succinimide ring opening to *N*-pivaloyl-Gly-Asp and *N*-pivaloyl-Gly-iso-Asp. The product ratio was pH-dependent, and a function of both the first (cyclization–elimination) and the second steps (succinimide hydrolysis). The rate of the first step was investigated in the pH range of 7.4–13. It was slowest at pH 7.4 ($k \approx 3 \times 10^{-8}$ s^{-1}, $t_{1/2}$ *ca.* 250 d), and increased by almost four orders of magnitude to pH 13.



6.75

6.76 **6.77**

Another factor that influences partitioning between *Pathways e* and *f* (*Fig. 6.29*) is substitution at the γ-amido group of asparagine. The reactivities of two octapeptide analogues, one containing an asparagine residue, the other a γ-*N*-methylasparagine residue (**6.78**, R″=H or Me, respectively, *Fig. 6.31*) [117], were compared. When R″=H, the ratio of *Pathways e/f* was 45:1 at pH 7.4. However, γ-*N*-methylation of Asn dramatically modified the ratio of *Pathways e/f*, which, in this case, was 1:3.

To summarize, the most frequently observed products of degradation at asparagine sites are:

Under acidic conditions, the *uncleaved aspartyl peptide* formed by direct *deamidation* (*Fig. 6.29*, *Pathway d*);

Under neutral or alkaline conditions, the *uncleaved but partly epimerized iso-aspartyl and aspartyl peptides* (*Fig. 6.29*, *Pathway e*).

Glutamine residues can undergo deamidation *via* formation of a six-membered ring (*i.e.*, a glutarimide ring) in a reaction analogous to *Fig. 6.29*, *Pathway e* [6][8][87]. However, the deamidation of glutamine is markedly slower than that of asparagine since formation of a six-membered glutari-

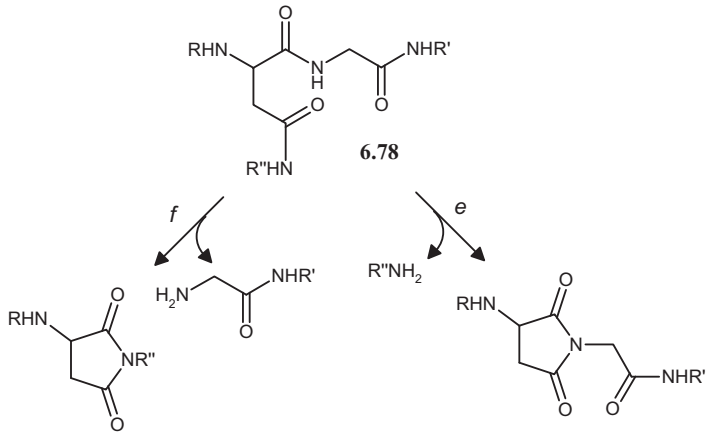


Fig. 6.31. *Formation of succinimidyl peptides from Asn peptides* (**6.78**, R″=H) *and* γ-N-*methyl-Asn peptides* (**6.78**, R″=Me). *Pathway e* was favored when R″=H, and *Pathway f* when R″=Me [117].

mide is entropically less favorable than that of a succinimide (see also above).

Direct deamidation of glutamine in analogy with *Pathway d* (*Fig. 6.29*) is also possible. This was observed for example with *glucagon*, a peptide hormone containing 29 amino acids, including one asparagine and three glutamine residues. Glucagon solutions degraded under acidic conditions at 60° for 70 h showed marked deamidation of all three glutamine residues (*i.e.*, Gln^{3}, Gln^{20}, and Gln^{24}), but, unexpectedly, not at Asn^{28} [118]. Other important breakdown reactions were aspartic acid cleavage at Asp^{9}-Tyr^{10}, Leu^{14}-Asp^{15}, Asp^{15}-Ser^{16}, and Asp^{21}-Phe^{22}.

A similar case is that of salmon *calcitonin* (see *Fig. 6.22* in *Sect. 6.3.2.4*). In acidic solution, this peptide undergoes deamidation of Gln^{14} and Gln^{20} to yield the corresponding (and active) [Glu^{14}]calcitonin and [Glu^{20}]calcitonin [85].

A case of very slow direct deamidation was reported for *Xaa-Gln dipeptides*, but, in contrast to *Pathway d* (*Fig. 6.29*), it was also base-catalyzed [119]. However, the relevance of this example for oligopeptides and proteins remains to be established.

6.3.4.3. *Structural Determinants*

As in the case of degradation at aspartic acid residues, the major structural factors that influence the reactivity of asparagine and glutamine residues

are *i*) the higher-order structure of the peptide, *i.e.*, its *secondary and tertiary structure*, and *ii*) its amino acid sequence (*i.e.*, *the nature of the flanking residues*) [90][113].

The influence of *secondary structure* on reactions of deamidation has been confirmed in a number of studies. Thus, deamidation was inversely proportional to the extent of α-helicity in model peptides [120]. Similarly, α-helices and β-turns were found to stabilize asparagine residues against deamidation, whereas the effect of β-sheets was unclear [114]. The *tertiary structure* of proteins is also a major determinant of chemical stability, in particular against deamidation [121], on the basis of several factors such as the stabilization of elements of secondary structure and restrictions to local *flexibility*, as also discussed for the reactivity of aspartic acid residues (*Sect. 6.3.3*). Furthermore, deamidation is markedly decreased in regions of *low polarity* in the interior of proteins because the formation of cyclic imides (*Fig. 6.29*, *Pathway e*) is favored by deprotonation of the nucleophilic backbone N-atom, which is markedly reduced in solvents of low polarity [100][112].

An example of this effect is provided by *ribonuclease A* (RNase A). At pH 8 and 37°, the rate of deamidation of Asn^{67} was more than 30-fold lower in the native than in the unfolded protein [111]. Deamidation of the native RNase A was also *ca.* 30-fold slower than of an octapeptide whose sequence is similar to that of the deamidation site, although the reaction mechanisms were similar [108][123].

In the case of the *influence of adjacent residues*, there are clear mechanistic analogies between activation of aspartic acid (*Sect. 6.3.3.2*) and asparagine sites. The presence of a C-flanking *glycine* residue consistently increases deamidation of peptides, for the reasons discussed in *Sect. 6.3.3.2* [6]. Replacement of glycine with a more bulky residue such as valine, leucine, or proline can decrease reactivity more than tenfold [99].

C-Flanking *serine* or *cysteine* residues can increase the rate of deamidation and, particularly, cleavage, in analogy with the mechanism discussed for aspartic acid [92]. Increased reactivity can also result from the presence of a C-flanking *histidine*, which increases the nucleophilicity of the Asn side-chain amido group and, thus, favors *Pathways f* and perhaps *d* in *Fig. 6.29* [124]. N-Flanking *lysine* was also found to facilitate *Pathway e* (*Fig. 6.29*) in a pH-dependent manner, likely by increasing the electrophilicity of the carbonyl C-atom in the Asn side chain [125].

A systematic study with two series of pentapeptides has afforded much information on the influence of flanking residues on asparagine reactivity [126]. In these two series, the central asparagine residue occurred in the sequences *Val-Xaa-Asn-Ser-Val* and *Val-Ser-Asn-Xaa-Val*, where Xaa is one of ten different residues. In acidic solutions, the Asp peptide was the only product found, and its rate of formation was independent of the nature of the

flanking residues. This provides additional proof of a mechanism of direct, proton-catalyzed hydrolysis of the amido group (*Fig. 6.29*, *Pathway d*).

In neutral and alkaline solutions, the iso-Asp/Asp-peptide products were always formed in a *ca.* 3:1 ratio. In the *Val-Xaa-Asn-Ser-Val* series, the nature of the N-flanking residue had little effect on the rate of deamidation, regardless of size. However, the actual effect may have been partly masked by cleavage of the Asn–Ser bond (*Sect. 6.3.2.4*), which proceeds at a rate *ca.* 10% that of deamidation. In the Val-Ser-Asn-Xaa-Val series, the nature of the C-flanking residue did have a major influence. As expected, the most unstable peptide was Val-Ser-Asn-Gly-Val ($t_{1/2}$ *ca.* 6 h at pH 7.3 and 60°). A second group of peptides had $t_{1/2}$ values in the range of 25–75 h, which increased in the order Xaa=His<Ser<Asp<Ala<Arg. The most stable peptides ($t_{1/2}$ 200–400 h) had Xaa=Leu<Trp<Ile<Val.

These results can be rationalized as follows. Cyclic imide formation is maximal with a Gly residue because of its minimal steric interference. In contrast, steric bulk (*i.e.*, size and branching) of the C-flanking residue is the major factor decreasing the reactivity of Asn to form a succinimide. Residues with a functionalized side chain, namely serine, threonine, and histidine, behaved as exceptions to this rule (see above).

A more quantitative comparison of the influence of flanking residues was carried out by means of multiple-regression analysis of a large set of 67 Asn-containing oligopeptides, whose rates of deamination at pH 7.4 and 37° were taken from the literature [125]. The average rate constant for oligopeptides containing the *Gly-Asn-Gly* motif was $k=4\times10^{-6}$ s^{-1} (log k=–5.4, standard deviation (SD) or 95% confidence limits (CL) were unfortunately not reported). Among the N-flanking residues, only leucine and isoleucine had a small deactivating effect, since they decreased log k by amounts of –0.3 and –0.7, respectively (again, no SD or 95% CL reported). The effect of the other N-flanking residues ranged from +0.2 to –0.2, which appears to be without statistical significance.

The effect of the C-flanking residues was much clearer and in line with expectations. All C-flanking residues included in the analysis decreased the value of log k (*i.e.*, decreased reactivity) by a decrement ranging from –0.8 (His) to –2.1 (Val). More concretely, the deactivating effect increased in the series His<Ser<Glu<Asp<Arg<Thr<Ala<Lys<Pro<Ile<Leu<Val.

From the above, a sharp distinction emerges between results obtained with small peptides and with proteins. Small peptides yield important information on the influence of flanking residues. However, the protein data presented above and below clearly show that a stable three-dimensional structure has overwhelming power to protect against deamidation of asparagine and glutamine residues. Asparagine and glutamine residues located in flexible regions on the periphery of a protein show reactivities approaching those

found for these residues in small peptides, whereas residues buried in stable α-helices or β-turns and/or hydrophobic regions in the protein interior may be highly resistant to deamidation.

To illustrate some of the above points, the degradation of a few selected bioactive peptides containing asparagine and glutamine residues will be described.

An interesting example of reactivity at asparagine sites is offered by *pramlintide* (*Fig. 6.20*) [79]. Among its 37 amino acids, this peptide contains six asparagines and one glutamine residue. When the peptide was stressed at pH 4 and 40° for 45 d, degradation occurred at four sites (Asn^{14}, Asn^{21}, Asn^{22}, and Asn^{35}), whereas three sites were unreactive (Asn^{3}, Asn^{31}, and Gln^{10}). All four reactive sites formed a succinimidyl peptide, plus the derived Asp and iso-Asp peptides in a second step (*Fig. 6.29*, *Pathway e*). Interestingly, *Pathway f* also occurred at residues Asn^{21} and Asn^{22}, giving rise to [Asu^{21}]pramlintide-(1–21) and [Asu^{22}]pramlintide-(1–22). These results are difficult to explain, especially in the absence of *N*-methylation and with all C-flanking being relatively bulky.

The degradation of *recombinant human parathyroid hormone* (rhPTH) at aspartic sites under acidic conditions has been discussed in *Sect. 6.3.3.1*. At pH 2, only one of the five asparagine residues (Asn^{76}) underwent proton-catalyzed deamidation (*Fig. 6.29*, *Pathway d*). Above pH 5, asparagine residues are the major sites of degradation, but differ in reactivity and products [97]. The sites Asn^{10}-Leu^{11} and Asn^{33}-Phe^{34} were unreactive presumably due to the bulky nature of their C-flanking residue. However, this explanation fails to account for deamination at Asn^{57}-Val^{58} and Asn^{76}-Val^{77}, yielding [Asp^{57}]-rhPTH and [Asp^{76}]rhPTH, respectively. The most reactive site appeared to be Asn^{16}-Ser^{17} to yield [iso-Asp^{16}]rhPTH and [Asp^{16}]rhPTH in a ratio of 3:1, plus the cleavage product rhPTH-(17–84) peptide.

Recombinant human tissue plasminogen activator consists of 527 amino acids and is glycosylated at several sites. Characterizing its degradation products was certainly no minor feat, yet it was possible to identify which of its eight asparagine residues were labile when the protein was allowed to age in solution at pH 7.3 and 37° [128]. Deamidation and formation of iso-aspartate were predominant at Asn^{58}-Gly^{59} and Asn^{177}-Ser^{178}, and were also detected at Asn^{37}-Ser^{38}. These sites appear to be located on the surface of the protein, and all three occur in regions of above-average backbone mobility. However, the combination of sequence and flexibility was not a perfect predictor of reactivity under mild conditions, since it was not clear why four other Asn residues that also met these criteria remained unmodified.

A protein whose degradation pattern is even more difficult to explain is *recombinant human interleukin 2* (rhIL-2), a lymphokine produced by T-cells and being developed as an immunomodulator. This protein, which consists

of 133 amino acids, including eight asparagine and six glutamine residues, is quite stable under the conditions of the study (pH 5, 25°, 6 months), and the only product of deamidation found was [Asp^{88}]rhIL-2 [129]. The Ser^{87}-Asn^{88}-Ile^{89} site is located in an α-helical region (Arg^{83}–Thr^{101}) on the exterior of the protein. It is, thus, far from clear why Asn^{88} is deaminated when other Asn and Gln residues that are equally likely candidates are not.

6.3.4.4. *Deamidation in the Solid State*

Peptide stability in solids has been briefly presented in *Sect. 6.3.2.5*. It is important to note here that deamidation reactions can also play a major role in the degradation of peptides in solid matrixes. While deamidation in the solid state has received less attention than deamidation in solution, there is enough evidence to suggest that the mechanisms and pathways are comparable if not similar in the two types of media [8][130].

A carefully investigated case is that of *human growth hormone* (hGH), a 22-kD protein known to undergo such degradation reactions as sulfoxidation of Met^{14}, deamidation (mainly at Asn^{149}) and aggregation to form oligomers. These reactions occurred on storage of freeze-dried solid, and were quite sensitive to formulation [131]. Formulation variables included O_2 concentration, the nature of excipients and their amorphous or crystalline character, and the pH and NaCl content of the solution prepared for freeze-drying.

With continuous development of systems for controlled drug release, new materials are being used whose influence on peptide stability must be carefully examined. Thus, the model hexapeptide *Val-Tyr-Pro-Asn-Gly-Ala* (*Fig. 6.30*) embedded in *poly(vinyl alcohol)* and *poly(vinyl pyrrolidone)* matrices had rates of deamidation that increased with increasing water content or water activity, and, hence, with decreasing glass transition temperature (T_g). However, the degradation behavior in the two polymers differed so that chemical reactivity could not be predicted from water content, water activity, or T_g alone. Furthermore, the hexapeptide was less stable in such hydrated polymeric matrices than in aqueous buffer or lyophilized polymer-free powders [132].

A very important medicinal peptide is *insulin* (*Fig. 6.32*), which contains 51 amino acids in two peptide chains (A and B) linked by two disulfide bonds. The most prevalent reaction of chemical degradation of insulin in *aqueous solutions* is deamidation. Six potential sites exist in the molecules, namely Gln^{A5}, Gln^{A15}, Asn^{A18}, Asn^{A21}, Asn^{B3}, and Gln^{B4} (*Fig. 6.32*). In acidic solution, extensive deamidation of human and porcine insulin occurred at Asn^{A21}, whereas, in neutral solution, deamidation proceeded at Asn^{B3} at a comparatively reduced rate under formation of a mixture of the iso-Asp and Asp peptides [133].

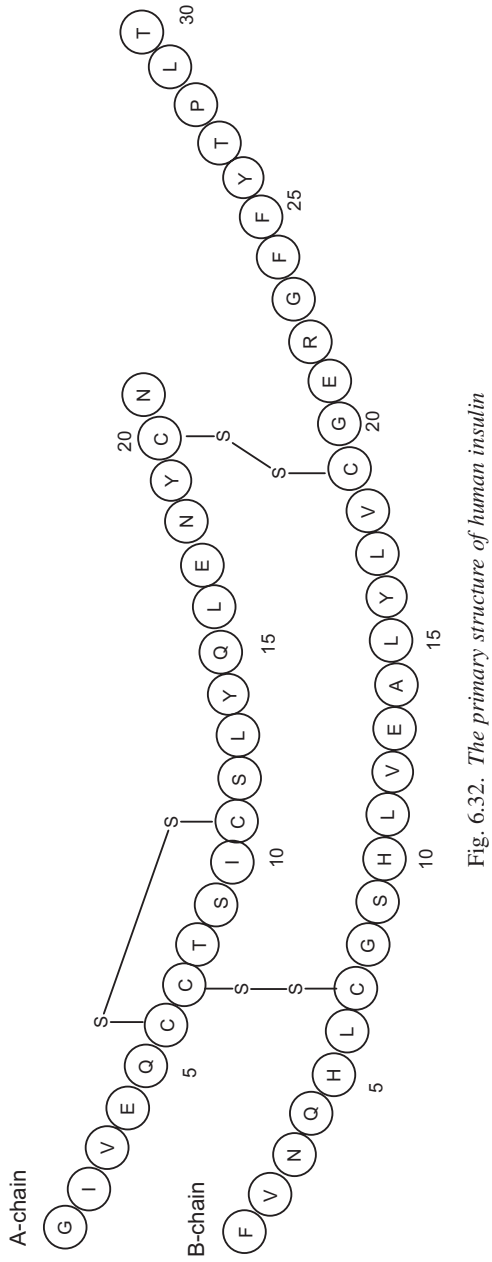


Fig. 6.32. *The primary structure of human insulin*

The differences in reactivity between the three Asn residues has been explained by their molecular environment [134]. Asn^{A18} appears protected from deamidation by being flanked at the C-terminal side with a bulky Tyr, and by being positioned in an α-helix and close to a disulfide bridge. In contrast, Asn^{A21} is at the C-terminus of chain A and appears readily accessible for acid catalysis. As for Asn^{B3}, it is located in a flexible part of the peptide sequence and can, thus, react at neutral pH to form the intermediate succinimide (*Fig. 6.29*, *Pathway e*).

In pharmaceutical *suspensions*, reactions of hydrolysis were faster in the amorphous than the crystalline forms. In addition to deamidation at Asn^{B3}, an unexpected hydrolytic backbone cleavage occurred at the Thr^8–Ser^9 bond [133][135]. This reaction takes place only in suspensions containing rhombohedral crystals and a relatively high content of free Zn ions. The reaction rate was also species-dependent (human < porcine < bovine (where Thr^8 is replaced by Ala^8)). The mechanism of this cleavage reaction was postulated to involve an adjacent insulin molecule in the stacking of hexamers.

Another degradation reaction observed in suspension was the formation of covalent insulin dimers [134][136]. These involve isopeptide links between two insulin molecules, that result from a transamidation reaction mainly between the B-chain N-terminus of one insulin molecule, and one of the four amide side chains in the A-chain (principally Asn^{A21}) of the second insulin molecule.

In insulin *powder* prepared by freeze drying, the reactions observed were deamidation at Asn^{A21} and dimer formation [137]. In contrast to suspensions, amorphous insulin in the powder form was more stable than crystalline insulin. In fact, the rate of degradation of crystalline insulin increased with increasing water content, whereas the rate of degradation of amorphous insulin was essentially independent of water content up to the maximum studied (*ca.* 15%).

6.4. Enzymatic Hydrolysis of Peptides That Contain only Common Amino Acids

In *Sect. 6.3*, we examined, in some detail, the chemical mechanisms of peptide hydrolysis and showed that, while all are relevant in a pharmaceutical context of production and storage, some are fast enough to shorten the half-life and duration of action of some peptides in the body. However, enzymatic reactions of hydrolysis play a much more important role than nonenzymatic ones in the metabolic degradation of peptides [7][14][138–141], as discussed in the remainder of this chapter.

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Peptidases (see *Chapt. 2* and *3*) are widely distributed in the body and can be subdivided into two main groups according to lower or higher degree of specificity [14]. *Peptidases with low substrate and product specificity* are found in the intestine and in the lysosomes of the cell. They rather indiscriminately degrade proteins and peptides to small fragments and amino acids. In contrast, *extracellular peptidases* are much more specific for substrates and cleavage sites. These extracellular peptidases can be divided into *circulating peptidases* (mainly in the plasma) and *ectopeptidases* (which are attached to the cell surface with the active site directed outwards.

We begin this presentation with peptides that contain only common amino acids as defined in *Table 6.1*, including peptides derivatized at the N-terminus, the C-terminus, and/or a functional side chain (*Sect. 6.4.3*). Peptides containing nonproteinogenic amino acids are surveyed in *Sect. 6.5*, whereas peptoids, pseudopeptides, and peptidomimetics are the focus of *Sect. 6.6*. Of course, most of the peptides that contain only common amino acids to be presented are endogenous compounds, but there are good reasons to include them here. Indeed, mechanisms of biotransformation of endogenous peptides afford valuable information on substrate specificity and regiospecificity of peptidases, information that may prove invaluable in designing xenobiotic peptides with improved biological properties.

6.4.1. Peptide Hydrolysis in Complex Biological Media

Numerous studies have been published on the *in vivo* metabolism of peptides. However, these studies are concerned mainly with assessment of pharmacokinetic parameters such as half-life and clearance. Only seldom is the *in vivo* biotransformation of peptides that contain only common amino acids investigated in any detail, due to the difficulty of monitoring products of proteolysis that are identical to endogenous peptides and amino acids. More importantly, such studies fail to yield mechanistic and biochemical insights. For this reason, we begin here with a discussion of the metabolism of just a few peptides in some selected tissues, namely portals of entry (mouth, gastro-intestinal tract, nose, and skin), plasma, organs of elimination (liver, kidney), and pharmacodynamic sites (brain and cerebrospinal fluid). These examples serve as introduction for the presentation in *Sect. 6.4.2* of the involvement of individual peptidases in peptide metabolism.

As illustrated below, the biotransformation of peptides in complex biological media is often due to contributions of only one or a very few enzymes. Indeed, the two main factors that govern the hydrolysis of a peptide in a given tissue are the cellular localization and level of peptidases (*i.e.*,

which peptidases are expressed in the tissue), and their substrate specificity [139].

The *oral cavity* is rich in metabolic enzymes, in particular oxidoreductases and hydrolases [141]. Peptides undergoing proteolysis range from oligopeptides like glutathione, thyrotropin-releasing hormone (TRH, *Fig. 6.21*), and enkephalin analogues, to more complex peptides such as calcitonin (*Fig. 6.22*) and insulin (*Fig. 6.32*). The enzymes involved include aminopeptidases, carboxypeptidases, dipeptidyl-peptidases, peptidyl-dipeptidases, endopeptidases, and various proteases. Aminopeptidase, dipeptidyl-peptidase, and peptidyl-dipeptidase activities were also found in human saliva with [Leu^5]enkephalin (Tyr-Gly-Gly-Phe-Leu) as a substrate [142].

The *gastrointestinal tract* is obviously rich in digestive proteases such as trypsin, chymotrypsin, mucosal peptidase, and pepsin. These enzymes were investigated for their activity toward angiotensin I and II, kentsin, [Met^5]enkephalin, and substance P [143]. As shown in *Table 6.6*, the shorter peptide kentsin was not a substrate. But, when a peptide was long enough to be a substrate, it was hydrolyzed at one or more sites by each enzyme. By combining these results, one can deduce that the peptide substrates would be broken down to a variety of small oligopeptides.

The brush border enzymes in the intestine play a major role in peptide hydrolysis. Thus, both aminopeptidase and endopeptidase activities were detected with [Leu^5]enkephalin as the substrate [146].

Nasal delivery of peptide drugs is an active field of biopharmaceutical research [156]. Here again, peptidases can play a role in drug-delivery failure and need to be better documented. Thus, sheep nasal preparations (homogenates, cellular fractions, wash preparations) were found to possess significant peptidase activity [157]. The hydrolysis of di- to pentapeptides was investigated and found to be significant, and to be mediated by both aminopeptidases and carboxypeptidases. Thus, an interesting and good substrate was [Leu^5]enkephalin.

Percutaneous absorption is another route of interest for the administration of peptides [158], with metabolism being a complicating factor [159]. Thus, [Leu^5]enkephalin and Tyr-Pro-Leu-Gly-NH_2 were rapidly degraded on the dermal side after penetration through rat skin preparations [160]. The use of inhibitors confirmed the involvement of serine proteases and metalloenzymes.

Another major site of peptide metabolism is the blood and especially blood *serum* and *plasma*. From an extensive compilation of peptide $t_{1/2}$ values (over 100 peptides, plus derivatized and cyclized analogues, D-amino acid stereoisomers, peptide bond isosteres, *etc.*), it appears that the differences between serum, heparinized plasma, and whole blood are fairly limited [161]. Interspecies differences are larger, particularly between humans and rats, with most human/rat $t_{1/2}$ ratios ranging from 1:1 to 25:1!

Table 6.6. *Substrate Regiospecificity of Selected Peptidases toward Some Peptides Containing only Common Amino Acids; only Main Site(s) of Cleavage Are Indicated.* See text for further details and additional references [139][143 – 155].

Enzyme	Peptide substrate	Site of cleavage
Aminopeptidases (EC 3.4.11) (remove N-terminal residue)		
Aminopeptidase (unspecified)	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	Asp^{1}-Arg^{2}
Aminopeptidase (unspecified)	[Leu^{5}]enkephalin (*Fig. 6.35*)	Tyr^{1}-Gly^{2}
Leucyl aminopeptidase (EC 3.4.11.1)	Insulin (*Fig. 6.32*)	Phe^{B1}-Val^{B2} Gly^{A1}-Ile^{A2}
Aminopeptidase N (EC 3.4.11.2, membrane alanyl aminopeptidase)	[Leu^{5}]enkephalin (*Fig. 6.35*) and [Met^{5}]enkephalin	Tyr^{1}-Gly^{2}
	Somatostatin (*Fig. 6.37*)	Ala^{1}-Gly^{2}
Cystinyl aminopeptidase (EC 3.4.11.3)	Oxytocin (*Fig. 6.36*)	Cys^{1}-Tyr^{2}
Aminopeptidase P (EC 3.4.11.9)	Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	Arg^{1}-Pro^{2}
Carboxypeptidases (EC 3.4.16, 3.4.17, 3.4.18) (remove C-terminal residue)		
Carboxypeptidase (unspecified)	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	C-terminus
	Corticotropin (ACTH; 39 residues)	Glu^{38}-Phe^{39} Leu^{37}-Glu^{38} Pro^{36}-Leu^{37}
Carboxypeptidase A (EC 3.4.17.1)	Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	Phe^{8}-Arg^{9}
	Insulin (*Fig. 6.32*)	Thr^{B30}-Lys^{B29} Asn^{A21}-Cys^{A19}
Dipeptidyl-peptidases and tripeptidyl-peptidases (EC 3.4.14) (remove N-terminal di- or tripeptide)		
Dipeptidyl-peptidase III (EC 3.4.14.4)	[Leu^{5}]enkephalin (*Fig. 6.35*)	Gly^{2}-Gly^{3}
Dipeptidyl-peptidase IV (EC 3.4.14.5)	Glucagon (29 residues)	Ser^{2}-Gln^{3}
	Growth hormone releasing hormone (GHRH; 44 residues)	Ala^{2}-Asp^{3}
	Neuropeptide Y (36 residues)	Pro^{2}-Ser^{3}
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Pro^{2}-Lys^{3}
Peptidyl-dipeptidases (EC 3.4.15) (remove C-terminal dipeptide)		
Angiotensin-converting enzyme (ACE) (EC 3.4.15.1, peptidyl-dipeptidase A)	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Phe^{8}-His^{9}
	Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	Pro^{7}-Phe^{8}
	[Leu^{5}]enkephalin (*Fig. 6.35*)	Gly^{3}-Phe^{4}
	Luteinizing hormone releasing hormone (LHRH, *Fig. 6.16*)	Trp^{3}-Ser^{4}
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Gly^{9}-Leu^{10} Phe^{8}-Gly^{9}

Table 6.6 (cont.)

Enzyme	Peptide substrate	Site of cleavage
Serine endopeptidases (EC 3.4.21)		
Chymotrypsin (EC 3.4.21.1)	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Tyr^4-Ile^5 Phe^8-His^9
	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	Tyr^4-Ile^5
	Kentsin (Thr-Pro-Arg-Lys)	none
	[Met^5]enkephalin (Tyr-Gly-Gly-Phe-Met)	Tyr^1-Gly^2 Phe^4-Met^5
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Phe^7-Phe^8
Chymotrypsin-like (unspecified, rat intestine)	Corticotropin (ACTH; 39 residues)	Phe^7-Arg^8
Serine-endopeptidase (unspecified)	Cholecystokinin-C-terminal octapeptide (CCK8) (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2)	Met^3-Gly^4 Met^6-Asp^7
Trypsin (EC 3.4.21.4)	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Arg^2-Val^3
	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	Arg^2-Val^3
	Kentsin (Thr-Pro-Arg-Lys)	none
	[Met^5]enkephalin (Tyr-Gly-Gly-Phe-Met)	none
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	none
Trypsin-like (brain)	Corticotropin (ACTH; 39 residues)	Lys^{16}-Arg^{17} Lys^{21}-Val^{22}
	[Arg^8]Vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2; disulfide bridge: 1-6)	Arg^8-Gly^9
Prolyl oligopeptidase (EC 3.4.21.26)	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	Pro^7-Phe^8
	Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	Pro^7-Phe^8
	Oxytocin (*Fig. 6.36*)	Pro^7-Leu^8
Tissue kallikrein (EC 3.4.21.35)	Somatostatin (*Fig. 6.37*)	Phe^7-Trp^8
Cysteine endopeptidases (EC 3.4.22)		
Cathepsin B1 (EC 3.4.22.1)	Human calcitonin (*Fig. 6.33*)	Met^8-Leu^9 Gly^{10}-Thr^{11} Thr^{21}-Phe^{22} Gln^{24}-Thr^{25} Thr^{25}-Ala^{26}
	Salmon calcitonin (*Fig. 6.22*)	Lys^{18}-Leu^{19}
Thiol endopeptidase (unspecified, brain)	Luteinizing hormone releasing hormone (LHRH, *Fig. 6.16*)	Tyr^5-Gly^6
	Neurotensin (Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)	Arg^8-Arg^9

Table 6.6 (cont.)

Enzyme	Peptide substrate	Site of cleavage
Aspartic endopeptidases (EC 3.4.23)		
Cathepsin D (EC 3.4.23.5)	Human calcitonin (*Fig. 6.33*)	Leu^{9}-Gly^{10} Gly^{10}-Thr^{11} Thr^{11}-Tyr^{12} Tyr^{12}-Thr^{13} Ala^{26}-Ile^{27}
	Salmon calcitonin (*Fig. 6.22*)	Leu^{9}-Gly^{10}
Pepsin (EC 3.4.23.1-2)	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Val^{3}-Tyr^{4}
	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	Val^{3}-Tyr^{4}
	Kentsin (Thr-Pro-Arg-Lys)	none
	[Met^{5}]enkephalin (Tyr-Gly-Gly-Phe-Met)	Gly^{3}-Phe^{4}
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Phe^{7}-Phe^{8}
Metalloendopeptidases (EC 3.4.24)		
Neprilysin (EC 3.4.24.11)	Atrial natriuretic factor (ANF; 28 residues)	Cys^{7}-Phe^{8} Ser^{25}-Phe^{26}
	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Pro^{7}-Phe^{8}
	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	Tyr^{4}-Ile^{5}
	Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	Pro^{7}-Phe^{8}
	Cholecystokinin-C-terminal octapeptide (CCK8) (Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH_2)	Gly^{4}-Trp^{5} Asp^{7}-Phe^{8}
	Gastrin (17 residues)	Gly^{13}-Trp^{14} Trp^{4}-Leu^{5} Ala^{11}-Tyr^{12}
	[Leu^{5}]enkephalin (*Fig. 6.35*) and [Met^{5}]enkephalin	Gly^{3}-Phe^{4}
	Luteinizing hormone releasing hormone (LHRH, *Fig. 6.16*)	Gly^{6}-Leu^{7} His^{2}-Trp^{3}
	Neurotensin (Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)	Pro^{10}-Tyr^{11} Tyr^{11}-Ile^{12}
	Oxytocin (*Fig. 6.36*)	Tyr^{2}-Ile^{3}
	Somatostatin (*Fig. 6.37*)	Phe^{6}-Phe^{7} Thr^{10}-Phe^{11} Asn^{5}-Phe^{6}
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Gln^{6}-Phe^{7} Phe^{7}-Phe^{8} Gly^{9}-Leu^{10}
Thimet oligopeptidase (EC 3.4.24.15)	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Pro^{7}-Phe^{8}
	Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	Phe^{5}-Ser^{6}

Table 6.6 (cont.)

Enzyme	Peptide substrate	Site of cleavage
Thimet oligopeptidase (EC 3.4.24.15) (cont.)	Luteinizing hormone releasing hormone (LHRH, *Fig. 6.16*)	Tyr^5-Gly^6
	Neurotensin (Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)	Arg^8-Arg^9
	Somatostatin (*Fig. 6.37*)	Phe^6-Phe^7 Thr^{10}-Phe^{11} Asn^5-Phe^6
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Gln^6-Phe^7
Neurolysin (EC 3.4.24.16)	Neurotensin (Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)	Pro^{10}-Tyr^{11} Arg^8-Arg^9
	Somatostatin (*Fig. 6.37*)	Phe^6-Phe^7 Thr^{10}-Phe^{11}
Endothelin-converting enzyme (EC 3.4.24.71)	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Pro^7-Phe^8
	Atrial natriuretic factor (ANF; 28 residues)	Cys^7-Phe^8 Ser^{25}-Phe^{26}
	Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)	Pro^7-Phe^8
	Neurotensin (Glp-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)	Leu^2-Tyr^3 Pro^{10}-Tyr^{11}
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Gln^6-Phe^7 Gly^9-Leu^{10}
Unspecified		
Mucosal peptidase (a preparation from porcine GI tract having proteolytic and aminopeptidase activities)	Angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)	Arg^2-Val^3 Tyr^4-Ile^5 Phe^8-His^9
	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	Arg^2-Val^3 Val^3-Tyr^4 Tyr^4-Ile^5
	Kentsin (Thr-Pro-Arg-Lys)	none
	[Met^5]enkephalin (Tyr-Gly-Gly-Phe-Met)	Tyr^1-Gly^2
	Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2)	Phe^7-Phe^8 Phe^8-Gly^9

A number of observations converge to indicate that much of plasma peptidase activity is due to *aminopeptidases*, with *N*-protection markedly increasing peptide stability in blood. Dipeptidyl-peptidase is another noteworthy peptidase in blood. In human plasma, some of the peptides showed very small $t_{1/2}$ values of only a few minutes, but a majority of $t_{1/2}$ values were on the order of 10–30 min.

An eloquent example of the biochemical complexity of peptide degradation in blood is afforded by *dynorphin A-(1–13)* (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys), a potent opioid peptide. When incubated at 37° with human plasma, dynorphin A-(1–13) had a $t_{1/2}$ value of <1 min, and

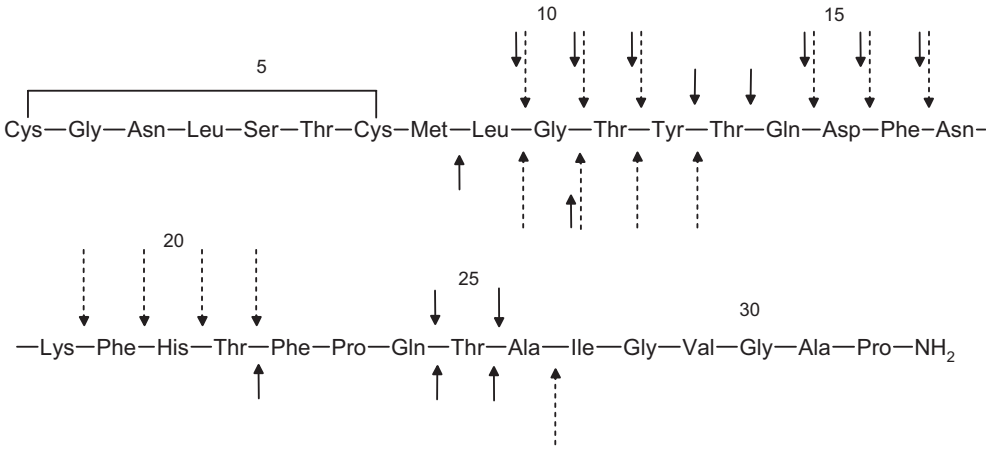


Fig. 6.33. *The structure of human calcitonin* (compare with salmon calcitonin in *Fig. 6.22*). The descending arrows indicate sites of cleavage in rat liver *lysosomal* fraction (full arrows) and rat liver and kidney *cytosolic* fractions (broken arrows). The ascending arrows indicate sites of cleavage by *cathepsin B1* (full arrows) and *cathepsin D* (broken arrows) [149].

its major metabolites were the fragments dynorphin A-(1–12), A-(2–12), A-(4–12), and A-(4–8) [162]. Further metabolites were A-(2–13), A-(3–13), A-(3–12), A-(5–12), A-(6–12), A-(7–12), A-(1–10), A-(2–10), A-(2–8), and A-(3–8). Despite this apparent complexity, it was shown that dynorphin A-(1–13) was primarily metabolized by *carboxypeptidases* to A-(1–12) (80%) and by *aminopeptidases* to A-(2–13) (15%), which were then further converted to A-(2–12). Subsequent reactions involved the same enzymes, plus *peptidyl-dipeptidase A* (EC 3.4.15.1) and *endopeptidases*.

As organs of elimination, the *liver* and *kidneys* play a major role in the degradation of peptides. This can be adequately illustrated with the degradation of human and salmon calcitonin (hCT and sCT, respectively) in rat subcellular preparations [150]. In the lysosomal fraction of rat liver and kidney, hCT was degraded 9–12 times faster than sCT, with many metabolites being produced from the former, and scarcely from the latter. The metabolites of hCT produced in the liver lysosomal fraction (downward-pointing full arrows) are shown in *Fig. 6.33*. These metabolites were formed principally by hydrolysis at positions between Leu^{9} and Asn^{17}, and between Gln^{24} and Ile^{27}, *i.e.*, endoproteolytically. Since both the N- and C-termini are blocked, calcitonin is considered to be resistant to exoproteolytic cleavage, as confirmed here. In the renal lysosomal fraction, hCT cleavage occurred only between Leu^{9} and Thr^{11}.

In the liver and kidney cytosolic fractions (downward-pointing broken arrows in *Fig. 6.33*), three positions of initial endoproteolytic cleavage were

found (Thr^{11}-Tyr^{12}, Phe^{16}-Asn^{17}, and Lys^{18}-Phe^{19}) that led to the production of additional fragments *via* subsequent exoproteolytic cleavage.

Given the numerous centrally active peptides, their degradation in the *brain* is of interest. Thus, human *corticotropin-releasing factor* (hCRF) was degraded in rat brain membrane fractions to a variety of peptides [163]. CRF is a 41-residue peptide whose activity is strongly decreased or abolished if its chain is shortened. In particular, the sequence (5–41) is accepted as being essential for activity. This may perhaps explain why most sites of hydrolysis were found in the more central part of the molecule. Indeed, Leu^{15}-Arg^{16} was the primary site of cleavage, with other major sites being Ser^{1}-Glu^{2}, Thr^{11}-Phe^{12}, His^{13}-Leu^{14}, Arg^{23}-Ala^{24}, Arg^{35}-Lys^{36}, and Lys^{36}-Leu^{37}. This metabolism was dominated by a metalloendopeptidase activity.

The concentrations of opioid peptides present in the human *cerebrospinal fluid* (hCSF) are altered in various pathological conditions, perhaps due to changes in the rates of degradation. The hydrolytic activity of hCSF toward opioid peptides was, therefore, examined with *dynorphin A* and fragments thereof as substrates [16]. Dynorphin A contains 17 residues (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln), fragment-(1–5) being *[Leu^5]enkephalin*. The metabolism of dynorphin A and its fragments in hCSF was mainly catalyzed by aminopeptidases. Interestingly, the overal rate of hydrolysis decreased by one order of between [Leu^5]enkephalin and dynorphin A, *i.e.*, [Leu^5]enkephalin>dynorphin A-(1–7)>dynorphin A-(1–9)>dynorphin A-(1–10)>dynorphin A-(1–13)>dynorphin A.

6.4.2. Hydrolysis of Individual Peptides by Purified Enzymes

In *Sect. 6.4.1*, the metabolic fate of a few peptides in selected tissues was summarized, pointing to the involvement of classes of peptidases. Here, we consider a number of important bioactive peptides (taken in alphabetical order) and examine the qualitative aspect of their cleavage by purified peptidases. The symmetrical viewpoint, namely the activity of peptidases toward various peptides, is presented in *Table 6.6*.

Bradykinin (*Fig. 6.34*) is a vasoactive nonapeptide that is hydrolyzed by a variety of peptidases. Its N-terminus is susceptible to cleavage, but only by aminopeptidase P (X-Pro aminopeptidase, EC 3.4.11.9). Dipeptidyl-peptidase IV can then cleave the N-terminus dipeptide of bradykinin-(2–9). However, most cleavage reactions have been found to occur at or close to the C-terminus, with angiotensin-converting enzyme (ACE, peptidyl-dipeptidase A, EC 3.4.15.1) playing an important role. In fact, aminopeptidase P and ACE accounted for *ca.* 30 and 70%, respectively, of total bradykininase activity in the isolated perfused rat heart [164]. As shown in *Fig. 6.34*, ACE

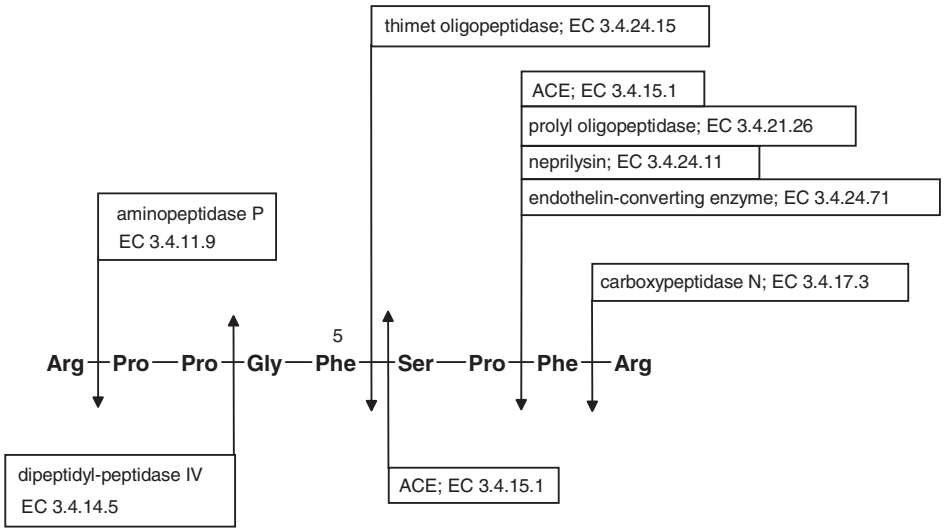


Fig. 6.34. *Major peptidases that act on bradykinin.* Descending arrows represent primary cleavage reactions, whereas ascending arrows indicate secondary reactions, *i.e.*, cleavage sites in shorter peptides.

cleaves the C-terminal dipeptide, but also the C-terminal dipeptide in the resulting bradykinin-(1–7).

Further susceptibilities of the nonapeptide are shown in *Fig. 6.34*. Thus, the Pro^7–Phe^8 bond is also cleaved by neprilysin (EC 3.4.24.11) and endothelin-converting enzyme (EC 3.4.24.71) [149][165]. The C-terminal residue is susceptible to cleavage by several carboxypeptidases, particularly carboxypeptidase N. Thimet oligopeptidase (EC 3.4.24.15) is also able to act on bradykinin, which is cleaved at the Phe^5–Ser^6 bond [144].

When infused into humans, bradykinin was so rapidly degraded that no intact compound could be detected in venous plasma sampled during infusion. Bradykinin-(1–5) was produced as a stable metabolite that cleared from blood with a terminal $t_{1/2}$ value of 86–100 min [166]. This stable metabolite probably results from two consecutive reactions of ACE.

The degradation of enkephalins (Tyr-Gly-Gly-Phe-Xaa) has received considerable attention given their biological significance and therapeutic potential [153][167]. Here, *[Leu^5]enkephalin* (*Fig. 6.35*) is taken as an example to illustrate the variety of hydrolytic reactions even a short peptide can be subjected to [168][169]. An important enzyme is obviously neprilysin (enkephalinase, endopeptidase-24.11, EC 3.4.24.11), which removes the C-terminal dipeptide. The same site is also cleaved by angiotensin-converting enzyme (EC 3.4.15.1). The N-terminal dipeptide can also be cleaved, dipeptidyl peptidase III (EC 3.4.14.4) being the enzyme responsible. The N-termi-

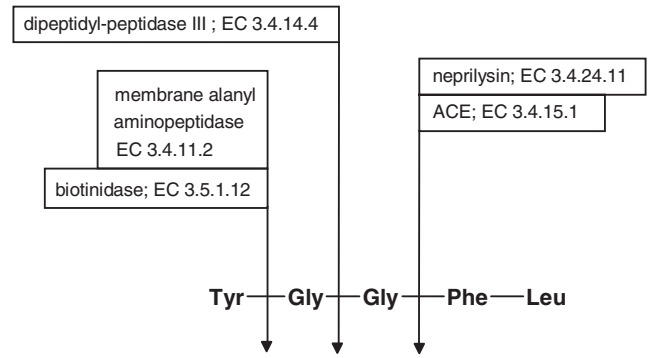


Fig. 6.35. *Major peptidases that act on [Leu^5]enkephalin and their primary sites of attack*

nal residue is also quite sensitive to hydrolytic cleavage by various aminopeptidases and particularly aminopeptidase N (membrane alanyl aminopeptidase, EC 3.4.11.2). It is interesting to note that the same cleavage is also carried out by an amidase present in human serum and known to possess aminopeptidase activity, namely *biotinidase* (biotin-amide amidohydrolase, EC 3.5.1.12) [170].

The pattern and efficiency of hydrolysis of [Leu^5]enkephalin, like that of any peptide, depends to a large extent on its compartmentalization. In other words, the qualitative and quantitative aspects of its degradation vary considerably as a function of species, tissue, and concentration profile. Thus, hydrolysis of [Leu^5]enkephalin in rat brain is catalyzed mainly by aminopeptidase, ACE, and neprilysin, in rat lungs by aminopeptidase and ACE, and in rat plasma by aminopeptidase, dipeptidyl peptidase III, and ACE [171][172].

The kinetics of disappearance from the circulation of intravenously administered *human insulin* (*Fig. 6.32*) is nonlinear [145]. Within a few minutes after injection, it becomes localized in the liver, heart, and kidneys, where it is rapidly metabolized. Indeed, the hepatic extraction could be as high as 70% on a single passage, whereas kidneys could account for 10–40% degradation. Enzymatic reduction of the disulfide bridges appears to be the first step in the *in vivo* metabolism of insulin, although this reaction appears of limited significance under *in vitro* conditions.

The *in vitro* hydrolysis of insulin has been shown to be catalyzed by exopeptidases and endopeptidases. Carboxypeptidase A (EC 3.4.17.1) cleaves the C-terminus of the B-chain (Thr^{B30}) and that of the A-chain (Asn^{A21}) [145]. Leucyl aminopeptidase (EC 3.4.11.1) cleaves the N-terminus of the B-chain (Phe^{B1}) and can continue to shorten it. But, leucyl aminopeptidase appears also able to cleave the N-terminus of the A-chain (Gly^{A1}). In addition to these exopeptidases, entire insulin is also cleaved by endopeptidases of the

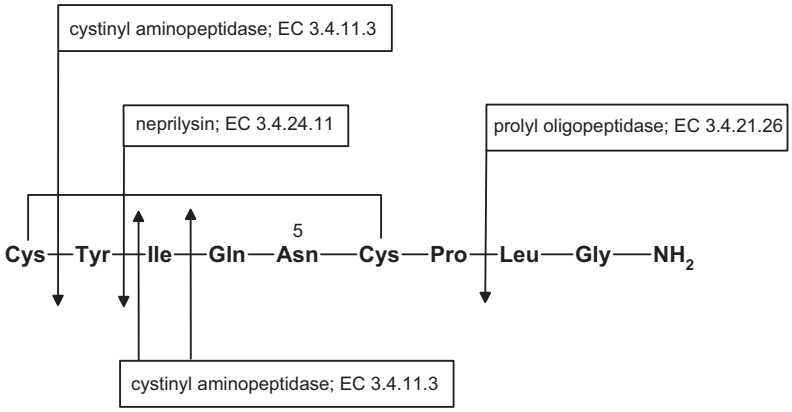


Fig. 6.36. *Major peptidases that act on oxytocin.* Descending arrows represent primary cleavage reactions, whereas ascending arrows indicate secondary reactions, *i.e.*, cleavage sites in shorter peptides.

digestive tract, namely chymotrypsin, trypsin, and elastase. Thus, elastase (EC 3.4.21.36) is able to cleave insulin at six sites in its A-chain and two sites in its B-chain. Chymotrypsin (EC 3.4.21.1) hydrolyzes insulin at five sites, and trypsin (EC 3.4.21.4) at two sites located at the C-terminus of the B-chain [173].

LHRH (*Fig. 6.16*) is a hypothalamic peptide that controls mammalian reproductive functions by releasing the gonadotropins from the pituitary. Because its N- and C-termini are protected by a pyroglutamic acid residue and a carboxamido group, respectively, LHRH is resistant to degradation by exopeptidases. Its enzymatic hydrolysis is, therefore, governed by endopeptidases [174]. These endopeptidases, which have been found in a number of tissues, include angiotensin-converting enzyme (EC 3.4.15.1), neprilysin (EC 3.4.24.11), and thimet oligopeptidase (EC 3.4.24.15), whose major or only cleavage sites are Trp^3-Ser^4, Gly^6-Leu^7, and Tyr^5-Gly^6, respectively [174–176].

We now turn our attention to *oxytocin* (*Fig. 6.36*), a potent and specific stimulant of myometrial contractions commonly used to induce labor. This peptide is of interest here because of its cyclic structure, which results from an intramolecular disulfide bridge. Here again, degradation by a variety of peptidases is documented. Thus, cleavage of the N-terminal cysteine is catalyzed by an aminopeptidase now known as cystinyl aminopeptidase (oxytocinase, EC 3.4.11.3), an enzyme found in the placenta and in the serum of pregnant women [177]. The enzyme acts efficiently to hydrolyze the Cys^1–Tyr^2 bond, thus opening the ring structure of oxytocin, and then cleaves successive residues from the N-terminal end.

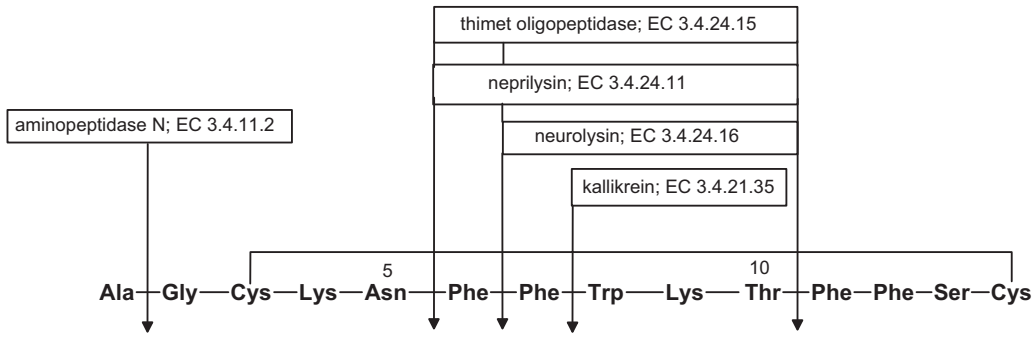


Fig. 6.37. *Major peptidases that act on somatostatin and their primary sites of attack*

At the C-terminus, prolyl oligopeptidase (postproline endopeptidase, EC 3.4.21.26) cleaves the C-terminal dipeptide [178]. The resulting oxytocin-(1–7) is also a substrate for aminopeptidase. Furthermore, neprilysin (EC 3.4.24.11) can also play a role in oxytocin degradation, although it seems to act with less efficiency than the two other enzymes.

The final peptide to be presented here, again a cyclic one, is *somatostatin* (*Fig. 6.37*), which is also a substrate of some aminopeptidases, particularly aminopeptidase N (EC 3.4.11.2) [17]. However, somatostatin degradation is catalyzed mainly by endopeptidases, as shown in *Fig. 6.37* [179][180]. Both neprilysin (EC 3.4.24.11) and thimet oligopeptidase (EC 3.4.24.15) cleave mainly the Phe^6–Phe^7 and Thr^{10}–Phe^{11} bonds, as well as the Asn^5–Phe^6 bond. Neurolysin (EC 3.4.24.16) digests somatostatin at two sites, Phe^6-Phe^7 and Thr^{10}-Phe^{11}, whereas tissue kallikrein (EC 3.4.21.35) is able to cleave the Phe^7–Trp^8 bond.

The above examples illustrate the versatility and overlapping substrate specificities of peptidases, but they also serve to explain the difficulties faced by medicinal chemists who try to design bioactive peptides that have improved pharmacokinetic properties. Clearly, general predictive rules and a global understanding of the *in vivo* fate of peptides are not in sight, but the sections below will show that medicinal chemists have developed various successful strategies of a rather empirical nature [7][181–188].

6.4.3. Derivatized Peptides and Peptide Prodrugs

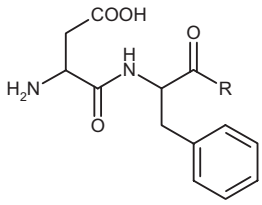
A first and apparently straightforward strategy to improve pharmacokinetic behavior of bioactive peptides is to derivatize the *N-terminus*, the *C-terminus*, and/or one or more *functionalized side chains*. Different biological consequences are conceivable depending on the stability and intrinsic activity of the derivative.

a) Derivatization can produce a compound that retains the bioactivity of the parent, and the former is superior to the latter when *in vivo* stability or pharmaceutical properties are, indeed, better.

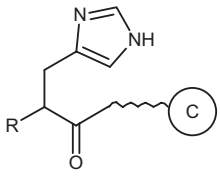
b) In contrast, a derivative that has lost the bioactivity of the parent will be of potential interest only when at least two conditions are met. First, derivatization must be bioreversible, *i.e.*, the parent peptide must be regenerated at a therapeutically suitable rate, and fulfill the other requisites of a prodrug (see below and *Chapt. 1*). Second, the bioactive peptide should be pharmacokinetically better behaved when released from the prodrug than when administered as such. The second condition is fulfilled, for example, when slow release maintains adequate levels of the peptide, when tissue-selective delivery is achieved, or when compartmentalized release protects the peptide against rapid inactivation.

The different outcomes summarized under *a*) and *b*) will be illustrated below with a few selected examples.

That derivatization may increase rather than decrease peptidase-catalyzed degradation is illustrated with *aspartame* (**6.79**, R = MeO), the C-terminal methyl ester of the dipeptide Asp-Phe. The metabolism of this artificial sweetener was compared to that of the underivatized dipeptide (**6.79**, R = H) and of the corresponding amide Asp-Phe-NH_2 (**6.79**, R = NH_2) in microvillar membranes obtained from human duodenum, jejunum, and ileum [189]. The activities monitored were clearly those of peptidases as shown by the effects of inhibitors. Whereas the peptide bond in Asp-Phe and Asp-Phe-NH_2 was hydrolyzed at a comparable rate, that in aspartame was hydrolyzed approximately twice as fast. This is an interesting and favorable situation, given that aspartame is expected to be degraded once it has elicited its effect in the buccal cavity.



6.79



6.80

Improved stability with simultaneous preservation of activity has been demonstrated with analogues of *glucagon-like peptide-1* and *gastric inhibitory polypeptide*, two hormones that stimulate the release of insulin. Glucagon-like peptide-1 (GLP-1) is a 30-residue gastrointestinal hormone, amidated at the C-terminus that has potential as a treatment for type-2 diabetes. However, GLP-1 is rapidly degraded by dipeptidyl-peptidase IV (EC

3.4.14.5), which removes the first two residues [190]. As a result of this lability, GLP-1 has a very short half-life – only a few minutes – that precludes its therapeutic use. In an attempt to improve stability, the N-terminal histidyl residue (**6.80**, R = NH_2) of GLP-1 was modified by *N*-methylation (**6.80**, R = MeNH), deamination (**6.80**, R = H), and replacement of the amino by a OH group (**6.80**, R = OH) [191]. All these analogues were hardly degraded by dipeptidyl-peptidase IV, and they all showed receptor affinity and *in vitro* activity comparable to GLP-1. These promising results seem to clear the way for the development of long-acting GLP-1 analogues for type-2 diabetes therapy.

Like GLP-1, gastric inhibitory polypeptide (GIP) is rapidly inactivated by dipeptidyl-dipeptidase IV. An attempt was made to stabilize this 42-residue peptide by glycation of its N-terminal Tyr residue [192]. This was achieved by allowing the NH_2 group to react with glucose under reducing conditions. The resulting Tyr^1-glucitol GIP was remarkably stable in the presence of dipeptidyl-peptidase IV or blood serum, and exhibited good glucose-lowering activity in challenged rats. This bioactivity is all the more noteworthy given that similarly derivatized GLP-1 (*i.e.*, His^1-glucitol GLP-1) was stable but inactive.

In recent years, significant progress has been made in developing *prodrug approaches* to improve the water solubility, membrane permeability, *in vivo* distribution, and stability of peptides [193 – 197].

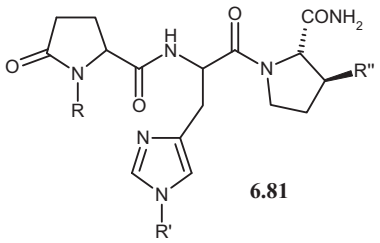
a) To improve water solubility, the focus has been on the bioreversible introduction of ionizable groups.
b) Lipophilicity has been optimized, and H-bonding capacity minimized, to obtain better membrane permeability.
c) Also, improved targeting to the site of action has been attempted by careful design of carrier moieties.

These objectives in the design of prodrugs will be discussed in more detail in the context of *Chapt. 8* (carboxylic acid prodrugs) and *11* (various hydrolyzable groups).

d) Here, we remain focused on the cleavage of peptide bonds and illustrate that a prodrug strategy may indeed slow peptide degradation by peptidases and, thus, achieve better stability and prolonged action. This objective may be reached by bioreversibly masking the terminal carboxy and/or amino groups (protection against exopeptidase-mediated hydrolysis), or by coupling the pro-moiety to a functionalized side chain and so obtaining a prodrug that will hopefully release the peptide at a rate that balances degradation, or in compartments where it is less prone to inactivation.

Thyrotropin-releasing hormone (TRH, **6.81**, R = R′ = R″ = H; see also *Fig. 6.21*) illustrates the difficulty in designing a prodrug able to decrease

degradation of the parent peptide. TRH is degraded in the blood and various tissues either by TRH-degrading pyroglutamyl aminopeptidase II (EC 3.4.19.6), which cleaves the Glp–His bond, or by prolyl oligopeptidase (EC 3.4.21.26), which deamidates the terminal prolylamide residue to yield TRF-OH (Glp-His-Pro). In serum or plasma, the degradation of TRH is catalyzed mainly by pyroglutamyl aminopeptidase II, whereas deamidation by intestinal prolyl oligopeptidase appears to severely limit absorption from the intestine.



6.81

A number of prodrugs were prepared and examined, namely a derivative of TRH that is acylated at the N-terminal pyroglutamic acid with lauric acid (**6.81**, R = lauroyl, R′ = R″ = H), and a series of carbamate esters with the promoiety attached to the imidazole ring of His[2] (**6.81**, R = R″ = H, R′ = CO–O–alkyl). The latter compounds included straight-chain alkyl, branched alkyl, and cycloalkyl carbamates [198][199] that exhibited good transcutaneous permeation but did not improve the penetration of TRH across small segments of rat intestine. From the point of view of stability, the carbamates were able to quantitatively liberate TRH and were resistant to pyroglutamyl aminopeptidase II. However, they were degraded faster than TRH by intestinal prolyl oligopeptidase, which explains the absence of intestinal absorption.

Another informative prodrug of TRH is its *N*-lauroyl derivative (**6.81**; R = lauroyl; R′ = R″ = H) [200][201], which was absorbed several-fold better by the intestine than TRH, indicating good stability toward intestinal peptidases. It was also far more stable than TRH in plasma. The *i.v.* administration of this prodrug to rats resulted in bioactivity that was not statistically different than that of TRH, indicating that it is activated *in situ* to TRH. Thus, lauroyl-TRH appears to be a more promising candidate than the series of carbamates, an outcome that appears impossible to forecast.

Enkephalins offer another example of prodrugs achieving peptide protection against some peptidases but not others. [Leu[5]]- and [Met[5]]enkephalins were condensed with various aldehydes and ketones to produce a series of imidazolidin-4-one derivatives, as exemplified in *Fig. 6.38* for [Met[5]]enkephalin [202]. These prodrugs were converted to the parent enkephalin *via*

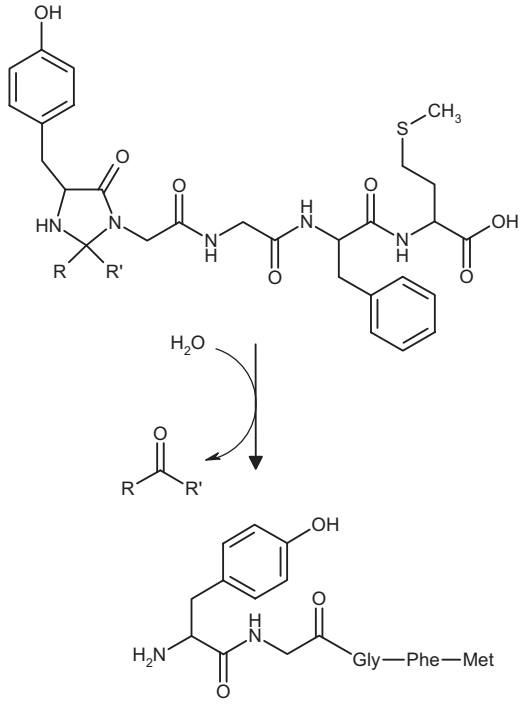
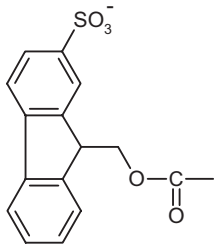


Fig. 6.38. *Imidazolidin-4-one derivatives of [Met^5]enkephalin and their nonenzymatic hydrolysis to liberate the neuropeptide* [202]

nonenzymatic hydrolysis, with rates of activation that were strongly dependent on the nature of the carbonyl pro-moiety. Thus, the prodrug prepared from cyclopentanone had a $t_{1/2}$ value for hydrolysis of *ca.* 3 h at pH 7.4 and 37°, whereas the derivative prepared from propionaldehyde had a $t_{1/2}$ value of *ca.* 150 h. Hydrolysis by peptidases was highly dependent on the enzyme examined. Whereas the enkephalins were rapidly hydrolyzed in the presence of a purified aminopeptidase, in human plasma or in rabbit intestinal homogenates, the prodrugs were almost totally resistant to enzymatic degradation in these media. On the other hand, the prodrugs were just as labile as enkephalins in the presence of carboxypeptidase A.

The last peptide discussed here is *insulin*, which might also benefit from a prodrug approach. Following earlier studies [145], interesting results were obtained with palmitoyl derivatives of insulin [203][204]. Two such prodrugs of bovine insulin were examined, namely monopalmitoyl-insulin (in which the amino group of Phe^{B1} is acylated), and dipalmitoyl-insulin (in which the side-chain amino group of Lys^{B29} is also acylated). After intravenous injection in rats, the monopalmitoyl prodrug was more active than the dipalmitoyl

derivative and produced a longer-lasting effect than native insulin. In a rat *in situ* model of intestinal absorption, the mono- and dipalmitoyl derivatives gave approximately threefold and sixfold higher plasma levels than insulin, respectively. This suggested greater stability of the prodrugs toward peptidases. Indeed, the two prodrugs were two- to threefold more stable than insulin when incubated in mucosal tissue homogenates of the large intestine.



6.82

A more recent approach has used a synthetic pro-moiety to derivatize insulin, namely a hydrophilic fluorenylsulfonic acid derivative (**6.82**) [205]. Three such pro-moieties were attached to the free amino group of Gly^{A1}, Phe^{B1}, and Lys^{B29}, yielding a water-soluble, long-acting insulin prodrug of particular interest. The prodrug was intrinsically inactive but could be quantitatively converted to insulin after being incubated for 20 h in pH 7.4 buffer at 37°. A single subcutaneous dose lowered glucose levels in rats for a prolonged period of more than 2 days compared to less than 5 h for insulin. Importantly, the prodrug was markedly more stable than insulin in the presence of a mixture of trypsin and chymotrypsin ($t_{1/2}$ 8.5 h, compared to 0.8 h). The prodrug persisted also for longer in the circulation since it evaded receptor-mediated endocytosis and degradation, and underwent a slow, spontaneous activation in the circulation. These facts demonstrate the feasibility of obtaining an insulin prodrug with a greatly improved pharmacokinetic behavior.

6.5. Enzymatic Hydrolysis of Peptides That Contain Nonproteinogenic Amino Acids

In *Sect. 6.1*, we defined a peptide as any compound produced by amide formation between a carboxy group of one amino acid and an amino group of another. Our definition is explicit as far as the relation between component residues is concerned, but is incomplete since the definition of amino acids is left open. In *Sect. 6.3* and *6.4*, we used a narrow definition of amino acids (*i.e.*, proteinogenic α-amino acids). In contrast, the peptides examined

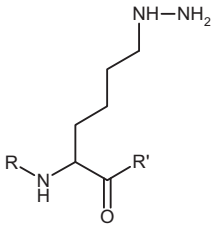
in this section are based on a broader definition of amino acids since we include *nonproteinogenic amino acids*, namely artificial amino acids, *N*-methyl amino acids, D-amino acids, and β-amino acids.

6.5.1. Peptides That Contain Artificial or D-Amino Acids

Many observations document that modifications of peptides by substitution with artificial amino acids or D-amino acids can often protect against enzymatic degradation. This is especially true when more than one modification is made. We begin here with two examples in which replacement of a single natural amino acid with an artificial one had a favorable impact on biological stability. Then, the influence of substitution with D-amino acids is described. Examples involving two or more modifications with artificial amino acids and D-amino acids combined are presented in the following section.

TRH (**6.81**, R = R′ = R″ = H) is extensively metabolized as discussed in *Sect. 6.4.3*. The replacement of the L-prolylamide residue with an artificial *trans*-3-methyl-prolylamide residue led to a peptide (**6.81**, R = R′ = H, R″ = Me) that had a markedly increased biological stability and showed qualitative differences in the nature of metabolites [206]. Thus, the $t_{1/2}$ values for hydrolysis of TRH and its analogue in rat brain homogenates were 30 and 110 min, respectively. The difference in rat plasma was even greater, since the $t_{1/2}$ of TRH was 12 min, whereas only 15% of the analogue was degraded in 120 min. This increased biological stability was consistent with a fourfold higher potency in mice.

Another structurally simple modification involves replacement of a Lys residue with Lys(NH_2) (**6.83**). Here, the amino group of lysine is replaced with a hydrazino group, which is less-basic by *ca.* 3 pK_a units. This modification allows model peptides to be stabilized against endopeptidases such as trypsin and thrombin [207]. Thus, the peptides Tyr-Gly-Xaa-Gly-Tyr-Ala-NH_2 with Xaa = Lys or Arg were very rapidly hydrolyzed by trypsin (half-



6.83

life of a few minutes), whereas the analogue with Xaa = Lys(NH_2) was remarkably stable ($t_{1/2}$ of up to several hours depending on pH). Similar observations were made with the peptides Tyr-Leu-Val-Pro-Xaa-Gly-Ser-Tyr-NH_2, which were rapidly hydrolyzed by trypsin or thrombin when Xaa was Arg or Lys, and 15 – 100 times more slowly when Xaa was Lys(NH_2). The same trend was seen in diluted human serum. This stabilization was postulated to be due to the markedly decreased basicity of *Lys(NH_2)-containing peptides*, which decreased the population of protonated forms at physiological pH and the affinity toward some endopeptidases.

Replacement of coded L-amino acids with D-*amino acids* is a common strategy in peptide drugs. However, it must be stressed that D-amino acids are far from unknown in nature and can even be found in animal peptides, where they are usually formed from L-amino acids by a post-translational reaction [208]. It appears that this mechanism increases the structural diversity of products that can be synthesized from one gene, thus increasing the scope of evolution by natural selection. In other words, the incorporation of D-amino acids into peptides is a strategy discovered by evolution and 'rediscovered' by chemists.

In a comprehensive review, *Fauchère* and *Thurieau* [139] have listed a number of modified peptides where replacement of one or more L-amino acids with D-amino acids resulted in preserved activity and increased metabolic stability. Examples include [D-Phe^7,D-Arg^8]ACTH-(4 – 10), [D-Ala^4]CCK8, [D-Trp^4]CCK8, [D-Tyr^{11}]neurotensin, [D-Phe^{11}]neurotensin, and [D-Trp^8]-somatostatin. However, many of the peptides compiled combine two or more types of modification, for example *N*-methyl amino acids, artificial amino acids, and D-amino acids. Indeed, this sort of multiple-modification strategy has been used in many recent studies on artificial peptides, as illustrated in the next section.

The possibility of stabilizing a peptide against N-terminal degradation has also been achieved with *glucagon*. This peptide hormone contains 29 amino acids and is a good substrate of dipeptidyl peptidase IV (see *Table 6.6*) to yield glucagon-(3 – 29) and, subsequently, glucagon-(5 – 29). A few analogues were prepared in an attempt to preserve the peptide against dipeptidyl peptidase IV degradation [209]. Of these, only [D-Ser^2]glucagon and [Gly^2]glucagon retained high *in vitro* activity, but only [D-Ser^2]glucagon was stable against the enzyme and showed enhanced *in vivo* activity. Interestingly, this analogue was also 10- to 20-fold more stable than other peptides investigated in human serum.

LHRH (see *Fig. 6.16*) is highly sensitive to proteolytic degradation. Enzymes with particular activity toward LHRH include angiotensin-converting enzyme, neprilysin, and thimet oligopeptidase (see *Sect. 6.4.2* and *Table 6.6*). D-Amino acid substitution in position 6, 7, or 10 has led to the de-

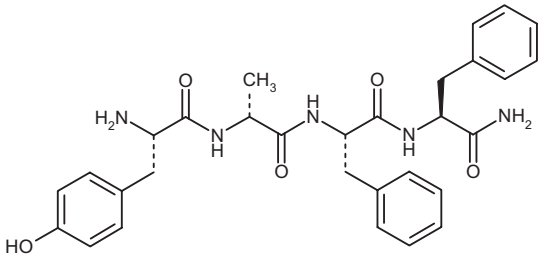
velopment of agonists with superactivity due to higher receptor affinity and higher stability toward enzymatic cleavage [210]. Thus, no intact LHRH was excreted in the urine of rats dosed *i.m.* with the peptide, whereas the recovery of [D-Phe^6]LHRH was *ca.* 1% and that of [D-Trp^3,D-Phe^6]LHRH *ca.* 25%. This order in increasing urinary excretion paralleled the observed increased metabolic stability toward membrane-bound proteolytic activity in the kidney (mainly neprilysin and mepryn A (EC 3.4.24.18)). In contrast, the three peptides exhibited similar susceptibility to hydrolysis in the presence of soluble fractions of rat tissues. These facts are consistent with the general role of membrane-bound enzyme in the *in vivo* degradation of peptides.

In another series of investigations, [D-Ala^6]LHRH was incubated with various cell types and found to have a rate of hydrolysis 3 – 8 times lower than that of LHRH [174][176]. The use of enzyme inhibitors showed [D-Ala^6]LHRH to be resistant to the endopeptidases neprilysin and thimet oligopeptidase, but to remain sensitive to the peptidyl-dipeptidase ACE.

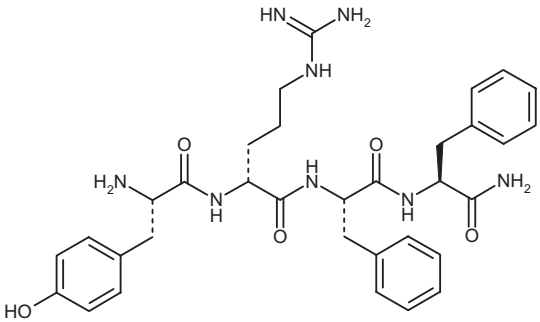
As discussed in *Sect. 6.4.1*, brush border aminopeptidases and endopeptidases in the intestine are highly active against *enkephalins*. Attempts were made to protect [Leu^5]enkephalin (Tyr-Gly-Gly-Phe-Leu) by substituting the second residue with D-alanine. The resulting [D-Ala^2,Leu^5]enkephalin was indeed strongly stabilized against aminopeptidase activity, but remained sensitive to cleavage of the Gly–Phe bond by endopeptidases [146].

Findings continue to accumulate in the field of endogenous opiates, as exemplified by two tetrapeptides isolated from mammalian brain and found to have high affinity and selectivity for μ-opioid receptors. These tetrapeptides are endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2). A number of synthetic analogues have been prepared with the view to improve their metabolic stability and, in some cases, to limit their access to peripheral opioid receptors. The three synthetic *endomorphin analogues* Tyr-D-Ala-Phe-Phe-NH_2 (**6.84**), Tyr-D-Arg-Phe-Phe-NH_2 (**6.85**), and Tyr-D-Arg-Phe-Ape-NH_2 (**6.86**), to be discussed in the next section, have potent antinociceptive effects in *in vivo* inflammatory tests but exhibit modest effects in the CNS. However, and despite the presence of a D-amino acid and a protected C-terminus, they remained sensitive to enzymatic hydrolysis [211][212].

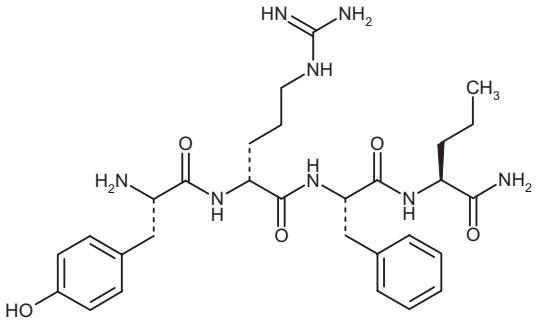
Indeed, all three peptides were rather rapidly degraded by rat jejunal homogenates with $t_{1/2}$ values of 12 – 32 min. The peptides **6.84** and **6.85** were also hydrolyzed in rat and human jejunal fluid, whereas **6.86** was less sensitive [211]. When examined in the presence of purified enzymes, the three peptides showed different reactivities. The peptides **6.84** and **6.85** were very rapidly degraded by chymotrypsin ($t_{1/2}$ *ca.* 1 min) and rapidly by trypsin ($t_{1/2}$ *ca.* 20 min) [212]. Hydrolysis by carboxypeptidase A was almost as fast as



Tyr D-Ala Phe Phe—NH_2 **6.84**



Tyr D-Arg Phe Phe—NH_2 **6.85**



Tyr D-Arg Phe Ape—NH_2 **6.86**

by trypsin, while other enzymes acted somewhat or much more slowly: aminopeptidase N (EC 3.4.11.2) > carboxylesterase (EC 3.1.1.1) > carboxypeptidase B (EC 3.4.17.2). The hydrolytic reactions catalyzed by these enzymes were C-terminus deamidation by chymotrypsin, trypsin, and carboxylesterase, Tyr^1–Xaa^2 cleavage by aminopeptidase N, and, in the case of **6.85**, Phe^3–Phe^4 cleavage by carboxypeptidases. Peptide **6.86** combines both artificial and D-amino substitution and is discussed in the next section.

6.5.2. Peptides That Contain Both Artificial and D-Amino Acids

We now turn our attention to synthetic *peptides that contain both artificial and* D*-amino acids*. These appear to be more common in the literature than peptides with only one type of modification.

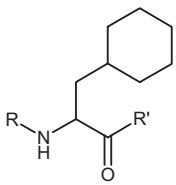
The *endomorphin analogue* Tyr-D-Arg-Phe-Ape-NH_2 (**6.86**) was markedly more resistant than its two congeners **6.84** and **6.85** (see above section), showing the influence of the presence of the artificial amino acid 2-aminovaleric acid (Ape, also known as norvaline). Analogue **6.86** was metabolized to any extent by only carboxypeptidase A ($t_{1/2}$ *ca.* 80 – 90 min) and chymotrypsin ($t_{1/2}$ *ca.* 2.5 h), the Phe–Ape bond being hydrolyzed by both enzymes and the CO–NH_2 bond by the latter. The conclusion of these studies was that the presence of the D-amino acid in position 2 largely protected the tetrapeptides against N-terminal cleavage, while the C-terminal amide did not stabilize them against deamidase and carboxypeptidase activities. Interestingly, the artificial Ape amino acid did have a global protective effect. We will have more to say about artificial amino acids later in this section.

An informative and medicinally relevant example is provided by *MDL 28,050* (**6.87**), an anticoagulant decapeptide [213]. This compound is an analogue of the C-terminal region of the anticoagulant hirudin, a 65-residue polypeptide. The ten residues at the C-terminus of hirudin were found to be the minimal requirement for binding to the noncatalytic binding site of thrombin and for inhibition of clotting. To stabilize against enzymatic degradation, various modifications at both the N- and C-termini were introduced. In the case of MDL 28,050, these modifications included protection of the N-terminus by a succinyl group, and replacements at the C-terminus by cyclohexylalanine (Cha, **6.88**) or D-glutaric acid. In other words, this decapeptide combines three artificial modifications, namely acylation of the N-terminus, and introduction of an artificial amino acid and a D-amino acid.

These structural changes did protect the peptide against exopeptidases, but left it sensitive to endopeptidases. Indeed, the compound was rapidly and

Succinyl—Tyr—Glu—Pro—Ile—Pro—Glu—Glu—Ala—Cha—D-Glu

6.87



6.88

extensively hydrolyzed in rats, such that only traces of the parent drug were present in urine 6 h after administration of the dose. The two primary reactions of hydrolysis occurred at the Ile^4–Pro^5 and Glu^6–Glu^7 bonds. The resulting fragments were degraded at the Ile–Pro, Pro–Glu, and Ala–Cha bonds.

Given that various D-amino acid analogues of LHRH (**6.89**, *Figs. 6.16* and *6.39*) were found to remain sensitive to angiotensin-converting enzyme (see previous section), combined modifications were investigated. *Leuprolide* and *deslorelin* (**6.90** and **6.91**, *Fig. 6.39*) are two LHRH analogues that contain one D-amino acid and a modified C-terminus. These two peptides are LHRH-like agonists of interest in the treatment of prostate cancer, endometriosis, and other sex-hormone-dependent indications. The same is true of *nafarelin* (**6.92**, *Fig. 6.39*), which contains an artificial amino acid of D-configuration in the position 6, namely D-2-naphthylalanine [D-2-Nal).

These three analogues are more metabolically stable than LHRH. Thus, the half-life of nafarelin in monkeys and rats after *i.v.* administration was four to five times longer than that of LHRH [214]. When incubated in monkey plasma, LHRH was readily degraded ($t_{1/2}$ *ca.* 2 h), whereas nafarelin was more resistant ($t_{1/2} > 160$ h). The metabolism of [^{14}C]nafarelin was also investigated in humans following subcutaneous administration [215]. As shown in *Fig. 6.39*, the first metabolic steps were cleavage in the 4–5, 5–6, and 7–8 positions. Because of the presence of the ^{14}C-label, the fate of D-2-Nal could be carefully monitored. The same metabolic pattern was observed in the rhesus monkey [216].

Despite being more metabolically stable than LHRH, leuprolide, deslorelin, and nafarelin are not orally active due to their sensitivity to gastrointestinal peptidases. In particular, they are sensitive to chymotrypsin, which cleaves the Trp^3–Ser^4 bond. In an effort to overcome this limitation, a number of modifications were introduced, in particular N-*methyl substitution at each peptide bond* separately [217]. A few of the *N*-methyl derivatives showed enhanced activity, others were inactive, and some were antagonists. In leuprolide, *N*-methyl substitution of Ser^4 or Tyr^5, or replacement of Glp^1 with *N*-Me-1-naphthylalanine (*N*-Me-1-Nal), rendered the peptide bond between residues 3 and 4 in these compounds completely stable toward chymotrypsin. It was shown that the NH groups of residues 3 and 5 are involved in H-bonding to chymotrypsin, and that *N*-methylation at these positions disrupted the H-bonding interactions and prevented the substrate from fitting in the active site. Several compounds also showed decreased clearance, and, hence, increased metabolic stability upon *i.v.* administration in the rat, *e.g.*, analogues of leuprolide, deslorelin, and nafarelin having an *N*-Me-Tyr^5 residue.

Our last two examples are intended to illustrate the chemical diversity of artificial amino acids that have found their way into two promising peptides,

Glp—His—Trp—Ser—Tyr—Gly—Leu—Arg—Pro—Gly—NH_2 **6.89**

Glp—His—Trp—Ser—Tyr—D-Leu—Leu—Arg—Pro—NHEt **6.90**

Glp—His—Trp—Ser—Tyr—D-Trp—Leu—Arg—Pro—NHEt **6.91**

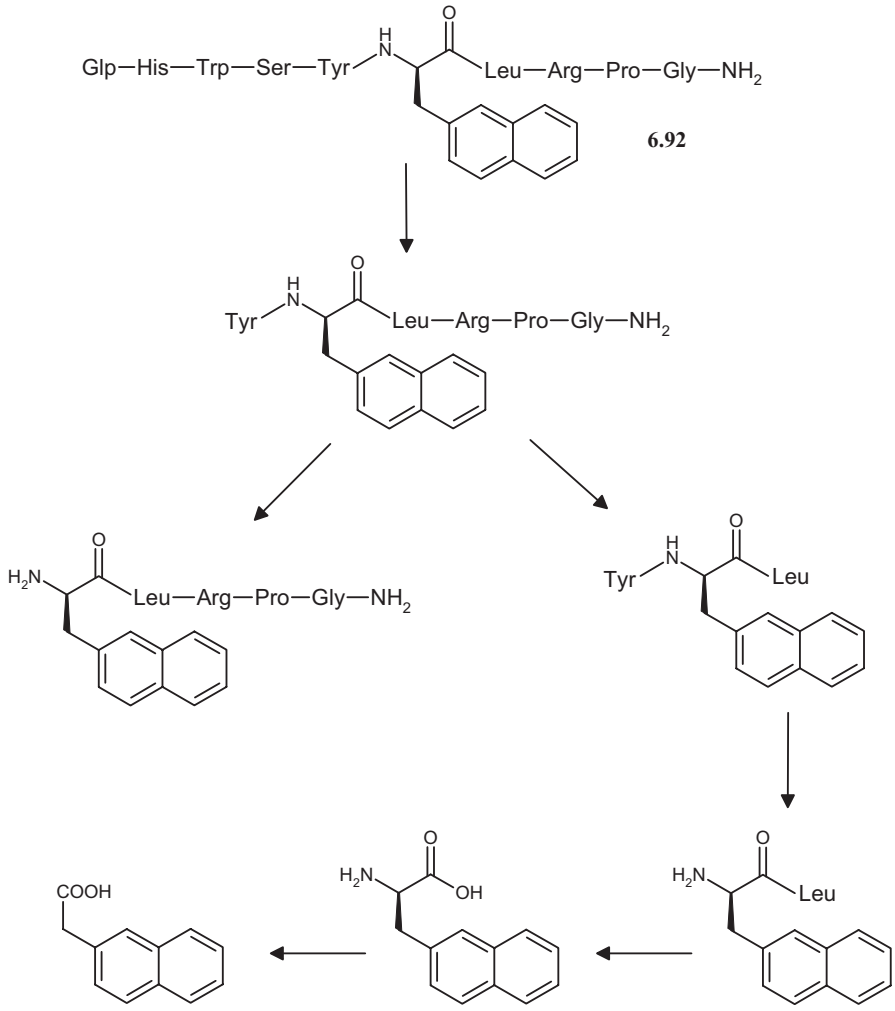
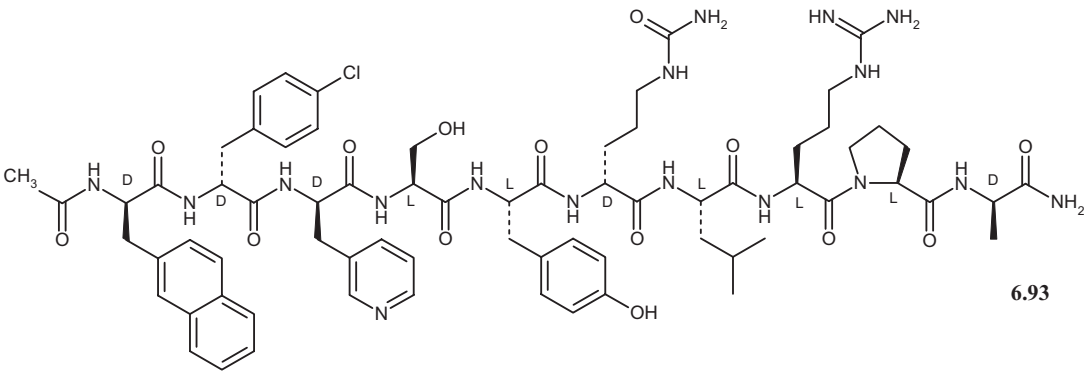
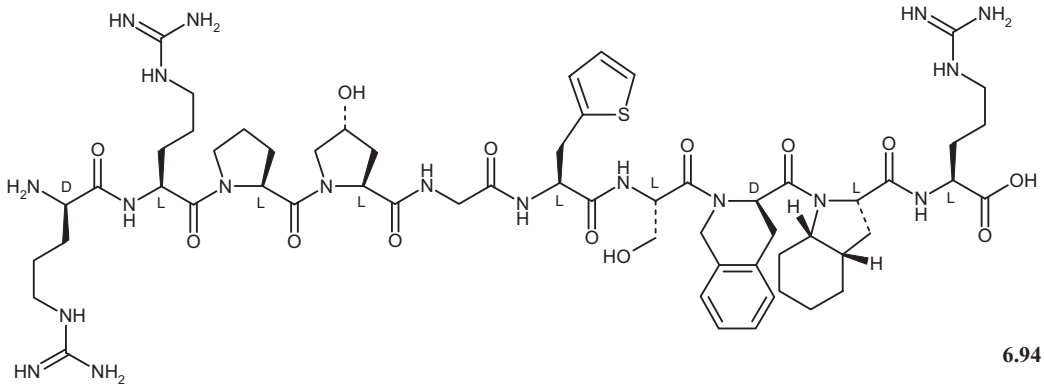


Fig. 6.39. *The structure of LHRH* (**6.89**)*, leuprolide* (**6.90**)*, deslorelin* (**6.91**)*, and nafarelin* (**6.92**). Also shown is the metabolism of nafarelin in humans [215].



6.93



6.94

namely *cetrorelix* and *Hoe 140*. Cetrorelix (**6.93**), a potent antagonist of LHRH receptors, is an *N*- and *C*-protected decapeptide containing D-Nal, D-(4-Cl)Phe, D-(pyridin-3-yl)Ala, D-Orn, and D-Ala as D-/artificial residues. Following subcutaneous administration, the $t_{1/2}$ values in plasma were 35 – 40 h in the rat and 100 – 130 h in the dog, indicating remarkable metabolic stability [218]. In both species, the urine contained only unchanged compound. Bile, on the other hand, contained up to four metabolites, namely the (1 – 9)-nonapeptide, the (1 – 7)-heptapeptide, the (1 – 6)-hexapeptide, and the (1 – 4)-tetrapeptide, indicating that only peptidase-modified products were identified. Interestingly, different relative abundances of the metabolites were found in bile and feces, indicating additional breakdown in the intestine by enteral peptidases or bacteria.

The decapeptide *Hoe 140* (**6.94**), its D- and/or artificial residues being D-Arg, ((4*R*)-hydroxy)Pro, (2-thienyl)Ala, D-[(1,2,3,4-tetrahydroisoquinolin-3-yl)carbonyl] and L-[(3a*S*,7a*S*)-octahydroindol-2-yl]carbonyl, is a potent and long-acting antagonist of bradykinin receptors [219][220]. This compound proved highly resistant to enzymatic degradation. It is not a substrate for kininase II and carboxypeptidases, and is only slowly degraded in human plasma.

6.5.3. Peptides That Contain β-Amino Acids

Whereas β-alanine (3-aminopropanoic acid, homoglycine) is found in nature, many other β-amino acids are not. Indeed, the development of β-peptides owes much to the pioneering work of *Seebach* and co-workers [221][222].

Some generic structures of β-amino acids are shown in *Fig. 6.40*. Since, in β-amino acids, two C-atoms separate the amino and carboxylate groups, there are two possible locations for attachment of a single side chain (*i.e.*, β^2 and β^3), or even two or more side chains (*e.g.*, $\beta^{2,3}$ and $\beta^{2,2,3}$, respectively). In a β-peptide, these symbols can be used as prefixes, *e.g.*, the $\beta^3\beta^2$-dipeptide in *Fig. 6.40* becomes β^3-HAla- β^2-HVal for R = Me and R′ = i-Pr. The stereodescriptors (*R*) and (*S*) should be used to specify the absolute configuration at the stereogenic centers. The same rules apply to γ-amino acids and γ-peptides.

The field of β-peptides remains markedly less investigated than that of α-peptides, yet much has been discovered regarding their synthesis and *con-*

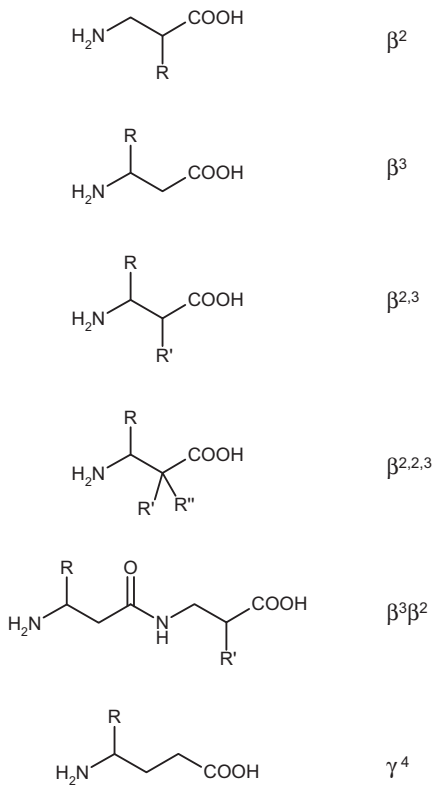


Fig. 6.40. *Examples of generic structures of β-amino acids, β-peptides, and γ-amino acids with the superscript(s) indicating the location of the side chain(s)*

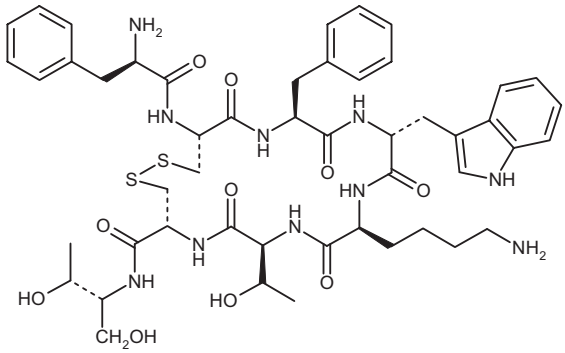
formational properties [221][223 – 225]. Whereas the formation of stable secondary structures in α-oligopeptides is not yet fully understood, it appears that β-oligopeptides have a more predictable capacity to fold into well-defined secondary structures such as turns, helices, and pleated sheets. This is true not only for cyclic β-peptides, but also for acyclic ones, even in the absence of cyclic residues.

In a pharmacological and biochemical perspective, β-peptides are of great interest due to their proven capacity to yield bioactive agents and to be highly resistant to peptidases. The capacity to be recognized by hormone receptors is nicely demonstrated by a study of *somatostatin analogues* [226]. The enzymatic degradation of this hormone has been discussed in *Sect. 6.4.2* (see *Fig. 6.37* and *Table 6.6*). Somatostatin, an inhibitor of growth hormone release, also inhibits the release of glucagon, insulin, gastrin, and secretin [227]. An important analogue is the cyclic α-octapeptide octreotide (**6.95**, also known as *Sandostatin*), which is used clinically to treat acromegaly and some intestinal tumors. A pharmacokinetic drawback of octreotide, however, is its rather short elimination half-life in patients (*ca.* 90 min), despite two D-residues and a modified C-terminus (**6.95**).

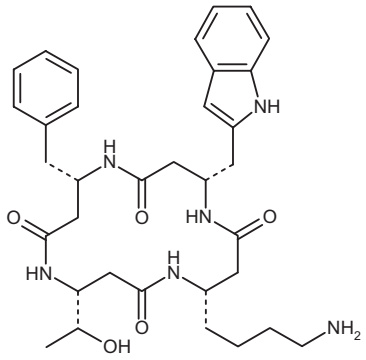
β-Peptide analogues were investigated in an attempt to improve on the *biological properties* of octreotide, a primary condition obviously being pharmacological activity [226]. A cyclic (all-*S*)-β-tetrapeptide (**6.96**) was designed with help from molecular modeling and shown to be superimposable on octreotide. Of significance here is that this β-peptide analogue had affinity for five human somatostatin receptors, although the actual affinities were approximately three orders of magnitude lower than those of octreotide. In other words, human hormone receptors can recognize β-peptides, but affinities similar to those of α-peptides may not be easy to realize.

Of greatest interest in our context is that β-peptides appear remarkably *resistant toward peptidases*. Thus, a number of β-peptides were incubated with proteinase K (a serine endopeptidase, EC 3.4.21.64) and 'pronase', a commercially available mixture of exo- and endopeptidases obtained from *Streptomyces griseus* [222]. The substrates were β-heptapeptides and included an (all-*S*)-(all-β^3)-peptide (**6.97**), an (all-β^3)-peptide containing both (*R*)- and (*S*)-centers (**6.98**), and an (all-*S*)-β-peptide containing both β^2- and β^3-residues (**6.99**). All tested β-peptides were stable for at least 23 h against the peptidases, in contrast to control α-peptides which were digested within 15 min. Compound **6.98** administered *i.v.* to rats was eliminated with a plasma half-life (β-phase) of *ca.* 3 h, whereas the half-life of α-peptide drugs is known to lie in the range of minutes.

As part of an extensive investigation, a large variety of β-peptides, plus some γ-peptides, were tested against proteinase K and 'pronase' [228]. The substrates included shorter and longer β^3-peptides, β^2-peptides, and a few γ-



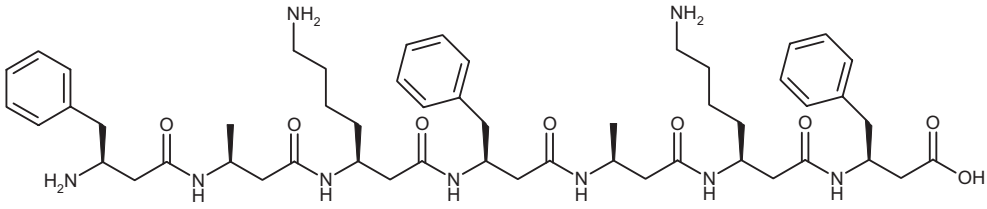
D-Phe—Cys—Phe—D-Trp—Lys—Thr—Cys—Thr-ol **6.95**



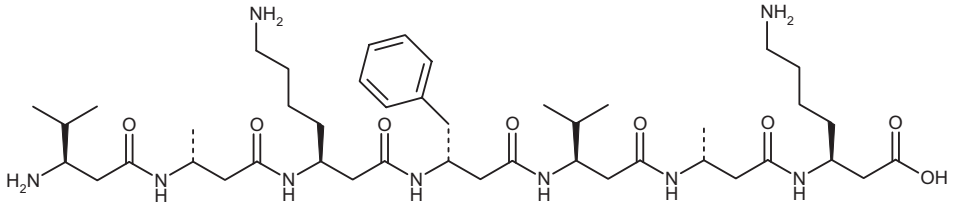
β^3-HPhe—β^3-HTrp

β^3-HThr—β^3-HLys **6.96**

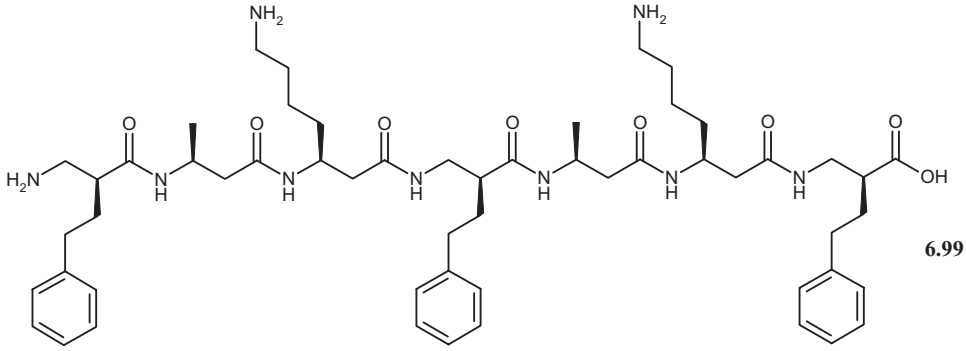
peptides substituted in various positions. No degradation was observed for any of the tested compounds, whereas control α-peptides were entirely degraded within a maximum period of 1 h. A few compounds (including the β^3-peptides **6.100**, **6.101**, and **6.102**, the β^2-peptide **6.103**, and the γ-peptide **6.104**) were also tested against a variety of proteolytic enzymes such as pepsin, chymotrypsin, trypsin, elastase, carboxypeptidase A, leucyl aminopeptidase, porcine intestinal mucosa peptidase, penicillin amidase, and β-lactamase. None of the β-peptides or γ-peptides was digested after 48 h of incubation, whereas a control α-peptide was entirely degraded within 1 h.



6.97



6.98

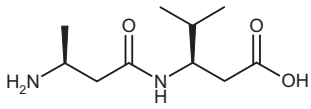


6.99

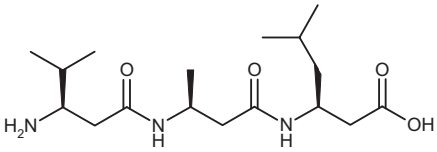
Such results are certainly very interesting, but the stability of β- and γ-peptides toward a greater variety of amidases (EC 3.5) and even esterases (EC 3.1) should also be examined in detail.

6.6. Peptoids, Pseudopeptides, and Peptidomimetics

In the previous section, we have examined peptides composed of nonproteinogenic amino acids, namely artificial, D-, β-, and γ-amino acids. In this section, we move even further afield from natural peptides. Given inconsistent usage in the literature, we begin by specifying the definitions used here [7][11][139][181][182][184 – 188][229].



6.100



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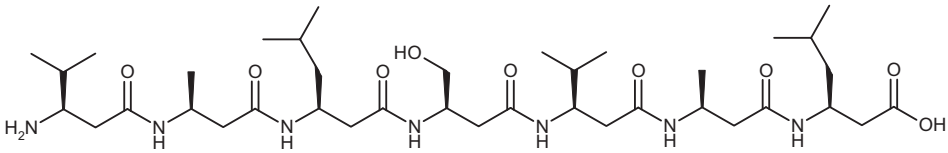
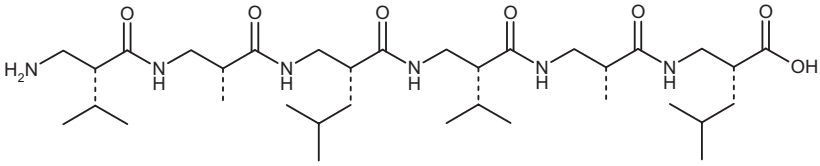


6.102



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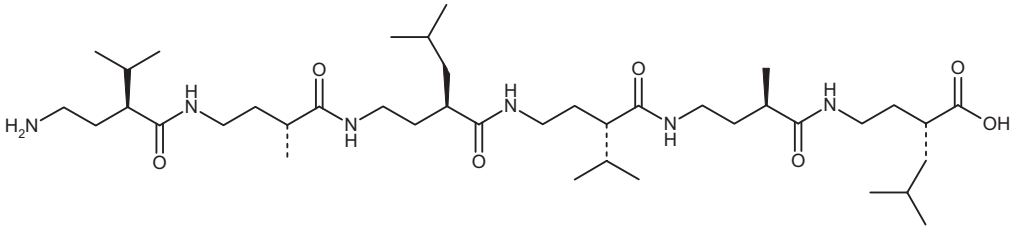


6.104

Peptoids are peptides in which the side chains are transposed from the backbone C^{α}-atom to the amide N-atom.

Pseudopeptides are not always clearly defined in the literature. Here, we use the term to mean compounds with a modified peptide backbone, namely with some or all peptide bonds replaced by bioisosteric surrogates.

Whereas *peptidomimetics* are sometimes taken to include all types of peptide analogues discussed in this *Sect. 6.6*, we restrict the term to two types of compounds. The first type includes moieties that mimic some elements of secondary structure, whereas the second type of peptidomimetics are mimics of a peptide's pharmacophore.

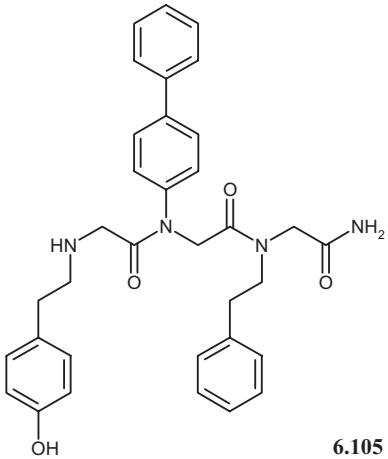
Much has been published on the design, synthesis, and activities of peptoids, pseudopeptides, and peptidomimetics. In contrast, published reports on their biological stability, metabolism, and pharmacokinetics are scarce, making it currently impossible to draw robust conclusions or even to delineate sound trends. This is an unfortunate situation given the vast amount of data that remains buried in industrial archives.

6.6.1. Peptoids

As defined above, peptoids are peptides in which the side chains are transposed from the backbone C^{α}-atom to the amide N-atom. In other words, peptoids are peptides composed of N-*monosubstituted glycine units*, *i.e.*, *N*-substituted polyglycine chains. These structural changes have a number of consequences for properties of peptoids relative to peptides [185][229]:

- Loss of chirality in the backbone;
- Loss of the H-bond-donor capacity of the backbone;
- The side chains are no longer in equivalent positions;
- Increased occurrence of *cis*-peptide bonds and differences in conformational behavior;
- Facilitated synthesis.

The resistance of peptoids toward peptidases has been verified in some cases, although the reported results tend to be preliminary. For example, the peptoid trimer *CHIR2279* (**6.105**), a high-affinity ligand of α_1-adrenoceptors, showed resistance to peptidases and remarkable *in vivo* activity in rats and guinea pigs [230][231]. These data afford indirect indication of good metabolic stability.



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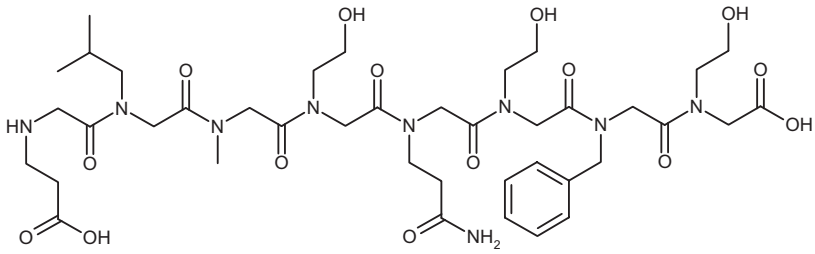

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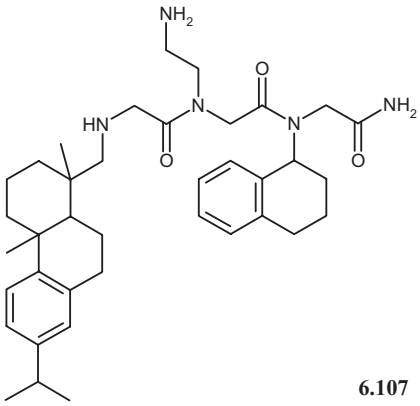
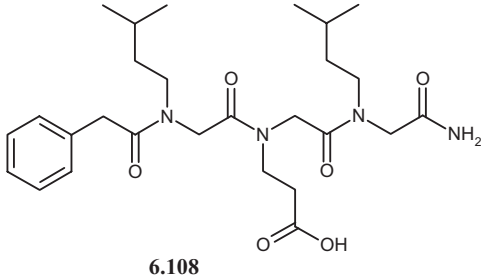


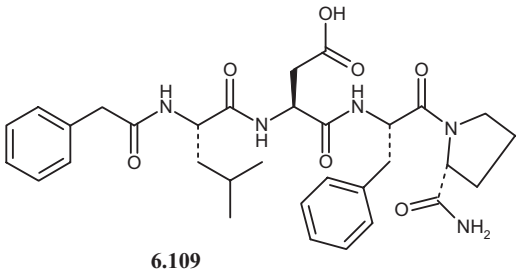
6.107

Resistance to peptidases was also reported for the octapeptoid **6.106** when incubated with papain, chymotrypsin, or thermolysin [229]. However, resistance to peptidases may not be synonymous with a long half-life *in vivo*, since many factors beside peptidases can be expected to contribute to the elimination of peptoids. An indirect indication of this effect can be found for antimicrobial peptoids and particularly compound *CHIR29498* (**6.107**) [232]. In mice infected with *Staphyllococus aureus*, this peptoid was less active when injected 2 h post-infection compared to 0 h or 0.5 h. The conclusion drawn by the authors was that the compound requires optimization for improved absorption or stability within the body.

A careful comparative pharmacokinetic study of the *tripeptoid* **6.108** and the tetrapeptide N-*phenylacetyl-Leu-Asp-Phe*-D-*Pro-amide* (**6.109**) in rats has provided insights on absorption and disposition [233]. The two compounds have comparable backbone structures but differ in the presence or absence of peptide bonds. They also have similar octanol/water partition coefficients, although the H-bonding capacity of the tetrapeptide is greater. In an *in vitro* model, the two compounds had comparable, and low, absorption clearances (6.7×10^{-4} *vs.* 4.8×10^{-4} ml min^{-1} cm^{-1} for the peptoid and the



6.108



6.109

peptide, respectively). The *in vivo* oral absorption results of the peptoid (3–8%) were in line with its low absorption clearance. In contrast, the peptide had an absorption of >75% suggestive of an active transport. A further limitation of the peptoid was its extensive biliary excretion, in fact its major route of elimination following *i.v.* administration.

A major and relevant difference between the two compounds was their *in vivo* biotransformation. Whereas the peptoid was metabolically stable (>90% excreted unchanged after *i.v.* administration), the peptide was extensively metabolized (*ca.* 2% excreted unchanged after *i.v.* administration). In conclusion, metabolic stability was achieved with the tripeptoid **6.108**, but its pharmacokinetic behavior was clearly unsatisfactory due to poor oral absorption and fast biliary excretion.

6.6.2. Pseudopeptides

As stated above, we define pseudopeptides as compounds having a modified peptide backbone, namely with at least one peptide bond replaced by a *bioisosteric surrogate* (summarized in *Table 6.7*) [139][181][234]. Such surrogate groups are nonhydrolyzable by nature, or hydrolyzable only under severe conditions in the case of the SO_2–NH bond. In the vast majority of published pseudopeptides, only one or a very few peptide bonds had been replaced and most monomeric units are amino acids, meaning that such pseudopeptides do qualify as peptides.

Table 6.7. *Examples of R–CONH–R′ Peptide-Bond Surrogates Used in Pseudopeptides*

R–CONH–R′ Surrogates	
$R–SO_2NH–R'$	$R–CH_2NH–R'$
$R–COCH_2–R'$	$R–CH(OH)CH_2–R'$
R–CH=CH–R′	$R–CH_2O–R'$
$R–CH_2S–R'$	$R–CH_2SO–R'$
$R–COCH_2NH–R'$	$R–CH_2SO_2NH–R'$

The peptide nomenclature has been extended to include such analogues. The CO–NH bridge that has been replaced can be indicated by placing ψ in square brackets before the name. The ψ is placed between superscripts indicating the residues modified, and is followed by a comma and the replacing group, *e.g.*, [$^3\psi^4$,CH_2–S]iupaciubin.

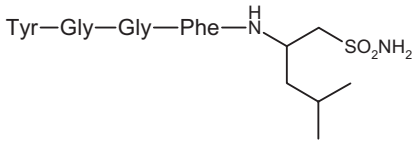
A review of the literature shows that replacement of a peptide bond can have a long-distance stabilizing effect on the enzymatic degradation of pseudopeptides [139]. Thus, analogues of *[Leu^5]enkephalin* containing a surrogate *CH_2S group* were evaluated with respect to resistance to hydrolysis in human serum [235]. The analogues with the modification at the 1-2, 2-3, 3-4, or 4-5 position had their $t_{1/2}$ values increased a factor of 10, 7, 11, and 25, respectively, compared to a $t_{1/2}$ value of 12 min for the parent peptide. This is comparable to the tenfold stabilization shown by [D-Ala^2,Leu^5]enkephalin. The significant increase in stability of the compound protected at the C-terminus might indicate that the sensitivity of serum aminopeptidases to backbone modifications is greater than previously recognized.

Other examples include *dermorphin*, which is cleaved at the 4,5-bond in rat brain homogenates. Whereas the metabolic stability of its [$^4\psi^5$,CH_2–S] analogue was increased 14-fold, that of the [$^3\psi^4$,CH_2–S] analogue was increased 7-fold.

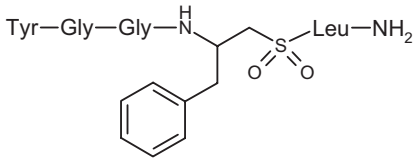
Eight analogues *of dynorphin A-(1 – 11)-NH_2* (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-NH_2, see *Sect. 6.4.1*) were prepared by replacing any of several peptide bonds with a CH_2–S surrogate [236]. Dynorphin A-(1 – 11) is hydrolyzed enzymatically at the 1-2, 5-6, 6-7, and 8-9 positions, and it exhibits a $t_{1/2}$ value of *ca.* 40 min in mouse brain homogenates. Replacing the 1,2-, 3,4-, 4,5-, 6,7-, or 9,10-peptide bond by a CH_2–S surrogate had only a modest effect on stability, whereas the 7,8- and 8,9-analogues had $t_{1/2}$ values of *ca.* 200 min, and the 5,6-analogue one of > 500 min.

An example of a pseudopeptide containing the *CH_2–NH group* is afforded by [$^8\psi^9$,CH_2–NH]bradykinin. This analogue was stabilized not only against carboxypeptidase, which cleaves bradykinin at the 8,9-position, but also against ACE, which cleaves it at the 7,8-position (see *Table 6.6*).

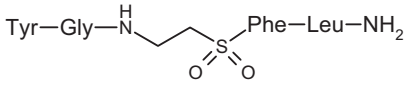
Comparable observations have been reported for pseudopeptide analogues of *[Leu^5]enkephalin amide* having a peptide bridge replaced by a *sulfonamido (CH_2–SO_2NH) group* [237]. [Leu^5]enkephalin amide itself was rapidly degraded by pepsin ($t_{1/2}$ *ca.* 0.5 h), which cleaves it preferentially at the Phe^4-Leu^5 position. In contrast, the analogue containing a CH_2–SO_2–NH_2 group in place of the $CONH_2$ terminus (**6.110**) was insensitive to pepsin, as were the two analogues in which the CONH bridge at the 4-5 and 3-4 positions was replaced with CH_2–SO_2–NH (**6.111** and **6.112**, respectively). When this replacement was made at the 2-3 position (**6.113**), however, stabilization was only modest ($t_{1/2}$ *ca.* 1.5 h).



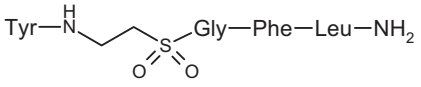
6.110



6.111



6.112



6.113

The above results reveal some trends. Replacing a CO–NH bridge with a surrogate protects the peptide at adjacent position as well as at the modified one. Effects further removed from the modified position might also be seen. However, the number of target sites and the variety of enzymes in the biological medium are all factors that may play a role, making it difficult to design peptidase-resistant pseudopeptides.

These difficulties are well illustrated by the metabolic fate of SK&F *107461* (benzyloxycarbonyl-Ala-Ala-Pheψ[$CHOHCH_2$]Gly-Val-Val-OMe, **6.114**, *Fig. 6.41*) in rats [238]. This pseudopeptide, which contains a $CHOHCH_2$ surrogate replacing the 3,4-peptide bridge, was developed as an

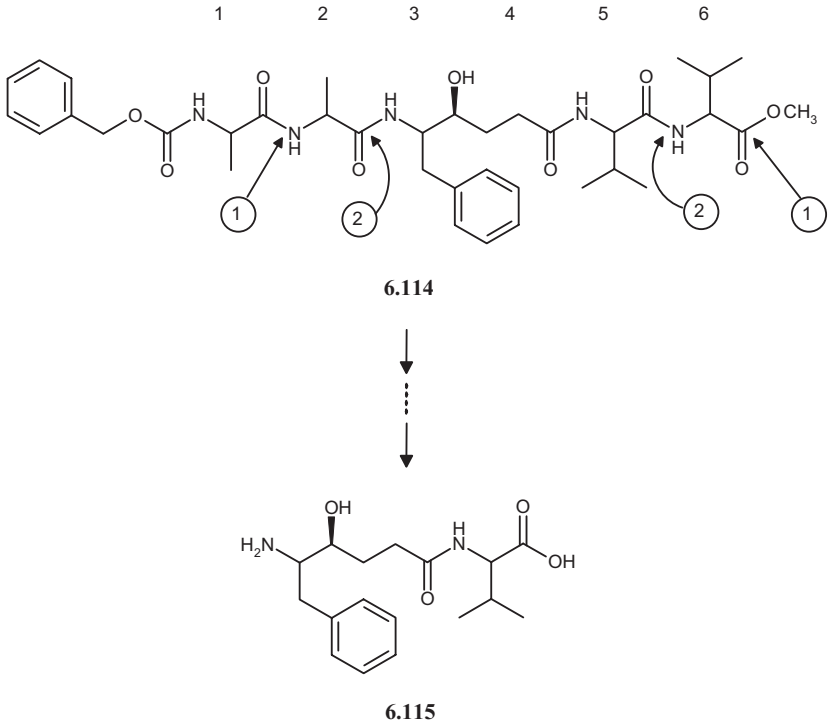


Fig. 6.41. *Metabolic fate of the pseudopeptide SK&F 107461* (Cbz-Ala-Ala-Pheψ[CHOHCH$_2$]-Gly-Val-Val-OMe, **6.114**) *in rats* [238]. The six residues are numbered as shown above the structure. The arrows marked *1* and *2* indicate the primary and subsequent sites of hydrolysis, respectively. Compound **6.115** was the smallest and most polar metabolite detected.

inhibitor of HIV1 protease. When injected in rats, it was rapidly and extensively metabolized. Two primary pathways were observed (*Fig. 6.41*, arrows labeled with 1), namely hydrolysis of the C-terminal ester group and cleavage of the Val^1–Val^2 peptide bond. The resulting metabolites underwent exopeptidase-catalyzed cleavage, leaving product **6.115** (Pheψ[CHOHCH$_2$]Gly-Val) as the smallest and most polar metabolite seen. Clearly, the centrally located surrogate group and an adjacent peptide bridge were resistant to hydrolysis, but both ends of *SK&F* 107461 were easy targets for hydrolases, most notably esterases and exopeptidases.

6.6.3. Peptidomimetics

Whereas *peptidomimetics* are sometimes taken to include all types of peptide analogues discussed in *Sect. 6.6*, we restrict the term to compounds,

which mimic peptides in part or totally. *Partial mimetics* contain moieties that mimic some elements of the secondary structure, *e.g.*, templates of α-helix segments, β-turns, or β-sheets, whereas the remainder of the molecule may be of a peptidic nature. In contrast, *full mimetics* are able to mimic the complete pharmacophore of a given peptide, but are not classifiable as peptides. In other words, they are recognized by the target receptor, enzyme, or other component by virtue of their stereoelectronic similarity to the peptide model.

Peptidomimetics containing templates of β-turns, α-helix segments, or other elements of the secondary structure are receiving considerable attention given their versatility in molecular modeling and chemical synthesis [14][139][183][184][186][187][239][240]. Two examples of templates modeled on β-turns are illustrated in *Fig. 6.42,b*. The mimicry of α-helix segments is often achieved by cyclization strategies that create conformational constraints, as exemplified by a tetrahydroisoquinolinyl and a cyclopropyl motif in *Fig. 6.42,c*. Such templates and constraints reduce the conformational space of peptidomimetics relative to peptides, the expected outcomes being *a*) insight into the active conformation, *b*) stabilization of the active conformation to increase affinity/activity, and/or *c*) reduced sensitivity toward peptidases. And indeed, a number of studies and reviews document the increased metabolic stability resulting from the presence of secondary structure templates.

It is interesting to note that the tetrahydroisoquinolinyl motif (see *Fig. 6.42,c*) has already been encountered in Hoe 140 (**6.94**) during discussion of artificial residues (*Sect. 6.5.2*). Similarly, the cyclopropane-derived peptidomimetic motif shown in *Fig. 6.42,c* [241] could also be classified as a pseudopeptide fragment. These examples show that some overlap in the definitions used here is unavoidable.

As discussed above, the 'full' peptidomimetics are compounds that cannot be classified as peptides, although some may contain peptide bonds. A number of bioactive compounds of natural origin are peptidomimetics, as exemplified by morphine. In recent years, the design of peptidomimetics has opened an apparently unlimited domain of molecular diversity that medicinal chemists are busy exploring and characterizing [182][187][188].

Inhibitors of angiotensin-converting enzyme are a clinically important class of peptidomimetic drugs [242]. The first drug in the series, *captopril* (**6.116**), undergoes oxidation of the thiol function as the primary pathway of metabolism, leading to captopril disulfide as well as to mixed disulfides of cysteine, glutathione, and cysteine residues in plasma proteins. The ethyl ester prodrug of enalaprilat, enalapril (**6.117**), in contrast to parent drug, is rapidly absorbed and efficiently hydrolyzed to the active agent by liver esterases (see also *Sect. 8.2.1*). No further metabolism was seen, indicating that en-

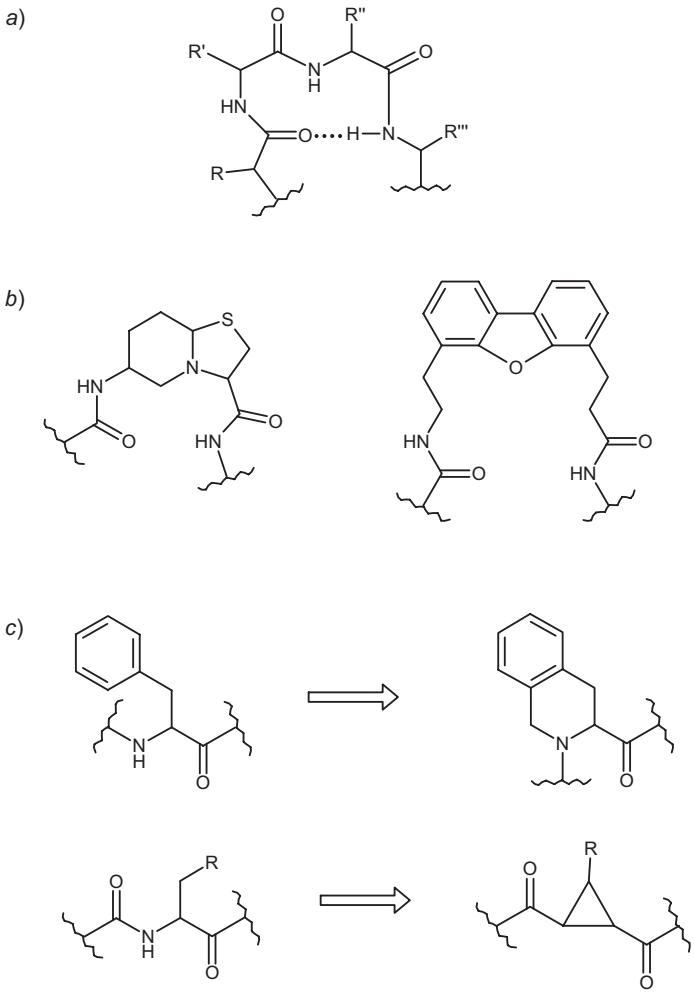
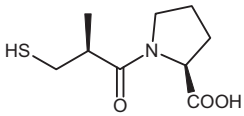


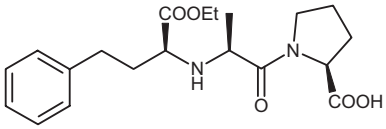
Fig. 6.42. a) *Schematic representation of a β-turn.* b) *Two templates used as β-turn mimics* [186][187]. c) *Two examples of cyclization to create conformational constraints* [186][241].

alaprilat is metabolically stable. Another drug, lisinopril (**6.118**), is modestly but sufficiently absorbed in humans (25%) and is slowly eliminated ($t_{1/2}$ of rapid phase is 12 h). These and other data confirm that ACE inhibitors appear stable to peptidases and amidases.

Our final peptidomimetic example is compound *FR190997* (**6.119**), a mimic of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, see also *Fig. 6.34*) that has high affinity and selectivity for the human B_2 receptor. Remarkably and in contrast to many analogues, FR190997 is a bradykinin



6.116



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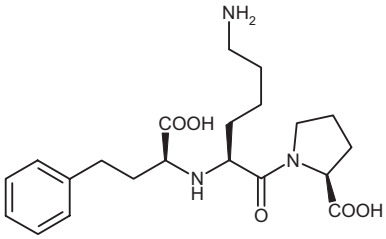
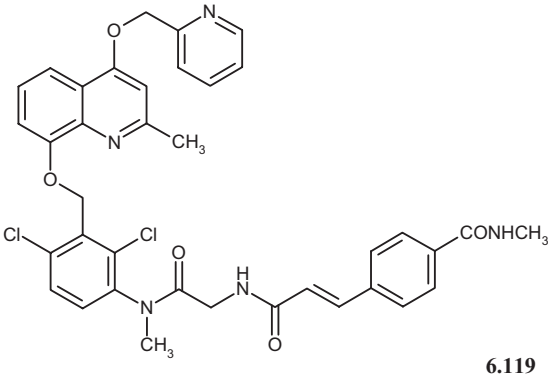


6.118



6.119

agonist [243]. Its *in vivo* behavior in rats proved particularly noteworthy when compared to bradykinin. Indeed, bradykinin injected *i.v.* produced a marked but brief (<2 h) decrease in arterial pressure, followed by longer-lasting (>15 h) hypertension, probably due to a compensatory adrenergic response. In contrast, FR190997 elicited a marked and long-lasting (*ca.* 10 h) hypotensive response with no rebound effect. This prolonged duration of action was taken as an indication that FR190997 is not susceptible to hydrolysis at any of its three amide bonds. However, the resistance to metabolism must extend beyond hydrolysis, since the compound contains a number of target sites for monooxygenases.

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Chapter 7

The Hydrolysis of Carboxylic Acid Esters

Contents

7.1. Introduction

That esters share with amides the property of being major substrates for hydrolases has been verified for endogenous as well as exogenous amides and esters. Because of the wide *chemical variety* of esters, the discussion of their hydrolysis requires extensive treatment under a large number of headings and subheadings. This chapter covers only the carboxylic acid esters, which may be model compounds, xenobiotics, or drugs, while prodrugs of

Hydrolysis in Drug and Prodrug Metabolism. Bernard Testa, Joachim M. Mayer

the carboxylic acid ester type will be treated separately in *Chapt. 8*. Esters of inorganic acids (be they model compounds, active xenobiotics, drugs, or prodrugs) will be discussed in *Chapt. 9*.

The chemical diversity of carboxylic acid esters (R–CO–O–R′) originates in both moieties, *i.e.*, the *acyl group* (R–CO–) and the *alkoxy or aryloxy group* (–OR′). Thus, the acyl group can be made up of aliphatic or aromatic carboxylic acids, carbamic acids, or carbonic acids, and the –OR′ moiety may be derived from an alcohol, an enol, or a phenol. When a thiol is involved, a thioester R–CO–S–R′ is formed. The model substrates to be discussed in *Sect. 7.3* will, thus, be classified according to the chemical nature of the –OR′ (or –SR′) moiety, *i.e.*, the alcohol, phenol, or thiol that is the first product to be released during the hydrolase-catalyzed reaction (see *Chapt. 3*). Diesters represent substrates of special interest and will be presented separately.

When discussing ester (and amide) biohydrolysis, an important question must always be the *nature of the enzyme(s)* catalyzing the reaction [1 – 3] (see *Chapt. 2* and *3*). Like *Chapt. 4 – 6*, this and the following chapter will provide relevant enzymatic information. In addition, most esters, but only few amides, are reactive enough to undergo *non-enzymatic (chemical) hydrolysis* to some extent under physiological conditions [4][5]. The following section offers a brief review of the catalytic mechanisms that underlie the chemical hydrolysis of esters under physiologically relevant conditions.

The relative contribution of the enzymatic and nonenzymatic reactions is frequently examined but seldom reported explicitly in *in vitro* studies, and is all but impossible to assess in *in vivo* investigations. Another factor complicating the interpretation of *in vivo* results is the contribution of gastrointestinal microflora, whose hydrolytic capacity is particularly marked and varied [6]. In contrast, *oxidative ester cleavage*, as discussed elsewhere (Chapt. 7 in [7]), seems at present to be restricted to a limited number of substrates (*e.g.*, some 1,4-dihydropyridine calcium antagonists) and should not be a complicating factor in the present chapters.

Considering the great chemical variety of substrates and the many enzymes involved, it is not an easy task to establish *structure–metabolism relationships* for the biohydrolysis of amides and esters. This can be done with relative success for closely related substrates investigated under well-defined conditions, but generalizing from an ensemble of such results remains a perilous exercise. The approach we have adopted for the carboxylic acid esters is, thus, the same as in *Chapt. 4 – 6*, namely the presentation of illustrative examples, which can be either representative series of compounds or single substrates of particular interest. Generating broadly predictive structure–metabolism relationship should be attempted only with great caution and with due consideration of the many classes of carboxylic acid esters (*e.g.*, model compounds, active esters, and prodrugs).

7.2. Chemical Mechanisms of Ester Hydrolysis

A number of catalytic mechanisms can operate in the chemical hydrolysis of esters [8 – 11], namely:

- Acid catalysis (specific or general) occurring in acidic solutions;
- Base catalysis (specific or general) occurring in neutral and basic solutions;
- Intramolecular catalysis;
- Spontaneous (uncatalyzed) hydrolysis.

The term 'acid catalysis' is often taken to mean 'proton catalysis' ('specific acid catalysis') in contrast to general acid catalysis. In this sense, *acid-catalyzed hydrolysis* begins with protonation of the carbonyl O-atom, which renders the carbonyl C-atom more susceptible to nucleophilic attack. The reaction continues as depicted in *Fig. 7.1,a* (*Pathway a*) with hydration of the carbonium ion to form a tetrahedral intermediate. This is followed by *acyl cleavage* (heterolytic cleavage of the acyl–O bond). *Pathway b* presents an S_N1 mechanism that can be observed in the presence of concentrated inorganic acids, but which appears irrelevant to hydrolysis under physiological conditions. The same is true for another mechanism of alkyl cleavage not shown in *Fig. 7.1,a*. All mechanisms of proton-catalyzed ester hydrolysis are reversible.

General acid catalysis is schematized in *Fig. 7.1,b*. Here, an acid A–H increases the polarity of the carbonyl group and, hence, the electrophilicity of the carbonyl C-atom. For entropy reasons, the reaction is greatly facilitated when it is an *intramolecular* one (*Fig. 7.1,b2*), in other words, when the general acid catalyst is favorably positioned within the molecule itself. Such a mechanism is the one exploited and refined by nature during the evolution of the hydrolases, with the general acid catalyst and the H_2O molecule replaced by adequate amino acid side chains, and the enzymatic transition state being *de facto* a supermolecule (see *Chapt. 3*).

The term 'base catalysis' is often taken to mean 'HO^- catalysis' ('specific base catalysis') in contrast to general base catalysis. In this sense, *base-catalyzed hydrolysis* begins with nucleophilic attack to form a tetrahedral intermediate (*Fig. 7.2,a*). The latter undergoes *acyl cleavage* (heterolytic cleavage of the acyl–O bond) with liberation of the alcoholate (or phenolate) anion. The HO^- anion is a very poor leaving group, thus, the reaction is driven very strongly to the right. The final proton transfer is an irreversible step. For these two reasons, base-catalyzed hydrolyses are generally not drawn with reversible arrows. Not shown in *Fig. 7.2,a* are an S_N1 mechanism with alkyl cleavage observed with some tertiary alkyl esters, and an S_N2 mechanism with alkyl cleavage sometimes observed with primary alkyl esters, particularly methyl esters.

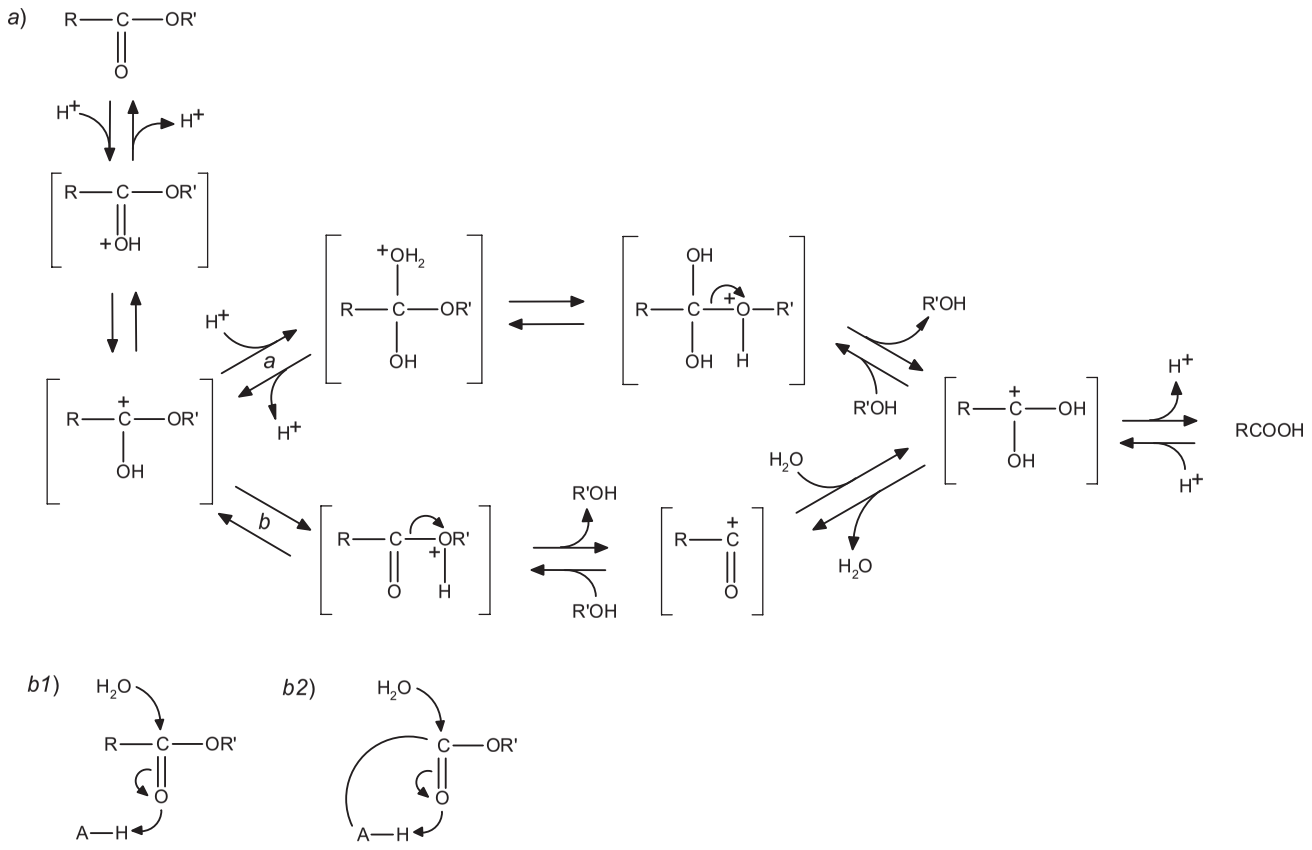


Fig. 7.1. a) *Specific acid catalysis* (proton catalysis) *with acyl cleavage in ester hydrolysis. Pathway a* is the common mechanism involving a tetrahedral intermediate. *Pathway b* is S_N1 mechanism observed in the presence of concentrated inorganic acids. Not shown here is a mechanism of alkyl cleavage, which can also be observed in the presence of concentrated inorganic acids. b) *Schematic mechanism of general acid catalysis in ester hydrolysis.* Intermolecular catalysis (*b1*) and intramolecular catalysis (*b2*).

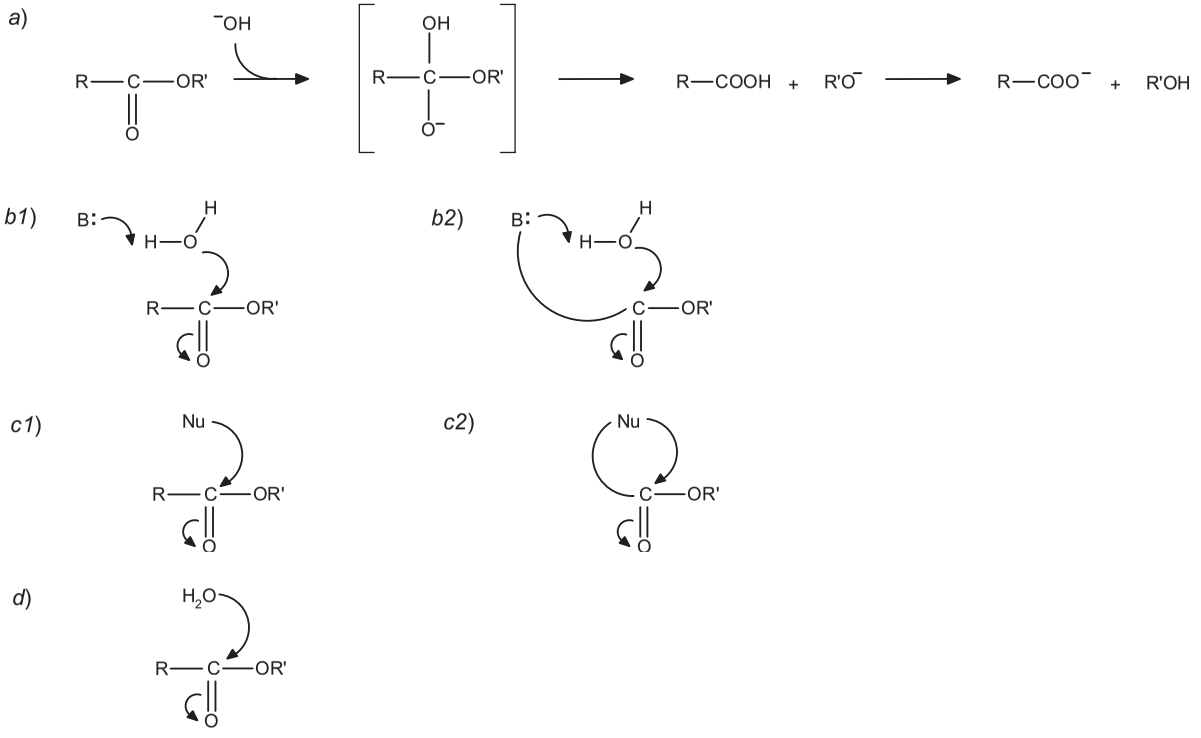


Fig. 7.2. a) *The most common mechanism of base-catalyzed ester hydrolysis, namely specific base catalysis* (HO^- catalysis) *with tetrahedral intermediate and acyl cleavage*. Not shown here are an S_N1 mechanism with alkyl cleavage observed with some tertiary alkyl esters, and an S_N2 mechanism with alkyl cleavage sometimes observed with primary alkyl esters, particularly methyl esters. b) *Schematic mechanism of general base catalysis in ester hydrolysis.* Intermolecular catalysis (*b1*) and intramolecular catalysis (*b2*). c) *The base-catalyzed hydrolysis of esters is but a particular case of nucleophilic attack.* Intermolecular (*c1*) and intramolecular (*c2*). d) *Spontaneous* (uncatalyzed) *hydrolysis*. This becomes possible when the R moiety is electrophilic enough to allow the nucleophilic attack by H_2O.

A scheme depicting g*eneral base catalysis* is shown in *Fig. 7.2,b*. Because the HO^- anion is more nucleophilic than any base-activated H_2O molecule, intermolecular general base catalysis (*Fig. 7.2,b1*) is all but impossible in water, except for highly reactive esters (see below). In contrast, entropy may greatly facilitate *intramolecular general base catalysis* (*Fig. 7.2,b2*) under conditions of very low HO^- anion concentrations. Alkaline ester hydrolysis is a particular case of intermolecular nucleophilic attack (*Fig. 7.2,c1*). Intramolecular nucleophilic attacks (*Fig. 7.2,c2*) are reactions of cyclization–elimination to be discussed in *Chapt. 8*.

Finally, there are cases of *uncatalyzed reactions of hydrolysis*. These can occur with esters whose acyl moiety contains an electron-withdrawing substituent able to render the carbonyl C-atom electrophilic enough toward a H_2O molecule (*Fig. 7.2,d*).

In ester hydrolysis, *plots of log* k_{obs} *(log of observed rate constant)* vs. *pH* show a common overall shape characterized by [5a]:

- A minimum in the pH region of *ca.* 5 – 6, corresponding to the region where the acid and base catalyses are minimal;
- At increasing pH values above *ca.* 6, a continuous and rapid increase of log k_{obs} due to base catalysis becoming more and more effective;
- At pH values decreasing in the acidic range, a progressive increase of log k_{obs} due to acid catalysis becomes increasingly efficient.

All external conditions being identical, the *level and width of the minimum* in log k_{obs} plots is highly substrate-dependent. Thus, a mechanism of spontaneous hydrolysis will increase the level and enlarge the width of this minimum. A comparable effect can be expected with intramolecular general base catalysis in the pH region of low acidity.

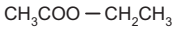
7.3. Model Compounds and Xenobiotics

In this section, we present a number of nonmedicinal esters that have been selected for discussion based on criteria of structural variety and interest of results. This will allow some chemical and biochemical concepts that will be of significance when presenting pharmacologically active esters and prodrugs to be illustrated.

7.3.1. Esters of Alcohols

Beginning the discussion with a very simple compound, we find *ethyl acetate* (**7.1**) to be a substrate that undergoes rapid hydrolysis both *in vitro* and *in vivo*. Thus, rat blood was shown to hydrolyze ethyl acetate to ethanol

and acetic acid with a $t_{1/2}$ value of reaction of *ca.* 1 h under physiological conditions of pH and temperature [12]. The reaction was much faster *in vivo* following injection to rats, with an estimated $t_{1/2}$ value of *ca.* 5 – 10 min. Such experiments demonstrate the involvement of blood and tissue esterases.



7.1

The *in vivo* metabolism of a homologous series of *alkyl carbamates* (**7.2**, *Fig. 7.3*) has yielded some informative results [13]. The hydrolysis of these esters liberates carbamic acid (**7.3**, *Fig. 7.3*), which breaks down spontaneously to CO_2 and NH_3, allowing the extent of hydrolysis to be determined conveniently and specifically by monitoring CO_2 production. When such substrates were administered to rats, there was an inverse relationship between side-chain hydroxylation and ester-bond hydrolysis. Thus, for compounds **7.2** the contribution of hydrolysis to total metabolism (90 – 95% of dose) decreased in the series R = Et (*ca.* 85 – 90%), Bu (*ca.* 60 – 65%), hexyl (*ca.* 45 – 50%), and octyl (*ca.* 30%). *Ethyl carbamate* (urethane) is of particular toxicological interest, being a well-established carcinogen in experimental animals. *In vitro* studies of adduct formation have confirmed the competition between oxidative toxification mediated by CYP2E1 and hydrolytic detoxification mediated by carboxylesterases [14].

7.2 **7.3**

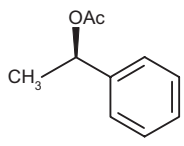
Fig. 7.3. *Hydrolysis of carbamate esters* (**7.2**) *to carbamic acid* (**7.3**), *and spontaneous breakdown of the latter*

This example documents the difficulty of rationalizing the results of *in vivo* investigations when competitive metabolic reactions are seen. In such cases, simpler *in vitro* systems may be more informative, as exemplified by the hydrolysis of *alkyl phenyl carbonates* (phenyl–O–CO–O–alkyl) catalyzed by pig pancreatic elastase (EC 3.4.21.36) [15]. The rate of hydrolysis was monitored by following CO_2 production as with the carbamates discussed above. Indeed, the enzyme-catalyzed hydrolysis yields the phenyl hemiester of carbonic acid (phenyl–O–COOH), which decomposes rapidly to produce CO_2 and phenol. With these carbonates, the rate of hydrolysis decreased in the series Bu > i-Bu > Et >> hexyl, the *t*-Bu and cyclohexyl derivatives being

unreactive. Note that in contrast to carbamic acids (RNH–COOH) and carbonate hemiesters (RO–COOH), carbamates (RNH–CO–OR′) and carbonates (RO–CO–OR′) are relatively stable in the neutral and slightly acidic pH range. A number of *other structure–metabolism relationship* studies are discussed in the following pages.

One of the most actively investigated aspects of the biohydrolysis of carboxylic acid esters is *enantioselectivity* (for a definition of the various stereochemical terms used here, see [7], particularly its Sect. 1.5) for two reasons, one practical (preparation of pure enantiomers for various applications) and one fundamental (investigations on the structure and function of hydrolases). The synthetic and preparative aspects of enantioselective biocatalysis by hydrolases have been extensively investigated for biotechnology applications but are of only secondary interest in our context (*e.g.*, [16 – 18], see *Sect. 7.3.5*). In contrast, the fundamental aspects of enantioselectivity in particular and of structure–metabolism relationships in general are central to our approach and are illustrated here with a number of selected examples.

Product enantioselectivity may be seen with diesters and will be described in *Sect. 7.3.5*. Here, we encounter *substrate enantioselectivity* as characteristic of the hydrolysis of esters displaying an element of chirality either in the alkyl or in the acyl moiety. Thus, a large number of *acetates of chiral secondary alcohols*, *e.g.*, **7.4** and **7.5**, were hydrolyzed by bovine pancreatic cholesterol esterase and a fungal lipase each displaying marked to high enantioselectivity [19]. These and other results could be summarized in the form of a simple topographical model showing the absolute configuration of the enantiomers being hydrolyzed faster by the hydrolases investigated (*Fig. 7.4*).



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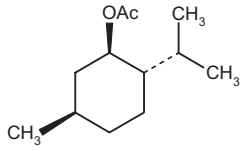

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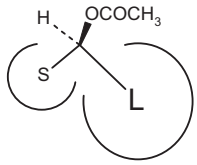
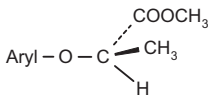


Fig. 7.4. *A simple topographical model showing the absolute configuration of the acetates reacting faster with cholesterol esterase and lipase* (modified from [19]). S = smaller group; L = larger group.

A large number of examples document the substrate enantioselective hydrolysis of *esters having an asymmetric acyl moiety*. Thus, a variety of methyl 2-(substituted phenoxy)propanoates were well-hydrolyzed by pig liver carboxylesterase (pig liver esterase, PLE), α-chymotrypsin (EC 3.4.21.1), porcine pancreatic lipase (EC 3.1.1.3), and a fungal lipase, the (*R*)-esters (**7.6**, aryl = phenyl, 2-chlorophenyl, 3-chlorophenyl, 4-chlorophenyl, 2,4-dichlorophenyl, *etc.*) being, in all cases, the preferred substrates [20]. Comparable results were obtained with 2-chloroethyl 2-(substituted phenyl)propanoates, whose hydrolysis by a fungal lipase was selective for the (*S*)-esters (**7.7**, aryl = phenyl, 4-nitrophenyl, 4-isobutylphenyl (*i.e.*, the 2-chloroethyl ester of ibuprofen)) [21]. Note that **7.6** and **7.7** have the same absolute 3D geometry, despite having the opposite descriptors (*R*) and (*S*) due to the inversion of the priority of substituents according to the sequence rule.



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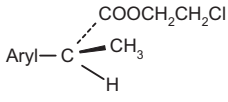

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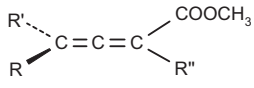


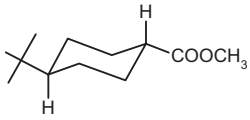
7.8

Another promising development is the enantioselective hydrolysis of various racemic xenobiotic esters in healthy and cancerous rat liver cell lines [22]. This has led to the design of anticancer prodrugs selectively activated by cancerous cell lines.

Interestingly, asymmetry originating in an axis rather than in a stereogenic center can also result in enantioselective hydrolysis. Thus, pig liver carboxylesterase has been shown to discriminate between the enantiomers of racemic *allenic esters* [23]. The preferred substrates (**7.8**) had the (*S*)-configuration when R and R′ were relatively small or acyclic, but the (*R*)-configuration when one substituent was cyclic. For example, a very high enantioselectivity was seen for R = Ph and R′ and R″ = Me or Et.

In the presence of *conformationally rigid* methyl cyclohexanecarboxylates (**7.9** – **7.12**), PLE displayed a marked preference for a carboxy group in an equatorial position. In fact, the rate of PLE-catalyzed hydrolysis decreased significantly in the series **7.9** > **7.10** > **7.11** > **7.12** [24]. Another element of complexity exists in this series of compounds since **7.10** and **7.12** are chiral, the structures shown being those of the preferred enantiomeric substrates. With these two chiral esters, however, the substrate enantiospecificity of PLE was found to be very modest.

Several observations of this type that have been published may appear confusing at first but have led to the design of useful topographical models



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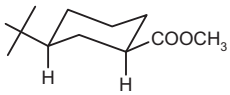

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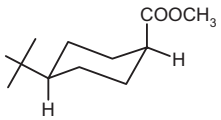

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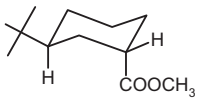


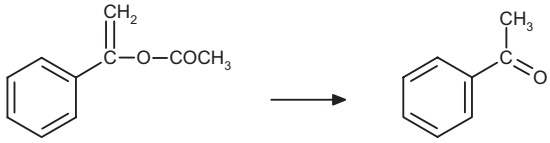
7.12

of the active site of hydrolases. Some of these models will be discussed in *Sect. 7.3.5* since they also take diesters into account.

7.3.2. Esters of Enols

Enol esters are distinct from other esters not because of a particular stability or lability toward hydrolases, but due to their hydrolysis releasing a 'ghost alcohol' (an enol), which may immediately tautomerize to the corresponding aldehyde or ketone. A well-studied example is that of *vinyl acetate* (CH_3–CO–O–CH=CH_2), a xenobiotic of great industrial importance that, upon hydrolysis, liberates acetic acid (CH_3–CO–OH) and acetaldehyde (CH_3–CHO), the stable tautomer of vinyl alcohol [25]. The results of two studies are compiled in *Table 7.1*, and demonstrate that vinyl acetate is a very good substrate of carboxylesterase (EC 3.1.1.1) but not of acetylcholinesterase (EC 3.1.1.7) or cholinesterase (EC 3.1.1.8). The presence of carboxylesterase in rat plasma but not in human plasma explains the difference between these two preparations, although the different experimental conditions in the two studies make further interpretation difficult.

Another example is that of α-acetoxystyrene (**7.13**), which yields acetic acid and acetophenone (**7.14**) upon hydrolysis. This compound is quite stable



7.13 **7.14**

Table 7.1. *Enzymatic Hydrolysis of Vinyl Acetate*[a])

Medium	$t_{1/2}$[b]) [min]	K_m[c]) [mM]	V_{max}[c]) [μmol min^{-1} (mg protein or mg preparation)$^{-1}$]
Buffer	270 – 300	–[d])	–[d])
Carboxylesterase	2.0	0.65	238
Acetylcholinesterase	48	55	1.7
Cholinesterase	61	77	4.6
Rat plasma	1.2	4.0	0.56
Rat red blood cells	5.6		
Rat blood	<1		
Human plasma	62	7.1	0.69
Human red blood cells	5.5		
Human blood	4.1		
Rat liver microsomes		0.73	23
Rat lung microsomes		6.1	6.2

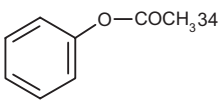
[a]) Compiled from [26][27]; see original publications for further experimental details. [b]) pH 7.4, 37°. [c]) pH 8.0, 37°. [d]) $t_{1/2}$ 180 min at pH 8.0.

in buffers of pH 7.4 ($t_{1/2}$ 57 h at 37°), but is readily hydrolyzed in human and rat plasma ($t_{1/2}$ values of a few minutes and a few seconds, respectively) [28]. Such results suggest that enol esters may be of interest as potential prodrugs of enolizable aldehydes and ketones. Other enol acetates were shown to be enantioselective substrates of lipase in reactions of preparative interest [29].

7.3.3. Esters of Phenols

A few *acetates of phenols* have been used extensively as probes to investigate esterases, *e.g.*, phenyl acetate (**7.15**), 4-nitrophenyl acetate (**7.16**), α-naphthyl acetate (**7.17**) and 7-acetoxy-4-methyl-2*H*-[1]benzopyran-2-one (4-methylumbelliferyl acetate, **7.18**). Such substrates are easy to handle and their phenolic metabolite is readily analyzed, allowing convenient monitoring of the reaction.

For example, the hydrolysis of *phenyl acetate* (**7.15**) by carboxylesterase isozymes was investigated over a broad pH range, allowing many insights into their catalytic mechanisms [30] (see *Chapt. 3*). This substrate was also used together with various inhibitors to characterize esterases in human and rat tissues [31]. Thus, the approximate values of K_m (in μM) and V_{max} (in μmol min^{-1} (g tissue)$^{-1}$) in human tissues were 300 and 60 in liver microsomes, 200 and 40 in liver cytosol, and 1500 and 250 in plasma, respective-



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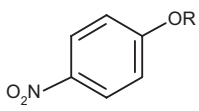
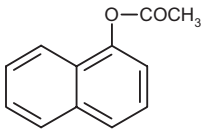


7.16 R = $COCH_3$

7.19 R = $CO(CH_2)_4CH_3$

7.20 R = $COC(CH_3)_3$



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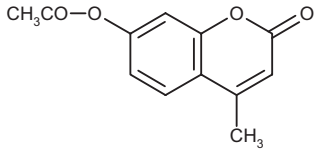


7.18

ly; no activity was detectable in red blood cells. The enzymes involved appear to be both carboxylesterases (EC 3.1.1.1) and arylesterases (EC 3.1.1.2) in the liver, but only the latter in plasma. *4-Nitrophenyl acetate* (**7.16**) has also yielded very useful results, for example in helping to assess the catalytic influence of various nucleophiles. This substrate has also helped to characterize the active site, reaction mechanism and regulations of acetylcholinesterase, carboxylesterases, and other hydrolases [32 – 37]. 4-Nitrophenyl acetate (**7.16**) and its two higher homologues were used together with a few medicinal substrates as probes of carboxylesterase in human liver microsomes, showing 5- to 40-fold ranges in activities [38].

Other 4-nitrophenyl esters have also been reported to be substrates of various hydrolases. For example, 4-nitrophenyl hexanoate (**7.19**) was hydrolyzed by bovine serum albumin [39]. The affinity of the substrate for the macromolecule was found to be high ($K_m/n = 0.040$ mM, where n is the number of sites), but the reaction itself was slow ($k_2 = 5 \cdot 10^{-3}$ s^{-1}, where k_2 is the first-order rate constant of the formation of the phenol product from the enzyme–substrate complex). Another ester, 4-nitrophenyl pivalate (**7.20**), was hydrolyzed by cytoplasmic aldehyde dehydrogenase at a maximum velocity *ca.* 1/3 and an affinity *ca.* 1/20 those of the acetate [40]. However, the rate-limiting steps were different for the two substrates, namely acylation of the enzyme for the pivalate, and acyl–enzyme hydrolysis for the acetate (see *Chapt. 3*).

Series of homologous esters have been investigated to try to establish *structure–metabolism relationships*, however partial and limited the latter may be. This aspect will be discussed again in the context of prodrugs (*Chapt. 8*). Here, we mention a few representative studies in which model substrates were used. *Table 7.2* documents the substrate specificity of a rabbit liver carboxylesterase (ES-1A) toward homologous series of methyl, 4-nitrophenyl, α-naphthyl, β-naphthyl, and 4-methylumbelliferyl esters [41]. In

Table 7.2. *Substrate specificity of rabbit hepatic carboxylesterase ES-1A*

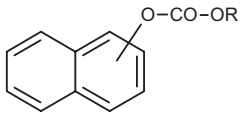
Substrate	Reaction velocity[a])	
	Absolute [nmol min^{-1} (mg protein)$^{-1}$]	Relative[b]) [%]
Methyl formate		2
Methyl acetate		7
Methyl propionate		11
Methyl butyrate		50
Methyl isobutyrate		29
Methyl valerate	250	100
Methyl isovalerate		7
Methyl pivalate		5
Phenyl acetate (**7.15**)	13600	
4-Nitrophenyl acetate (**7.16**)		40
4-Nitrophenyl propionate		65
4-Nitrophenyl butyrate		76
4-Nitrophenyl valerate	8800	100
4-Nitrophenyl hexanoate (**7.19**)		91
4-Nitrophenyl octanoate		55
4-Nitrophenyl decanoate		10
4-Nitrophenyl dodecanoate		4
α-Naphthyl acetate (**7.17**)		88
α-Naphthyl propionate		89
α-Naphthyl butyrate		84
α-Naphthyl valerate		80
α-Naphthyl hexanoate		93
α-Naphthyl octanoate	3700	100
α-Naphthyl decanoate		96
β-Naphthyl acetate		78
β-Naphthyl propionate		81
β-Naphthyl butyrate		32
β-Naphthyl valerate		92
β-Naphthyl hexanoate		100
β-Naphthyl octanoate	32100	100
4-Methylumbelliferyl acetate (**7.18**)		17
4-Methylumbelliferyl propionate		45
4-Methylumbelliferyl butyrate	9400	100
4-Methylumbelliferyl heptanoate		4

[a]) At pH 7.1 and 37° [41]. [b]) Relative to the most reactive member in the homologous series.

some series, the reactivity as a function of acyl chain length increases up to an optimum beyond which it decreases (*e.g.*, methyl, 4-nitrophenyl and 4-methylumbelliferyl esters). In the other series, the reactivity shows little (α-naphthyl esters) or complex (β-naphthyl esters) sensitivity to chain length. In addition, it is informative to compare the various series, whose reactivity decreases by two to three orders of magnitude in the sequence β-naphthyl > Ph > 4-nitrophenyl ≅ α-naphthyl ≅ 4-methylumbelliferyl > Me. Such studies and their conclusions are limited for a variety of reasons. Thus, the

structural variations are monotonous and explore only a limited range of structural properties (*e.g.*, steric/bulk factors). More significantly perhaps, the rates of reaction may not be comparable since the transition-state structure and the rate-limiting step can differ among homologues, as seen, for example, in the hydrolysis of 4-nitrophenyl esters (acetate to dodecanoate) by sterol esterase (EC 3.1.1.13) [42].

In comprehensive studies, the hydrolysis of some 30 naphthyl esters by human, rat, and mouse liver carboxylesterases was investigated [43]. A general trend that was apparent was that the rate of hydrolysis of α- and β-naphthyl carbonates (**7.21**, R = alkyl or arylalkyl) catalyzed by human microsomes or rat hydrolases showed a tendency to decrease with increasing lipophilicity (range *ca.* 2 to 5). A similar trend was not seen with naphthyl aryl carbonates nor with α-naphthyl carboxylates. These results tell us that, even with purified enzymes and large series of substrates, it is very difficult indeed to discern sound structure–hydrolysis relationships due to the complexity of the structural and enzymatic factors involved.

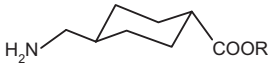


7.21

7.3.4. Basic Esters

Esters containing a basic function (either in the acyl or in the alkyl part of the molecule) form an important group. Such compounds, which can be of interest as prodrugs (see *Chapt. 8*), often show good water solubility and sometimes undergo relatively facile chemical hydrolysis at physiological pH. They are also relatively poor substrates for carboxylesterases, but may be hydrolyzed by cholinesterases. For example, a large series of *trans*-4-(aminomethyl)cyclohexanecarboxylates (**7.22**, R = aryl or arylmethyl) were hydrolyzed by rat, pig, rabbit, and guinea pig liver microsomal carboxylesterases at rates 3 – 4 orders of magnitude lower than those of aryl acetates [44]. Another relevant example is afforded by amino acid 4-nitrophenyl esters of amino acids [45]. These compounds had a lower affinity for, and a lower reactivity toward, human serum albumin than their more lipophilic analogues with a protected amino group.

In the above compounds, the amino and ester groups are not positioned to allow optimal through-space interactions. This steric factor is of major significance in the *chemical hydrolysis of amino esters*, where intramolecular



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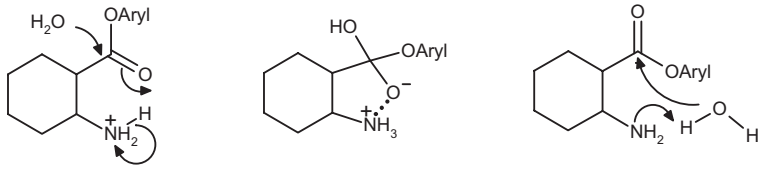


A **B** **C**

7.23

interactions between the amino and ester group may strongly affect the rates of hydrolysis. Thus, *aryl esters of 2-aminocarboxylic acid* (*e.g.*, 4-nitrophenyl 2-aminocyclohexanecarboxylate; **7.23**, Aryl = 4-NO_2–C_6H_4) showed rates of hydrolysis in the neutral pH range that were some orders of magnitude faster than that of the analogue lacking an amino group [46]. More generally, scattered examples in the literature document the peculiar pH–rate profile of carboxylates that have a basic side chain located such that it may affect the apparent rate of hydrolysis:

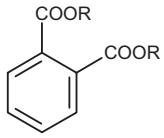
- In the acidic range, the rate constant of hydrolysis is low;
- At pH values in the weakly acidic range and close to neutrality, the rate constant is increased by orders of magnitude and reaches higher than usual values;
- In the alkaline pH range, the rate constant shows the usual high values [46][47].
- This is remarkably different from the behavior of other esters discussed in *Sect. 7.2*.

A number of *mechanisms* may account for this increased stability in acidic media and decreased stability in weakly acidic and neutral media [46]. In strongly acidic media, the protonated amino group might prevent protonation of the carbonyl O-atom and, thus, also proton-catalyzed hydrolysis. In slightly acidic media, where the amino group is still protonated, intramolecular general acid catalysis by the neighboring ammonium group (**7.23A** and *Fig. 7.1,b2*) or electrostatic catalysis by stabilization of the tetrahedral intermediate (**7.23B**) have been postulated to explain the increased nucleophilicity of the carbonyl group and, hence, the decreased stability of the ester. In neutral media, intramolecular general base catalysis by the nonprotonated amino group may accelerate hydrolysis (**7.23C** and *Fig. 7.2,b2*). More information on esters incorporating aminoalkyl groups may be found in *Sect. 7.4.4* and in *Chapt. 8*.

7.3.5. Diesters

Investigations of the hydrolysis of diesters has unveiled a wealth of information on various aspects of the specificity of hydrolases, in particular their substrate and product enantiospecificities, which are discussed in this section. But, first, the *substrate specificity* of these enzymes will be briefly introduced.

Benzenedicarboxylic acid diesters offer an interesting example of *substrate regioselective hydrolysis*, as documented for the dibutyl esters of *phthalic acid* (**7.24**, R = Bu), *isophthalic acid* (**7.25**), and *terephthalic acid* (**7.26**). The former compound is a widely used plasticizer with known testicular toxicity, whereas its *ortho*- and *meta*-isomers appear to be less toxic. The hydrolysis of the three regioisomers was compared in various rat tissue preparations, and showed that the isophthalates and terephthalates were relatively poor substrates, whereas their hemiesters (the monobutyl esters) were rapidly hydrolyzed to the corresponding acid. In contrast, dibutylphthalate was rapidly transformed to the monoester, but the latter was not hydrolyzed to any detectable extent, leading to the accumulation of the monoester [48]. It has been postulated that this regioselectivity might be related to the differences in toxicity. Comparable results were seen when bis(2-ethylhexyl) phthalate (**7.24**, $R = CH_2CH(CH_2CH_3)CH_2CH_2CH_2CH_3$) and bis(2-ethylhexyl) terephthalate (a general purpose plasticizer) were incubated with rat gut homogenate fractions [49]. Here again, the *ortho*-diester was hydrolyzed faster than its *para* isomer, but only to the monoester, while the *para*-diester gave the diacid.



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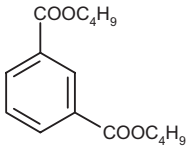

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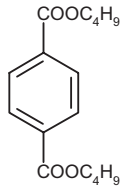
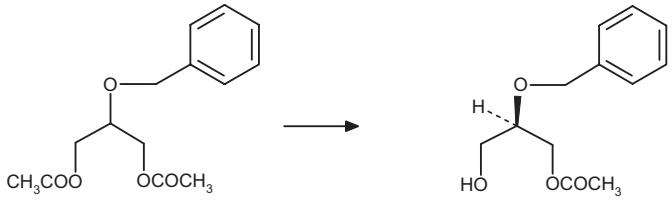


7.26

In an homologous series of phthalate diesters (**7.24**, R = Me, Et, allyl, 2-methoxyethyl, Bu, i-Bu, and $PhCH_2$), hydrolysis by some human and rat liver carboxylesterases was fastest for the diethyl and diallyl esters [50]. This suggested a maximal rate of hydrolysis for phthalate esters with a lipophilicity (log *P* in the octanol/water system) of 3 – 4. Interestingly, and compatible with this interpretation, monoesters were not hydrolyzed. However, that regioisomeric monoesters are hydrolyzed clearly indicates that factors other than lipophilicity (*e.g.*, steric factors) are also influential.

One of the most intriguing and best explored specificities of hydrolases is their *product enantiospecificity*, a property that is not restricted to the biotransformation of xenobiotics since it is displayed by lipases acting on their physiological substrates. Indeed, *prochiral triglycerides* have been found to be hydrolyzed with marked product enantioselectivity by various lipases [51][52]. Such specificity can hardly be fortuitous and must have a physiological significance, which remains to be understood.

The product enantiospecificity of hydrolases towards diesters is of considerable *synthetic value* for the preparation of optically active synthons from prochiral substrates. Such reactions are also of interest in the present context because of the insights they afford into the functioning of hydrolases. Thus, the *glyceryl diacetate* **7.27** derivative was hydrolyzed by pig pancreatic lipase to the monoester **7.28** having the (*R*)-configuration (88% enantiomeric excess (ee)) [53]. Most published data, however, pertain to *prochiral diesters of α,ω-dicarboxylic acids*, as seen for example with functionalized *dimethyl malonates* of general structure **7.29** [54]. These compounds were hydrolyzed by PLE to the monoester with a enantioselectivity that depended on the size of the alkyl group (*i.e.*, the R group in structure **7.29**); when R was bulky (*e.g.*, $PhCH_2$ or *t*-Bu), the monoester **7.30** had the (*R*)-configuration (65 – 95% ee). In contrast, R = H or Me resulted in low ee values for the monoester of (*S*)-configuration.



7.27 **7.28**

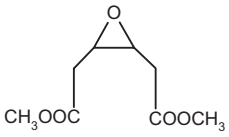
7.29 **7.30**

Esters of higher diacids (*e.g.*, *dimethyl glutarates*) have also proven quite informative. Thus, dimethyl 3-hydroxyglutarate (**7.31**) was hydrolyzed by PLE to the monoester of (*S*)-configuration (**7.32**), but the ee was low (*ca.* 20%) [55]. In contrast, α-chymotrypsin yielded the (*R*)-monoester with ee values of 60 – 70%, indicating large differences in the active sites of these

two enzymes. The transformation of **7.31** can be gainfully compared with that of dimethyl *meso*-3,4-epoxyadipate (**7.33**), another prochiral substrate whose PLE-catalyzed hydrolysis gave the monoester of the configuration shown in **7.34**. As in **7.32**, the stereogenic C-atom closer to the site of hydrolysis had the (*S*)-configuration, but the enantioselectivity of the reaction was much higher (99% *vs.* 20% ee) [55].

7.31

7.32



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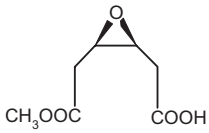

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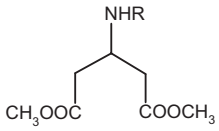
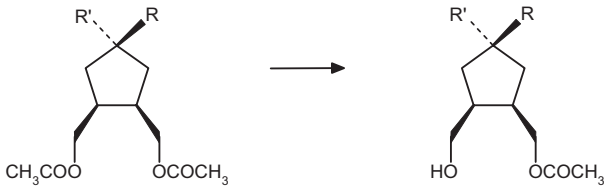


7.35

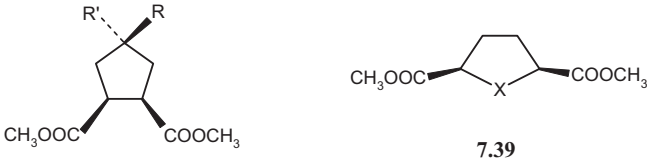
Even more confusing is the comparison with the PLE-catalyzed hydrolysis of dimethyl 3-aminoglutarates (**7.35**). Depending on the nature and size of the substituent R (*e.g.*, Ac, PhCO, and $PhCH_2$), the monoester produced had the (*R*)- or (*S*)-configuration, with ee values ranging from very modest to very high [56]. Clearly, the steric and perhaps also electronic properties of the various groups in such compounds must have a major influence in orienting the substrate in the enzyme active site (in other words, in directing its binding modes) and, hence, in controlling the enantioselectivity of the reaction. Such a rationale has led some authors to propose topographical models of active sites, as illustrated below.

Diesters of alicyclic dicarboxylic acids are also valuable as tools to study the specificity of hydrolases. Such substrates can be achiral (*e.g.*, *cis*-1,2-dicarboxylates) or chiral (*e.g.*, *trans*-1,2-dicarboxylates), allowing product-enantioselective hydrolysis or substrate-enantioselective hydrolysis, respectively, to take place. Considering first cis-*1,2*- and cis-*1,3-diesters* (*e.g.*, **7.36**,

7.38, and **7.39**), we note that these are achiral due to the existence of a plane of symmetry (hence their designation as *meso* stereoisomers), whereas their monoester metabolites are chiral [57]. Many reports confirm that the enzymatic hydrolysis of such substrates occurs with product enantioselectivity. For example, the hydrolysis of *meso*-diacetates of structure **7.36** (R = OH, *etc.*; R′ = H, *etc.*) by pig pancreatic lipase yielded the monoesters shown as **7.37** with good to high ee values [58]. Comparable results were obtained in the PLE-catalyzed hydrolysis of various dimethyl *meso*-cyclopentyl-1,2-dicarboxylates (**7.38**) and dimethyl *meso*-1,3-dicarboxylates of structure **7.39** (X = CH_2, NH–CH_2Ph, O, S) [59 – 61].



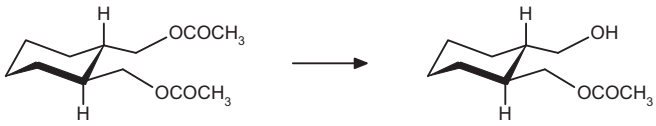
7.36 **7.37**



7.38 **7.39**

Substrate enantioselectivity is also documented, *e.g.*, in the metabolism of chiral trans-*1,2*- and trans-*1,3-diesters*. Thus, *trans*-cyclohexane-1,2-diyl diacetate was hydrolyzed by pig liver esterase with modest but genuine substrate enantioselectivity, the (*S*,*S*)-enantiomer (**7.40**) being preferred [62]. For reasons of symmetry (the two acetoxy groups are regiochemically indistinguishable), each enantiomeric diester can only form one monoester, *e.g.*, **7.41** formed from **7.40**. Interestingly, the monoester was further hydrolyzed to the diol, the reaction again being selective for the (*S*,*S*)-enantiomer. In a number of cases, monoesters are not substrates of hydrolases due to their high polarity. Here, however, we note that this monoester (**7.41**) does not contain a free carboxylate group and may, therefore, be of sufficient lipophilicity to bind to the enzyme.

Similar findings were reported following incubation of PLE with some *trans*-diesters of structure **7.42** and **7.43** (X = CH_2, NH, N–CH_2Ph, S) [63].



7.40 **7.41**

7.42 **7.43**

For compounds **7.42**, the preferred substrate always had the 3D geometry shown here, which corresponded to an (*R*)- or (*S*)-absolute configuration, depending on priorities assigned by the sequence rule, *e.g.*, the (*S*,*S*)-enantiomer for $X = CH_2$. The situation is not so clear-cut with compounds **7.43**, with which contradictory results were obtained.

The many examples published in the literature, a few of which have been considered in the previous paragraphs, may reasonably appear to be a large heap of data generating more confusion than understanding. Fortunately, a number of authors have been able to make sense out of sets of data and to bring partial order to this apparent disorder by unraveling *structure–metabolism relationships* (SMRs). Such rationalizations can take a number of forms such as regression equations (quantitative SMRs, QSMRs), three-dimensional QSMRs (3D-QSMRs), approximately defined (qualitative) or rigorously defined (quantitative) topographical models of enzymatic active sites, or molecular modeling of such sites [7][64 – 66]. Here, we illustrate the topographical approach with three *models of the active site of pig liver esterase*, going from the simplest to the most informative.

To rationalize the stereospecificity of PLE toward a large variety of monocarboxylic and dicarboxylic esters, *Tamm* and co-workers have proposed the general formula displayed in *Fig. 7.5* [55][67]. Here, no representation of the active site is implied, but the model does rationalize numerous data and allows some qualitative predictions. A qualitative topographical model of the active site of PLE has been proposed by *Jones* and co-workers [68][69]. As shown in *Fig. 7.6*, substrate binding is defined by a carboxylate group that interacts with the catalytic serine residue, and by one or two hydrophobic groups that bind to sites 1 and/or 2.

Perhaps the most elaborate and successful topographical model of PLE currently available is that also conceived by *Jones* and co-workers and shown

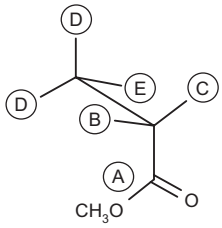


Fig. 7.5. *General formula summarizing the structural and stereochemical requirements, which an ester should meet to fit into the active site of pig liver esterase.* A: nucleophilic attack by the serine OH group seemingly occurs from this side; B: no or only small substituents allowed here; C: groups of small to medium size allowed in this area; D: space allowed in these areas for carbon chains, with polar substituents (*e.g.*, another ester group) preferred in the upper part; E: only small, nonpolar substituents (*e.g.*, Me) allowed here [67].

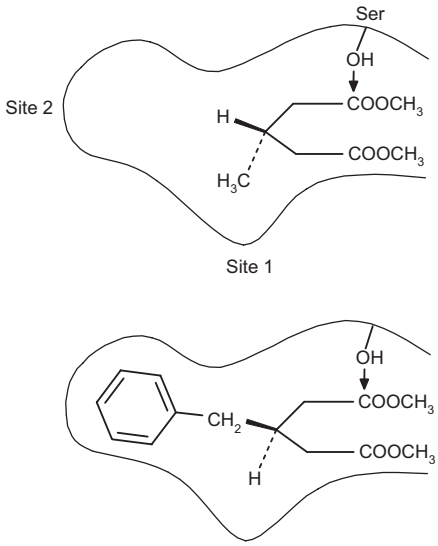


Fig. 7.6. *Topographical model of the active site of pig liver esterase showing the catalytic OH group and two binding sites* (1 and 2) *capable of accommodating hydrophobic groups of the substrate.* Binding to site 1 is stronger and, thus, dominates until the steric dimensions of the site are exceeded. The model shows two substrates in position, namely dimethyl 3-methylglutarate (*top*) and dimethyl 3-benzylglutarate (*bottom*), which are hydrolyzed preferentially to the (*R*)- and (*S*)-monoester, respectively [68].

in *Fig. 7.7* [70][71]. Two hydrophobic pockets (H_L and H_S) and two polar binding sites (P_F and P_B) are postulated (*Fig. 7.7,a*). The hydrophobic pockets can bind aliphatic and aromatic groups, halogens substituents, and even ether functions. More polar moieties (*e.g.*, OH, amino, carbonyl, or NO_2) are excluded from the hydrophobic pockets but will bind to the polar sites. Ac-

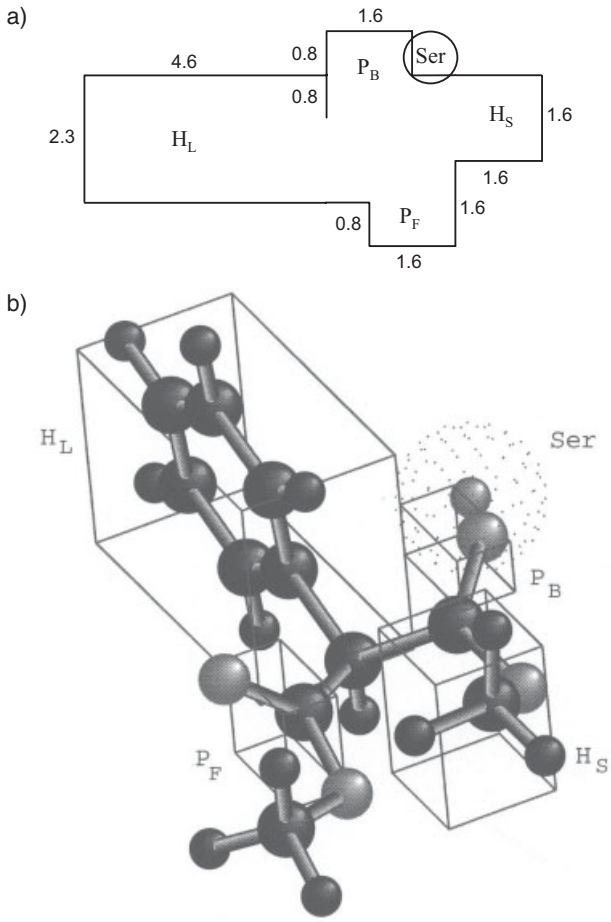


Fig. 7.7. *Topographical model of the active site of pig liver esterase as proposed by* Jones *and co-workers* [70][71]. The model postulates two hydrophobic sites, one large (H_L) and one small (H_S), and two polar binding sites, one in the front (P_F) and one in the back (P_B). The serine sphere shows the approximate zone of action of the catalytic OH group. *a*) A view from the top with the dimensions in Å; the sites H_L, H_S, and P_F are at 'ground level' and have an elevation of 3.1 Å, 2.3 Å, and 1.6 Å, respectively, while P_B is located 1.5 Å above 'ground level' and has an elevation of 0.8 Å. *b*) A computer-generated perspective view with dimethyl phenylmalonate positioned to have its *pro-S* ester group close to the catalytic site [72a].

cording to later results, H_L may be larger than originally postulated [70]. The area above the model is open to any substrate moiety. A perspective view is also shown in *Fig. 7.7,b* with dimethyl phenylmalonate bound with its *pro-S* ester group close to the catalytic site. Interestingly, this substrate is hydrolyzed by PLE without any notable product enantioselectivity, implying that the other binding mode (*pro-R* ester group close to the catalytic site, not shown in

Fig. 7.7) is equally favored [72]. Independent research teams have confirmed the validity of this model (*e.g.*, [73]).

Topographical models such as those discussed above are valuable attempts to deduce an indirect and simplified picture of enzymatic sites from the steric properties of substrates. A direct approach has also become practical, namely the determination by X-ray crystallography of the three-dimensional structure of hydrolase–ligand complexes [74].

7.4. Pharmacologically Active Esters

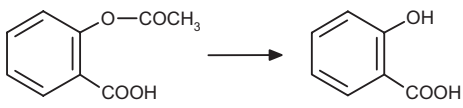
The innumerable drugs that are active *per se* must be carefully distinguished from *prodrugs* (see *Chapt. 8*), which are themselves inactive or only poorly active and become active only after having been transformed into one or more metabolite(s). One of the implications of this essential discrimination is that drugs acting both directly *and via* one or more active metabolite(s) cannot be classified as prodrugs. In fact, a few of the drugs discussed below are examples of such a therapeutic profile.

Pharmacologically active esters represent a relatively modest proportion of all the directly acting drugs. This is due, in large part, to the generally rapid metabolic hydrolysis of ester drugs, which results in their speedy inactivation except when an active metabolite is formed.

The structural variety of biologically active esters (mostly but not exclusively drugs) is sufficiently great to allow their classification into representative groups, as discussed below. Over the years, a number of published reviews have presented biological aspects of ester hydrolysis and discussed some selected examples [1][2][75][76].

7.4.1. Esters of Acetic Acid

A simple compound to begin our presentation is *acetylsalicylic acid* (aspirin, **7.44**), the well-known analgesic and anti-inflammatory drug whose primary metabolite, salicylic acid (**7.45**), is also an anti-inflammatory agent but not an analgesic. Extensive kinetic data have been published on the chemical hydrolysis of acetylsalicylic acid as a function of temperature and



7.44 **7.45**

pH [77]. Thus, the enthalpy and entropy of activation at pH 7.0 were found to be $\Delta H^{\neq} = 71$ kJ mol^{-1} and $\Delta S^{\neq} = -110$ J K^{-1} mol^{-1}, respectively.

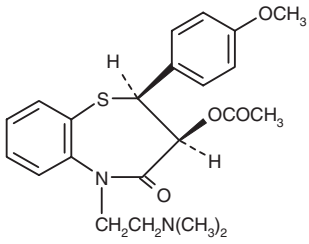
Following oral administration, acetylsalicylic acid undergoes a significant first-pass hydrolysis in the intestinal wall and liver (*ca.* 1/3 of a standard dose in humans), followed by extensive hydrolysis in the blood (serum and red cells) [76a]. As a result, no intact drug is detectable in plasma *ca.* 2 h after administration of a standard dose [78]. Similarly, no unchanged drug is detectable in the urine of patients whatever the administered dose [79]. The further metabolism of salicylic acid is mainly *via* conjugation with glycine and glucuronic acid, and to a small extent by oxidation to 2,3- and 2,5-dihydroxybenzoic acid [79 – 81].

A variety of hydrolases catalyze the hydrolysis of acetylsalicylic acid. In humans, high activities have been seen with membrane-bound and cytosolic carboxylesterases (EC 3.1.1.1), plasma cholinesterase (EC 3.1.1.8), and red blood cell arylesterases (EC 3.1.1.2), whereas nonenzymatic hydrolysis appears to contribute to a small percentage of the total salicylic acid formed [76a][82]. A solution of *serum albumin* also displayed weak hydrolytic activity toward the drug, but, under the conditions of the study, binding to serum albumin decreased chemical hydrolysis at 37° and pH 7.4 from $t_{1/2}$ 12 ± 1 h when unbound to 27 ± 3 h for the fully bound drug [83]. In contrast, binding to serum albumin increased by >50% the rate of carboxylesterase-catalyzed hydrolysis, as seen in buffers containing the hydrolase with or without albumin. It has been postulated that either bound acetylsalicylic acid is more susceptible to enzyme hydrolysis, or the protein directly activates the enzyme.

Many studies have been performed in laboratory animals to better characterize the distribution, nature, regulation, kinetic properties, and substrate specificity of aspirin hydrolases, as they are sometimes designated (*e.g.*, [41][84 – 86]).

Diltiazem (**7.46**) is also an acetic acid ester, but of an alcohol rather than of a phenol like aspirin. The compound is extensively metabolized in humans and animals, producing a wealth of metabolites [87][88]. The major routes of metabolism are oxidative (deamination, *N*-demethylation, *O*-demethylation, hydroxylation), but the formation of most metabolites identified so far also involves hydrolytic deacetylation. Thus, hydrolysis of the acetate moiety is globally a major pathway, particularly in the rat where the deacetylated metabolites accounted for an approximately tenfold higher proportion of the dose than those retaining the acetyl group. In humans also, the proportion of deacetylated metabolites appears to predominate, but the results are fragmentary and harder to interpret. Note that the primary deacetylated metabolite of diltiazem is pharmacologically active, albeit less so than the parent drug.

An example of particular importance is *heroin* (3,6-diacetylmorphine, **7.47**). Following *i.v.* administration to human subjects, most of the dose was



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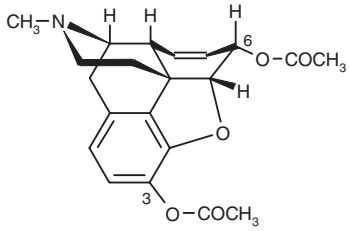


7.47

recovered in the urine as 6-acetylmorphine, morphine, and their glucuronides [89]. Only trace amounts of the parent compound were excreted. Thus, *in vivo* hydrolysis of heroin in humans involves first the 3-Ac group (*i.e.*, the acetylated phenol group), and only then the 6-Ac group (*i.e.*, the acetylated alcohol group). Studies in human plasma and aqueous buffer confirmed the preferential hydrolysis of the 3-Ac moiety [90]. In pH 7.4 buffer at 37°, hydrolysis of the 3-ester group occurs with a $t_{1/2}$ value of *ca.* 33 h, whereas the hydrolysis of 6-acetylmorphine to morphine is slower by one order of magnitude. In the neutral and weakly basic pH range, *N*-methylheroin (a model compound of protonated heroin) was hydrolyzed twelve times faster at the 3- than at the 6-ester group [91].

In human blood, only 6-acetylmorphine was formed from heroin, with no 3-acetylmorphine or morphine being detected. Four kinetically distinct enzyme activities were seen, namely one in plasma, one in the cytosol of red blood cells, and two in red blood cell membranes [92]. In human plasma at 37°, hydrolysis to 6-acetylmorphine occurs with a $t_{1/2}$ value of some minutes, the enzyme responsible being cholinesterase [90][93]. These and other results tend to indicate that the formation of morphine from 6-acetylmorphine is due to tissue carboxylesterases, in particular cerebral enzymes [94].

The metabolism of heroin is of interest in connection with its pharmacological activities. Earlier opiate μ-receptor binding studies led to the belief that heroin is a prodrug acting through its metabolites 6-acetylmorphine and morphine [95]. However, heroin is now known to activate δ-receptors, whereas morphine activates μ-receptor and 6-acetylmorphine acts at both receptor types [96]. Thus, the pharmacodynamic profile of heroin results from both direct and metabolite-mediated effects.

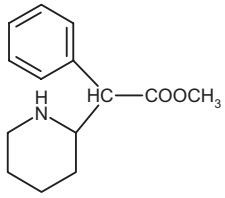
The *rifamycins* are a group of antibiotics of large size (M_r close to 1,000), which, among other chemical similarities, have an acetyl group at position 25. Deacetylation by carboxylesterases is a major metabolic pathway in animals and humans, as seen for example with rifampin, rifabutin, and rifalazil [97][98].

7.4.2. Esters That Incorporate Simple Alkyl Substituents

A number of drugs are alkyl esters. As such, they undergo metabolic hydrolysis to variable extents, as illustrated here with a few examples. *Methylphenidate* (**7.48**), a representative methyl ester, is a psychoactive drug used as the racemic *threo*-diastereoisomer. Its major metabolic route is by deesterification, but the reaction is relatively slow in human blood at 37° (*ca.* 50% hydrolysis after 8 h) and only moderately enantioselective; both plasma and red blood cells are active [99]. Rat blood showed the highest hydrolytic activity among all animal species investigated. The *transesterification* of methylphenidate to ethylphenidate is another carboxylesterase-mediated pathway affecting this drug according to a mechanism discussed in *Sect. 7.4.4* [100].

An ethyl ester easily hydrolyzed by human liver carboxylesterase is the benzodiazepine antagonist *flumazenil* [101]. Here, we focus on another ethyl ester, namely the synthetic opioid *pethidine* (meperidine, **7.49**), whose metabolism shows analogies and differences with that of methylphenidate. Both drugs are inactivated by hydrolysis, but pethidine is not hydrolyzed in the blood of humans and other animal species. The reaction occurs mainly in the liver, the human enzyme being a microsomal carboxylesterase designated hCE-1 [75][102][103]. A rather intriguing analogy with methylphenidate is the transesterification undergone by pethidine in the presence of carboxylesterase and ethanol [100a]. But because pethidine is an ethyl ester, its product of *transesterification* is identical with the substrate and its formation could only be demonstrated in the presence of [$^2H_6$]ethanol.

Higher alkyl esters are also cleaved, as shown here with the propyl ester propanidid and the butyl ester *fluazifop-butyl*. *Propanidid* (**7.50**) is a short-acting anesthetic agent whose duration of action is controlled by its rate of



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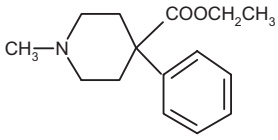

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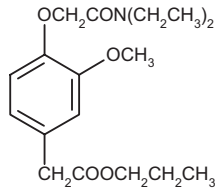

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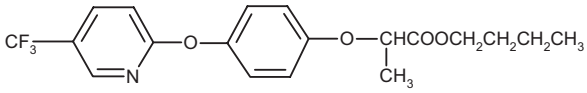


7.51

hydrolysis. In rats, the reaction occurs mainly in hepatic cytosol, and is slow in hepatic cytosol and plasma [37]. As for the herbicide fluazifop-butyl (**7.51**), it was found to be hydrolyzed rapidly by human liver carboxylesterases located in microsomes and cytosol [29a]. Plasma esterase activity was *ca.* 1/100 that of liver microsomes and cytosol, with little of the activity found in the red blood cells.

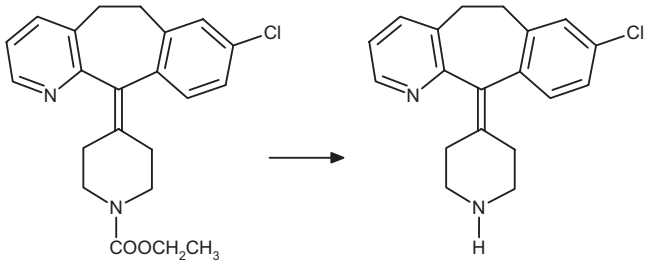
The above examples are purely phenomenological and do not allow any generalization. Their brief presentation in this section simply aims to suggest the structural diversity of biologically active alkyl esters and some metabolic differences in their hydrolytic inactivation.

7.4.3. Carbamates

Although a good number of prodrugs or prodrug candidates are alkyl carbamates (*Chapt. 8*), few drugs are esters of carbamic acid. An example worthy of attention is that of *felbamate* (**7.52**). This anticonvulsant agent is oxidized mainly in rat liver microsomes, with small amounts of the monocarbamate metabolite also being formed. In humans, more than half of a dose of felbamate is excreted unchanged, while a major urinary metabolite is the acid **7.53**, resulting from hydrolysis of the ester followed by dehydrogenation of the intermediate alcohol [104][105]. These results confirm that hydrolysis of a carbamate group in felbamate to form the monoester is a major pathway in humans.

7.52 **7.53**

Another important example is that of *loratadine* (**7.54**), a potent antihistaminic drug exhibiting few sedative side-effects. The compound is metabolized to descarboxyethoxyloratadine (**7.55**), which is also a potent H_1-receptor antagonist whose production probably accounts for the long duration of action of loratadine [106][107]. Although hydrolysis, followed by spontaneous decarboxylation, is a likely mechanism leading to descarboxyethoxyloratadine, there is evidence that, in human liver microsomes, its formation is catalyzed by cytochrome P450, primarily the CYP3A4 and CYP2D6 isozymes [108]. Given the therapeutic significance of the formation of descarboxyethoxyloratadine, it would be of value to have a better understanding of the possible involvement of hydrolases in other human tissues.



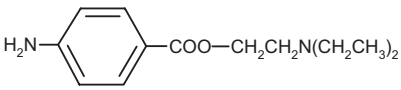
7.54 **7.55**

Another recently reported case is that of the antiarrhythmic agent *moricizine*, an ethyl carbamate derivative of a primary aromatic amine (R–NH–CO–O–CH_2CH_3) [109]. Hydrolysis to the carbamic acid (R–NH–COOH), followed by decarboxylation to the aromatic amine, was an important pathway in humans. However, in contrast to loratadine, the carbamic acid metabolite appeared to be sufficiently stable to be detectable in fair amounts in human urine. It can be postulated that the aromatic nature of the amine accounts for the relative stability of its carbamic acid derivative.

7.4.4. Esters That Incorporate Aminoalkyl Groups

Several drugs, in particular neuropharmacological agents, feature a carboxylate group esterified to an aminoalkyl moiety. As a rule, such lipophilic compounds are good substrates for hydrolases, and their duration of action is influenced by their rate of hydrolysis (see also *Sect. 7.3.4*). A simple example is that of *procaine* (**7.56**), which is rapidly inactivated by hydrolysis [41][76a]. Various hydrolases catalyze the reaction, in particular plasma cholinesterase and cellular carboxylesterases. As often reported, *atropine* and *scopolamine* are rapidly hydrolyzed by plasma carboxylesterases in rabbits (with very large differences between individual animals), but are stable in human plasma [1][75][76a][110].

(–)-Cocaine (**7.57**, *Fig. 7.8*) deserves much attention following unexpected insights into its metabolism and molecular toxicology [111][112]. This xenobiotic is also of interest due to the presence of two ester groups. Studies in humans and laboratory animals have shown cocaine to be metabolized



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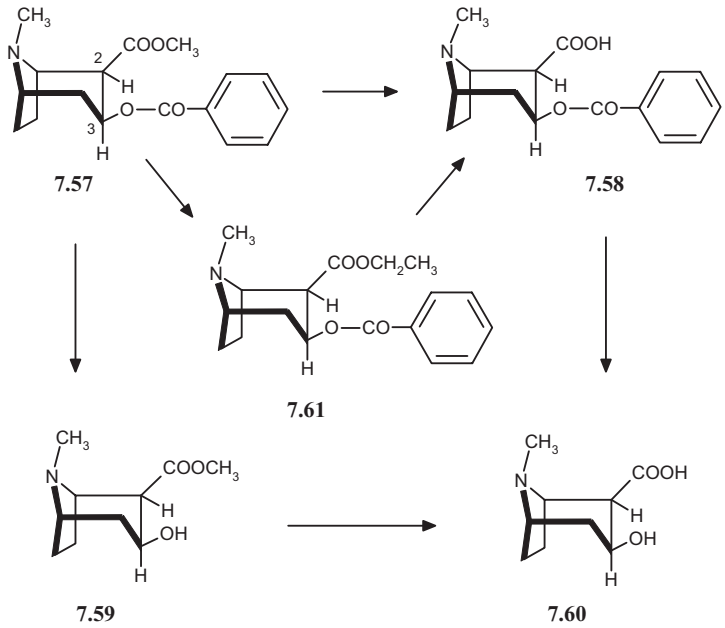


Fig. 7.8. *The hydrolytic metabolism of cocaine* (**7.57**) *to form benzoylecgonine* (**7.58**)*, ecgonine methyl ester* (**7.59**)*, and ecgonine* (**7.60**). In the presence of ethanol, benzoylecgonine ethylester (**7.61**, cocaethylene) is also formed enzymatically as discussed in the text.

by various routes, including *N*-demethylation (to form norcocaine), *N*-oxygenation, aromatic hydroxylation, and hydrolysis. The latter route is particularly important, as it represents a global route of detoxification, and accounts for as much as 90% of the dose in humans. Humans excrete high urinary concentrations of benzoylecgonine (**7.58**, *Fig. 7.8*), ecgonine methyl ester (**7.59**, *Fig. 7.8*), and ecgonine (**7.60**, *Fig. 7.8*) [113]. In mice, the areas-under-the-curve (AUC) for hepatic concentrations were approximately equal for cocaine, ecgonine methyl ester, and ecgonine, whereas the AUC of benzoylecgonine was five- to ninefold larger [114].

Studies on human serum and liver showed enzymatic hydrolysis of the benzoyloxy bridge, whereas indirect evidence suggested the nonenzymatic hydrolysis of the methyl ester group [115]. Indeed, cocaine undergoes slow, nonenzymatic hydrolysis at the methyl ester group (30 – 40% at pH 7.4 and 37°) [116]. However, the metabolic hydrolysis of the methyl ester group also has a significant enzymatic component since sodium fluoride, a well-known inhibitor of esterases, markedly decreased the formation of benzoylecgonine in hepatic preparations from mice and humans [114]. Furthermore, marked tissue differences in the formation of benzoylecgonine from cocaine were seen in the rat, with more than one enzyme activity being involved [117]. Three human en-

zymes are now known to be involved in the hydrolysis of cocaine. One is a liver carboxylesterase (designated hCE-1), which catalyzes the hydrolysis of the methyl ester group. As for the benzoyloxy group, it is hydrolyzed by the liver carboxylesterase hCE-2 and serum cholinesterase [118–120]. Both hCE-1 and hCE-2 also play an important role in the hydrolysis of heroin (**7.47**) to 6-monoacetylmorphine and then to morphine [103] (see *Sect. 7.4.1*).

Natural (–)-cocaine (**7.57**, *Fig. 7.8*), which has the (2*R*,3*S*)-configuration, is a relatively poor substrate for hepatic carboxylesterases and plasma cholinesterase (EC 3.1.1.8), and also a potent competitive inhibitor of the latter enzyme [116][121]. In contrast, the unnatural enantiomer, (+)-(2*S*,3*R*)-cocaine, is a good substrate for carboxylesterases and cholinesterase. Because hydrolysis is a route of detoxification for cocaine and its stereoisomers, such metabolic differences have a major import on their monooxygenase-catalyzed toxification, a reaction of particular effectiveness for (–)-cocaine.

There is an additional factor contributing to the toxicity of cocaine, namely its interaction with ethanol [122][123]. Many cocaine (ab)users simultaneously ingest ethanol, probably to experience potentiation of effects and attenuation of headaches. It is now known that ethanol interferes in two ways with the metabolism of cocaine, first by inhibiting its hydrolysis and second by allowing transesterification to form benzoylecgonine ethyl ester (**7.61**, *Fig. 7.8*) commonly known as *cocaethylene*. These metabolic effects are illustrated by studies in the rat (*Table 7.3*), with ethanol inhibiting the formation of

Table 7.3. *Effect of Acute Ethanol Administration on Tissue Concentrations of Cocaine and Its Major Metabolites in the Rat*[a])

Compound	Treatment[b])	Liver [nmol g^{-1}]	Brain [nmol g^{-1}]	Serum [nmol ml^{-1}]
Cocaine (**7.57**)	Control	1.9 ± 0.5	7.5 ± 0.7	5.7 ± 0.81
	+ Ethanol	3.6 ± 0.9[c])	7 ± 1	5.0 ± 0.7
Norcocaine	Control	1.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
	+ Ethanol	1.7 ± 0.6	0.8 ± 0.3	0.7 ± 0.1
Benzoylecgonine (**7.58**)	Control	6 ± 1	0.4 ± 0.1	14 ± 1
	+ Ethanol	1.7 ± 0.7[c])	0.4 ± 0.1	4 ± 1[c])
Benzoylnorecgonine	Control	3 ± 1	n.d.[d])	n.d.[d])
	+ Ethanol	5 ± 1[c])	n.d.[d])	9 ± 3
Cocaethylene (**7.61**)	Control	n.d.[d])	n.d.[d])	n.d.[d])
	+ Ethanol	0.8 ± 0.3	0.6 ± 0.1	0.5 ± 0.1
Norcocaethylene	Control	n.d.[d])	n.d.[d])	n.d.[d])
	+ Ethanol	0.6 ± 0.3	0.4 ± 0.1	0.4 ± 0.1

[a]) Data from [122]. [b]) Ethanol (2 g kg^{-1}) or saline was administered *i.p.* 30 min prior to *i.p.* administration of cocaine (25 mg kg^{-1}); sacrifice was 15 min later. [c]) Statistically significant difference from control ($P < 0.002$). [d]) Not detectable (<0.08 nmol g^{-1}).

benzoylecgonine and allowing the production of cocaethylene and norcocaethylene [122]. These results are significant since cocaethylene retains the pharmacological and toxicological properties of cocaine, in particular its CNS and hepatic effects.

The *transesterification* of cocaine to cocaethylene is an enzymatic reaction catalyzed by microsomal carboxylesterases and blocked by inhibitors of serine hydrolases [124][125]. In *Chapt. 3*, we have discussed the mechanism of serine hydrolases, showing how a H_2O molecule enters the catalytic cycle to hydrolyze the acylated serine residue in the active site of the enzyme. In the case of cocaine, the acyl group is the benzoylecgoninyl moiety (*Fig. 7.9,d′*), which undergoes esterification with ethanol according to *Steps e′* and *f′* (*Fig. 7.9*).

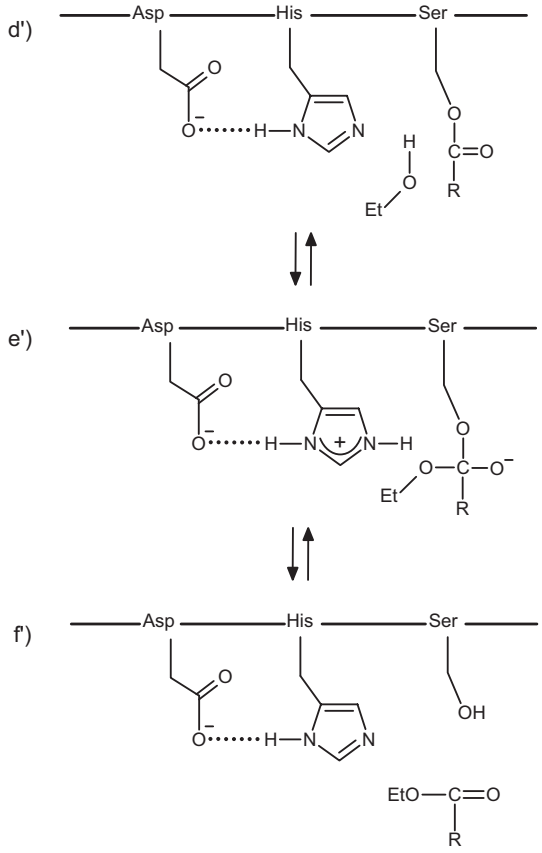


Fig. 7.9. *Mechanism of carboxylesterase-catalyzed transesterification of cocaine to cocaethylene, schematized in analogy to* Fig. 3.3,d – f (*Chapt. 3*). Ethanol enters the catalytic cycle at Step *d′* and reacts with the benzoylecgoninyl moiety (*Steps e′* and *f′*) to form benzoylecgonine ethyl ester [125].

Other basic ester drugs that undergo transesterification include methylphenidate and pethidine (*Sect. 7.4.2*). A lipophilic, nonbasic substrate metabolized by ethyl esterification is *acitretin*, a retinoid derivative with a free carboxylic group. The reaction, which forms the retinoid etretinate, was seen in trace amounts in human liver microsomes in the presence of ethanol [126]. Given the very long tissue retention of etretinate and its teratogenic potential, the reaction has clear clinical significance.

It, thus, appears that the capacity to catalyze *reactions of transesterification* and *esterification* is a characteristic of various hydrolases (*Chapt. 3*). Apart from the carboxylesterases discussed here, lipoprotein lipase has the capacity to synthesize fatty acid ethyl esters from ethanol and triglycerides, or even fatty acids [127]. Ethanol, 2-chloroethanol, and other primary alcohols serve to esterify endogenous fatty acids and a number of xenobiotic acids [128 – 130]. In this context, it is interesting to note that the same human liver carboxylesterase was able to catalyze the hydrolysis of cocaine to benzoylecgonine, the transesterification of cocaine, and the ethyl esterification of fatty acids [131].

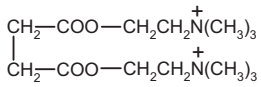
7.4.5. Soft Drugs

Bodor, who pioneered the concept and coined the term, has defined soft drugs as:

> '*biologically active compounds (drugs) characterized by a predictable* in vivo *metabolism to non-toxic moieties, after they achieve their therapeutic role. … The metabolic disposition of the soft drugs takes place with a controllable rate in a predictable manner.*' [132] (see also Chapt. 1 in [7]).

In practice, soft drugs are *a*) bioisosteres of known drugs, *b*) contain a labile bridge (often an ester group), and *c*) are cleaved to metabolites known to lack activity and toxicity. In other words, soft drugs are stereoelectronically closely analogous to the target drugs, but rapid breakdown to inactive metabolites is programmed into their chemical structures [133]. However, caution is required with such terms as 'predictable manner' and 'controllable rate' used to describe the metabolic breakdown of soft drugs. What can, indeed, be predicted and controlled is, in qualitative terms, the site of cleavage, and in semiquantitative terms, the approximate lability of this site. What cannot be predicted and controlled in a quantitative manner is the actual *in vivo* rate of breakdown. This is due to the many biological factors involved, in particular the nature, distribution, and levels of the inactivating enzymes, as well as the disposition of the soft drugs themselves, for example, the extent of their binding to plasma proteins.

A typical example is *succinylcholine* (suxamethonium, **7.62**), although the discovery of this agent predates by decades the concept of soft drugs. In most individuals, this curarimimetic agent is very rapidly hydrolyzed to choline by plasma cholinesterase with a $t_{1/2}$ value of *ca.* 4 min [76][134].



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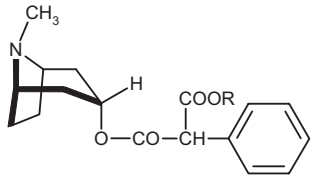
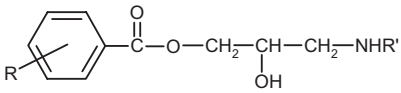


7.63

Ad hoc designed soft drugs may be found in a variety of therapeutic classes, two of which, soft anticholinergics and soft β-blockers, will be presented here. Thus, a number of potential *soft anticholinergic agents* (**7.63**), bioisosteres of atropine, were prepared, with the $-CH_2OH$ group being replaced by a labile –COOR group [135]. *In vitro*, the most-active analogue in the series **7.63** was the isopropyl ester. The rates of chemical and enzymatic hydrolysis were found to decrease with the size of the R substituent in **7.63**. For example, the $t_{1/2}$ values in rat liver homogenates at 37° were *ca.* 3.5, 7, and 19 min for R = Et, i-Pr, and cyclohexyl, respectively, while the corresponding $t_{1/2}$ values in human plasma were much longer (*ca.* 80, 190, and 270 h, respectively), suggesting preferential rapid hydrolysis by carboxylesterases. These studies were extended to scopolamine analogues, with promising *in vivo* results (mydriatic activity) [136].

Soft β-blockers have a particular therapeutic role to play, accounting for the amount of attention they have received. For example, a comprehensive study of *ca.* 80 analogues of general structure **7.64** has established the *in vitro* and *in vivo* activity of many of these compounds and confirmed that their enzymatic hydrolysis is highly sensitive to structural effects [137]. Here, the labile moiety is incorporated into the side chain, with the consequence that hydrolysis destroys the pharmacophore.

Another class of soft β-blockers characterized by an ester group in the substituent *para* to the 3-amino-2-hydroxypropoxycarbonyl side chain has been reported [138][139]. In this series, the *para*-substituent has intermediate polarity, compatible with good receptor affinity, but hydrolysis unmasks a carboxy group whose polarity is too high for receptor binding. A promising compound in this series appears to be *esmolol* (**7.65**), whose ester bond is hydrolyzed by an esterase in the cytosol of red blood cells [140]. The $t_{1/2}$ value of esmolol in whole blood is *ca.* 1, 2, 13, and 27 min in guinea pigs, rats, dogs, and humans, respectively. In humans, the plasma $t_{1/2}$ value of the drug



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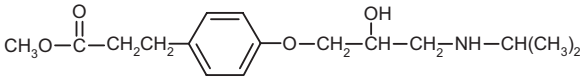


7.65

in vivo is *ca.* 9 min, the recovery from β-blockade beginning 2 min after discontinuation of infusion and being complete at 18 min [2][141].

Recent results suggest a promising application of soft drugs in the field of topical corticosteroids (*e.g.*, loteprednol etabonate [142]) and calcium antagonists (*e.g.*, clevipidine [143]).

7.5. Thioesters

7.5.1. Introduction

Thioesters (also sometimes called thiolesters) differ electronically from 'oxyesters' both in their ground and transition states [144]. As shown in *Fig. 7.10*, the carbonyl O-atom is much more polarized in methyl acetate than in *S*-methyl thioacetate, while the bridging O-atom is more electron-rich than the S-atom. These and geometric differences may be postulated to play a role in the relative *binding affinities* of oxyesters and thioesters toward hydrolases. In addition, oxyesters and thioesters behave differently upon deprotonation to form the corresponding anions, a reaction that is facilitated by *ca.* 20 kcal mol^{-1} in thioesters. This is apparently due to a difference in the delocalization of the negative charge, with the carbonyl O-atom acting as an electron sink much more strongly in thioesters than in oxyesters. Such an effect may be postulated to account for part or much of the *higher reactivity of thioesters* toward base-catalyzed hydrolysis and, hence, toward enzymatic

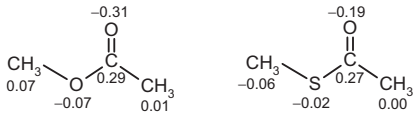


Fig. 7.10. *Approximate charge distribution in methyl acetate and S-methyl thioacetate, as calculated by the INDO quantum-mechanical method* [144]

hydrolysis (consider the tetrahedral transition state resulting from the nucleophilic addition of HO^- or Ser–O^-, as discussed in *Chapt. 3*).

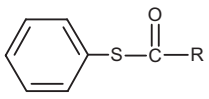
Thioesters play a paramount biochemical role in the metabolism of fatty acids and lipids. Indeed, *fatty acyl-coenzyme A thioesters* are pivotal in fatty acid anabolism and catabolism, in protein acylation, and in the synthesis of triacylglycerols, phospholipids and cholesterol esters [145]. It is in these reactions that the peculiar reactivity of thioesters is of such significance. Many hydrolases, and mainly mitochondrial *thiolester hydrolases* (EC 3.1.2), are able to cleave thioesters. In addition, cholinesterases and carboxylesterases show some activity, but this is not a constant property of these enzymes since, for example, carboxylesterases from human monocytes were found to be inactive toward some endogenous thioesters [35][146]. In contrast, allococaine benzoyl thioester was found to be a good substrate of pig liver esterase, human and mouse butyrylcholinesterase, and mouse acetylcholinesterase [147].

7.5.2. Model Compounds and Therapeutic Agents

A number of synthetic fatty acid thioesters, notably derivatives of branched fatty acids or thioester-containing phospholipids, have been used as kinetic and catalytic probes of various hydrolases [148][149]. However, such compounds are of limited interest in our context.

Simple alkyl or aryl thioesters are commonly assayed as substrates of hydrolases, witness the hydrolysis of *phenyl thioesters* by horse serum carboxylesterase [150]. For most substrates investigated, *e.g.*, phenyl thioacetate, phenyl thiopropionate, and phenyl thiobutyrate (**7.66**, R = Me, Et, and Bu, respectively), k_{cat} values were found, which were a few times larger than those of corresponding nitrophenyl esters, whereas the affinities were lower by approximately one order of magnitude. Methyl and phenyl esters of various linear thioacids were also found to be good substrates of mammalian liver carboxylesterases and serum cholinesterases [151].

Interesting findings have been reported for the hydrolysis of *α- and β-naphthyl thiocarbonates* (**7.67**, R = Me or Et) by mammalian liver carboxylesterases [43]. In comparison with their carbonate analogues (**7.21**), the thiocarbonates were more lipophilic by one log *P* unit and were hydrolyzed at rates modestly to markedly slower. These two findings are apparently unrelated.



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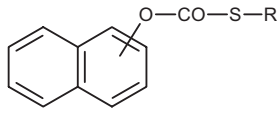

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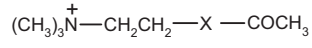


7.68

7.69 **7.70**

Among pharmacologically active compounds and other therapeutic agents, various examples of thioesters are worth mentioning. Thus, *acetylthiocholine* (**7.68**, X = S) is hydrolyzed by acetylcholinesterase (EC 3.1.1.7) with bimolecular rates comparable to those of acetylcholine (**7.68**, X = O), *i.e.*, k_{cat}/K_m values in the order of 10^9 M^{-1} min^{-1} [32]. These rates are among the highest known. A few other thiocholine esters such as succinyldithiocholine are good substrates of human plasma cholinesterase [152]. Another simple compound is S-*acetyl*-N-*glycylcysteamine* (**7.69**), an agent that requires *S*-deacetylation to liberate a free SH group before it can manifest radioprotective effects. When administered to mice, this compound was indeed hydrolyzed mainly at the thioester group but also at the amide bond, to generate the active metabolites *N*-acetylcysteamine and cysteamine (**7.70**) [153].

The compound *S*-(1-{[(2,3,4,5-tetrahydro-2-oxothiophen-3-yl)amino]carbonyl}ethyl) thiophene-2-carbothioate (*MR 889*, **7.71**, *Fig. 7.11*) is a regulator of bronchial secretion that also exhibits antiemphysema, mucolytic, and expectorant activities. This agent is a thioester that undergoes extensive hydrolysis in rats to liberate homocysteine thiolactone thiolactamide (**7.72**, *Fig. 7.11*) and thiophene-2-carboxylic acid (**7.73**, *Fig. 7.11*) as the primary metabolites [154]. The thiol metabolite **7.72** is believed to be responsible for part or most of the mucolytic activity. This metabolite is further transformed by hydrolysis of the amide bond (*Fig. 7.11*); the metabolites **7.72** and **7.73** undergo *S*-methylation and glycine conjugation, respectively.

An important drug in the present context is the mineralocorticoid receptor antagonist *spironolactone* (**7.74**, *Fig. 7.12*). Among its many metabolic reactions, spironolactone is readily hydrolyzed at the thioester bond (*Fig. 7.12*, *Reaction a*) to form deacetyl-spironolactone (**7.75**, *Fig. 7.12*), a metabolite found in a variety of tissues [155 – 157]. This thiol compound, which is also a potent mineralocorticoid antagonist, promotes the mechanism-based inactivation of hepatic, adrenal, and testicular cytochrome P450 isozymes. There is now good evidence to indicate that this behavior is the result of microsomal *S*-oxidation (see Chapt. 7 in [7]). When spironolactone was incubated with liver microsomes from rats pretreated with dexamethasone (an inducer of CYP3A), the sulfinic and sulfonic acid derivatives were characterized [158]. Perhaps the importance of the *S*-deacetylation of spironolactone

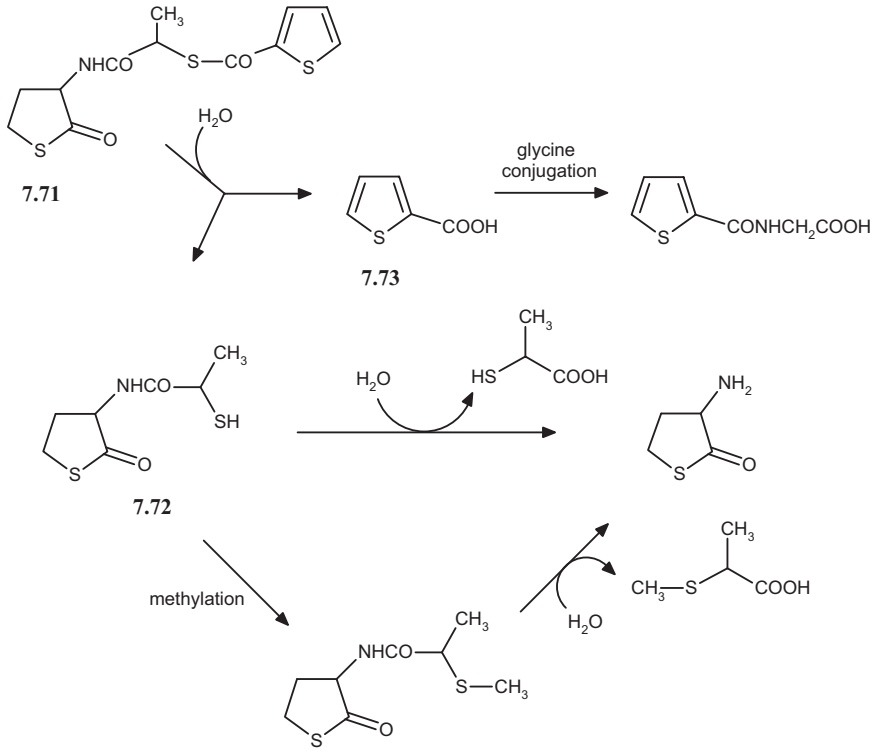


Fig. 7.11. *Metabolism of S-(1-{[(2,3,4,5-tetrahydro-2-oxothiophen-3-yl)amino]carbonyl}ethyl) thiophene-2-carbothioate* (MR 889, **7.71**) *in rats to the active thiol metabolite homocysteine thiolactone thiolactamide* (**7.72**) *and to thiophene-2-carboxylic acid* (**7.73**) [154]. Subsequent reactions of hydrolysis and conjugation are also shown.

would be more apparent were it not in competition with dethioacetylation (*Fig. 7.12*, *Reaction b*) to be discussed in *Chapt. 11*.

7.6. Lactones

7.6.1. Peculiarities of Lactone Hydrolysis

Lactone hydrolysis is similar to ester hydrolysis in terms of catalytic mechanisms, but differs as far as reaction kinetics and products are concerned. Whereas esters are hydrolyzed to two metabolites/products, lactones generate a hydroxy acid as the sole metabolite/product of hydrolysis. In fact, the reaction should be designated as *hydration* rather than hydrolysis according to the definitions given in *Chapt. 1*. Another characteristic of hydrolytic

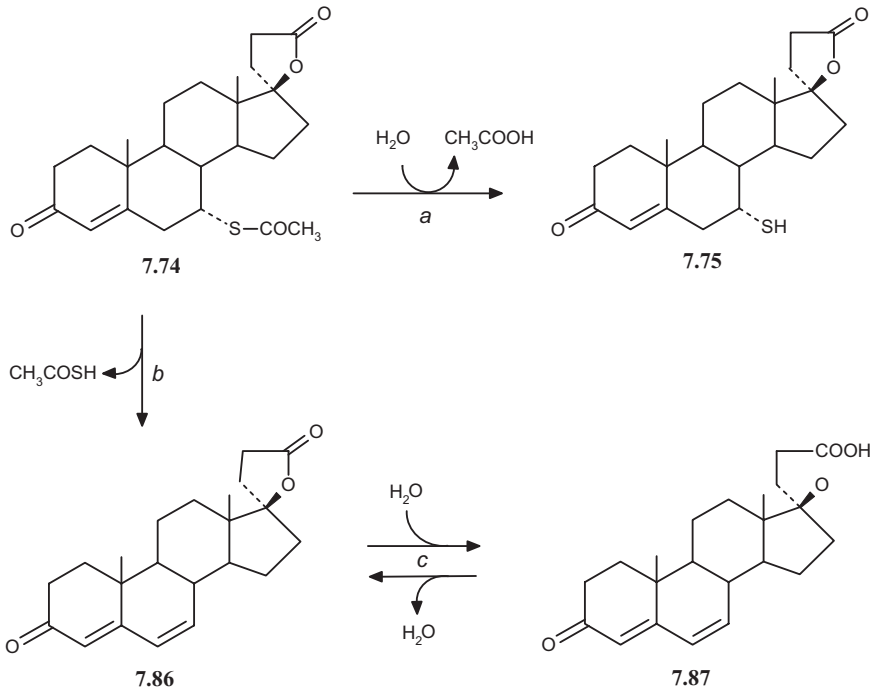


Fig. 7.12. *Some reactions in the metabolism of spironolactone* (**7.74**). Deacetylation (*Reaction a*) is discussed in this section, while the equilibrium between canrenone (**7.86**) and canrenoic acid (**7.87**) (*Reaction c*) is presented in *Sect. 7.6*. Dethioacetylation (*Reaction b*) to form canrenone will be discussed in *Chapt. 11*.

lactone opening is its *reversibility*, *lactonization* (ring closure with elimination of H_2O) occurring at rates and to an extent dependent on substrate and conditions. A number of informative examples are discussed below. Cases also exist of lactone hydration being followed by lactonization to a product different from the starting compound, as discussed in *Chapt. 11*.

Lactone ring opening can be both *enzymatic* and *nonenzymatic*, but it appears that the two mechanistic routes are seldom distinguished in metabolic studies [2]. Thus, a number of the reports published on metabolism of lactones assume enzymatic hydration and do not examine what the relative contribution of nonenzymatic processes may be. Like for lactonization reactions, only very few *in vitro* biochemical studies address the question of relative enzymatic contribution and mechanism.

The significance, reversibility, and mechanisms of *nonenzymatic lactone hydration* may be aptly illustrated with *pilocarpine*, an extensively investigated drug whose stability in aqueous solution is of great pharmaceutical relevance. Pilocarpine (**7.76**, *Fig. 7.13*), whose absolute configuration is

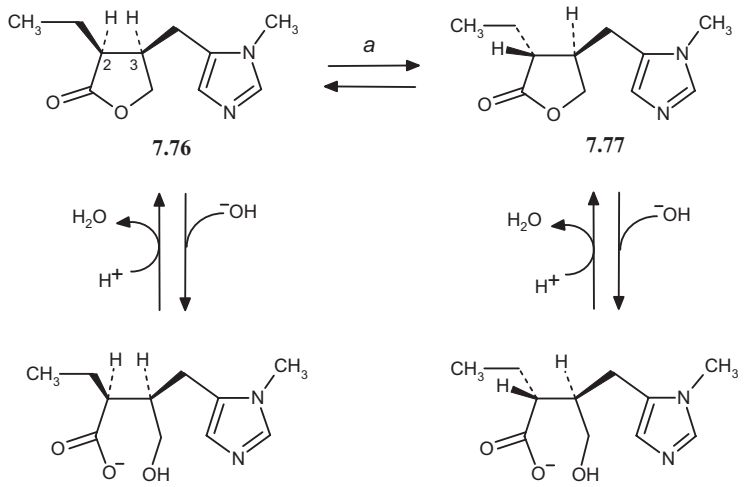
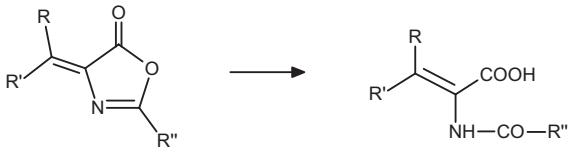


Fig. 7.13. *HO⁻-Catalyzed ring opening of pilocarpine* (**7.76**) *and isopilocarpine* (**7.77**) *to pilocarpic acid and isopilocarpic acid, respectively, and proton-catalyzed lactonization of the two acids to the respective lactone.* Note that pilocarpine and isopilocarpine interconvert by a base-catalyzed reaction of epimerization (*Reaction a*).

(2*S*,3*R*), epimerizes to (2*R*,3*R*)-isopilocarpine (**7.77**, *Fig. 7.13*) in a base-catalyzed reaction (*Fig. 7.13*, *Reaction a*) [159]. The activation energy of this reaction was found to be *ca.* 119 kJ mol^{-1}, and, in alkaline solutions at 35°, *ca.* 26% of pilocarpine underwent epimerization [160]. Epimerization is in competition with hydrolytic ring opening, a reaction catalyzed by the HO$^-$ ion (activation energy 105 kJ mol^{-1}) to form pilocarpic acid or, more precisely, its anion (*Fig. 7.13*).

The reverse reaction of *lactonization* of pilocarpic acid is proton-catalyzed and, hence, favored at low pH. Thus, the pilocarpine/pilocarpic acid ratio was 1.0, 1.6, 2.7, 4.0, 5.1, and 6.7 at pH values of 6.0, 5.6, 5.2, 4.8, 4.4, and 4.0, respectively [161]. Interestingly, the lactonization of isopilocarpic acid is *ca.* 17-fold faster than the lactonization of pilocarpic acid, since the second-order rate constants of proton-catalyzed lactonization at 25° were 0.96 and 0.055 м$^{-1}$ min^{-1} for isopilocarpine and pilocarpine, respectively [162]. A lack of planarity in the lactone ring of pilocarpine, and a more lactone-like planarity in isopilocarpine, appear to account for this difference in reactivity between the two epimers.

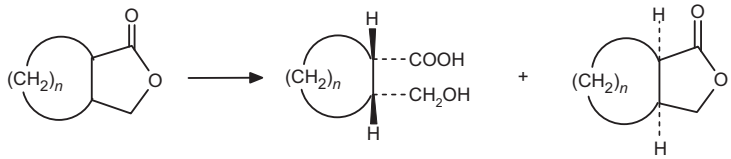
The *enzymatic hydration* of lactones is also documented, a variety of hydrolases having demonstrated activity. Very detailed kinetic studies have, for example, been published on the hydrolysis of oxazolones (**7.78**, R = H or Me, R′ = Me or aryl, R″ = Me or Ph) catalyzed by α-chymotrypsin [163]. These compounds are interesting from a chemical point of view, being enolic lac-



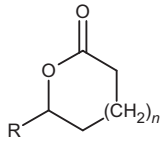
7.78 **7.79**

tones whose hydrolytic ring opening yields a carbinolimine, which tautomerizes to an amide (**7.79**). Their interaction with α-chymotrypsin revealed high affinities but relatively low k_{cat} values. Their high selectivity for α-chymotrypsin permitted active site titration in the presence of other hydrolases.

One proof of particular elegance for enzymatic hydration is the demonstration of a *substrate enantioselectivity* in the reaction [164]. Racemic cis-*fused bicyclic γ-butyrolactones* (**7.80**, $n = 1-4$) offer such an example [165]. When incubated for a few hours with horse liver *carboxylesterase*, these model compounds were hydrated to the hydroxy acid with (1*S*) absolute configuration (**7.81**), the remaining lactone having the (1*R*)-configuration (**7.82**). Other examples were seen with chiral lactones of structure **7.83** ($n = 0-2$, R = Et, Pr, pentyl, or heptyl), which were hydrated by pig pancreatic lipase or liver carboxylesterase with high enantioselectivity [166]. Had these reactions been entirely or mainly nonenzymatic, none or only a modest enantioselectivity would have been expected. In contrast, the high ee values reported indicate that the nonenzymatic component was moderate at best. In fact, a number of *lactonases* (lactonohydrolases) are known to act on lactones besides the above-mentioned hydrolases, *e.g.*, 1,4-lactonase (EC 3.1.1.25), 4-pyridoxolactonase (EC 3.1.1.27), limonin-D-ring-lactonase (EC 3.1.1.36), steroid-lactonase (EC 3.1.1.37), and actinomycin lactonase (EC 3.1.1.39).



7.80 **7.81** **7.82**



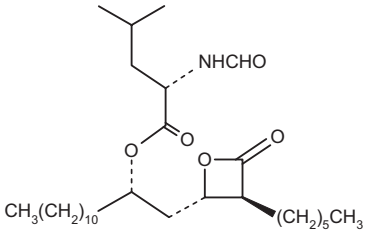
7.83

Of great interest is the recent finding that *human serum paraoxonase* (EC 3.1.8.1, which belongs to the class of A-esterases), is very active in hydrolyzing a range of four-, five-, six-, and seven-membered lactones [167]. Some cyclic carbonates (see *Sect. 7.6.3*) were also substrates.

7.6.2. Lactones of Medicinal or Toxicological Interest

Lactones of increasing ring size are discussed here, with some additional examples to be examined in *Chapt. 8* (prodrugs) and *11* (hydration followed by closure to a different ring).

A *β-lactone* (four-membered ring) of medicinal interest is *tetrahydrolipstatin* (orlistat, **7.84**), a specific inhibitor of triacylglycerol lipases of therapeutic value as an antiobesity agent [168]. When incubated with purified human carboxyl-ester lipase (HCEL, EC not specified), the compound underwent extensive lactone hydration. The first step in the reaction is believed to be a nucleophilic attack of the active-site serine at the carbonyl C-atom of the lactone ring, resulting in formation of an acylated HCEL derivative and inhibition of the enzyme. This inhibition, however, is only transitory since the serine ester is subsequently cleaved to regenerate the fully active enzyme and to liberate the ring-opened metabolite as the main product. The ring-opened metabolite is a further substrate of the enzyme, yielding a variety of products formed by reactions of hydration, acyl migration, and lactonization.



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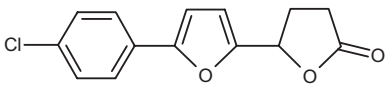
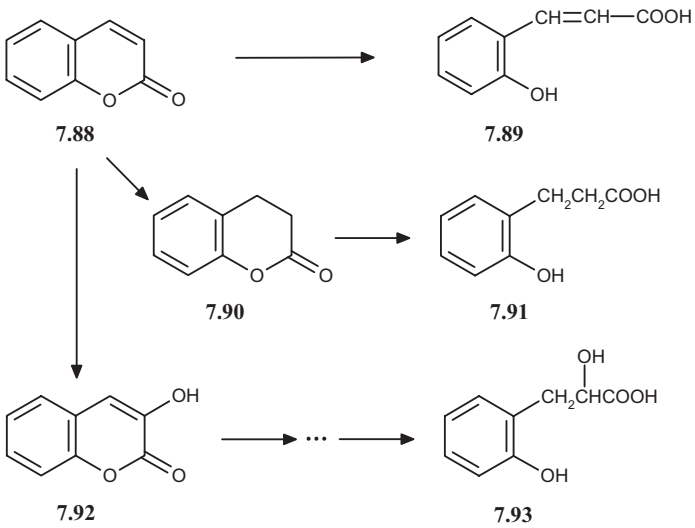


7.85

The compound *5-[5-(4-chlorophenyl)furan-2-yl]-2,3,4,5-tetrahydrofuran-2-one* (F-1044, **7.85**) is a nonsteroidal anti-inflammatory agent possibly acting *via* a ring-opened hydroxybutyric acid metabolite. To examine this hypothesis, F-1044 was submitted to extensive *in vivo* testing, which revealed potent activities and a unique pharmacological profile markedly different from that of acidic nonsteroidal anti-inflammatory drugs [169]. These results have been interpreted to mean that part or most of the observed effects of F-

1044 must be due to the drug itself and not to a ring-opened metabolite, implying a noteworthy metabolic stability of the *γ-lactone* (five-membered) ring in this agent. A therapeutically important γ-lactone derivative is *spironolactone* (**7.74**, *Fig. 7.12*) whose thioester bond hydrolysis has been discussed in *Sect. 7.5.2*. Spironolactone also undergoes dethioacetylation (*Fig. 7.12*, *Reaction b*) to be discussed in *Chapt. 11*. This is the major metabolic reaction of spironolactone in humans, and *ca.* 80% of an oral dose of the drug is in the form of metabolites lacking the thioacetyl function [155]. The primary metabolite thus formed, called *canrenone* (**7.86**, *Fig. 7.12*), is also a mineralocorticoid antagonist. Of interest in the present context is the fact that canrenone undergoes reversible lactone hydration to canrenoic acid (**7.87**, *Fig. 7.12*, *Reaction c*). In buffer solutions under physiological conditions, the canrenone/canrenoic acid equilibrium was reached with a $t_{1/2}$ value of *ca.* 24 d, whereas, in humans injected with potassium canrenoate, the equilibrium was established in *ca.* 3 h, giving similar plasma concentrations of the lactone and the acid. This finding suggests that, *in vivo*, the reversible reaction of lactone hydration is enzyme-catalyzed, and it is tempting to speculate that the enzyme involved might be or resemble steroid-lactonase.

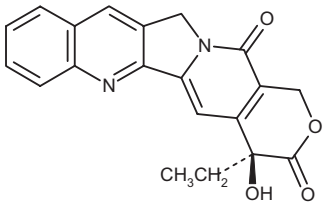
Coumarin (**7.88**) is a well-known *δ-lactone* (six-membered ring) of natural origin found in various preparations such as some tobaccos, alcoholic beverages, and cosmetics. Besides reactions of oxidation, reduction, and conjugation, coumarin is also subject to lactone hydration *in vivo* and in the presence of microsomes [170 – 174]. The resulting metabolites include *ortho*-coumaric acid (**7.89**) formed directly from coumarin, 3-(2-hydroxyphenyl)-propionic acid (**7.91**) formed following reduction of coumarin to dihydrocou-



7.88 **7.89** **7.90** **7.91** **7.92** **7.93**

marin (**7.90**), and 3-(2-hydroxyphenyl)lactic acid (**7.93**) formed after hydroxylation of coumarin to 3-hydroxycoumarin (**7.92**).

*(20*S*)-Camptothecin* (**7.94**) is a naturally occurring antineoplastic agent, which, under physiological conditions, exists in a rapid equilibrium between the lactone and carboxylate forms [175]. Interestingly, the two forms are very different in pharmacodynamic and pharmacokinetic terms. The lactone is both poorly water-soluble and intrinsically active, whereas the carboxylate is inactive *per se* but is well water-soluble. Despite expectations, the carboxylate was only poorly effective and did not appear to be useful as a prodrug of the lactone. Recent studies in rats showed that the intravenously administered carboxylate underwent significant urinary and biliary excretion (*ca.* 40 and 25% of a 1 mg kg^{-1} dose, respectively). In contrast, camptothecin itself gave much higher plasma concentrations of the active lactone form while only limited amounts were excreted (*ca.* 10% in urine and 7% in bile) [176].



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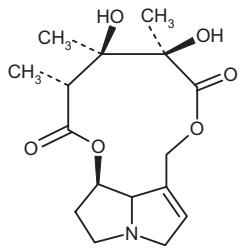


7.95

The final example to be discussed here is that of a chemically more-complex dilactone *monocrotaline* (**7.95**). This pyrrolizidine alkaloid is an important phytotoxin with large interspecies differences in the susceptibility to its toxic effects. Thus, the rat is highly sensitive to monocrotaline intoxication, whereas the guinea-pig is resistant. Since pyrrolizidine alkaloids act *via* oxidized metabolites, it was believed that interspecies differences in susceptibility to intoxication were caused by different capacities to oxidize the compound. It now appears, at least in the case of rats and guinea pigs, that large differences exist in hydrolytic capacities, which underlie the differences in susceptibility [177]. Indeed, carboxylesterases accounted for *ca.* 90% of the metabolism of monocrotaline in guinea pig liver microsomes, resulting in the hydrolysis of both ester groups. In contrast, no enzyme-catalyzed hydrolysis was seen in rat hepatic microsomes.

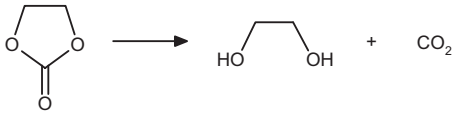
These various examples underline various aspects of the metabolism and disposition of lactones in connection with their pharmacological or toxicological activities. Other cases also exist, namely the lactone prodrugs to be presented in *Chapt. 8*.

7.6.3. Analogues of Lactones

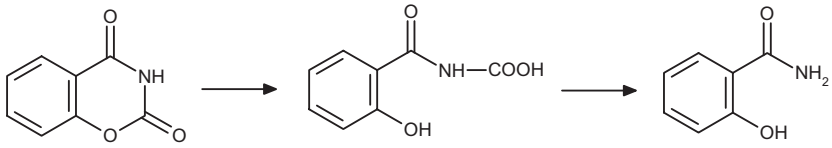
In this short section, cyclic compounds are discussed that are not strictly lactones since they contain an endocyclic structural motif of the type –O–CO–X– or –X–O–CO–. However, these compounds share with lactones the possibility to be hydrolyzed at the endocyclic –CO–O– bond. This is the case for *cyclic diesters of carbonic acid* (–O–CO–O–), *cyclic esters of carbamic acid* (–O–CO–NH–), and *cyclic anhydrides* (–CO–O–CO–). One example of each class is presented here.

A simple cyclic diester of carbonic acid is *ethylene carbonate* (**7.96**), a chemical with a toxicity profile resembling that of ethylene glycol (**7.97**). Metabolic studies have confirmed that ethylene carbonate is hydrolyzed very rapidly to ethylene glycol (whose oxidation is discussed in Chapt. 2 in [7]) and CO_2. Indeed, rats given an oral dose of ethylene carbonate did not excrete the unchanged xenobiotic in detectable amounts, and blood levels of the diester were *ca.* 100-fold smaller than those of ethylene glycol [178].

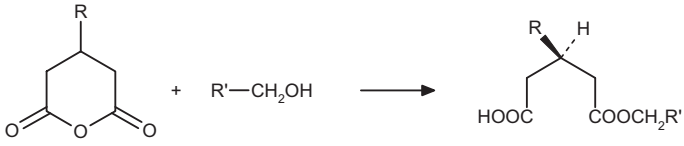
*2*H-*1,3-Benzoxazine-2,4(3*H)*-dione* (**7.98**) is an interesting cyclic carbamate. Hydrolysis of the ester group likely yields the ring-opened carbamic acid **7.99** as an undetected intermediate, which very rapidly decarboxylates to form the product salicylamide (**7.100**). When the cyclic carbamate was ad-



7.96 **7.97**



7.98 **7.99** **7.100**



7.101 **7.102**

ministered to rabbits, extensive hydrolysis to salicylamide was indeed observed [179]. The enzymatic nature of the reaction was also indicated, since the $t_{1/2}$ values of **7.98** at pH 7.4 and 37° was 17 h, 4.6 h, and 0.4 h in buffer, 80% rabbit plasma, and a rabbit liver homogenate, respectively.

Our final example is that of cyclic anhydrides, namely prochiral *3-substituted glutaric anhydrides* (**7.101**, R = Me, Et, or Pr). When incubated with lipase in an inert solvent in the presence of an alcohol (methanol, butan-1-ol, *etc.*), these compounds underwent nucleophilic ring opening with formation of a hemiester (**7.102**) of (*R*)-configuration (60 – 90% ee) [180]. This product enantioselectivity and, of course, the lack of reactivity in the absence of lipase show the enzymatic nature of the reaction.

Such examples bring additional evidence for the very broad substrate specificity and the catalytic versatility of hydrolases.

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Chapter 8

The Hydrolysis of Carboxylic Acid Ester Prodrugs

Contents

Hydrolysis in Drug and Prodrug Metabolism. Bernard Testa, Joachim M. Mayer

8.1. Introduction

Prodrugs, which occupy a significant position in the scope of the present volume, were defined and discussed in *Chapt. 1*. In addition to this chapter, prodrugs activated by *reactions of hydrolysis* receive further attention in *Chapt. 4*, *6*, *9*, and *11*.

The present chapter is dedicated to the very large group of prodrugs that incorporate a carboxylic acid ester function. The classification used here is mainly a chemical one, since we consider, in turn, prodrugs formed from active carboxylic acids, alcohols, phenols, amines, and amides, as well as lactone prodrugs. The subdivision within these classes is also based on chemical arguments, with the discussion centering mainly on catalytic, therapeutic, and toxicological considerations whenever relevant and available.

Ester prodrugs, and the other prodrug types discussed elsewhere in this volume, can be subdivided in two major categories depending whether the pro-moiety released by hydrolysis is stable or breaks down into fragments. As discussed and exemplified in various sections, such a criterion of classification is relevant to the mechanism of activation of these prodrugs, as well as to the possibility of toxicogenicity, *e.g.*, when a toxic fragment such as formaldehyde is liberated.

8.2. Prodrugs of Active Carboxylic Acids That Incorporate a Simple or Functionalized Alkyl Pro-Moiety

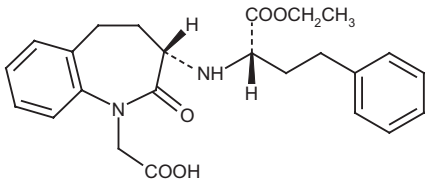
A carboxylic acid group, being ionized in the physiological pH range, will contribute significantly to reducing the lipophilicity of compounds containing this moiety. As a result, a large number of pharmacologically active carboxylic acids may display unfavorable pharmacokinetic properties such as low bioavailability, a problem of particular concern for compounds that contain other moieties or a skeleton of high polarity. Thus, a major aim of prodrug design is to improve the pharmacokinetic behavior of active carboxylic acids, explaining why the majority of marketed or experimental prodrugs are derived from such acids. In some cases, prodrugs of this type were developed to correct some pharmacokinetic defect(s) of established carboxylic drugs (*post hoc* prodrugs). In other cases also illustrated below, the problem was identified during development and *ad hoc* prodrugs were designed.

8.2.1. Prodrugs That Incorporate a Simple Alkyl Pro-Moiety

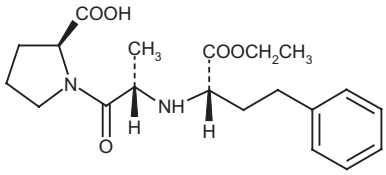
8.2.1.1. *Ethyl Esters*

A classical therapeutic group illustrating *ad hoc* prodrug design is that of *angiotensin-converting enzyme (ACE) inhibitors* [1]. Captopril, the first marketed drug in this class, contains one carboxylate group, while more recent agents contain two carboxylate groups as important features of the pharmacophore. However, the polarity of these groups prevents good gastrointestinal

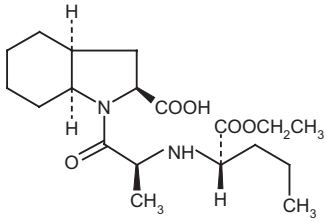
absorption after oral administration. As a result, most dicarboxylate ACE inhibitors in the literature have been developed as prodrugs, these being ethyl monoesters, *e.g.*, benazepril (**8.1**), enalapril (**8.2**), and perindopril (**8.3**).



8.1



8.2



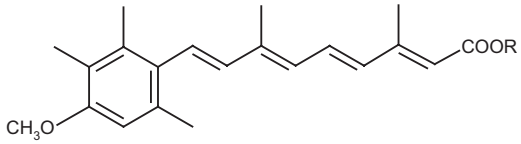
8.3

Benazepril (**8.1**) is illustrative of the metabolism and disposition of ACE inhibitor prodrugs. The compound is rapidly and well absorbed perorally in rats, dogs, baboons, and humans [2]. In contrast, its acidic metabolite (known as benazeprilate) was very poorly absorbed after oral administration to rats. The hydrolysis of benazepril to benazeprilate occurred very rapidly. In rat plasma collected 10 min after an oral dose of benazepril, over 90% of the radioactivity represented the active metabolite with only traces of the unchanged prodrug. In humans, fast activation was also seen, although seemingly not as fast as in rats. After oral administration of benazepril, the plasma AUC (area-under-the-curve) of benazeprilate was at least tenfold greater than that of the prodrug. Comparable results were reported for such ACE inhibitors as imidapril, perindopril (**8.3**), and ramipril [3 – 6].

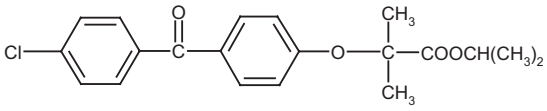
In vitro studies were also conducted to discover the enzymes responsible for bioactivation of benazepril. With different preparations and eserine as an inhibitor, the involvement of arylesterases (EC 3.1.1.2) and cholinesterases (EC 3.1.1.8) was ruled out, while that of carboxylesterases (EC 3.1.1.1) was

demonstrated [2a]. The latter enzymes are found in mammalian liver and in rat plasma, but are absent in the plasma of humans and other mammals. This difference might explain some interspecies variations in the rate of activation of ACE inhibitors.

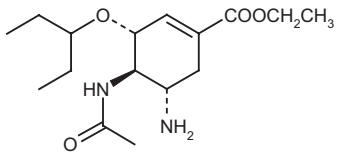
Some *retinoids* offer examples of prodrugs being activated to the active metabolite. Thus, the orally effective retinoid *etretinate* (**8.4**, R = Et) is rapidly hydrolyzed to acitretin (**8.4**, R = H) in humans and animals prior to further extensive metabolism by isomerization, chain shortening, and conjugation [7]. The pharmacokinetic behavior of the two compounds is quite different, absorption being good for the prodrug as expected, but variable and incomplete for acitretin, while accumulation in adipose tissues occurs only for the ester [8]. Interestingly, one of the metabolic reactions of acitretin first discovered in patients was its ethyl esterification to etretinate. This finding has now been confirmed in rats *in vivo*, as well as in the 12,000 *g* supernatant from human and rat liver homogenates [9]. Addition of EtOH to *in vitro* hepatic preparations, or co-administration of EtOH and acitretin to rats, consistently led to increased ethyl esterification. The reaction was clearly an enzymatic one, and, under *in vivo* conditions, occurred systemically rather than during absorption. Such a reaction of esterification may appear to be a curiosity, but it is neither novel nor rare, as discussed in *Chapt. 3* and *7*. Interestingly, and in addition to the reaction of carboxylesterase-catalyzed ethyl esterification discussed in these chapters, another mechanism of esterification involving acitretin has been discovered in human liver microsomes [10]. This pathway involves the ATP-dependent conjugation of acitretin to coenzyme A, followed by esterification of this intermediate with an unbranched alkanol (MeOH, EtOH, PrOH, BuOH, or hexanol) added to the incubations.



8.4



8.5



8.6

Another therapeutic class to be briefly discussed is that of the lipid-lowering agents known as *fibrates*, *e.g.*, *clofibrate* and *fenofibrate* (**8.5**). Here also, the acidic metabolite is the active form: clofibrate (an ethyl ester) is rapidly hydrolyzed to clofibric acid by liver carboxylesterases and blood esterases [11]. Human metabolic studies of fenofibrate (**8.5**), the isopropyl ester of fenofibric acid, showed incomplete absorption after oral administration, while hydrolysis of the absorbed fraction was quantitative [12]. This was followed by other reactions of biotransformation, mainly glucuronidation of the carboxylic acid group.

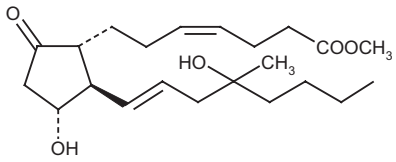
Two final examples where formation of the ethyl ester has strongly increased bioavailability are those of the thiol-containing tripeptide *glutathione* and of *Ro-640802*. In rats, the plasma concentrations of glutathione were *ca.* 250 times higher following *i.v.* administration of the ethyl ester than of glutathione itself [13]. An even more telling case is that of *oseltamivir* (**8.6**), the ethyl ester prodrug of *Ro-640802*. The latter is a potent neuraminidase inhibitor showing valuable effects against influenza A and B viruses. However, its high polarity, which appears indispensable for affinity toward neuraminidase, results in low oral bioavailability. The problem was solved elegantly by development of a prodrug strategy, which has achieved good gastrointestinal absorption and rapid enzymatic activation by hydrolysis [14 – 16].

8.2.1.2. *Other Alkyl Esters*

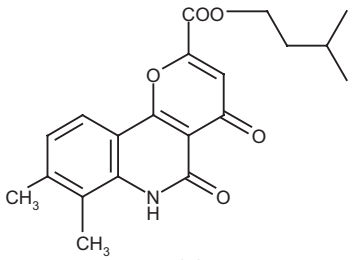
Most of the prodrugs discussed above are ethyl esters, but it would be wrong to conclude that ethyl esters alone constitute ester prodrugs. Indeed, a number of active carboxylic acids that are under investigation or are being used as esters incorporate other alkyl substituents. Thus, *enisoprost* (**8.7**) is the methyl ester of a synthetic prostaglandin E_1 analogue. Following oral administration to humans, it was rapidly absorbed, but no unchanged prodrug was detectable in plasma. In contrast, the active carboxylic acid reached peak plasma concentrations within 20 min of dosing [17]. Its further transformation was extensive and involved β-oxidation, ω-oxidation, and keto reduction.

An isopentyl group is seen in *repirinast* (**8.8**), the orally effective prodrug of a cromoglycate-like anti-allergy agent. In humans dosed with this prodrug, peak plasma concentrations of the active metabolite were seen *ca.* 1 h after dosing [18]. Interestingly, the plasma AUC values of the metabolite were approximately doubled when a meal was taken immediately before or after dosing, an effect due, at least in part, to enhanced dissolution in the gastrointestinal tract. Other alkyl groups are found in prodrugs of *indomethacin* (**8.9**, R = OH) aimed at reducing gastrointestinal irritation, for example the butyl

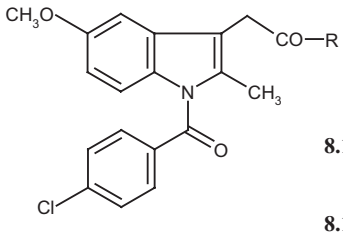
and octyl esters [19]. A more complex alkyl group is found in *indomethacin farnesil* (**8.10**), another derivative designed to reduce the occurrence of side effects. In rats, absorption of an oral dose occurred mainly *via* the thoracic lymph duct, followed by a seemingly improved distribution into inflamed tissues [20]. Some activation occurred in the plasma and pancreatic juice of rats but not dogs or humans. Taken globally, the results of this study indicate that indomethacin farnesil is hydrolyzed relatively slowly in the periphery, and that an important fraction is transported intact into tissues, including the sites of action, where the active drug is liberated.



8.7

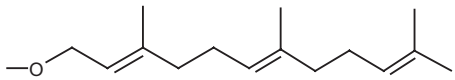


8.8

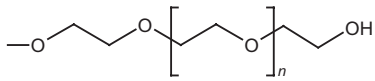


8.9

8.10 R = —O



8.11 R = —O



Even more complex potential prodrugs of indomethacin were examined, namely its limonenyl, perillyl, bornyl, and menthyl esters, *i.e.*, terpenoid derivatives [21]. These highly lipophilic esters showed rapid enzymatic hydrolysis, and the limonenyl prodrug assayed in humans had an interesting delayed and sustained cutaneous anti-inflammatory activity.

8.2.2. Prodrugs That Incorporate an Oxyalkyl, Hydroxyalkyl, or Arylalkyl Pro-Moiety

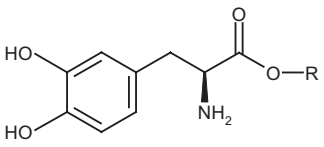
A large number of studies in the literature describe prodrugs of carboxylic acids incorporating alkyl groups functionalized with oxygenated, nitro-

genated, or aromatic substituents. Here and in the next three sections, we consider esterifying groups that do not break down upon hydrolysis but are recovered as *functionalized alkanols or phenols*. Particular representatives of this category are the lipid-like derivatives as examined in *Sect. 8.2.5*. Oxygenated and nitrogenated substituents that break down upon hydrolysis will be treated separately in *Sect. 8.3*.

A promising example of oxygenated alkyl groups is offered by a series of *oligoethylene esters of indomethacin* (**8.11**, $n = 0$, 1, 2, 3, or 4) [22][23]. These esters were relatively stable in a pH 7.4 buffer ($t_{1/2}$ of hydrolysis at 32° *ca.* 500 – 600 h), but were readily hydrolyzed in human plasma at pH 7.4 and 37° ($t_{1/2}$ *ca.* 3 h). The esters were slightly less potent than indomethacin as anti-inflammatory agents, but they exhibited analgesic activities similar to or better than that of indomethacin, and their ulcerogenic potential was considerably less. The same pro-moieties were also used to prepare dermal prodrugs of ketoprofen, naproxen, and diclofenac with promising results [24].

L-*Dopa esters* (**8.12**) are used here to illustrate some of the functionalized alkyl groups that have been used in the design of prodrugs of carboxylic acids. L-Dopa (L-3,4-dihydroxyphenylalanine, **8.13**) is used extensively to alleviate the symptoms of *Parkinsonism*. Being the amino acid bioprecursor of the neurotransmitter dopamine, L-dopa is itself a prodrug. Despite a fair gastrointestinal absorption, L-dopa displays a number of pharmacokinetic and pharmacodynamic defects that justify the many efforts made toward improving its therapeutic profile. Thus, a variety of prodrugs of L-dopa have been prepared and tested, sometimes with promising but never striking results [25 – 27].

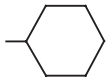
Here, we present the hydrolysis of a series of L-dopa esters whose pharmacodynamic behavior has also been reported [28 – 31]. As shown in *Table 8.1*, the compounds are esters incorporating a simple primary, secondary, or



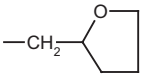
8.12

8.13 R = —H

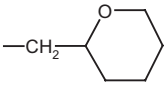
8.14 R =



8.15 R =



8.16 R =



tertiary alkyl group, an alkyl group containing an ether or alcohol function, or an arylalkyl moiety. When examined in buffer solutions under physiological conditions of pH and temperature, their hydrolysis followed pseudo-first-order kinetics, the $t_{1/2}$ values of which are reported in *Table 8.1*. In contrast to another example discussed in *Sect. 8.2.4*, no quantitative structure–hydrolysis relationship could be derived. However, *qualitative structure–hydrolysis relationships* were apparent in that the rate of chemical hydrolysis of alkyl esters decreased with increasing size of the alkyl group, suggesting a role for steric hindrance. Esters with an oxygenated substituent were hydrolyzed much faster than expected from the size of the substituent. The same was true, but to a smaller extent, when the substituent contained an aromatic moiety.

The L-dopa esters were also examined for their enzymatic hydrolysis in human plasma and/or by purified pig liver carboxylesterase (EC 3.1.1.1; *Table 8.1*). In human plasma under the conditions of study, hydrolysis again followed pseudo-first-order kinetics. In all but two cases examined, enzymatic hydrolysis was slightly faster than chemical hydrolysis. For the methyl ester, rates of chemical and enzymatic hydrolysis were comparable, whereas the *tert*-butyl ester was not hydrolyzed in plasma and was protected from chemical hydrolysis presumably by becoming bound to plasma proteins.

Table 8.1. *Chemical and Enzymatic Hydrolysis of* L-*Dopa Esters* (**8.12**) *at pH 7.4 and 37°* [28][30a]

R	Buffer	80% Human plasma	Carboxylesterase	
	$t_{1/2}$ [min][a])	$t_{1/2}$ [min][b])	K_m [mM][c])	V_{max} [μmol U^{-1} min^{-1}][d])
CH_3	620	800	5.2	0.45
CH_3CH_2	2500	770	2.5	0.42
$CH_3CH_2CH_2$	1600	[e])	1.1	0.36
$(CH_3)_2CH$	8500	2000	[e])	[e])
$CH_3CH_2CH(CH_3)$	14000	1200	[e])	[e])
$(CH_3)_3C$	7400	9100[f])	[e])	[e])
8.14	6300	[e])	1.5	0.24
$CH_3OCH_2CH(CH_3)$	1700	[e])	[e])	[e])
8.15	670	140	[e])	[e])
8.16	950	250	7.4	0.92
$CH_3CH(OH)CH_2$	530	[e])	13.0	0.37
$PhCH_2$	2100	530	[e])	[e])
$PhCH_2CH_2$	620	[e])	7.1	1.95
4-CH_3O–$C_6H_4CH_2CH_2$	1500	170	[e])	[e])
$PhOCH_2CH_2$	600	50	5.9	1.54

[a]) SD 2 – 18%. [b]) SD 3 – 23%. Values are for enzymatic hydrolysis, *i.e.*, total minus chemical hydrolysis. [c]) SD 13 – 39%. [d]) SD 8 – 28%. [e]) Not determined. [f]) This is the total plasma hydrolysis, which, due to protein binding, is slower than chemical hydrolysis; enzymatic hydrolysis is effectively nil.

There was a trend for hydrolysis by plasma enzymes to increase with increasing polarity of the substituent, or, more precisely, with the polar surface area of the substituent.

In the case of carboxylesterase-catalyzed hydrolysis (*Table 8.1*), the *Michaelis* constant consistently indicated relatively low affinity for the enzyme, with a variation between substrates of one order of magnitude. Even less variation was seen in the maximal velocity of the reaction. It is interesting to note that, for the four compounds where comparisons are possible, a direct relationship exists between the rate of hydrolysis in plasma and the V_{max} of carboxylesterase hydrolysis, suggesting comparable catalytic mechanisms.

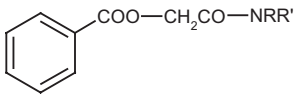
There has been an interest in recent years for *cyclodextrins as carriers for colon-targeting drugs*. Indeed, cyclodextrin conjugates are slowly hydrolyzable in media of moderately acidic pH, essentially stable at other pH values, and resistant to enzymatic hydrolysis. In contrast, they are fermented to small saccharides by the colon microflora. As a result of this behavior, they remain mostly intact and poorly absorbed in the stomach and intestine, only to be broken down to liberate the active drug in the colon. For example, [1,1′-biphenyl]-4-acetic acid was coupled to α-, β-, and γ-cyclodextrin through an ester bond [32][33]. All three prodrugs released [1,1′-biphenyl]-4-acetic acid when incubated with rat cecal and colonic contents, but not in the presence of rat stomach or intestine contents, intestinal or hepatic homogenates, or rat blood. This led to the suggestion that cyclodextrin could indeed be a valuable carrier for colon-specific delivery.

8.2.3. Carboxylic Acid Esters That Incorporate a Carbamoylmethyl, Aminoalkyl, or Amidoalkyl Pro-Moiety

In *Sect. 7.3* and *7.4*, we have emphasized that an amino group in an ester molecule contributes to increased water solubility and may, in some cases, facilitate enzymatic hydrolysis. In the present section, this argument is further illustrated and is extended to esters containing nonbasic nitrogenated pro-moieties, *e.g.*, amidoalkyl or carbamoylalkyl substituents. Substituents liable to undergo fragmentation (*i.e.*, pro-moieties that will break down during hydrolysis) will be discussed separately in *Sect. 8.3*.

8.2.3.1. *Carbamoylmethyl Esters*

Variously N-*substituted glycolamide esters* (*N*-substituted carbamoylmethyl esters) of carboxylic acids have been proposed as prodrugs of particular

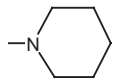


8.17

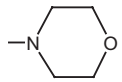
8.18 —NRR' =

8.19 —NRR' =

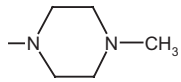
8.20 —NRR' =



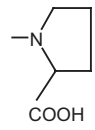
8.21 —NRR' =



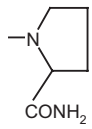
8.22 —NRR' =



8.23 —NRR' =



8.24 —NRR' =



interest with respect to water solubility, rate of enzymatic hydrolysis, and innocuity of the pro-moiety. Extensive investigations were reported for *glycolamides* (= 2-hydroxyacetamides) *of benzoic acid* (= carbamoylmethyl benzoates, **8.17**) as model prodrugs [34][35]. A compilation of a large number of such esters is given in *Table 8.2* together with rate constants for hydrolysis in human plasma. The original publications contain many other relevant data such as water solubility, rate constant of HO^--catalyzed hydrolysis, and K_m and V_{max} values.

It is obvious when examining *Table 8.2* that the hydrolysis in human plasma is generally very fast. For the carbamoylmethyl benzoates with neutral substituents, the $t_{1/2}$ values of hydrolysis ranged from *ca.* 5 s (!) to *ca.* 1 h, indicating that the variations in reactivity were strongly structure-dependent. *N*,*N*-Dialkyl-substituted derivatives underwent the fastest hydrolysis, whereas the *N*-monoalkyl-substituted derivatives had $t_{1/2}$ values of a few minutes. Polar *N*-substituents tended to slow hydrolysis, this being very marked with a carboxylate group ($t_{1/2}$ values in the order of hours or even days).

A very interesting comparison between methyl and (*N*,*N*-diethylcarbamoyl)methyl prodrug benzoates of *nonsteroidal anti-inflammatory agents*

Table 8.2. *Carbamoylmethyl Benzoates* (**8.17**) *as Model Prodrugs*. Hydrolysis in 50% human plasma at pH 7.4 and 37° [34][35].

R	R′	$t_{1/2}$ [min][a])
H	H	31
CH_3	H	9.3
CH_3CH_2	H	11.5
H_2NCOCH_2	H	25
$H_2NCOCH(CH_3)$	H	42.5
$(HOCH_2)_3C$	H	69.1
CH_3	CH_3	0.15
CH_3CH_2	CH_3CH_2	0.08
$CH_3CH_2CH_2$	$CH_3CH_2CH_2$	0.14
$(CH_3)_2CH$	$(CH_3)_2CH$	0.08
$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	3.1
$HOCH_2CH_2$	CH_3	0.20
H_2NCOCH_2	CH_3	0.13
$CH_3CH_2OCOCH_2$	CH_3	0.22
$HOCOCH_2$	CH_3	104
$(CH_3)_2NCH_2CH_2$	CH_3	0.12
$HOCH_2CH_2$	CH_3CH_2	0.16
$HOCH_2CH_2$	$HOCH_2CH_2$	0.42
$CH_3OCH_2CH_2$	$CH_3OCH_2CH_2$	0.25
8.18		0.83
8.19		5.7
8.20		2.5
8.21		4.9
8.22		12.7
8.23		>24 h
8.24		2.3

[a]) Total hydrolysis observed (chemical plus enzymatic).

(NSAIDs) has been reported [36]. As shown in *Table 8.3*, the hydrolysis of the methyl esters in human plasma proceeds far too slowly to be of therapeutic interest, with $t_{1/2}$ values ranging from *ca.* 5 to 150 h. In contrast, the carbamoylmethyl benzoates are hydrolyzed very rapidly (perhaps too rapidly?) with $t_{1/2}$ values in the order of minutes. But *Table 8.3* also illustrates another important point, namely the marked influence of the acyl moiety on the rate of hydrolysis, with a more than 500-fold difference between the smallest and the largest acid (salicylic acid and sulindac, respectively).

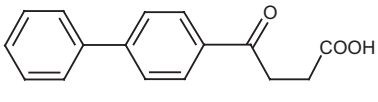
These studies have been extended and confirmed with *N*-monosubstituted and *N*,*N*-disubstituted carbamoylmethyl esters of *6-methoxy-2-naphthylacetic acid* [38]. This compound, a close analogue of naproxen (**8.26**), is the active metabolite of the anti-inflammatory agent *nabumetone*. The *N*-substituents investigated were mostly lower alkyl and oxygenated alkyl groups. The compounds were quite stable in buffer under physiological

Table 8.3. *Prodrug Esters of Nonsteroidal Anti-Inflammatory Acids.* Hydrolysis in 80% human plasma at pH 7.4 and 37° [36][37].

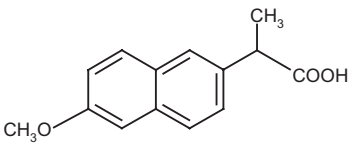
Drug	(*N*,*N*-Diethylcarbamoyl)methyl ester $t_{1/2}$ [min]	Methyl ester $t_{1/2}$ [h]
Aspirin	9	3.2 min[a])
Fenbufen (**8.25**)	3.8	4.7
Flurbiprofen	4.7	[b])
Ibuprofen (**8.28**)	4.0	
Indomethacin (**8.9**, R = OH)	25	150
Ketoprofen	0.5	>20
Naproxen (**8.26**)	0.6	20
Salicylic acid	0.05	17.6
Sulindac	26	
Tolfenamic acid (**8.27**)	5.0	100

[a]) In 10% human plasma at pH 7.4 and 37°. [b]) Not determined.

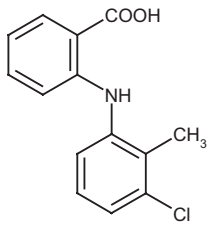
conditions of pH and temperature, with $t_{1/2}$ values ranging from 16 to 470 h. As above, enzymatic hydrolysis in human plasma was very fast, with the chemically more stable *N*,*N*-disubstituted carbamoylmethyl esters having $t_{1/2}$ values of 7 – 80 s, while the *N*-monosubstituted had $t_{1/2}$ values of 170–800 s.



8.25



8.26



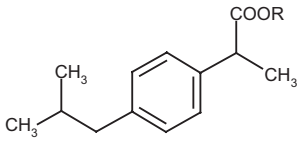
8.27

The case of *aspirin* in *Table 8.3* is of special interest. Indeed, its acetyl ester group is particularly labile to enzymatic and nonenzymatic hydrolysis (see *Sect. 7.4*), and the reaction is even faster when the carboxy group is neutralized by esterification. A true ester prodrug of acetylsalicylic acid must fulfill the condition that its hydrolysis liberates aspirin rather than a prodrug of salicylic acid. An investigation of several aspirin prodrugs confirmed the interest of carbamoylmethyl esters and showed the (*N*,*N*-diethylcarbamoyl)methyl ester (*Table 8.3*) to liberate the highest proportion (*ca.* 60%) of aspirin [37]. In

contrast, no aspirin was formed from simple alkyl or aryl esters. While such a prodrug may have some pharmaceutical advantages, its great lability casts doubts on any potential therapeutic advantage over the drug itself.

8.2.3.2. *Aminoalkyl Esters*

A comparison between carbamoylmethyl and aminoalkyl esters has been reported for a small series of prodrugs of *ibuprofen* (**8.28**) [39]. A further interest of this work is the systematic comparison made between the enantiomers of ibuprofen, *i.e.*, the active (+)-(*S*)-form and the inactive (–)-(*R*)-form. As shown in *Table 8.4*, the ethyl esters were hydrolyzed very slowly in human plasma, with $t_{1/2}$ values in the order of 100 – 200 h. In contrast, the hydrolysis of the carbamoylmethyl and aminoalkyl esters occurred with $t_{1/2}$ values in the order of some or several minutes. The carbamoylmethyl esters were hydrolyzed very rapidly with low substrate enantioselectivity, while the 2-(imidazol-1-yl)ethyl esters were hydrolyzed somewhat more slowly and with marked substrate enantioselectivity.



8.28 R = —H

8.29 R = —CH_2CH_2—N

8.30 R = —CH_2CH_2—N

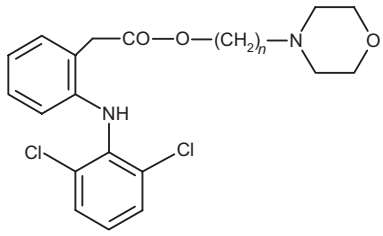
A well-designed series of *N*,*N*-disubstituted 2-aminoethyl and 3-aminopropyl esters of *indomethacin* (**8.9**, R = OH) was examined for its chemical and enzymatic (human plasma) rates of hydrolysis [40]. The 3-(diethylamino)propyl ester had a transcutaneous permeability coefficient *ca.* 100-times

Table 8.4. *Prodrug Esters of the Enantiomers of Ibuprofen* (**8.28**). Hydrolysis in 80% human plasma at pH 7.4 and 37° [39].

R	(*S*)-Enantiomer $t_{1/2}$ [min]	(*R*)-Enantiomer $t_{1/2}$ [min]
CH_3CH_2	5580	15900
$(CH_3)_2NCOCH_2$	1.5	7.0
$(CH_3CH_2)_2NCOCH_2$	1.0	1.4
$(CH_3)_2NCH_2CH_2$	1.7	2.6
8.29	19	960
8.30	5.2	135

greater than that of indomethacin itself, indicating the potential of such prodrugs for transdermal penetration.

Morpholinoalkyl esters appear to be valuable prodrugs of carboxylic acids, being characterized by good lipophilicity and fast rates of enzymatic hydrolysis. Here again, particular efforts have been made to improve absorption and decrease side effects of nonsteroidal anti-inflammatory agents. Thus, three homologous *esters of diclofenac* (**8.31**, $n = 2$, 3, or 4) were examined for their physicochemical properties, rates of chemical and enzymatic hydrolysis, bioavailability and irritancy in rats [41]. At 37°, the three compounds were relatively stable in neutral buffer ($t_{1/2}$ *ca.* 3, 14, and 34 h, respectively) and in simulated gastric fluid of pH 1.3 ($t_{1/2}$ *ca.* 73, 40, and 33 h, respectively). In rat plasma, however, the $t_{1/2}$ values for hydrolysis were *ca.* 5, 23, and 14 min, respectively. The extent, but not the rate, of oral absorption of the 2-(morpholino)ethyl ester (**8.31**, $n = 2$) was significantly greater than that of unesterified diclofenac. Following single and repeated oral administration, gastric irritation was significantly less after the prodrug.



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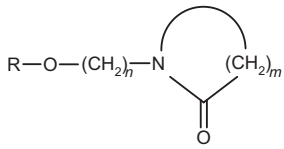


8.32

A comparable approach was used to design (piperazine-1-yl)alkyl prodrugs of naproxen (**8.26**), some of which improved severalfold the skin permeability of the drug [42].

8.2.3.3. *Amidoalkyl Esters*

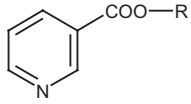
Examples of amidoalkyl esters are provided by *1-alkyl-1-azacycloalkan-2-ones*, *i.e.*, esters with a lactam ring at the end of an alkyl chain (**8.32**, $n = 1$ or 2; $m = 3$, 4, 5, or 6). Amidoalkyl esters of indomethacin (**8.9**, R = OH) and naproxen (**8.26**) have been described [43]. The prodrugs in the series $n = 1$ underwent facile chemical hydrolysis, but, since they may liberate formaldehyde, they belong to the fragmentable amidomethyl derivatives discussed in *Sect. 8.3.3*. In the series $n = 2$ (**8.32**), high stability was seen ($t_{1/2}$ 3 – 20 d). No obvious relation exists between the size of the lactam ring and

the rate of chemical hydrolysis. In contrast to their high chemical stability, the prodrugs in the series $n = 2$ were good substrates of pig liver esterase, with $t_{1/2}$ values of reaction of *ca.* 2 – 5 h under the study conditions. When compared to indomethacin and naproxen, the prodrugs in the series $n = 2$ displayed good permeability across excised human skin, with a dependence on lipophilicity. Some *N*-acyllactam esters of indomethacin, *i.e.*, *N*-acyl analogues of prodrugs **8.32**, also exhibited moderately enhanced *in vitro* skin permeation [44].

In summary, the various examples presented in this section illustrate the diversity of molecular factors that influence the many physicochemical and biochemical properties of significance in prodrug design. Many items of information are reported in this section, but the partial and fragmentary character hinders comprehensive understanding. Whether and how much such information can be rationalized is discussed in the next section.

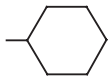
8.2.4. Quantitative Structure–Metabolism Relationships in Prodrug Design

The development of quantitative structure–metabolism relationships prodrug studies will be illustrated here with a series of *esters of nicotinic acid* (**8.33**). This simple molecule displays a range of interesting pharmacological effects such as vasodilation, fibrinolysis, and reduction of high blood levels of triglycerides, cholesterol, and lipoproteins associated with atherosclerosis. Rapid elimination (mainly by biotransformation), a short half-life (20 – 45 min), the necessity for high doses (2 – 8 g/d), and the occurrence of side effects have limited the use of nicotinic acid. In contrast, its bioprecursor β-pyridylcarbinol and some esters (*e.g.*, inositol hexanicotinate, sorbitol nicotinate, and tocopherol nicotinate) have therapeutic utility.

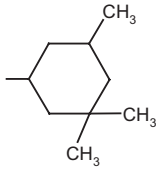


8.33 R = —H

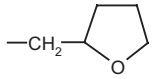
8.34 R =



8.35 R =



8.36 R =



An examination of some of the factors influencing the chemical and enzymatic hydrolysis of nicotinic acid esters was undertaken [45 – 48]. Twenty-five esters of nicotinic acid were prepared whose pro-moiety was a simple alkyl (unbranched, *sec-,* or *tert*-alkyl), a chloroalkyl, a hydroxyalkyl, an alkoxyalkyl, a carbamoylmethyl, an aminoalkyl, a cycloalkyl, an arylalkyl, or an aryl group (*Table 8.5*).

The chemical hydrolysis of the 25 nicotinates at pH 7.4 and 37° followed pseudo-first-order kinetics, with the rate constant k (in min^{-1}) ranging from $0.08 \cdot 10^{-4}$ ($t_{1/2}$ 1390 h) to $41.1 \cdot 10^{-4}$ ($t_{1/2}$ 2.8 h; *Table 8.5*). Four compounds, which, interestingly, were the least soluble, appeared highly resistant to hydrolysis under the conditions of study. Good correlation between the rate constant of hydrolysis (expressed as log k) and two electronic parameters is notable. Thus, *Taft*'s parameter σ^* (a measure of polarity) was available for eleven esters and gave the following regression equation (*Eqn. 8.1*):

$$\log k = 0.47\ (\pm\ 0.11)\ \sigma^* - 0.74\ (\pm\ 0.08)$$
$$n = 11;\ r^2 = 0.91;\ s = 0.088;\ F = 95 \qquad (8.1)$$

In this and later regression equations, the 95% confidence limits are given in parentheses. *Eqn. 8.1* quantitates the facilitation of hydrolysis by electron-withdrawing substituents. Because such substituents decrease the electron density on the carbonyl C-atom and render it more susceptible to nucleophilic attack, *Eqn. 8.1* is compatible with a base-catalyzed reaction, as indeed shown. *Eqn. 8.1*, thus, leads to mechanistic insights, but its predictive power is narrow since the σ^* parameter is available for only a few substituents.

Such a restriction does not exist for a readily accessible experimental parameter such as the chemical shift of the carbonyl C-atom ($\Delta\delta$). This parameter, as measured by ^{13}C-NMR, expresses a complex mixture of electronic and steric effects, some of which may not be relevant to the mechanism of hydrolysis. Its correlation with log k is not as good as that of *Taft*'s polarity parameter, yet it is of sufficient quality and includes enough variation between substituents to have fair predictive value (*Eqn. 8.2*):

$$\log k = +\ 0.76\ (\pm\ 0.23)\ \Delta\delta - 5.0\ (\pm\ 0.3)$$
$$n = 19;\ r^2 = 0.73;\ s = 0.34;\ \mathrm{F} = 47 \qquad (8.2)$$

In contrast to the σ^* and $\Delta\delta$ parameters, log k was not related to lipophilicity, despite the unreactivity of the poorly water-soluble esters.

Table 8.5 also lists some of the results obtained for the enzymatic hydrolysis of the nicotinates [45a]. Other results not discussed here include hydrolysis by rat liver and rat brain microsomes, cytosol, and mitochondria, as well as binding to and hydrolysis by human serum albumin [45b][46][47]. The

Table 8.5. *Chemical and Enzymatic Hydrolysis of Ester Prodrugs of Nicotinic Acid* (**8.33**) *at pH 7.4 and 37°* [45][48]

R	Buffer	Carboxylesterase[b])		Human plasma[b])[c])	
	$t_{1/2}$ [min][a])	K_m [mM][d])	V_{max} [mmol (mg protein)$^{-1}$ min^{-1}][e])	K_m [mM][f])	V_{max} [μmol (mg protein)$^{-1}$ min^{-1}][g])
CH_3	295	0.70	24.0	78.0	46.0
CH_3CH_2	760	0.35	18.0	5.4	2.2
$CH_3CH_2CH_2$	735	0.35	30.0	3.5	16.5
$(CH_3)_2CH$	1390	0.75	98.0	2.2	0.20
$CH_3CH_2CH_2CH_2$	785	0.63	71.0	2.2	39.0
$(CH_3)_2CHCH_2$	875	2.4	120	0.88	11.5
$(CH_3)_3C$	135	0.20	0.020	[i])	
$CH_3(CH_2)_5$	[h])	1.1	44.0	2.4	4.2
$CH_3(CH_2)_7$	[h])	0.050	420	7.3	17.0
$ClCH_2CH_2$	185	2.0	70.0	2.2	13.0
$HOCH_2CH_2$	305	4.6	55.0	9.8	10.3
$HOCH_2CH_2CH_2$	410	5.4	21.0	7.0	15.5
$CH_3OCH_2CH_2$	330	1.2	42.0	5.9	44.0
$CH_3(CH_2)_3OCH_2CH_2$	245	1.5	87.0	2.0	150
H_2NCOCH_2	125	5.3	1.9	6.1	19.0
$H_2NCOCH(CH_3)$	105	1.6	3.5	[i])	
$(CH_3)_2NCH_2CH_2$	2.8	12.5	1.4	4.0	2400
$H_2NCH_2CH_2CH_2$	94	6.3	0.050	1.5	45.0
8.34	[h])	1.5	12.0	6.7	9.5
8.35	[h])	0.080	0.60	17.0	0.06
8.36	385	0.63	110	1.4	145
$PhCH_2$	225	1.4	90.0	1.6	46.0
$PhOCH_2CH_2$	325	1.8	140	0.45	27.5
4-Cl–C_6H_4	17.0	0.16	53.0	0.73	4.4
4-NO_2–C_6H_4	2.9	0.006	1.4	0.47	5.9

[a]) SD 4 – 26%. [b]) Enzymatic hydrolysis, *i.e.*, chemical hydrolysis subtracted from total hydrolysis observed. [c]) When [S] ≪ K_m, $t_{1/2} = 0.693/(V_{max}/K_m)$. [d]) SD 6 – 33%. [e]) SD 1 – 45%. [f]) SD 10 – 35%. [g]) SD 3 – 30%. [h]) No quantifiable hydrolysis (< 2% in 5 weeks). [i]) Too slow to allow determination of K_m and V_{max}.

prodrugs showed a 2000-fold range in affinity, and a 7000-fold range in maximal velocity toward purified carboxylesterase. As judged by the V_{max}/K_m parameter, the best substrates were the following esters: octyl ≫ 4-chlorophenyl > 4-nitrophenyl > tetrahydrofurfuryl > i-Pr > Bu. No correlation was found for V_{max} and V_{max}/K_m values (which express high-energy catalytic processes of bond cleavage and bond formation), presumably because these biological activities are influenced by structural and electronic properties not described by the investigated parameters.

In contrast to V_{max}, the K_m parameter (which describes the low-energy process of binding to the catalytic site) was correlated with various structu-

ral and physicochemical descriptors containing information on intermolecular interactions. These descriptors are MR (molar refractivity, a descriptor of substituent size), log k_w^o (log of the capacity factor in reversed-phase HPLC, a measure of the lipophilicity of the neutral compound), $\Delta\delta$ (chemical shift of the carbonyl C-atom as measured by ^{13}C-NMR), and B_5, the maximal width of the substituent (one of the geometric dimensions, a measure of steric hindrance).

The best correlation equations obtained for the K_m values in the presence of carboxylesterase (CE) or human plasma (HP) are given below as *Eqns. 8.3* and *8.4*, respectively. The statistical quality of the equations can be assessed by r^2, the squared correlation coefficient, and q^2, the cross-validated correlation coefficient (a measure of the predictive power of the equation, which is considered as acceptable when $q^2 > 0.4$). Both equations are statistically sound and have acceptable predictive power.

$$\begin{aligned} pK_{m(CE)} = &+ 0.002\,(\pm 0.001)\,(MR)^2 - 0.14(\pm 0.09)\,MR \\ &+ 0.51\,(\pm 0.23)\log k_w^o + 0.30\,(\pm 0.10)\,\Delta\delta^2 + 3.6\,(\pm 1.1) \\ &n = 24;\ r^2 = 0.78;\ q^2 = 0.60;\ s = 0.38;\ F = 16.9 \end{aligned} \tag{8.3}$$

Eqn. 8.3 indicates that the binding of nicotinates to the catalytic site of carboxylesterase depends first and mainly on substituent bulk (optimal MR = 35, which corresponds to a quite large substituent such as heptyl or 2-phenoxyethyl). In addition, affinity increases with increasing lipophilicity. The influence of $\Delta\delta$ appears complex and difficult to interpret, perhaps suggesting that the interaction of the carbonyl C-atom with the catalytic OH group contributes to affinity.

$$\begin{aligned} pK_{m(HP)} = &- 0.24\,(\pm 0.10)\,(\log k_w^o)^2 + 1.1\,(\pm 0.5)\log k_w^o + 0.18\,(\pm 0.12)\,B_5 \\ &+ 0.13\,(\pm 0.08)\,\Delta\delta^2 + 0.64\,(\pm 0.70) \\ &n = 22;\ r^2 = 0.69;\ q^2 = 0.50;\ s = 0.32;\ F = 9.5 \end{aligned} \tag{8.4}$$

The interaction of nicotinates with human plasma hydrolases (*Eqn. 8.4*) is determined principally by their lipophilicity (optimal $\log k_w^o = 2.3$). Affinity also increases with increasing width of the substituent, and shows the same complex dependence on $\Delta\delta$ as in *Eqn. 8.3*.

In summary, the above discussion illustrates how metabolic data for large series of analogous compounds may be amenable to quantitative structure–metabolism relationships. In ideal cases, such regression equations may even have some predictive power and can lead to mechanistic insights.

8.2.5. Carboxylic Acid Esters of Acylglycerols and Bis(acylamino)propan-2-ols

A number of recent and older studies have examined the potential of glyceryl esters to increase intestinal absorption, to target the lymphatic route or such organs as the heart or liver, and to deliver neuroactive acids to the brain [49]. However, the results are difficult to rationalize at present, as a given pro-moiety gives promising results with one drug and is disappointing with another, depending on both the drug and the biological system. In many cases, the utility of a brain-targeted prodrug is examined by comparing its *in vivo* CNS activity with that of the drug itself, a global assessment that offers little mechanistic understanding and is not always easy to extrapolate to humans [50].

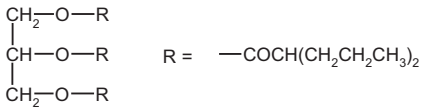
The prodrugs to be discussed in this section are:

- Hybrid triglycerides ($RCOOCH_2–CH(OCOR')–CH_2OCOR''$), *i.e.*, containing zero, one, or two natural fatty acyl substituents, and three, two, or one active carboxylate moieties;
- Hybrid pseudolipid analogues such as 1,3-bis(acylamino)prop-2-yl esters ($RCONHCH_2–CH(OCOR')–CH_2NHCOR$), where R is a fatty acyl substituent and OCOR′ is the active carboxylate moiety.

8.2.5.1. *Hybrid Triglycerides*

The first hybrid triglyceride to be discussed is *glyceryl trivalproate* (**8.37**), a triple prodrug of the anti-epileptic agent *valproic acid*. This compound was found to be inactive, as were 1,3-dipalmitoyl-2-valproylglycerol (see below) and 1,2-dipalmitoyl-3-valproylglycerol [51][52]. In contrast, promising results were reported for two prodrugs of *GABA* (γ-aminobutyric acid, a neurotransmitter that exhibits anticonvulsant effects). Indeed, the 2,3-dilinolenoyl-1-glyceryl GABA monoester (**8.38**) and the corresponding 3-linolenoyl-1,2-glyceryl GABA diester demonstrated high brain penetration and selectivity, and CNS depressant activity [50][53]. The triacylglyceryl lipid **8.39** was even more interesting, being a *mutual prodrug* of both GABA and γ-vinyl-GABA, a specific and irreversibly inhibitor of GABA-transaminase (GABA-T). This double prodrug had a good brain penetration, and *in vivo* had a 300-fold higher activity than γ-vinyl-GABA [54].

A few interesting *glyceride prodrugs of peptides* can be found in the literature. For example, the pentapeptide renin inhibitor isovaleryl-Phe-Nle-Sta-Ala-Sta was derivatized to the 1,3-dipalmitoyl-2-glyceryl ester (**8.40**) in the hope of improving oral bioavailability [55]. The *in vitro* results were



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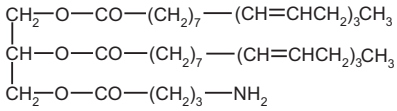

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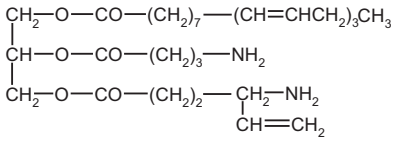

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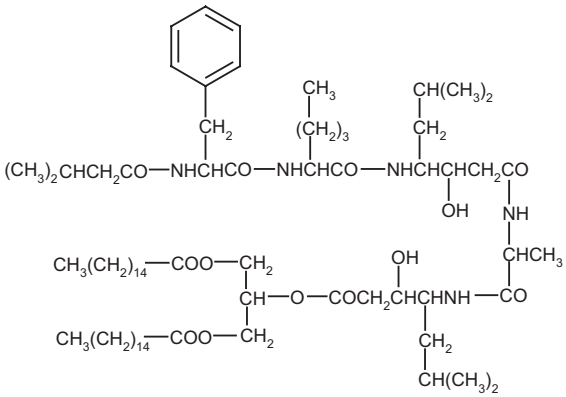


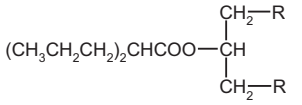
8.40

promising and showed in particular that when properly solubilized, this prodrug is a substrate of pancreatic lipase. However, *in vivo* studies in rats failed to demonstrate the expected enteral and lymphatic absorption after oral administration.

8.2.5.2. *Hybrid Pseudolipid Analogues*

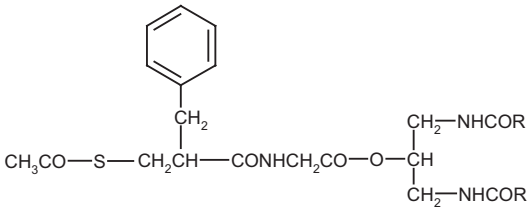
In an attempt to improve the pharmacokinetic properties of hybrid triglyceride prodrugs, a number of hybrid pseudolipid analogues were prepared with the glyceryl moiety replaced by a *1,3-diaminopropyl moiety*. As

mentioned above, hydride triglycerides of valproic acid, *e.g.*, 1,3-dipalmitoyl-2-valproylglycerol (**8.41**), proved disappointing. In contrast, its isostere 1,3-bis(palmitoylamino)-2-(valproyloxy)propane (**8.42**) was far more active than valproic acid as an anticonvulsant in mice, and its peak activity was considerably delayed [51]. That this pseudolipid prodrug was only 2 – 3 times less active after oral than after *i.p.* administration suggests good bioavailability.



8.41 R = $-OCO-(CH_2)_{14}CH_3$

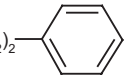
8.42 R = $-NHCO-(CH_2)_{14}CH_3$

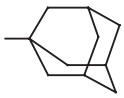


8.43 R = $-(CH_2)_{14}CH_3$

8.44 R = $-(CH_2)_8CH_3$

8.45 R = 

8.46 R = 

8.47 R = 

Following this promising result, other drugs were coupled to 1,3-(diacylamino)propan-2-ol, and a number of acyl groups were used. One example is that of glycine prodrugs, which, when administered *i.p.* to mice, were effective anticonvulsants in a test where glycine itself was inactive [56]. Another example is provided by S-*acetylthiorphan*, an enkephalinase inhibitor of interest as an antinociceptive agent. The prodrugs of structure **8.43** – **8.47** all exhibited a significant antinociceptive activity superior to that of the parent compound. The derivatives with cyclic side chains (**8.45** – **8.47**) were more bioavailable and somewhat more active than those containing linear acyl groups (**8.43** and **8.44**) [57]; however, they were also more toxic. In fact, de-

rivatives with cyclic side chains demonstrated unexpected pharmacodynamic effects caused by the pro-moiety itself, which may limit their usefulness [58].

8.3. Prodrugs of Active Carboxylic Acids That Incorporate a Fragmentable Pro-Moiety

This section discusses prodrugs whose hydrolysis (be it chemical and/or enzymatic) is followed by breakdown of the pro-moiety with liberation of a carbonyl compound. In the glycolic acid (= hydroxyacetic acid) esters briefly examined in *Sect. 8.3.1*, the pro-moiety once cleaved breaks down enzymatically. In the rest of the section, however, the cleaved pro-moiety will be seen to break down spontaneously.

8.3.1. Glycolic Acid Esters

Active carboxylic acids esterified with a *monoester of glycolic acid* (**8.48**, *Fig. 8.1*) may represent a promising, but as yet poorly explored, approach to prodrugs with controllable properties. As shown in *Fig. 8.1*, such prodrugs can undergo hydrolysis at the two ester bridges. *Reaction a* yields the drug and the glycolic acid monoester carrier. In contrast, *Reaction b* must be considered an unwanted side reaction since it produces an acidic prodrug whose hydrolysis to the drug and glycolic acid (*Fig. 8.1*, *Reaction c*) is predictably slow (see *Chapt. 7*).

Interesting data are available for three *glycolic acid esters of benzoic acid*, namely methyl, ethyl, and benzyl *O*-benzoylglycolate (**8.48**, R = Ph,

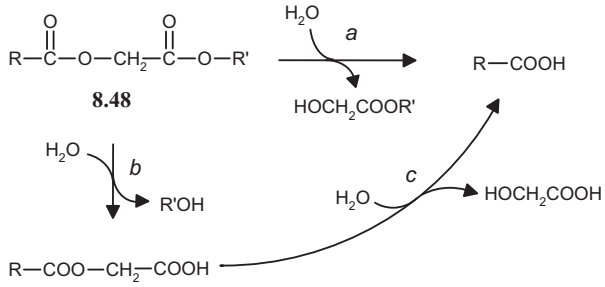
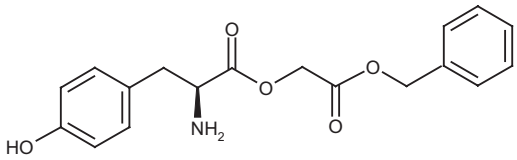


Fig. 8.1. *Hydrolytic pathways in the activation of glycolic acid esters as prodrugs of active carboxylic acids. Reaction a* is the desirable, direct activation, whereas *Reaction b* must be seen as parasitic in view of the relative slowness of *Reaction c*.

R′ = Me, Et, and $PhCH_2$, respectively; *Fig. 8.1*). In 80% human plasma at pH 7.4 and 37°, these model prodrugs were hydrolyzed with $t_{1/2}$ values of 3.5, 16, and 2.6 min, respectively [59]. Such rates of enzymatic hydrolysis are comparable to those of various carbamoylmethyl esters of benzoic acid (*Table 8.2*). It is important to note that the direct liberation of benzoic acid by *Reaction a* (*Fig. 8.1*) was severalfold faster than the competitive *Reaction b*. *Reaction c* was very slow in human plasma ($t_{1/2} > 100$ h). In HO^--catalyzed hydrolysis, the opposite regioselectivity was seen, with the terminal ester bridge being cleaved markedly faster than the central one. No data appears to be available on chemical hydrolysis at neutral pH.

The L-tyrosine ester of benzyl glycolate (**8.49**) is another relevant example. Here, chemical hydrolysis was investigated at pH 7.4, with the central ester bridge being cleaved approximately six times faster than the terminal ester [60]. This regioselectivity was attributed to intramolecular catalysis by the protonated amino group since, at higher pH, the terminal ester bridge was hydrolyzed faster as also observed for benzoyl esters (see above). In 80% human plasma at pH 7.4 and 37°, the $t_{1/2}$ value was 6 min, and cleavage of the central ester bridge to liberate L-tyrosine was the predominant reaction.

Compared to the carbamoylmethyl esters discussed in *Sect. 8.2.3*, glycolic acid esters appear somewhat disadvantageous because of the possibility of a parasitic reaction. The fact that the chemical hydrolysis of glycolic acid esters is faster while their enzymatic hydrolysis in human plasma is slower may not necessarily be detrimental.



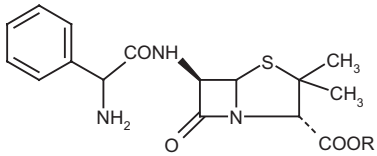
8.49

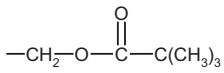
8.3.2. Prodrugs That Incorporate an (Acyloxy)alkyl or [(Alkoxycarbonyl)oxy]methyl Pro-Moiety

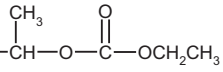
8.3.2.1. *(Acyloxy)alkyl Esters*

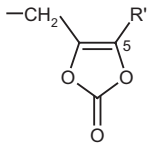
A few well-established prodrugs contain an (acyloxy)methyl or an [(alkoxycarbonyl)oxy]methyl group. Classical examples of the former are two prodrugs of β-lactam antibiotics, namely *pivampicillin* (**8.50**), the (pivaloyloxy)methyl ester of ampicillin, and *cefuroxime axetil* (**8.51**), the

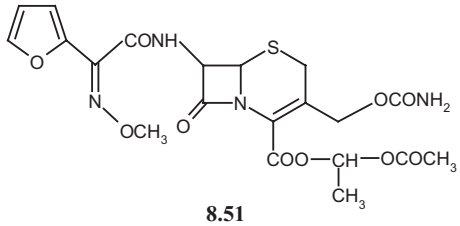
1-acetoxyethyl ester of cefuroxime. An example of an [(alkoxycarbonyl)oxy]-methyl prodrug (more precisely a 1-[(alkoxycarbonyl)oxy]alkyl prodrug) is afforded by *bacampicillin* (**8.52**), the 1-[(ethoxycarbonyl)oxy]ethyl ester prodrug of ampicillin [61 – 63].



8.50 R = 

8.52 R = 

8.63 R = 



8.51

The hydrolytic pathways by which prodrugs of these types are activated can be found in *Fig. 8.2*. Depending on whether the –CH(R′)– bridge is unbranched (R′ = H in *Fig. 8.2*) or branched (usually a 1-ethyl group, *i.e.*, R′ = Me in *Fig. 8.2*), hydrolysis will liberate *formaldehyde* or *acetaldehyde*, two aldehydes of very different toxicities [62]. Thus, while pivampicillin is known to be more bioavailable than ampicillin itself, the formaldehyde it generates may account for some side effects of the prodrug. This is certainly the reason why a 1-ethyl bridge should be preferred by medicinal chemists over a $–CH_2–$ bridge, despite the somewhat greater synthetic difficulties.

A potentially interesting application of (acyloxy)methyl ester prodrugs can be found in delivery through skin of antibacterial agents. *Nalidixic acid* (**8.53**, R = H), a topoisomerase II inhibitor, showed promising activity in the treatment of antiproliferative skin disorders, notably psoriasis. To improve the

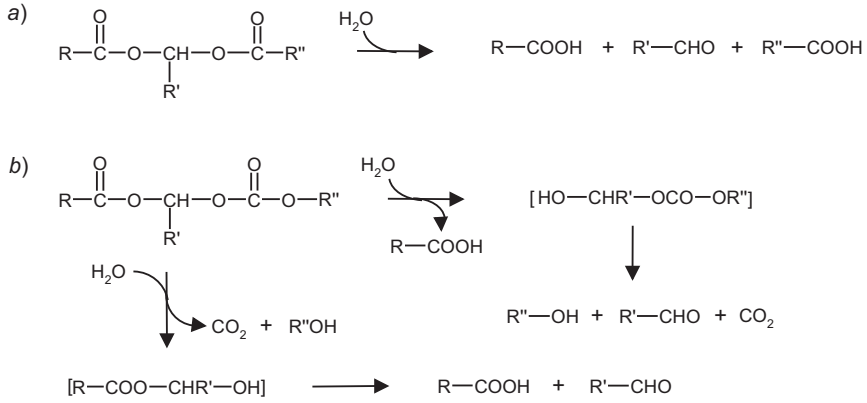
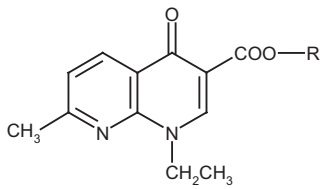


Fig. 8.2. *Hydrolytic pathways in the activation of prodrugs of carboxylic acids that contain* a) *an (acyloxy)methyl or* b) *an [(alkoxycarbonyl)oxy]methyl group*. Liberation of the active acid occurs with comparable rates whatever the initial site of hydrolytic attack is. Formaldehyde or acetaldehyde is liberated when R′ = H or Me, respectively.

skin permeation of topically administered nalidixic acid, a small number of prodrugs were prepared, all of which were highly resistant to chemical hydrolysis (*Table 8.6*) [64]. Furthermore, the methyl ester and the (*N*,*N*-diethylcarbamoyl)methyl ester were very poor substrates of plasma enzymes, whereas the four (acyloxy)methyl esters were readily hydrolyzed to nalidixic acid.



8.53

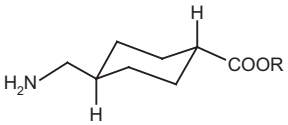
Table 8.6. *Chemical and Enzymatic Hydrolysis of Ester Prodrugs of Nalidixic Acid* (**8.53**) *at pH 7.4 and 37°* [64]

R	Buffer $t_{1/2}$ [h]	80% Human plasma $t_{1/2}$ [min]
CH_3	3100	[a])
$(CH_3CH_2)_2NCOCH_2$	3600	[a])
$CH_3CO–OCH_2$	87	18.2
$CH_3CH_2CO–OCH_2$	89	8.9
$CH_3CH_2CH_2CO–OCH_2$	114	7.8
$(CH_3)_2CHCO–OCH_2$	130	15.5

[a]) Not measurable (< 5% hydrolysis in 24 h).

Since the use of human plasma as a model for skin esterases is debatable, the relevance of the biological results in *Table 8.6* need validation as far as skin delivery is concerned. Indeed, permeation experiments across excised human skin showed that administration of prodrugs markedly improved the cutaneous delivery of nalidixic acid, with complete hydrolysis of the (acyloxy)methyl esters and significant, albeit incomplete, hydrolysis of the methyl and carbamoylmethyl esters [64].

Another informative example is provided by prodrugs of the antifibrinolytic agent *tranexamic acid* (**8.54**, R = H). This highly polar compound exists as a zwitterion at physiological pH and is incompletely absorbed from the gastrointestinal tract. A comparison of some (acyloxy)methyl and [(alkoxycarbonyl)oxy]methyl esters is given in *Table 8.7* [65]. These derivatives were quite stable in neutral buffer ($t_{1/2}$ in the order of a few days) but underwent facile hydrolysis in human plasma ($t_{1/2}$ of a few minutes). Both chemical and enzymatic hydrolyses were much faster than those of the corresponding ethyl ester. Replacing the $-CH_2-$ bridge with a 1-ethyl bridge slightly decreased reactivity. Similarly, steric hindrance at the terminal acyl or alkoxy group modulated the rate of hydrolysis, but the number of observations was too limited to permit meaningful conclusions. All prodrugs in *Table 8.7* showed greatly improved gastrointestinal absorption compared to the drug itself, as assessed by the percent of tranexamic acid recovered in rat urine after oral administration.



8.54

Table 8.7. *Chemical and Enzymatic Hydrolysis of Ester Prodrugs of Tranexamic Acid* (**8.54**) *at 37°* [65]

R	Buffer pH 7.5 $t_{1/2}$ [h]	90% Human plasma[a]) $t_{1/2}$ [min]
CH_3CH_2	[b])	240[c])
$CH_3CO–OCH(CH_3)$	68	6 – 12
$(CH_3)_2CHCO–OCH_2$	36	0.5
$(CH_3)_2CHCO–OCH(CH_3)$	140	[d])
$(CH_3)_3CCO–OCH_2$	147	2 – 9
$CH_3CH_2OCO–OCH(CH_3)$	90	1.5
$(CH_3)_2CHOCO–OCH(CH_3)$	144	[d])

[a]) Unbuffered. [b]) Very slow hydrolysis, 88% intact after 21 d. [c]) Human blood. [d]) Not determined.

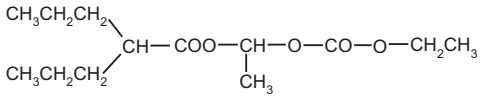
(Acyloxy)alkyl esters have also been examined as dermal prodrugs of anti-inflammatory agents, for example acetoxyalkyl esters of *naproxen* (**8.26**) and *ketoprofen* having the general structure R–COO–$(CH_2)_n$–$OCOCH_3$ with $n = 1 - 4$ [66]. These compounds are of particular interest given the variable length of the spacer between the two ester groups, allowing the hydrolytic behavior of (acyloxy)alkyl esters to be better understood. In phosphate buffer of pH 7.4 at 37°, the rate of hydrolysis decreased strongly in the homologous series, with $t_{1/2}$ values of 20 and 1900 h, respectively, for the acetoxymethyl and acetoxybutyl esters of naproxen. Hydrolysis in human serum was much faster, with $t_{1/2}$ values of 33, 82, 137, and 68 min for the four homologous esters of naproxen. The same trends and comparable values were shown by the ketoprofen derivatives.

This study is of further interest since it not only reports the half-life for disappearance of the prodrugs in human serum (*i.e.*, the result of *Reactions a* and *b* in *Fig. 8.1*), as presented above, but also compares it to the time needed for 50% of the drug to be formed in human serum (*i.e.*, the result of *Reactions a*, *b* and *c* in *Fig. 8.1*). The latter values were equal to, or slightly slower (up to threefold) than, the $t_{1/2}$ values for disappearance.

8.3.2.2. *[(Alkoxycarbonyl)oxy]methyl Esters*

Some interesting examples of 1-[(ethoxycarbonyl)oxy]ethyl prodrugs have been reported in the preparation of *valdice* (**8.55**), a prodrug of valproic acid. In an extensive pharmacokinetic investigation, this compound was compared with two commercially available enteric coated formulations of valproic acid [67]. In humans and dogs, biotransformation was complete, as no prodrug was detected in plasma following oral administration. Bioavailability was essentially 100% in humans, like that of valproic acid itself. In fact, the only advantage of the prodrug over enteric coated tablets of valproic acid was its slower absorption rate, which, however, was not as slow as that of sustained-release formulations of the drug. Thus, the therapeutic gain offered by this prodrug compared to valproic acid appears to be modest.

Ibuprofen (**8.28**) and naproxen (**8.26**) were derivatized with the same 1-[(ethoxycarbonyl)oxy]ethyl pro-moiety to yield two prodrugs designated as



8.55

ibudice and *napdice* [68]. When examined in dogs, the two prodrugs had pharmacokinetic profiles practically identical to that of the parent drugs. No sustained-release effect or pharmacodynamic advantage was noted.

8.3.3. Prodrugs That Incorporate an Amidomethyl, an Aminomethyl, or Another Fragmentable Pro-Moiety That Contains an Amino Group

In *Sect. 8.2.3*, we have discussed 1-alkyl-1-azacycloalkan-2-ones (**8.32**, $n = 1$ or 2; $m = 3$, 4, 5 or 6) as a group of *amidoalkyl esters*, taking prodrugs of indomethacin (**8.9**, R = OH) and naproxen (**8.26**) as examples [43a,b]. For both compounds, the prodrugs in the series $n = 1$ were rapidly hydrolyzed ($t_{1/2}$ *ca.* 0.5 – 1 h in pH 7.4 buffer at 32°). Furthermore, the carbinolamides liberated were not fully stable and broke down by hydrolysis to produce the toxic formaldehyde (*Fig. 8.3*) [62]. Thus, the half-life of decomposition of *N*-(hydroxymethyl)benzamide ($HOCH_2$–NH–CO–C_6H_5), a pro-moiety of **8.56** discussed below, was 160 h at pH 7.4 and 37° [69]. This means that a small amount of formaldehyde may be formed *in vivo*.

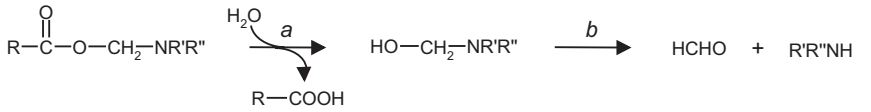
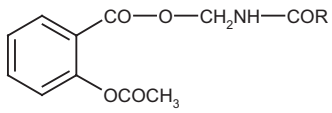


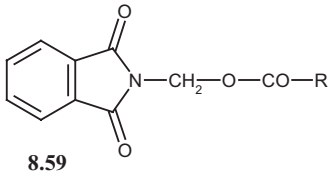
Fig. 8.3. *Hydrolytic reactions in the activation of prodrugs of carboxylic acids that incorporate an aminomethyl* (R′ = H or alkyl, R″ = H or alkyl) *or amidomethyl group* (R′ = acyl, R″ = H or alkyl). *Reaction a* (chemical and/or enzymatic) liberates the drug RCOOH and a carbinolamine or carbinolamide, which then breaks down to formaldehyde and an amine or amide (*Reaction b*).

The relatively rapid chemical hydrolysis, combined with the potential production of formaldehyde, is the main reason that prodrugs of this type are seldom investigated. One interesting exception is a group of *aspirin prodrugs* that incorporate an amidomethyl group (**8.56**). In contrast to the carbamoylmethyl esters discussed in *Sect. 8.2.3* and *Table 8.3*, the esters **8.56** liberated practically 100% aspirin due to the extreme lability of the amidomethyl ester moiety [69]. Indeed, the $t_{1/2}$ values for nonenzymatic degradation of the acetamide and benzamide derivatives at pH 7.4 and 37° (**8.56**, R = Me and Ph, respectively) were 1.1 min and 8 s, respectively. This is markedly faster than the enzymatic hydrolysis of the acetyl ester moiety in human plasma and explains the selective formation of aspirin. But, as discussed in *Sect. 8.2.3*, the advantages of such labile prodrugs are not always obvious.



8.56

8.57 **8.58**



8.59

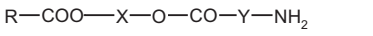
More recently, systematic studies have shown the interest of the carrier **8.58** derived from *ethyl hippurate* [70][71]. The corresponding prodrugs **8.57** were prepared for, *e.g.*, penicillin G, dicloxacillin, valproic acid, clofibric acid, ibuprofen, diclofenac, and naproxen. These prodrugs showed a U-shaped pH–rate profile of chemical hydrolysis, with a plateau of greatest stability in the unusually broad region of pH 3 – 8 or even 3 – 11 (pH-independent region of hydrolysis). There is considerable variability in rates of both chemical and enzymatic hydrolysis. At pH 7.4 and 37°, the $t_{1/2}$ values for chemical hydrolysis ranged from 25 to 4900 min; the corresponding $t_{1/2}$ values in 80% plasma were smaller by a factor of 1.1 – 32. However, some rationalization is possible, since the chemical stability of these esters is mostly dependent on the pK_a of the carboxylate leaving group. The esters of carboxylic agents with p$K_a > 4$ were very stable in aqueous buffer, whereas those with p$K_a < 4$ were the least stable ones. As for the rates of hydrolysis in plasma, they showed a tendency to increase with increasing lipophilicity of the substrate.

Of interest is also that the rate of liberation of penicillin G from the prodrug was much faster than the breakdown of the former by opening of its β-lactam ring. Furthermore, no breakdown of the pro-moiety *N*-(hydroxymethyl)-*N*-[(ethoxycarbonyl)methyl]benzamide (**8.58**) to liberate formaldehyde was detected over the timescale of ester hydrolysis.

The *phthalimidomethyl* moiety in **8.59** is another carrier examined during the design of prodrugs of nonsteroidal anti-inflammatory acids [72]. The pro-

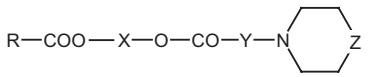
drugs of ibuprofen, indomethacin, and naproxen had $t_{1/2}$ values for chemical hydrolysis at pH 7.4 and 37° of 240, 300, and 170 min, respectively. The corresponding values in 80% rabbit plasma at 37° were 5, 11, and 9 min, respectively, indicating *ca.* 20- to 50-fold acceleration. Some liberation of formaldehyde from *N*-hydroxyphthalimide was reported. However, the prodrugs were significantly less irritating to rat stomach mucosa than the parent NSAIDs.

More complex fragmentable pro-moieties containing a basic function have also been examined to improve the topical delivery of *naproxen* [73]. These are [(aminoacyl)oxy]alkyl esters (**8.60**), [(morpholinylacyl)oxy]alkyl esters (**8.61**, Z = O), and {[(methylpiperazinyl)acyl]oxy}alkyl (**8.61**, Z = MeN) esters. In general, these prodrugs showed a better water solubility and a somewhat higher lipid solubility compared to naproxen, and were rapidly hydrolyzed to naproxen in human plasma ($t_{1/2}$ of a few to several minutes under physiological conditions). Skin permeation was also improved relative to the drug.



8.60 X = $(CH_2)_n$, *n* = 2 to 4

$CO—Y—NH_2$ = aminoacyl



8.61 X = $(CH_2)_n$, *n* = 2, 4

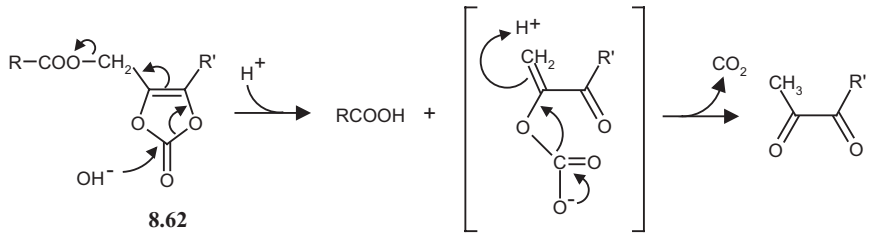
Y = $(CH_2)_n$, *n* = 1 to 3

Z = O, NCH_3

8.3.4. Prodrugs That Incorporate an (Oxodioxolyl)methyl Pro-Moiety

An esterifying substituent of value in prodrug design is the (2-oxo-1,3-dioxol-4-yl)methyl group (**8.62** in *Fig. 8.4*). The interest in this moiety was first documented with prodrugs of antibiotics, as discussed below. To the best of our knowledge, no detailed or systematic studies on the *mechanism of activation* of (oxodioxolyl)methyl derivatives have been published. The available evidence supports a clean reaction of base-catalyzed hydrolysis as shown in *Fig. 8.4*, although the actual mechanism may be more complex and/or condition-dependent [74][75]. Briefly, hydrolysis liberates the pro-

moiety as an unstable carbonate monoester, which decarboxylates and generates a diketone. The hydrolytic activation of (oxodioxolyl)methyl esters was originally believed to be purely nonenzymatic. However, more recent studies presented below have demonstrated that enzymatic hydrolysis may also be involved, at least in some cases.

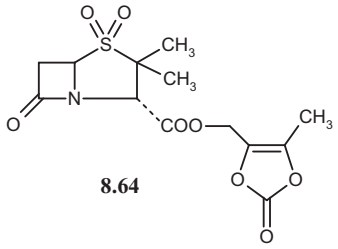


8.62

Fig. 8.4. *Postulated mechanism of the base-catalyzed activation of (2-oxo-1,3-dioxol-4-yl)methyl ester prodrugs* (**8.62**) *of carboxylic acids* [74][75]. After hydrolysis, the pro-moiety is liberated as a nonisolatable carbonate monoester intermediate, which is decarboxylated rapidly to a diketone.

(Oxodioxolyl)methyl esters (daloxates) of ampicillin (**8.63**, R′ = H, Me, *t*-Bu, or Ph; see structure in *Sect. 8.3.2.1*) have demonstrated that the substituent at C(5) has some influence on the rate of chemical hydrolysis. Indeed, while the rates of hydrolysis of the three prodrugs having R′ = H, Me, or Ph were comparable at acidic and neutral pH, the *tert*-butyl derivative was approximately tenfold more stable [63][75a]. This suggests that the rate of hydrolysis can be modulated by the substituent at C(5) of the dioxolone ring (see also below). Following oral administration to mice, the (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester of ampicillin (**8.63**, R′ = Me) gave the highest serum concentrations of ampicillin (approximately fourfold higher levels compared to direct administration of the drug). Comparable results were obtained with the (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl esters of the antibiotic *mecillinam* and of the β-lactamase inhibitor *sulbactam* (**8.64**) [76][77].

Some *quinolone antibacterial agents* may also exhibit unsatisfactory bioavailability by the oral route, suggesting that administration in prodrug form might be fruitful. Thus, *norfloxacin* (**8.65**, R = R′ = H) was derivatized on its carboxylic group to the (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl ester, with disappointing pharmacokinetic results [75b]. Indeed, serum concentrations of the drug were markedly lower in mice dosed orally with the prodrug than with the drug. The reason for this was not established and is all the more surprising considering the promising behavior of the prodrug with an (oxodioxolyl)methyl moiety attached to the basic N-atom (see *Sect. 8.7*). Here, we note with interest that serum paraoxonase (PON, EC 3.1.8.1) has been shown



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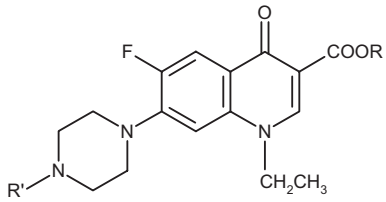

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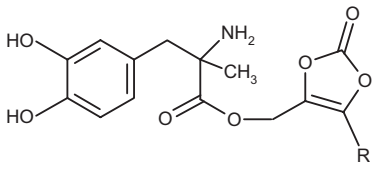


8.66

to play a major role in the hydrolytic activation of *prulifloxacin*, a prodrug of the quinolone antibacterial agent NM394 [78].

Prodrugs of *methyldopa* (**8.66**) were among the first series of (oxodioxolyl)methyl esters to be published [74]. The two prodrugs examined in this study (R = Me or *t*-Bu) yielded significant plasma levels of methyldopa in dogs, and produced an antihypertensive response that was comparable or somewhat higher than that of methyldopa. In buffers of pH 7.4 and 37°, the half-life for hydrolysis of the methyl and *tert*-butyl derivatives was 3.7 ± 2.3 h and 11.6 ± 4.1 h, respectively. This, again, suggests that the rate of hydrolysis is modulated by the substituent at C(5) of the dioxolone ring.

Recent studies with *quinoline-4-carboxylic acid angiotensin II receptor antagonists* have confirmed the interest of (oxodioxolyl)methyl esters as prodrugs with improved oral bioavailability and efficacy in laboratory animals [79]. *Olmesartan*, another angiotensin II receptor antagonist, has also been derivatized to a (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl carboxylate designated as olmesartan medoxomil [80]. In this case, both human serum albumin and arylesterase (presumably EC 3.1.1.2, although paraoxonase cannot be excluded) were shown to be involved in hydrolysis.

In summary, (oxodioxolyl)methyl esters of carboxylic acid drugs appear to be generally useful as prodrugs. However, more studies are needed to document the structure–metabolism relationships, the relative contribution of enzymatic *vs.* nonenzymatic reactions in their *in vivo* activation, the reasons of some failures, their toxic potential, and their pharmacokinetic behavior in humans.

8.4. Prodrugs of Active Carboxylic Acids Esterified with a Phenol or Hydroxylamine

For insufficiently understood reasons, there exist relatively few reports on the use of phenols and hydroxylamines as pro-moieties of active carboxylic acids. Medicinal chemists perhaps perceive these pro-moieties as potential sources of toxicity problems (see below). Furthermore, an aryl pro-moiety may unfavorably influence on solubility.

8.4.1. Carboxylic Acid Esters of Phenols

Most data available on such prodrugs concern esters in which the phenol moiety is the pharmacologically active one (see *Sect. 8.5*). Here, we present some of the few studies that describe aryl esters of active carboxylic acids.

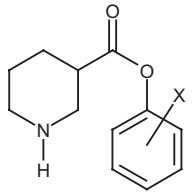
The *toxicological potential* of the phenol pro-moiety should not be underestimated, especially for oral prodrugs requiring relatively high doses. Indeed, the phenol carrier, once liberated by hydrolysis, may, in some cases, undergo oxidation to 1,2- or 1,4-diphenols and, then, to the corresponding quinones (Chapt. 4 and 7 in [81]; [82]). These reactions result in toxicogenicity, which, although attenuated or prevented by detoxifying reactions of (*e.g.*, conjugations), may explain why prodrug designers appear reluctant to use phenol carriers.

Two examples of aryl esters are given in *Table 8.5*, namely the 4-chlorophenyl and 4-nitrophenyl esters of *nicotinic acid* (**8.33**). Under physiological conditions of pH and temperature, these two compounds were clearly much more susceptible to chemical hydrolysis than the alkyl and arylalkyl esters in *Table 8.5*. Their affinity for carboxylesterase and human plasma hydrolases, as assessed by the *Michaelis* constant K_m, was generally higher, while nothing can be said regarding V_{max} values.

A systematic study on the chemical and enzymatic hydrolysis of *phenyl esters of nipecotic acid* (**8.67**) suggests some conclusions regarding not only structure–hydrolysis relationships, but also the methodology of such investigations. The substituents in compounds **8.67** (usually in the *para*-position) were some lower alkyls, oxygenated alkyls, halogens, and nitrogenated groups [83]. At physiological pH and temperature, the $t_{1/2}$ values for chemical hydrolysis (rounded values) ranged between 22 min (4-NO_2) and 650 min (4-NH_2) and could be correlated with the *Hammett* substituent constant σ^- by means of the regression *Eqn. 8.5*:

$$\log t_{1/2} = -0.87\,(\pm 0.15)\,\sigma^- + 2.56\,(\pm 0.08)$$
$$n = 17;\ r^2 = 0.91;\ s = 0.135 \qquad (8.5)$$

The *Hammett* substituent constant σ^- is a measure of the electron-withdrawing capacity of substituents directly conjugated to the reaction center [84]. Since the values of this parameter were obtained from the rate constants of base-catalyzed hydrolysis of other aryl esters, it can be concluded from *Eqn. 8.5* that the same electronic factors are involved in both cases.



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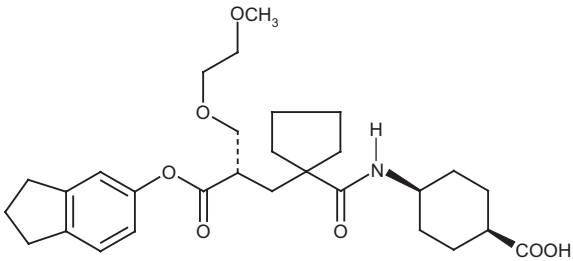


8.68

The hydrolysis of the phenyl nipecotates was also examined in 10% human serum at pH 7.4 and 37°. The $t_{1/2}$ values, which ranged from *ca.* 8 min (4-NO_2) to *ca.* 570 min (4-NH_2), were only slightly smaller than those observed for chemical hydrolysis. This indicates that the hydrolysis of phenyl nipecotates in 10% human serum was essentially nonenzymatic.

A few of the phenyl esters of nipecotic acid were also examined for their anticonvulsant effects and found to be active. The most stable ester was also the most active, but more labile esters were also effective. Because nipecotic acid itself was not used for comparison, no conclusion can be derived regarding the pharmacological value of such prodrugs.

One of the interests of aromatic pro-moieties is the *variety of substituents* they can carry. This allows prodrug designers to modulate the physicochemical and even pharmacokinetic behavior of such prodrugs. An example of a rather unusual phenol pro-moiety is provided by *candoxatril* (**8.68**), the prodrug of the neutral endopeptidase inhibitor candoxatrilat [85]. Following oral administration to mice, rats, rabbits, dogs, and humans, the ester bridge was

rapidly cleaved to release candoxatrilat and the carrier 5-indanol, with systemic bioavailability of the active drug of 88, 53, 42, 17, and 32%, respectively. The latter was defined as:

$$\frac{100 \times (\text{total AUC of candoxatrilat following oral candoxatril})}{(\text{total AUC of candoxatrilat following } \textit{i.v.} \text{ candoxatril})}$$

In addition to and like innumerable other examples, these values are taken to reflect to some extent species differences in esterase activity and demonstrate how biological variability can complicate prodrug design.

Aryl esters of retinoids have also received some interest. The 4-(acetamido)phenyl ester of (all-*E*)-retinoic acid, for example, showed topical activity in various animal models but was ineffective for the local treatment of acne in patients [86]. This difference is probably due to the prodrug being readily hydrolyzed in mice skin homogenates but not in human skin preparations.

8.4.2. Carboxylic Acid Esters of Hydroxylamines

Esters of N,N-*dialkylhydroxylamines* ((acyloxy)amines) appear to be possible candidates for prodrugs of carboxylic acids, but more studies must be published before any firm conclusion can be drawn. First, there are indications of low acute toxicity for *N*,*N*-dialkylhydroxylamines [87]. Whether the same applies (acutely and chronically) to the pro-moieties after their release from the prodrugs is not known. A second argument is the low basicity of hydroxylamines (the pK_a of *N*,*N*-dimethylhydroxylamine is 5.2), and the expected lower basicity of *O*-acylated hydroxylamines. As a result, esters of hydroxylamines will be in the unprotonated, more lipophilic form at physiological pH and should be absorbed more readily than the corresponding carboxylic acid [88].

An interesting study, one of the very few dealing with this type of prodrug, compared the topical activity of *indomethacin* (**8.9**, R = OH) and its *N*,*N*-diethylhydroxylamine ester (**8.9**, $R = Et_2NO$) [87]. Rates of hydrolysis were not provided, but the prodrug was found to be stable in aprotic solvents used as topical excipients, and sensitive to protic impurities in such vehicles. In diffusion cell tests, twice as much indomethacin was delivered by the prodrug than by the drug itself, and only indomethacin was observed on the receptor side of the diffusion cells. This is a good indication of an adequate rate of hydrolysis. When applied in aprotic solvents, the prodrug was two to three times more active than indomethacin as a local anti-inflammatory agent. Thus (acyloxy)amines might have value as topical prodrugs but appear to be too labile in protic solvents to be used orally.

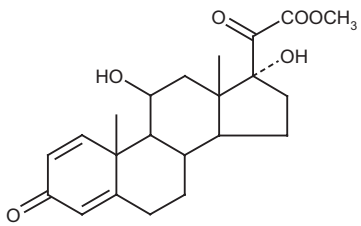
8.5. Prodrugs of Active Alcohols and Phenols

Prodrugs of active alcohols and phenols have received nearly as much attention in the literature as prodrugs of active carboxylic acids. However, this symmetry does not seem to apply to prodrugs in clinical use, where one has the distinct if unproven feeling that the majority of marketed ester prodrugs derive from active carboxylic acids. The reason for this may be that the improvement in pharmacokinetic properties is generally greater when masking the highly polar carboxylate rather than the less polar OH group. However, it is clear that a number of active alcohols and phenols are gainfully used in therapy as ester prodrugs. The sections to follow consider a selection of the common and less common acyl groups that are being used or have been examined as carrier groups in prodrug design.

8.5.1. Prodrugs That Incorporate an Alkanoyl Pro-Moiety

8.5.1.1. *Acetic Acid Esters*

Like in *Chapt. 7*, we begin the discussion with acetates, since acetic acid is the simplest nontoxic acyl group, formic acid being less innocuous. An informative study was carried out to compare the kinetics of hydrolysis of two types of *corticosteroid esters*, namely methyl steroid-21-oates (which are active *per se*) and acetyl steroid-21-ols (which are prodrugs), as exemplified by methyl prednisolonate (**8.69**) and prednisolone-21-acetate (**8.70**), respectively [89]. In the presence of rat liver microsomes, the rate of hydrolytic inactivation of methyl steroid-21-oates was much slower than the rate of hydrolytic activation of acetyl steroid-21-ols. Thus, while the K_m values were *ca.* 0.1 – 0.3 mM for all substrates, the acetic acid ester prodrugs and the methyl ester drugs had V_{max} values of *ca.* 20 and 0.15 nmol min^{-1} mg^{-1}, respectively. It can be postulated that the observed rates of hydrolysis were determined by the acyl moiety, in other words by the liberation of the carboxylic acid from the acyl–enzyme intermediate (see *Chapt. 3*).



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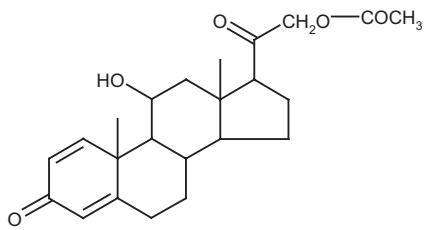
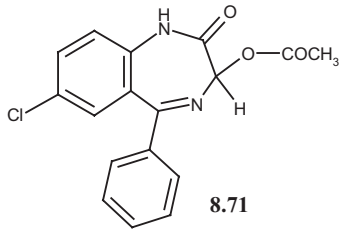


8.70

One ester that received some attention is O-*acetyloxazepam* (**8.71**), a chiral compound of interest as a biochemical probe [90]. The enantiomers of *O*-acetyloxazepam showed the expected enantioselective enzymatic hydrolysis, but the magnitude and direction of the phenomenon were noteworthy. Indeed, depending on the species, and the tissue and subcellular origin of the esterase preparation, opposite substrate enantioselectivities of some complexity were seen. In preparations from rat intestinal mucosa and pig liver, for example, microsomal and cytosolic esterases were severalfold more selective for the (*R*)- and (*S*)-ester, respectively [91]. Interestingly, the microsomal and cytosolic esterases that selectively hydrolyzed (*R*)-*O*-acetyloxazepam were different protein entities [92]. Human and rat liver microsomes were also highly selective toward the (*R*)-ester. In addition, an enantiomeric interaction occurred such that the (*R*)-ester stimulated the hydrolysis of the (*S*)-ester, whereas the latter inhibited the hydrolysis of the former [93]. Clearly oxazepam 3-acetate is a substrate whose enantiomers are discriminated very differently by various esterase proteins, with probable differences in the kinetics of both binding and catalysis.



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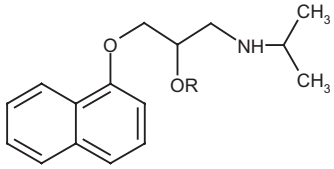

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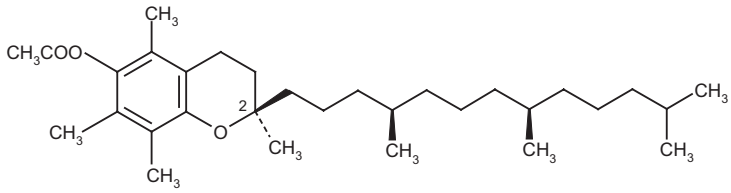
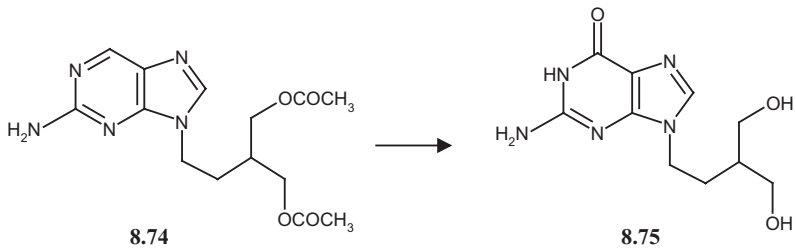


8.73

Although less extensively investigated, O-*acetylpropranolol* (**8.72**, R = Ac) afforded comparable results [94]. In human and rat serum, hydrolysis was selective for the (*R*)-enantiomer, whereas, in rat intestine and liver tissue homogenates (9,000 *g* supernatant), the opposite enantioselectivity was seen. A slight degree of mutual inhibition was observed in some cases. Reports of oxazepam- and propranolol-acetates are of significance since they confirm that substrate enantioselectivity should be taken into account when designing prodrug esters of racemic drugs.

All of the above examples are acetates of active alcohols. Here, we also mention the acetate of a phenol, namely the provitamin *α-tocopheryl acetate*, whose natural enantiomer of absolute configuration (2*R*,4′*R*,8′*R*) is shown as **8.73**. α-Tocopheryl acetate is a substrate of cholesterol esterase (EC 3.1.1.13), and was hydrolyzed in rats faster than its (2*S*,4′*R*,8′*R*)-epimer. *In vitro* experiments required α-tocopheryl acetate to be dispersed as a micellar pseudosolution, and the nature of the bile salt used to prepare micelles had a profound effect on the substrate stereoselectivity of the reaction [95][96]. Only when the micelle composition approximated that of the gastrointestinal tract did the *in vitro* substrate stereoselectivity resemble that seen *in vivo*.

In a few cases, chemists have found it useful to prepare *diacetates* or *triacetates* as prodrugs of diols or catechols. An example of a diacetate is that of *famciclovir* (**8.74**), a prodrug of the antiviral agent penciclovir (**8.75**) [97]. Penciclovir, like other synthetic nucleoside analogues, is poorly absorbed orally, and is, thus, an ideal candidate for prodrug design. Following oral administration to human subjects, famciclovir was totally absorbed and underwent extensive first-pass metabolism. The intermediate diol metabolite and the drug reached peak plasma concentrations at 0.5 and 0.75 h, respectively, indicating rapid deacetylation and 6-hydroxylation to form the active agent as the major metabolite. Indeed, excretion of penciclovir accounted for *ca.* 2/3 of an oral dose of the prodrug.



8.74 **8.75**

An example of a protected catechol is provided by the diacetyl prodrug of *ABT-431*, a potent D_1-receptor agonist. The chemical stability of the prodrug was markedly better than that of the drug. Hydrolysis of the former in rat plasma occurred with a $t_{1/2}$ value of less than a minute [98]. An example of a *triacetate* is *2′,3′,5′-triacetyl-6-azauridine*, an orally active prodrug of the antipsoriasis and antineoplastic drug 6-azauridine [99].

8.5.1.2. *Unbranched Alkanoic Acid Esters*

A variety of acyl groups other than acetyl have been used in prodrug design, several of which have already been presented in *Chapt. 7*. Branched and

cyclic alkanoyl groups, as well as functionalized alkanoyl and aromatic acyl groups, will be discussed in subsequent sections. First, we review selected examples of simple unbranched alkanoyl groups.

Linear alkanoyl groups used in prodrug design range from the lower acyl to the fatty acyl groups. Thus, homologous acyl esters of propranolol (**8.72**, R = alkanoyl) were examined for their enzymatic hydrolysis by purified carboxylesterase and rabbit serum [100]. With both enzyme preparations, the rates of hydrolysis increased in the sequence acetyl < propanoyl < decanoyl < octanoyl < butanoyl < pentanoyl < hexanoyl. In other words, the rates of hydrolysis increased up to the hexanoyl ester and then decreased sharply for higher homologues. Tissue-specific enantioselectivity was observed in the hydrolysis of the *O*-acetyl (see above), *O*-propionyl, and *O*-butyryl esters of propranolol [101][102]. Interesting qualitative structure–metabolism relationships were also reported for series of retinoates and 2′-esters of acyclovir [103][104].

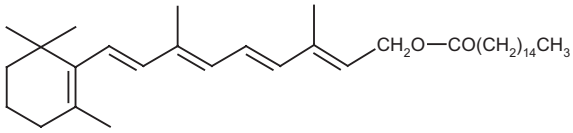
An interesting study examined the 3-acetyl, 3-propionyl, and 3-butyryl prodrugs of the potent opiate agonist *morphine-6-sulfate*, with the aim to obtain higher potency and longer duration of action as a result of improved brain penetration [105]. Chemical hydrolysis under physiological conditions was slow ($t_{1/2}$ of a few days), whereas enzymatic hydrolysis was fast in 10% rat blood ($t_{1/2}$ 1 – 2 h) and 10% rat brain homogenate ($t_{1/2}$ *ca.* 0.5 h). This fast activation, together with increased lipophilicity relative to morphine-6-sulfate, suggested that such prodrugs may be of value as analgesic agents. Indeed, depot injections of the 3-propionyl prodrug had a significantly longer antinociceptive effect than morphine-6-sulfate or the 3-acetyl prodrug.

In the therapeutic class of *steroid hormones*, prodrugs were also examined to obtain improved transdermal delivery. Thus, the highly active progestin *gestodene* was derivatized on its 17β-OH group to the acetate, propionate, butyrate, valerate, and caproate [106]. Gestodene caproate proved to be of particular interest, showing high solubility in the matrix of the transdermal drug delivery systems, significantly higher transdermal penetration rate relative to gestodene, and a high degree of hydrolysis during passage through the skin.

A number of prodrugs in clinical use are *esters of fatty acids*. For example, haloperidol decanoate is of interest in slow-release preparations. This compound was hydrolyzed by such hydrolases as purified carboxylesterase but was reported to be stable in human blood or plasma and in a variety of rat tissue homogenates [107]. The source of this apparent lack of reactivity was competitive binding to blood and tissue proteins. In other words, protein binding sequesters this very lipophilic prodrug and prevents enzymatic hydrolysis, thereby slowing its activation and prolonging its *in vivo* effects.

Some esters of palmitic acid are well known, *e.g.*, chloramphenicol palmitate and *retinyl palmitate* (**8.76**) [108][109]. The latter is a provitamin of

wide use in cosmetic products. It is a good substrate for cutaneous esterases, as demonstrated in human and guinea pig skin preparations, and gives significant delivery of retinol into the skin [108]. Steroid hormones can also be used in derivatized form, *e.g.*, testosterone undecanoate, an orally active prohormone, that is well absorbed and is then distributed *via* the lymphatic system. In this context, it is interesting to note that *fatty acid esters of some steroid hormones* are in fact endogenous compounds synthesized and stored *in vivo* to serve as a reservoir of preformed hormones [110].



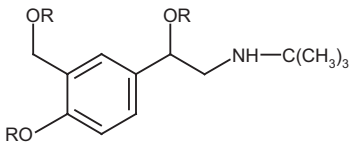
8.76

8.5.1.3. *Branched Alkanoic Acid Esters*

In prodrug design, esterification with branched alkanoyl groups is seemingly as common as with linear ones, one reason being that the slower chemical and enzymatic hydrolysis of branched acyl groups may be useful in prodrug design. For example, O-*isovalerylpropranolol* (**8.72**, R = (i-Bu)CO) given orally to dogs was absorbed faster and yielded higher plasma levels of propranolol than a corresponding dose of the drug itself [111]. Hydrolysis was selective (by factors of 3 – 4) for the (*R*)-enantiomer in dog plasma and liver preparations, with some degree of mutual inhibition by each enantiomer. Thus, slower hydrolysis of the prodrug seemingly decreased the first-pass clearance and increased the bioavailability of the pharmacologically active (*S*)-propranolol (see also above the case of *O*-acetylpropranolol).

A branched carboxylic acid frequently used to derivatize active alcohols and phenols is *pivalic acid*, *i.e.*, trimethylacetic acid. A reason for such a choice may well be found in the relatively high lipophilicity and resistance of pivaloyl esters toward enzymatic and chemical hydrolysis. Steric hindrance mainly accounts for the usually slow hydrolysis, as also found for esters of *tert*-butanol (see for example the *tert*-butyl esters of L-dopa and nicotinic acid in *Tables 8.1* and *8.5*, respectively). In some cases, however, too-slow activation may be unfavorable, but pivaloyl esters remain valuable as research tools that broaden the range of explorable rates of hydrolysis. An illustrative example is provided by prodrugs of *albuterol* (**8.77**), a β_2-receptor stimulant that has been investigated for possible ophthalmic use against glaucoma. It contains three OH functions, namely primary and secondary alcohol groups, and a phenol group. The drug was compared with its triacetate (**8.78**),

triisobutyrate (**8.79**), and tripivaloate (**8.80**) [112]. In buffers at physiological pH and temperature, the three prodrugs liberated albuterol with $t_{1/2}$ values of *ca.* 5.2, 13, and 61 d, respectively. As expected, lipophilicity also increased in the same order. Following ocular administration to rabbits, the hypotensive response was lowest with albuterol, and strongest and longest with the tripivaloate. This indicates good corneal permeation and hydrolysis of the triesters, with the tripivaloyl ester being endowed with the best properties in the series.

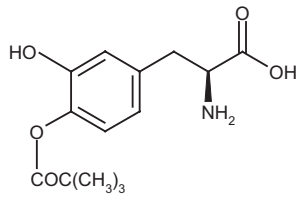


8.77 R = −H

8.78 R = $-COCH_3$

8.79 R = $-COCH(CH_3)_2$

8.80 R = $-COC(CH_3)_3$



8.81

The hydrolysis of a number of esters of *oxprenolol* was compared under different biological conditions, yielding quantitative insights into the relative stability of pivaloyl esters [113]. Oxprenolol is a β-blocker that resembles propranolol. In buffer solution at pH 7.4 and 37°, its *O*-acetyl, *O*-propionyl, *O*-butyryl, and *O*-valeryl esters had $t_{1/2}$ values in the range of 10 – 20 min, whereas the corresponding value for the *O*-pivaloyl ester was 2000 min! Comparably higher (*ca.* 20- to 100-fold) stabilities were seen in 30% human plasma ($t_{1/2}$ *ca.* 260 min), aqueous humor ($t_{1/2}$ *ca.* 700 min), and corneal extract ($t_{1/2}$ *ca.* 380 min). It was concluded that the *O*-acetyl ester might be too unstable, and the *O*-pivaloyl ester too stable, for ocular use against glaucoma.

One must be careful not to generalize from the above discussion that hydrolysis of pivaloyl esters will always be slow. Indeed, a notable exception may well exist for *monoesters of catechols*, where intramolecular catalysis accelerates hydrolysis. This was seen for L-3-[3-hydroxy-4-(pivaloyloxy)phenyl]alanine (**8.81**; *4-pivaloyl-*L*-dopa*), a potentially valuable prodrug of L-dopa [114]. When given to rats and dogs, 4-pivaloyl-L-dopa displayed markedly longer duration of action and a higher bioavailability of L-dopa than the drug itself; complete conversion to L-dopa was noted in rat. The

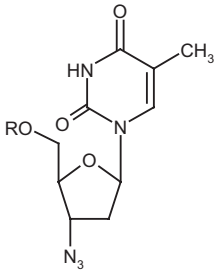
point of relevance here is that the chemical hydrolysis of 4-pivaloyl-L-dopa as investigated *in vitro* was not typical of pivaloyl esters, being rapid compared with pivaloyl esters of phenols. Thus, the $t_{1/2}$ for hydrolysis at pH 7.4 and 37° was 22 min for **8.81**, but neither the 4-pivaloyl ester of tyrosine (*i.e.*, the analogue without the 3-OH group) nor 3,4-dipivaloyl-L-dopa was hydrolyzed. In weakly or strongly acidic buffers, however, 4-pivaloyl-L-dopa underwent little or no hydrolysis, respectively. The hydrolysis of 4-pivaloyl-L-dopa is discussed further in the next section in connection with intramolecular acyl migration.

8.5.1.4. *Cycloalkanoic Acid Esters*

Comparatively little has been published on cycloalkanoic acid esters of active alcohols and phenols resembling cycloalkyl esters of active acids (see for example compounds **8.14**, **8.34**, and **8.35**). One interesting example is that of O-*cyclopropane carboxylic acid esters* of various β-blockers such as propranolol (**8.72**, R = C_3H_5CO) [115]. At pH 7.4 and 37°, these esters underwent chemical hydrolysis with $t_{1/2}$ values ranging from 4 h (timolol prodrug) to *ca.* 1 d (alprenolol prodrug); the $t_{1/2}$ values in human plasma were lower (1 – 7 h). The increased lipophilicity of the esters relative to the free drugs resulted in a higher permeability across *Caco-2* cells; this valuable effect was marked for the hydrophilic β-blockers (*e.g.*, acebutolol) and modest for the more lipophilic ones (*e.g.*, propranolol). Cyclopropane carboxylic acid appears interesting in prodrug design due to its unique steric, electronic and lipophilic characteristics.

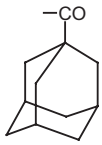
Adamantane-1-carboxylic acid also appears promising as a pro-moiety to enhance brain penetration of polar drugs. A convincing example of this potential has been reported for the anti-HIV drug *zidovudine* (AZT, azidothymidine; **8.82**, R = H) [116]. While AZT is transported through the blood/cerebrospinal fluid barrier into the cerebrospinal fluid, it cannot pass the blood–brain barrier to reach the brain parenchyma and act in HIV-infected brain cells. When administered *i.v.* to rats, prodrugs of AZT coupled to adamantine-1-carboxylate (**8.83**) or (adamandan-1-yl)acetate (**8.84**), gave total brain levels (AZT plus prodrug) that were *ca.* 7- to 18-fold higher than after AZT administration. Hydrolysis occurred readily in rat plasma but was slower in human plasma. Clearly, such results are encouraging for the treatment of retroviral infections of the CNS.

To conclude this section, we compare large series of potential prodrugs of β-blockers, comprising esters of linear, branched, and cyclic alkanoic acids, as well as benzoic acid esters to be discussed in *Sect. 8.5.6*. In the case of esters of *timolol* (**8.85**, R = H), the $t_{1/2}$ values of chemical hydrolysis at

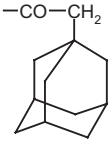


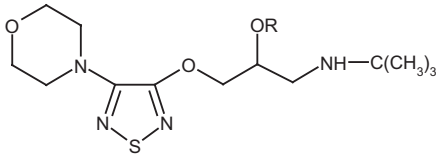
8.82

8.83 R = —CO



8.84 R = —CO—CH$_2$





8.85

pH 7.4 at 37° was 0.5 – 23 h for the alkanoates and cycloalkanoates (*Table 8.8*) [117]. *Table 8.8* also reports the intriguing finding that, for most of these esters, hydrolysis was slower in human plasma (by a factor of 1 – 6) than in phosphate buffer at the same pH and temperature. This rate-retarding effect increased with lipophilicity for the aliphatic esters, but only for them. In more-dilute plasma solutions, the rate-retarding effect disappeared, and hydrolysis was, in fact, accelerated. These observations are compatible with the hypothesis that binding to plasma proteins retards hydrolysis.

When comparing the behavior of various esters of *propranolol* (**8.72**, R = H) in rat and dog plasma, liver microsomes and liver cytosol, the global trend emerged that the rates of hydrolysis decreased in the series linear alkanoates > cycloalkanoates > branched alkanoates [118][119]. Such an observation may be of interest to prodrug designers since it affords a continuum of foreseeable rates of activation. Furthermore, a clear substrate enantioselectivity was seen, in that hydrolysis of the (*R*)-propranolol esters was consistently faster than that of their antipodes. The same was true in mouse skin.

8.5.2. Reactions of Intramolecular Acyl Transfer

The hydrolysis of 4-pivaloyl-L-dopa is accompanied by rapid and reversible isomerization to 3-pivaloyl-L-dopa, a reaction of *O-acyl migration* that

Table 8.8. *Chemical and Enzymatic Hydrolysis of Ester Prodrugs of Timolol* (**8.85**, R = H) *at pH 7.4 and 37°* [117]

R	Buffer $t_{1/2}$ [h]	80% Human plasma $t_{1/2}$ [h]
CH_3CO	0.47	0.58
CH_3CH_2CO	0.67	0.75
$CH_3CH_2CH_2CO$	0.83	1.80
$(CH_3)_2CHCO$	0.94	1.30
$CH_3(CH_2)_3CO$	1.0	2.40
$(CH_3)_3CCO$	3.6	8.8
$(CH_3CH_2)_2CHCO$	23	[a])
$(CH_3)_3CCH_2CO$	23	105
$CH_3(CH_2)_4CO$	1.3	3.1
$CH_3(CH_2)_6CO$	1.2	7.4
Cyclopropanecarbonyl	4.1	4.9
1-Methylcyclopropanecarbonyl	9.9	11.5
2-Methylcyclopropanecarbonyl	12.1	13.2
Cyclobutanecarbonyl	0.35	0.65
Cyclopentanecarbonyl	0.92	2.2
Cyclohexanecarbonyl	1.6	4.5
PhCO	2.0	4.4
2-CH_3–C_6H_4CO	10	52
4-CH_3–C_6H_4CO	4.9	20
2-CH_3O–C_6H_4CO	4.1	7.3
4-CH_3O–C_6H_4CO	4.9	20
2-CH_3COO–C_6H_4CO	0.51	[a])
2-$PhCOOCH_2$–C_6H_4CO	4.5	[a])
2-NH_2–C_6H_4CO	36.6	70
2-CH_3NH–C_6H_4CO	41.8	[a])
Thiophene-3-carbonyl	3.8	7.2

[a]) Not reported.

is competitive with and significantly faster than hydrolysis under physiological pH and temperature conditions. In addition, 4-pivaloyl-L-dopa is a poor substrate for rat intestinal and pancreatic esterases, the observed hydrolysis being mainly nonenzymatic. The high and long-lasting blood levels of L-dopa seen after administration of its 4-pivaloyl derivative were, thus, suggested to be due to its stability in and slow absorption from the intestinal tract, followed by its rapid hydrolysis in blood [114a].

These findings are compatible with a *mechanism of intramolecular catalysis* for both acyl migration and hydrolysis, as proposed in *Fig. 8.5*. Also, the possibility that both reactions share a common intermediate is emphasized. *Reactions a* and *b* in *Fig. 8.5* involve a first step of deprotonation, in agreement with the observed specific base catalysis. Intramolecular nucleophilic attack (*Reactions c* and *d*) generates a tetrahedral intermediate that can result in acyl migration or hydrolysis (*Reaction e*).

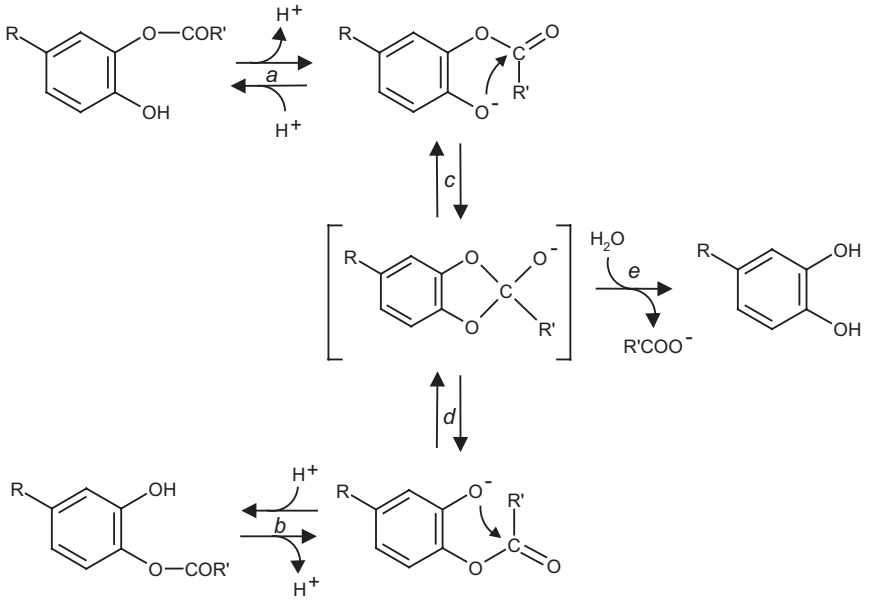


Fig. 8.5. *Mechanism postulated for competitive, specific base catalyzed hydrolysis and acyl migration of catechol monoesters, as seen with 4-pivaloyl-*L*-dopa* (**8.81**) [114a]. Deprotonation (*Reactions a* and *b*) accelerates intramolecular nucleophilic attack (*Reactions c* and *d*) to form a tetrahedral transition state. The latter is postulated to be the intermediate common to hydrolysis (*Reaction e*) and acyl migration.

It is of interest that the mechanism shown in *Fig. 8.5* should be applicable to all catechol monoesters, but this is only seen when hydrolysis is comparatively slow. More studies are needed to examine the mechanistic hypothesis presented in *Fig. 8.5* and the pharmacokinetic consequences of intramolecular acyl migration in monoesters of catechols.

Another type of intramolecular transfer is that of *O* → *N migration*, as documented for *O*-acyl-*β*-hydroxy amines such as ester prodrugs of propranolol (**8.72**, R = acyl). This reaction of intramolecular aminolysis, which forms a stable *N*-acyl derivative, was seen with the *O*-acetyl, *O*-isobutyryl, and *O*-cyclopropanoyl esters of propranolol, but not with its *O*-pivaloyl ester. In a homologous series of *O*-acyl esters of propranolol, the rate of aminolysis decreased with increasing length of the unbranched acyl group [120][121]. The reaction occurred only at pH > 7, becoming increasingly important at elevated pH values. For propranolol *O*-cyclopropanecarboxylate (**8.72**, R = C_3H_5CO), the rate constants for hydrolysis and rearrangement at pH 9.9 and 37° were $12.3 \cdot 10^{-3}$ and $6.7 \cdot 10^{-4}$ min^{-1}, respectively, *i.e.*, a ratio of *ca.* 18 : 1.

The few examples of acyl migrations discussed above may have pharmacokinetic and pharmacodynamic consequences, but these are insufficiently understood at present.

8.5.3. Prodrugs That Incorporate an Aromatic Acyl Pro-Moiety

Aromatic acyl groups used in prodrug design derive from aromatic or arylalkanoic acids. *Table 8.8* reports a number of *esters of benzoic acids* examined as potential prodrugs of timolol (**8.85**, R = H). No conclusions can be drawn on the relationships between chemical structure and rates of chemical hydrolysis, and the phenomenon of protein binding is again suspected to mask or prevent hydrolysis by plasma hydrolases. However, *Table 8.8* is of interest in that it illustrates some of the substituted benzoic acids that have been examined in prodrug design.

In a series of ten *morphine 3-benzoates*, large differences in rates of enzymatic hydrolysis were seen [122]. In 80% human plasma at pH 7.4 and 37°, the unsubstituted 3-benzoate had a $t_{1/2}$ value of 0.6 h, whereas esters of 2,6-disubstituted benzoic acid were much more resistant to enzymatic attack ($t_{1/2}$ ranging from 60 h for the dimethylbenzoate to 300 h for the dichloro- and dimethoxybenzoates). Although these results point to marked steric hindrance, electronic effects cannot be excluded but escape characterization because of the limited series. Furthermore, and as mentioned repeatedly in this text, the possibility of binding to plasma proteins is a complicating factor that should be kept in mind.

A complex example of activation, aimed at improving ocular delivery, has been reported for prodrugs of *pilocarpine* (**8.87**, *Fig. 8.6*) [123][124]. The prodrugs are, in fact, lipophilic diesters of pilocarpic acid (**8.86**, *Fig. 8.6*). The first step is enzymatic *O*-acyl hydrolysis to remove the acyl carrier (*Fig. 8.6*, *Reaction a*). In a second step, intramolecular nucleophilic attack leads to loss of the alcohol carrier and ring closure to pilocarpine (*Fig. 8.6*, *Reaction b*).

It is significant that no enzymatic attack occurred at the *O*-alkyl ester group. The acyl pro-moieties investigated included some low-molecular-weight alkanoyl groups and various substituted benzoyl groups. *Reaction a* (*Fig. 8.6*) is of relevance here: chemical hydrolysis was very slow at pH 7.4 and 37° ($t_{1/2}$ $10^2 - 7 \cdot 10^4$ h). In contrast, enzymatic hydrolysis in 75% human plasma at pH 7.4 and 37° was fast, with $t_{1/2}$ values for *Reaction a* ranging from 3 min (RCO = butyryl) to 33 min (RCO = pivaloyl). The aromatic esters (RCO = benzoyl, 2-chlorobenzoyl, and nicotinyl) had $t_{1/2}$ values of enzymatic hydrolysis in the range of 6 – 25 min, *i.e.*, comparable to those of the alkanoyl esters. Efficient enzymatic hydrolysis was also seen in various ocular

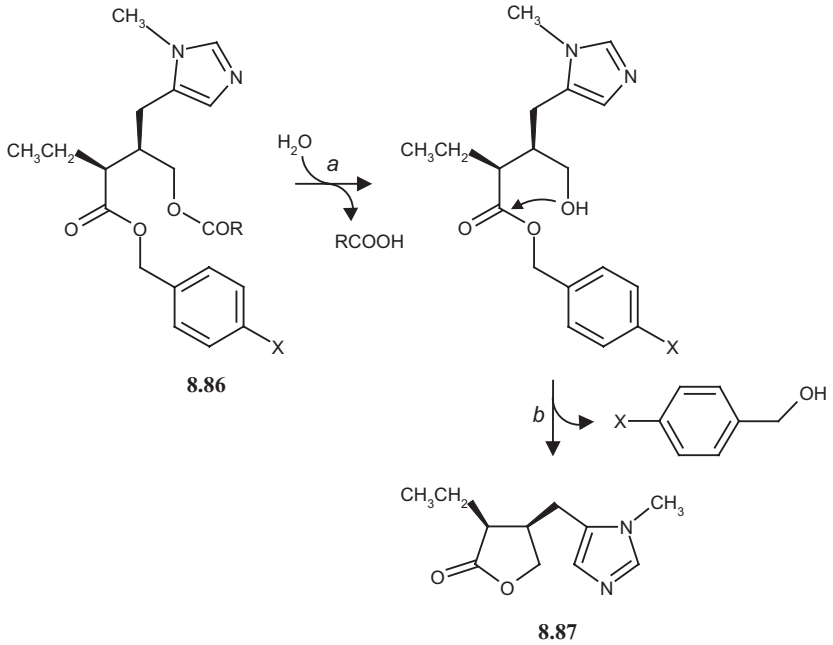
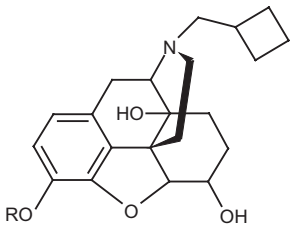


Fig. 8.6. *Two-step activation of pilocarpine prodrugs* [123]. The prodrugs are diesters of pilocarpic acid (**8.86**). Enzymatic hydrolysis (*Reaction a*) cleaves the acyl carrier group. The product is a monoester of pilocarpic acid that undergoes cyclization to pilocarpine (**8.87**) upon intramolecular nucleophilic attack and loss of the alcohol carrier.

tissue preparations, confirming the potential of these diesters as prodrugs for ocular delivery.

In the above example, a substituted benzoic acid and another aromatic acid were used. Aromatic acids are popular in prodrug design since the properties of the prodrug can, to some extent, be tailored to requirements by substituents on the aromatic ring, as also suggested by earlier examples. What is missing, however, is enough published evidence to support such design. A few additional and promising aromatic acids are illustrated below.

The narcotic agonist/antagonist *nalbuphine* (**8.88**) is poorly bioavailable in humans (*ca.* 10%), mostly due to extensive first-pass metabolism. Two prodrugs were examined, namely the acetylsalicylate (**8.89**) and the anthranilate (**8.90**) [125]. The hydrolysis of these compounds in rat plasma was fast ($t_{1/2}$ values of some minutes and 1 – 2 h, respectively), while it was slow in dog plasma ($t_{1/2}$ *ca.* 3 h and 15 h, respectively) and in human plasma ($t_{1/2}$ *ca.* 7 – 10 h and 50 – 60 h, respectively). Hydrolysis in dog tissue homogenates was also markedly slower than in rat. The dog was, thus, considered to be a fair animal model, and it is of interest that, in this species, the oral bioavailability of **8.88**, **8.89**, and **8.90** were *ca.* 6, 20, and 50%, respectively.



8.88 R = —H

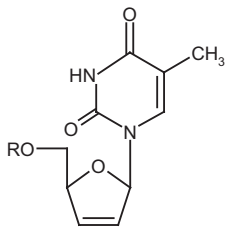
8.89 R = —CO— CH_3OCO

8.90 R = —CO— H_2N

Aromatic acids with a basic side chain are also of considerable interest. Since the determining feature is the basic group, prodrugs derived from such acids are discussed in *Sect. 8.5.5.2*.

8.5.4. Prodrugs That Incorporate an Oxygenated Alkanoyl Pro-Moiety

A number of attempts have been made to improve the pharmaceutical and pharmacokinetic properties of ester prodrugs with alkanoic acids containing an oxygenated function. Some examples are given by prodrugs of 2′,3′-didehydro-3′-deoxythymidine (*stavudine*, **8.91**). This anti-HIV agent displayed good pharmaceutical, pharmacodynamic, and pharmacokinetic properties, but attempts to improve them were carried out nevertheless. Thus, esters of alkanoic acids (**8.92** – **8.94**), of benzoic acid (**8.95**), and of oxygenated acids (**8.96** – **8.99**) were prepared and evaluated [126][127]. The solubility of the



8.91 R = —H

8.92 R = —$COCH_3$

8.93 R = —$CO(CH_2)_6CH_3$

8.94 R = —$COC(CH_3)_3$

8.95 R = —COC_6H_5

8.96 R = —$COCH_2OCH_3$

8.97 R = —$CO(CH_2)_2COOH$

8.98 R = —$CO(CH_2)_3COOH$

8.99 R = —CO—O—

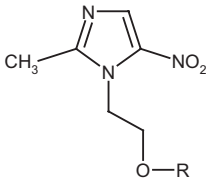
prodrug was always lower than that of the drug itself, and the increase in lipophilicity depended on the polarity of the acyl moiety. Enzymatic hydrolysis was most pronounced in rat liver and duodenum homogenates, with very high rates for the octanoate (**8.93**) and remarkable stability of the succinyl and glutaryl monoesters (**8.97** and **8.98**). None of the compounds tested in rats had improved oral bioavailability relative to that of stavudine, and for some it was lower. However, administration of the prodrugs prolonged retention of stavudine in plasma, and this can be viewed as an advantage afforded by their use.

The results from compounds **8.92 – 8.99** are too fragmentary to allow robust conclusions to be drawn regarding their pharmaceutical and pharmacokinetic properties. However, prodrugs **8.96 – 8.99** are of interest because they illustrate some of the oxygenated acyl moieties available to prodrug designers. Compound **8.99** is a carbonate, a group of prodrugs discussed in *Sect. 8.5.6.2.* Here, we focus mainly on *hemiester prodrugs of diacids*, a number of which have been described in the literature (see also *Chapt. 7*). For example, the hydrolysis of hydrocortisone hemisuccinate was mediated by two distinct carboxylesterases in rat liver microsomes [128]. When administered orally to rats, the hemisuccinate prodrug of propranolol (**8.72**, R = $HOCOCH_2CH_2CO$), like the acetic acid ester, underwent reduced first-pass metabolism, resulting in two- to threefold higher bioavailability of the drug [129].

One drug that has attracted marked attention for prodrug design is *metronidazole* (**8.100**, R = H). At 37° and pH 7.4, the hemisuccinic acid ester (**8.101**) hydrolyzed with $t_{1/2}$ values of 600 – 700 h in phosphate buffer and in human plasma, indicating the absence of enzymatic catalysis in this biological medium. Under the same conditions, similar results were obtained with the hemimaleinate (**8.103**), with $t_{1/2}$ values of *ca.* 250 – 350 h. In contrast, the hemiglutarate (**8.102**) was hydrolyzed much faster in human plasma ($t_{1/2}$ *ca.* 16 h) than in phosphate buffer ($t_{1/2}$ *ca.* 800 h). This provides valuable information on the substrate selectivity of human plasma hydrolases, but further study of a variety of other hemiglutarates as compared to hemisuccinates is required [130 – 132].

It is also interesting to note that the three hemiesters of metronidazole are good substrates of pig liver esterases. The $t_{1/2}$ values for hydrolysis of the hemisuccinate, hemiglutarate, and hemimaleinate were 0.2, 2.3, and 2.0 h, respectively, in 5% pig liver homogenate at 37° and pH 7.4.

In summary, the scattered data available indicate that hemiester prodrugs generally undergo negligible hydrolysis at neutral pH in buffered solutions and in human plasma. In contrast, hydrolysis appears relatively fast in the presence of hepatic hydrolases. This would suggest limited first-pass metabolism and activation mediated mainly by liver hydrolases.



8.100 R = H

8.101 R = $-COCH_2CH_2COOH$

8.102 R = $-COCH_2CH_2CH_2COOH$

8.103 R = $-CO-CH=CH-COOH$

8.107 R = $-COCH_2-N\langle O$

8.108 R = $-COCH_2-N\langle N-CH_3$

8.109 R = $-CO-C_6H_4-CH_2N(CH_3)_2$

8.110 R = $-CO-C_6H_4-CH_2N(CH_3)_2$

8.111 R = $-CO-C_6H_4-CH_2-N\langle O$

8.112 R = $-CO-C_6H_4-CH_2-N\langle O$

8.113 R = $-CO-C_6H_4-CH_2-N\langle N-CH_3$

8.114 R = $-CO-C_6H_4-CH_2-N\langle N-CH_3$

8.115 R = $-CO-C_6H_4-CH_2-\overset{+}{N}(CH_3)_3$

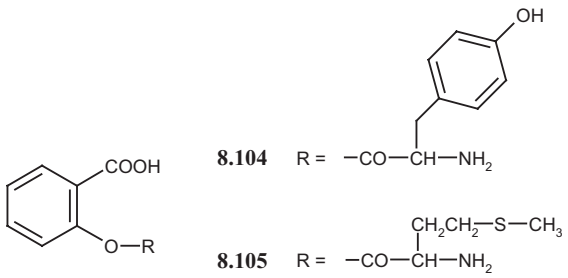
Another feature of diacids of interest in prodrug design is that the free carboxy group of a drug R–COOH can be used to attach an additional carrier group R′OH, *e.g.*, R–CO–CH_2–CH_2–CO–OR′. An example of this type has been reported for a lipid conjugate of testosterone, with R′ = 2-(1,3-dipalmitoyl)glyceryl [133] (see also *Sect. 8.2.5*).

8.5.5. Prodrugs That Incorporate an Amino Acyl Pro-Moiety

Prodrugs of alcohols and phenols incorporating an amino acid carrier, and, hence, a *basic side chain*, usually combine good water solubility with rapid enzymatic hydrolysis (see *Sects. 7.3* and *7.4*). Such prodrugs are, thus, of potential interest for parenteral administration and offer a contrast to the hemiesters, which combine good water solubility with slower hydrolysis (see above and *Sect. 7.3*). The carrier moiety conjugated to the drug can be an α-amino acid, a β-amino acid, or an analogue with a greater distance between the carboxy and amino functions. Further variety may come from *N*-substituents.

8.5.5.1. *Esters of α-Amino Acids*

Most published examples of prodrugs of relevance in the present context contain an *α-amino acyl moiety*. A number of reasons may explain this fact, such as the lack of toxicity of these natural compounds, the large differences in lipophilicity and other properties between amino acids, and the variability afforded by *N*-substituents. Interesting examples are provided by salicylic acid and metronidazole. Thus, the hydrolysis of tyrosine and methionine prodrugs of *salicylic acid* (**8.104** and **8.105**, respectively) was examined in rabbits after intraduodenal and intracecal administration [134]. The former ester, but not the latter, was hydrolyzed in the mucosa of the small intestine. In addition, both prodrugs underwent marked hydrolysis by intestinal microflora.



Similarly, chemical hydrolysis of a number of α-amino acyl prodrugs of *metronidazole* (**8.100**, R = H; see *Sect. 8.5.4*) was compared to the serum-catalyzed reaction [135][136]. The amino acids used for esterification included alanine, glycine, isoleucine, leucine, lysine, phenylalanine, and valine. Under physiological conditions of pH and temperature, $t_{1/2}$ values for hydrolysis in human serum ranged from 4.5 min for the Phe ester to 96 h for the Ile ester. A good linear relationship was established between the log of the rate constant of enzymatic hydrolysis and the log of the rate constant of HO^--cata-

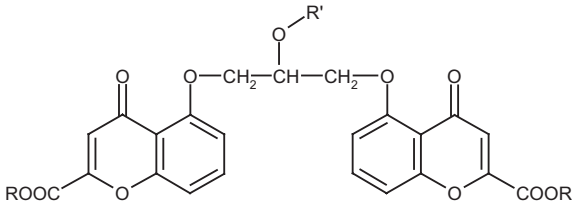
lyzed hydrolysis of the protonated prodrugs. In other words, the rate of chemical hydrolysis was a good predictor of the rate of enzymatic hydrolysis. This renders the involvement of a plasmatic enzyme such as cholinesterase unlikely, but could suggest an albumin-mediated reaction.

The activation under physiological conditions of a variety of other amino acyl esters of metronidazole was investigated; the results presented in *Table 8.9* are discussed here and in *Sect. 8.5.5.2* [137]. This series contains the glycine ester, various *N*-substituted glycinates, two esters of β-alanine and one of 4-aminobutyric acid. The glycine ester and its *N*-substituted analogues of relevance here were hydrolyzed relatively rapidly in plasma, the exception being the piperazinyl derivative discussed below.

Various promising applications of α-amino acid esters have been published in recent years, such as *valaciclovir*, the L-valyl ester of acyclovir, which showed encouraging pharmacokinetic results in the rat and monkey [139 – 141]. For example, the oral bioavailability of acyclovir after prodrug administration was 67 ± 13% in the monkey, a significant improvement over the limited availability of the drug administered as such. Interestingly, the rat liver enzyme that hydrolyzes valaciclovir, a newly described hydrolase of unknown physiological function, shows high selectivity for various amino acid esters of acyclovir, different from typical esterases and peptidases [142]. Recently, the L-aspartate β-ester, and L-lysyl and L-phenylalanyl esters of acyclovir were prepared and examined as prodrugs for nasal absorption [143]. The aspartate β-ester was the most stable, and was also the only one to be detectably absorbed, seemingly by active uptake.

Another application is the esterification of *menahydroquinone-4*, a water-insoluble vitamin K, with *N*,*N*-dimethylglycine [144]. The 1-mono, 4-mono, and 1,4-bis esters were found to be water-soluble and rapidly hydrolyzable by liver and plasma esterases. A rapid pharmacodynamic response was seen after intravenous administration of the prodrugs.

Multiple esterification, as exemplified above, is not uncommon in prodrug design and has been referred to earlier. Here, we discuss another interesting case, namely α-amino acid prodrugs of *cromoglycate* (**8.106**, R = R′ = H). This potent anti-allergic agent suffers from a major pharmacokinetic defect, namely extremely low oral bioavailability due to its high acidity and



8.106

Table 8.9. *Chemical and Enzymatic Hydrolysis of Ester Prodrugs of Metronidazole* (**8.100**, R = H) *at pH 7.4 and 37°* [137][138]

R	Buffer $t_{1/2}$ [min]	80% Human plasma $t_{1/2}$ [min]
H_2NCH_2CO	115	41
$CH_3CH_2CH_2NHCH_2CO$	90	8
$(CH_3)_2NCH_2CO$	250	12
8.107	1880	30
$H_2NCH_2CH_2CO$	315	210
$(CH_3)_2NCH_2CH_2CO$	52	46
$(CH_3)_2NCH_2CH_2CH_2CO$	580	330
8.108	1720	520
8.109	[a])	4.7
8.110	[a])	5.1
8.111	[a])	0.4
8.112	[a])	5.0
8.113	[a])	2.4
8.114	[a])	0.6
8.115	[a])	51

[a]) Not reported.

high polarity. Esterifying both carboxy groups did indeed increase lipophilicity, but at the expense of water solubility. Another approach was, therefore, investigated, namely esterification of the two carboxylate groups with alkyl moieties, and derivatization of the OH group with an amino acid [145]. With R = Et in **8.106**, and R′ = L-Ala, Gly, L-Ile, L-Leu, L-Pro, or L-Val, satisfactory oral bioavailability of cromoglycate was obtained. The best prodrug was the L-lysyl ester, which, of all bifunctional prodrugs investigated, had good water solubility and the best oral bioavailability (*ca.* 30% of the dose in rabbits). A good anti-anaphylactic response was seen in rats after oral administration.

8.5.5.2. *Esters of Other Amino Acids*

Higher homologues of glycine have also been evaluated as pro-moieties of active alcohols or phenols. Thus, the highly lipophilic *α-tocopherol* was derivatized with a series of *ω*-aminoalkanecarboxylic acids in the search for a water-soluble, injectable provitamin E [146]. Good to high water solubility was indeed achieved. The esters were substrates of liver esterases and showed marked structure-dependent differences in their rate of enzymatic hydrolysis.

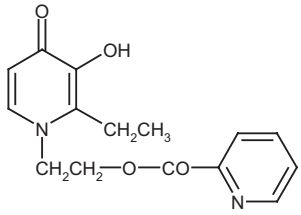
In addition to esters of *α*-amino acids, *Table 8.9* also lists four prodrugs of metronidazole where the pro-moieties are *longer-chain amino acids*, namely

two aminopropanoates and one aminobutanoate, together with the piperazinyl derivative **8.108**. Indeed, this compound, in the strict sense, is a substituted glycinic acid ester, but, from a physicochemical viewpoint, five atoms separate the ester bridge from the more-basic, distal N-atom. As shown in *Table 8.9*, these four prodrugs, with the exception of the 3-(dimethylamino)propanoate, were more stable in plasma than the glycinic acid esters. The latter compound underwent rapid chemical activation (the fastest in the series) but, seemingly, no enzymatic hydrolysis. Intramolecular catalysis probably accounts for this low stability (see *Sect. 7.3*). The piperazinyl derivative was quite stable chemically, and its hydrolysis was accelerated only threefold in plasma [137].

The 4-(dimethylamino)butanoate pro-moiety was also used to produce a prodrug of *testosterone* exhibiting greatly improved rates of skin penetration [147]. In fact, the rate of diffusion across human skin was *ca.* 60 times faster for the prodrug. Similarly, the plasma levels of testosterone after topical administration to hairless mice were *ca.* 7 times higher after the prodrug. These results may appear unexpected considering the polarity of the protonated pro-moiety. However, the determining feature was certainly the overall lipophilicity of the prodrug, as well as its reduced H-bond-donating capacity compared to testosterone.

Benzoic acids substituted with a basic side chain also are also of interest as pro-moieties whose physicochemical properties and rates of enzymatic hydrolysis can readily be modulated. A number of drugs have been converted to prodrugs with this type of pro-moiety, *e.g.*, hydrocortisone, prednisolone, acyclovir, chloramphenicol, and paracetamol [148][149]. These prodrugs appear well suited as parenteral formulations, being water-soluble, stable in slightly acidic solution, and readily hydrolyzed enzymatically. As examples, we consider here the hydrolysis in human plasma of a number of (aminomethyl)benzoates of metronidazole (**8.109** – **8.115**, *Sect. 8.5.5.1*, *Table 8.*9) [138]. These prodrugs are very rapidly activated, which may be beneficial for parenteral administration. However, this type of pro-moiety may be cleaved too rapidly after oral administration to be of interest for poorly absorbed drugs.

A few *heterocyclic aromatic acids* such as nicotinic acid and regioisomers have also been examined as pro-moieties for esterification of OH func-



8.116

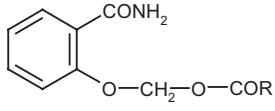
tions. Thus, the iron chelator CP102 saw its oral efficacy markedly improved when administered *in vivo* as the picolinic ester (**8.116**) to an ^{59}Fe-ferritin loaded rat model [150]. Prodrugs of estradiol were also prepared by esterification of its phenolic group with a basic aromatic acid, *e.g.*, isonicotinic acid and two benzoic acid derivatives having a basic side chain [151]. Whereas the former prodrug was disappointing, oral administration to rats of the latter two prodrugs markedly improved the bioavailability of estradiol. Interestingly, the most-promising prodrugs in this study were the 3-glutarate-17-succinate and 3-benzyl-17-succinate derivatives.

8.5.6. Prodrugs That Incorporate a Fragmentable Acyl Pro-Moiety

Fragmentable pro-moieties esterifying active carboxylic acids have been discussed in *Sect. 8.3*. Acyl groups of comparable lability can be used to derivatize active alcohols and phenols, as summarized in this section. Fragmentable pro-moieties used in preparing prodrugs of active alcohols and phenols include (acyloxy)methyl, alkoxycarbonyl, and *N*-substituted carbamoyl groups. The latter two types of pro-moiety form carbonates and carbamates, respectively.

8.5.6.1. O-*[(Acyloxy)methyl] Derivatives*

There are few reports of *O*-[(acyloxy)methyl] derivatives in prodrug design. One example is that of *salicylamide*, whose *O*-(acetoxymethyl), *O*-[(butyryloxy)methyl], and *O*-[(pivaloyloxy)methyl] derivatives (**8.117**, R = Me, Pr, and *t*-Bu, respectively) were examined for the kinetics and mechanism of their



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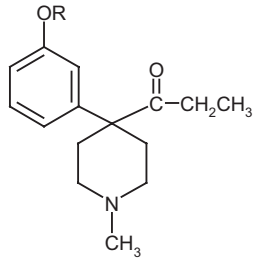


8.118 R = —H

8.119 R = —CO—$CH_2C(CH_3)_3$

8.120 R = —CO—O—CH_2CH_3

8.121 R = —CO—O—$CH(CH_3)_2$

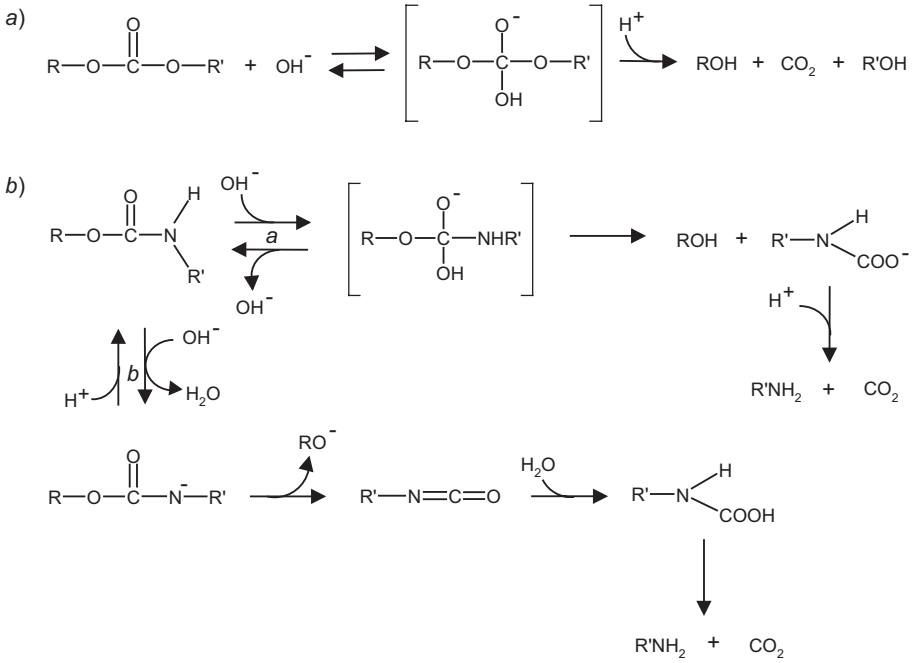


Fig. 8.7. *a*) *Mechanism of the HO⁻-catalyzed* (and presumably also of the enzyme-catalyzed) *hydrolysis of carbonic acid esters. b*) *Alternative mechanisms of the HO⁻-catalyzed hydrolysis of* N-*carbamates. Reaction b* is restricted to monosubstituted carbamates, whereas *Reaction a* is also possible for *N,N*-disubstituted carbamates.

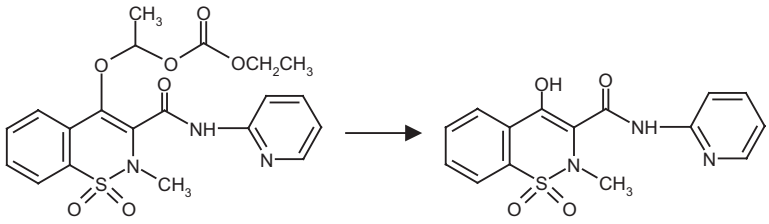
hydrolysis [152]. The $t_{1/2}$ values for hydrolysis in aqueous buffer at pH 7.4 and 37° were *ca.* 50 h, 100 h, and 1300 h, respectively, but the reaction was much faster in 80% human plasma ($t_{1/2}$ *ca.* 16, 6, and 160 min, respectively). This indicates a strong enzymatic component in plasma hydrolysis, and marked steric effects in both the chemical and enzymatic reactions. However, the release of formaldehyde from prodrugs of this type is a serious drawback, especially for drugs used in high doses (see also *Sect. 8.3*).

8.5.6.2. *Alkoxycarbonyl Derivatives*

Carbonic acid esters (alkoxycarbonyl derivatives) are diesters of general formula R–O–CO–O–R′. A single mechanism operates in the HO^--catalyzed (and presumably also in the enzyme-catalyzed) hydrolysis of carbonic acid esters, namely a rate-determining addition of the base to the carbonyl C-atom to form an intermediate whose breakdown yields the drug (ROH), CO_2, and an alcohol (R′OH) (*Fig. 8.7,a*) [153].

In *Sect. 8.5.4*, we mentioned the carbonic acid cyclohexyl ester of stavudine (**8.99**). This prodrug gave rise to lower bioavailability of the drug in rats than the acetate or methoxyacetate (**8.92** and **8.96**, respectively), but the plasma concentrations of the drug were somewhat prolonged, suggesting relatively slow hydrolysis [127]. Other carbonic acid esters have been reported for the narcotic analgesic *ketobemidone* (**8.118**). In the search for prodrugs suitable for buccal delivery, their hydrolysis was examined in human saliva [154]. The carbonic acid esters **8.120** and **8.121** were good substrates for salivary esterases, displaying V_{max}/K_m ratios 25 – 100 times higher than those of the 3,3-dimethylbutanoate **8.119**. Extrapolation to the clinical situation would suggest that only a few percent of **8.120** and **8.121** would be hydrolyzed in the mouth after 5 min, whereas **8.119** would be inert. This behavior, compatible with high buccal bioavailability, was considered desirable.

No decisive advantage was reported for the carbonic acid esters **8.99**, **8.120**, and **8.121**, but only fragmentary data are available. More extensive investigations were reported for ampiroxicam (**8.122**), a prodrug of the anti-inflammatory piroxicam (**8.123**) [155]. Chemically, this compound is both a carbonate and an *O*-[(acyloxy)methyl] derivative (see above), and its breakdown will yield EtOH, acetaldehyde, CO_2, and piroxicam. When administered to humans, ampiroxicam was completely and rapidly converted to piroxicam, probably in the intestinal wall during the absorption process. As a result, most pharmacokinetic parameters for piroxocam (AUC, maximal plasma concentration C_{max}, mean $t_{1/2}$) were similar after administration of drug or prodrug. Only the time to reach C_{max} (t_{max}) was notably prolonged after administration of the prodrug, presumably because the lower solubility of ampiroxicam compared to piroxicam resulted in slower dissolution in the gastrointestinal tract before absorption.



8.122 **8.123**

+ CO_2 + CH_3CHO + CH_3CH_2OH

A somewhat comparable conclusion emerges for *penciclovir* (**8.75**). When administered orally to mice and rats, various carbonic acid alkyl esters afforded bioavailability of the drug similar to but not better than that seen

after administration of the diacetic acid ester famciclovir (**8.74**) (see *Sect. 8.5.1.1*) [156].

Taken together, these results do not reveal any major advantage of ampiroxicam over a drug already characterized by its favorable dispositional behavior. Thus, no decisive advantage appears to emerge for the (acyloxy)methyl and carbonate derivatives mentioned above, but extended structure–stability studies might prove more encouraging. This appears to be the case with O-*(ω-hydroxyalkyl) carbonate* derivatives of zidovudine (**8.82**, R = HO–$(CH_2)_n$–CO) [157]. These prodrugs were rearranged through an intramolecular cyclic process during their enzymatic hydrolysis, and they demonstrated severalfold higher activities than the parent drug in anti-HIV screens.

8.5.6.3. *Carbamic Acid Esters*

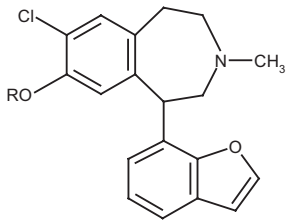
The potential of carbamic acid ester prodrugs (R–O–CO–NR′R″) is well-established by a number of studies on their stability and metabolism. Upon hydrolysis, they liberate the active agent ROH and the carbamic acid R′R″N–COOH, which, being unstable, breaks down to the amine R′R″NH and CO_2 (see also *Sect. 8.7*). The *mechanism* of HO^--catalyzed hydrolysis of carbamates, summarized in *Fig. 8.7,b*, is more complex than that of carbonates [153][158][159]. In the case of N,N-*disubstituted carbamates* (R–O–CO–NR′R″), the only possible mechanism is as for carbonates (*Fig. 8.7,b*, *Reaction a*), which is, presumably, also the mechanism of enzyme-catalyzed hydrolysis for all carbamates.

In the case of N-*monosubstituted carbamates* (R–O–CO–NHR′) where RO^- is a good leaving group (*i.e.*, having a $pK_a < ca.$ 12, in practical terms a phenol), another mechanism will operate (*Fig. 8.7,b*, *Reaction b*). Here, the carbamate is *N*-deprotonated, and the conjugate base splits spontaneously in a rate-determining step to give the phenolate RO^- and an isocyanate R′–N=C=O. The latter hydrates rapidly to the carbamic acid, which, in turn, breaks down to an amine and CO_2. This mechanism is, thus, characteristic for the nonenzymatic hydrolysis of aromatic *N*-monosubstituted carbamates, and it is usually several orders of magnitude faster than the mechanism of HO^- addition discussed above.

Thus, there will be a major difference in the design of *carbamate prodrugs of phenols and alcohols*, as illustrated below. In the case of alcohols, both *N*-monosubstituted and *N*,*N*-disubstituted carbamates will *a priori* be stable. For phenols, *N*,*N*-disubstituted carbamates are also stable toward chemical hydrolysis, whereas the *N*-monosubstituted carbamates are, as a rule, more labile toward chemical hydrolysis. However, some notable exceptions are discussed below. In addition, many other factors beside chem-

ical stability influence the bioavailability and pharmacokinetic behavior of a prodrug, *e.g.*, enzymatic hydrolysis and lipophilicity. As the examples below will show, a comparison between *N*-monosubstituted and *N*,*N*-disubstituted carbamates of phenols is quite confusing and prevents any generalization.

An interesting illustration of the behavior of carbamates is provided by prodrugs of two dopaminergic *7-hydroxy[3]benzazepines* (**8.124** and **8.125**, R = H) [160]. Because these drug candidates were poorly bioavailable, a prodrug approach was investigated. A number of *N*-monosubstituted carbamates were prepared (**8.124** and **8.125**, R = allyl–NH–CO, $PhCH_2$–NH–CO, i-Pr–NH–CO, *etc.*), but, as expected, these esters proved quite labile at pH 7.4 and 37°, with $t_{1/2}$ values for hydrolysis ranging from 4 to 40 min in buffer, and from 0.7 to 4.6 min in human plasma. This lack of stability at neutral pH was taken to imply intestinal hydrolysis and disqualified the *N*-monosubstituted carbamates from further consideration. However, the comparison between hydrolysis in buffer and plasma is of interest since it indicates the occurrence of enzymatic hydrolysis and the absence of enzymatic inhibition (see below).



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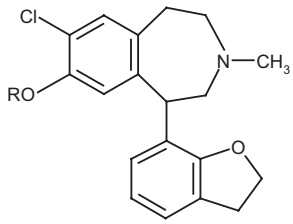
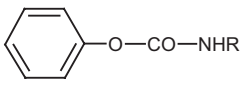


8.125

In contrast to the *N*-monosubstituted carbamates, the *N*,*N*-disubstituted analogues (**8.124** and **8.125**, R = R′R″NCO; R′ = Me or Et; R″ = Me, Et, i-Pr, *etc.*) proved very stable at pH 7.4 in both buffer and plasma, with less than 5% degradation in 4 d. In fact, these compounds were potent inhibitors of plasma cholinesterase (EC 3.1.1.8), with K_i values ranging from 600 to 3 nM. Although these carbamates were stable in plasma, they underwent rapid bioactivation in liver, as demonstrated with mouse and rat liver microsomes. For example, the *N*,*N*-dimethylcarbamate (**8.124**, R = Me_2NCO) was bioactivated in rat liver microsomes with $t_{1/2}$ of *ca.* 30 min. Two routes of bioactivation were postulated, namely direct carboxylesterase-catalyzed hydrolysis, and cytochrome P450 mediated *N*-dealkylation to a more labile *N*-monosubstituted carbamate.

It would be wrong to conclude from the above that all *N*-monosubstituted carbamates of phenols are unstable. Indeed, model carbamates of structure **8.126**, in which NHR is an amino acyl moiety, had $t_{1/2}$ values ranging from 6 to 200 min in buffer at pH 7.4 and 37° [161]. The greatest stability was seen when the aminoacyl moiety had a free carboxy group (*e.g.*, $NHCH(CH_3)COOH$). However, hydrolysis in human plasma was fast ($t_{1/2}$ 1 – 20 min), again demonstrating enzymatic hydrolysis and lack of inhibition of cholinesterase. The marked effect of ring substitution on the rate of hydrolysis was also documented in this study.



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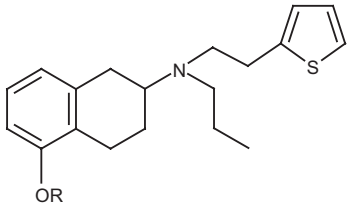
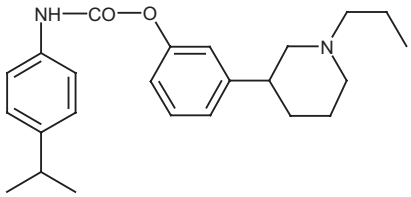


8.127 R = —H

8.128 R = —CONHR'

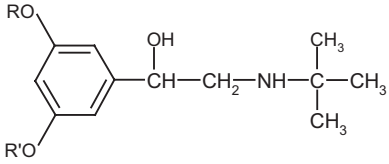
The first example of a *N*-monosubstituted carbamate of pharmacological interest to be illustrated here is a small series of prodrugs of the dopamine agonist *N-0437* (**8.127**) [162]. In rat serum at 37°, the *N*-propylcarbamate (**8.128**, R′ = propyl) was found to be very stable, while various *N*-arylcarbamates (**8.128**, R′ = substituted phenyl) had $t_{1/2}$ values for hydrolysis of 2 – 6 h. In an *in vivo* model of oral administration of a dopaminergic agonist, some of the *N*-arylcarbamates exhibited less-acute but considerably prolonged effects compared to those of the directly administered drug (5 – 7 h). The most interesting compound in this respect was the *N*-(2,4-dimethylphenyl)carbamate, whose activity was sustained for 20 h. This pharmacodynamic behavior indicates that the active drug is delivered by sustained release and should be of value in chronic therapy.

The second illustration of the interest of *N*-monosubstituted carbamates is in prodrugs of *(–)-3-(3-hydroxyphenyl)*-N-*propylpiperidine*, also known as (–)-3-PPP [163]. This presynaptic dopamine autoreceptor agonist readily crosses the blood–brain barrier but is orally poorly bioavailable. The bioavailability of the drug was not improved in the majority of a large and structurally very diverse series of prodrugs. However, a few *N*-(substituted phenyl)carbamates stood out as remarkable exceptions. While the *N*-phenylcarbamate and *N*-(4-chlorophenyl)carbamates were poorly bioavailable, the *N*-(4-isopropylphenyl)carbamate (**8.129**), *N*-(4-ethoxyphenyl)carbamate, and *N*-(3,4-dimethoxyphenyl)carbamate each exhibited good bioavailability. Pro-



8.129

drug **8.129** was particularly noteworthy, since, after an oral dose to rats, it produced plasma levels of (–)-3-PPP that were up to 90 times higher than after direct administration of the drug. Interestingly, a few *N*,*N*-dialkylcarbamates were poor prodrugs, in marked contrast to other examples discussed here. Much remains to be understood before the rational design of carbamate prodrugs can be undertaken.



8.130 R = R' = —H

8.131 R = R' = —CON(CH$_3$)$_2$

One of the best examples of a clinically successful prodrug is, again, a *N*,*N*-disubstituted carbamate, namely the bronchodilator *bambuterol* (**8.131**), the bis(*N*,*N*-dimethylcarbamoyl) derivative of *terbutaline* (**8.130**) and a β_2-adrenoreceptor agonist. To increase the duration of action and decrease the side effects usually seen with drugs of this type, a prodrug strategy was adopted and a substantial research program undertaken [164b]. Simple *O*-alkanoyl esters were too unstable to survive first-pass hydrolysis, but their increased lung affinity was encouraging. Some benzoates gave very good results in dogs, but behaved disappointingly in humans. The discovery of bambuterol fulfilled clinical expectations. Compared to terbutaline 5 mg taken three times daily, bambuterol 20 mg taken once daily generated smooth and sustained plasma levels of terbutaline in patients and provided greater symptomatic relief of asthma with a lower incidence of side effects [165].

The phase-I metabolism of bambuterol is complex and involves both oxidation and hydrolysis. In human plasma, hydrolysis rapidly yields the monocarbamate metabolite, and then, slowly, terbutaline [164a]. The reaction is catalyzed mainly by the nonspecific cholinesterase (EC 3.1.1.8) found in plasma [166]. In addition, cytochrome P450 catalyzed hydroxylation of an

N-Me group is partly followed by *N*-demethylation, and the oxidation products can also undergo ester hydrolysis. As a result, several metabolic pathways convert bambuterol to terbutaline, and both plasma and liver microsomal enzymes may be involved.

These results alone do not explain the slow hydrolysis of bambuterol and the resulting long duration of action of terbutaline. Bambuterol is also characterized by a high inhibitory capacity toward its activating enzyme, cholinesterase, with an IC_{50} value of 17 nM [167a]. The monocarbamate is ten times less potent. During the course of enzymatic hydrolysis of bambuterol, a catalytic serine residue becomes carbamoylated (see *Chapt. 3*). Liberation of the carbamic acid to regenerate active enzyme occur very slowly, thus, inhibition of cholinesterase by bambuterol is of long duration but fully reversible, with 2 d or more being necessary for complete recovery. Another important feature of the inhibition is its selectivity toward nonspecific cholinesterase, since inhibition of acetylcholinesterase (EC 3.1.1.7) would lead to increased levels of acetylcholine and exacerbation of asthmatic symptoms. As it turns out, bambuterol is 2000 times less effective as an inhibitor of acetylcholinesterase ($IC_{50} = 41$ μM) than of cholinesterase [167a].

8.5.7. Cyclization-Activated Carboxylic Acid Ester Prodrugs

The design of prodrugs that are activated by intramolecular reactions, *i.e.*, prodrugs that are partly or completely activated without the need for enzymatic contribution, is an area of great current interest. As outlined in *Chapt. 1*, this approach can lead to a decrease in biological variability that facilitates the development of clinically useful prodrugs. One important condition, however, is that intramolecular catalysis should not be so fast that it results in poor bioavailability.

A few examples of ester prodrugs that are activated by *intramolecular reactions* have been mentioned in *Sect. 8.3.1*, *8.5.1*, and *8.5.2*. Here, we discuss the special case of some carboxylic acid esters of active alcohols or phenols that are released following an intramolecular cyclization–elimination reaction [168]. The general reaction scheme of such reactions is shown in *Fig. 8.8*.

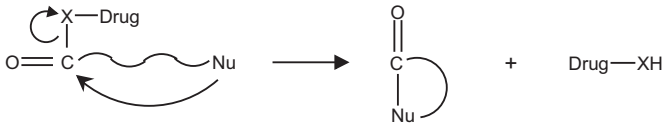


Fig. 8.8. *General reaction for the intramolecular activation of prodrugs by cyclization–elimination* [168][169]

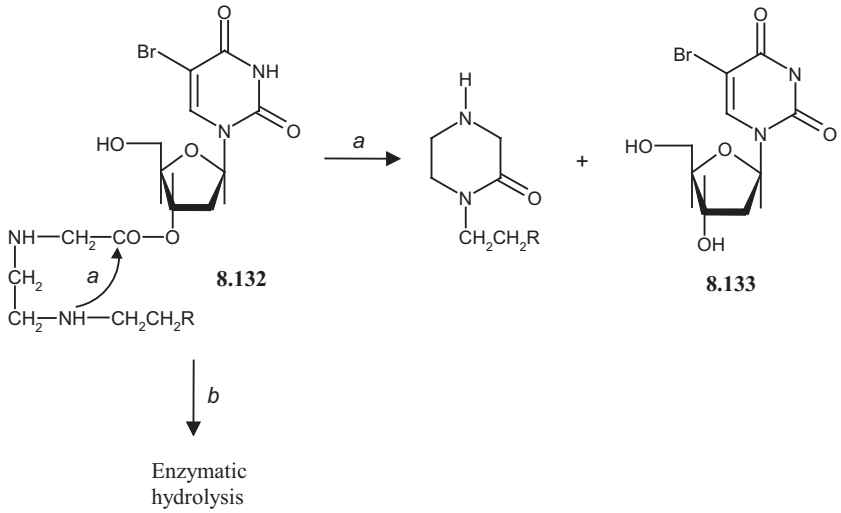
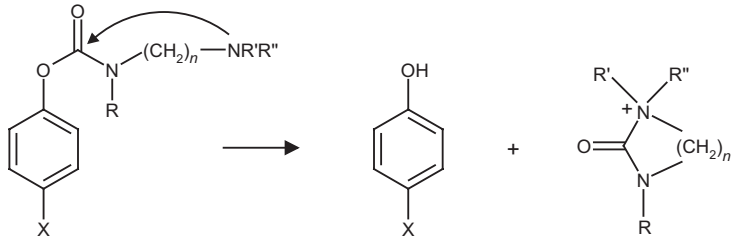


Fig. 8.9. *Activation of basic ester prodrugs of 5-bromo-2′-deoxyuridine* (**8.132**) *by cyclization of the pro-moiety* (*Reaction a*) *and by enzymatic hydrolysis* (*Reaction b*) [170]

8.5.7.1. *Nucleophilic Attack by a Basic Group*

Nucleophilic attack by a basic group is a useful strategy for cyclization. Thus, the radiation sensitizer *5-bromo-2′-deoxyuridine* (**8.133**, *Fig. 8.9*) was derivatized with *diamino acids* to obtain prodrugs **8.132** (R = H or cyclohexyl) [170]. Cyclization proceeded cleanly to yield the drug and a piperazinone derivative without any other detectable product being formed (*Fig. 8.9*, *Reaction a*). No hydrolysis was observed under acidic conditions. In pH 7.4 buffer at 37°, the $t_{1/2}$ values for hydrolysis of the two prodrugs (**8.132**, R = H or cyclohexyl, *Fig. 8.9*) were 23 and 30 min, respectively. The corresponding values in human plasma under the same conditions were 70 and 47 min, respectively, suggesting that protein binding protects the prodrugs from breakdown. In rat plasma, the $t_{1/2}$ value of 47 min for the first compound again suggests protection, but the cyclohexyl derivative had a $t_{1/2}$ value of only 5 min, which suggests that carboxylesterases can carry out enzymatic hydrolysis in rat plasma. Similar behavior was observed with the positional isomer in which the pro-moiety was attached to the 5′-OH group. The structure of the pro-moiety released by enzymatic cleavage (*Fig. 8.9*, *Reaction b*) is not expected to be a piperazinone.

Thus, intramolecular activation (cyclization–elimination) in this series is modulated by steric factors. In addition, hydrolysis may be enzyme-catalyzed, depending on substrates and biological conditions.



8.134

Fig. 8.10. *Activation of basic carbamates of phenols* (**8.134**) *by cyclization–elimination of the pro-moiety* (X = H or MeO, *n* = 2 or 3) [171][172]

Table 8.10. *Activation of Basic Carbamic Acid Esters* (**8.134**, *Fig. 8.10*) *of 4-Hydroxyanisole* (X = MeO) *or Phenol* (X = H) *by Cyclization of the Pro-Moiety at pH 7.4 and 37°* [171][172].

X	*n*	R	R′	R″	Buffer $t_{1/2}$ [min]
CH_3O	2	H	H	H	724
CH_3O	2	CH_3	H	H	304
CH_3O	2	H	CH_3	H	335
CH_3O	2	CH_3	CH_3	H	36
CH_3O	2	CH_3	CH_3	CH_3	40
CH_3O	2	CH_3CH_2	CH_3CH_2	H	118
CH_3O	3	H	H	H	910
CH_3O	3	CH_3	CH_3	H	942
H	2	CH_3	CH_3	CH_3	30
H	2	CH_3CH_2	CH_3CH_2	CH_3CH_2	2800
H	2	CH_3	CH_3CH_2	CH_3CH_2	2900
H	2	CH_3CH_2	CH_3	CH_3	30
H	2	CH_3	CH_3	H	24
H	2	CH_3CH_2	CH_3CH_2	H	120
H	2	H	CH_3	CH_3	167
H	2	H	CH_3	H	170
H	2	H	H	H	273

A similar type of intramolecular reaction was achieved with *basic carbamates of 4-hydroxyanisole* (**8.134**, X = MeO, *n* = 2 or 3, *Fig. 8.10*) [171]. The drug itself is a clinically effective melanocytotoxic agent. Intramolecular nucleophilic attack again resulted in cyclization–elimination, the pro-moiety being recovered as an imidazolidinone (*Fig. 8.10*, *n* = 2). In the series examined, the compounds were stable at pH 4, and became more reactive at higher pH values. At pH 7.4 and 37°, chain length and substitution at the two N-atoms had a marked influence on the $t_{1/2}$ values for hydrolysis (*Table 8.10*). First, it is clear that a shorter chain (*Fig. 8.10*, *n* = 2) favors intramolecular attack, but a decrease in $t_{1/2}$ values for hydrolysis for three compared

to two CH_2 groups was not consistently observed. More information on the influence of the amido and amino substituents is available. The fastest intramolecular reaction was seen for the *N*,*N*′-dimethyl and *N*,*N*′,*N*′-trimethyl analogues, whereas the two *N*- or *N*′-monosubstituted analogues were approximately ten times less reactive. The *N*,*N*′-unsubstituted compound was even more stable. Thus, two Me groups are a favorable feature for fast activation. Increasing the size of the substituents to Et created steric hindrance and decreased reactivity.

When the *N*,*N*′-dimethylcarbamate (**8.134**, X = MeO, $n = 2$, R = R′ = Me, *Fig. 8.10*) was incubated with rat plasma, no enzymatic hydrolysis was observed [171], which does not rule out enzymatic activation but, certainly, makes it less likely.

Valuable insights into this problem are given by an informative study on the reactivity of *phenyl carbamates of ethylenediamines* (**8.134**, X = H, $n = 2$, *Fig. 8.10*) [172]. The $t_{1/2}$ values for chemical activation at pH 7.4 and 37° reported in *Table 8.10* show remarkable consistency with the values for the 4-hydroxyanisole prodrugs. Here again, *N*,*N*′-dimethyl and *N*,*N*′,*N*′-trimethyl substitution were the optimal patterns, whereas the N-atoms being unsubstituted or substituted with an Et group markedly decreased the rate of activation. The other major interesting result of this study was that it provided proof that hydrolysis of the phenyl carbamates in *Fig. 8.10* occurs nonenzymatically. Indeed, when these compounds were incubated with human plasma, pig liver homogenates, or rat liver homogenates, the $t_{1/2}$ values for release of the phenol were either identical to those in buffer, or slightly higher due to protein binding.

8.5.7.2. *Intramolecular Attack by an Anionic N-Atom*

While the examples outlined in the previous section all pertain to attack by a basic N-atom, another possibility is intramolecular attack by an acidic N-atom, *i.e.*, a deprotonated amide. For example, in *N*-(2-carbamoylphenyl)carbamates of model phenols (**8.135**, X = H, Cl, or MeO, *Fig. 8.11*), the deprotonated carboxamido group attacks the carbamate carbonyl C-atom to form a quinazoline-2,4-dione with release of the phenol [173]. In acidic media, formation of the quinazoline-2,4-dione is decreased by competitive breakdown of the intermediate to an anthranilate and CO_2 in addition to the phenol (not shown).

The stability of a large series of *N*-(2-carbamoylphenyl)carbamates was explored in buffer and in diluted human plasma. As shown in *Table 8.11*, the rate of nonenzymatic cyclization–elimination was very sensitive to the nature of the carboxamido substituent (R′ in **8.135**, *Fig. 8.11*). Alkyl substituents

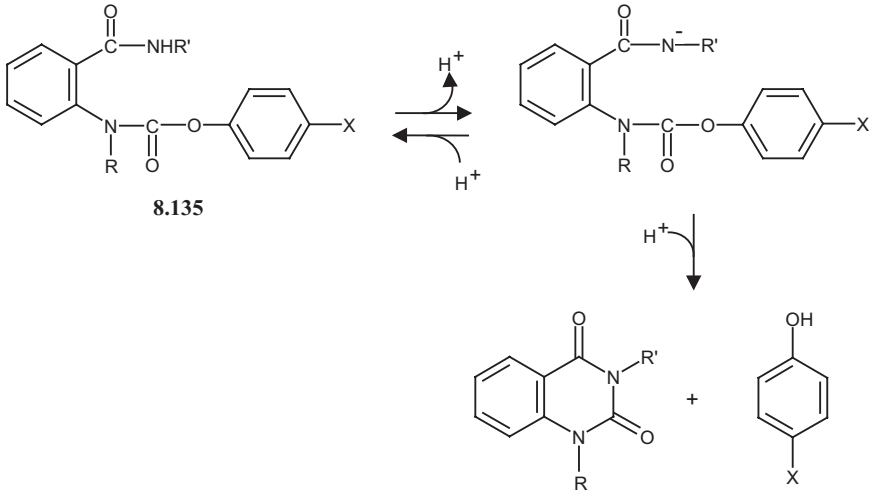


Fig. 8.11. *Simplified reaction mechanism of intramolecular cyclization–elimination of anthranilamide phenylcarbamates* (**8.135**) [173]

larger than Me markedly decreased reactivity, presumably by steric hindrance, while, in contrast, facilitated deprotonation by electron-withdrawing substituents increased reactivity. These very significant effects gave rise to rates that span four orders of magnitude.

In *Table 8.11* are also reported rates of activation in human plasma. Here, in most cases, the reaction in plasma (*i.e.*, chemical plus enzymatic activation) was approximately twofold faster than in buffer, indicating that any enzymatic hydrolysis in human plasma was modest at best. For only three prodrugs with unsubstituted carboxamido group (**8.135**, R′ = H, *Fig. 8.11*) was the enzymatic reaction severalfold faster than intramolecular catalysis, suggesting that these compounds are substrates for plasma hydrolases. With one exception, rabbit liver homogenates did not demonstrate any catalysis. In conclusion, the *N*-(2-carbamoylphenyl)carbamate pro-moiety was shown to undergo highly modulatable intramolecular activation, with no significant enzymatic cleavage.

8.5.7.3. *Intramolecular Attack by a Carboxylate Group*

Activation by intramolecular cyclization is not restricted to nucleophilic attack by acidic and basic N-atoms, but can also be catalyzed by carboxylate groups. This has been demonstrated with *hemiester prodrugs* of phenol and paracetamol (**8.136**, R = H and MeCONH, respectively, *Fig. 8.12*) [174]. In

Table 8.11. *Activation of Phenyl* N-*(2-Carbamoylphenyl)carbamates* (**8.135**, *Fig. 8.11*) *at pH 7.4 and 37°* [173]

X	R	R′	Buffer $t_{1/2}$ [min]	40% Human plasma $t_{1/2}$ [min]
H	CH_3	H	485	125 (65[a]))
Cl	CH_3	H	260	20[a])
CH_3O	CH_3	H	520	44[a])
H	CH_3	CH_3	120	70 (55[a]))
Cl	CH_3	CH_3	60	25
CH_3O	CH_3	CH_3	110	55
H	CH_3	CH_3CH_2	610	440
H	CH_3	$CH_3CH_2CH_2$	1500	[b])
H	CH_3	$(CH_3)_2CH$	130000	[b])
H	CH_3	$CH_3CH_2CH_2CH_2$	1800	580
H	CH_3	$CH_3CH_2OCOCH_2$	11	13
H	CH_3	H_2NCOCH_2	10	5
H	CH_3	$H_2NCOCH(CH_3)$	625	360
H	CH_3	$CH_3CH_2OCOCH_2CH_2$	165	85
H	CH_3CH_2	CH_3	145	85
H	CH_3CH_2	H_2NCOCH_2	12	5
H	H	H	9	5

[a]) 80% human plasma. [b]) Not reported.

addition to activation by enzymatic hydrolysis, three mechanisms of chemical hydrolysis could be distinguished, namely acid-catalyzed, base-catalyzed, and cyclization–elimination *via* intramolecular nucleophilic attack (*Fig. 8.12*, *Reactions a*, *b*, and *c*, respectively). In buffered solution, the relative importance of these three pathways was clearly pH-dependent. At physiological pH, cyclization–elimination was the predominant reaction, with $t_{1/2}$ values ranging from 1 to 350 min at 37°. Reactivity at this pH was markedly influenced by the length and degree of substitution of the pro-moiety, and by the pK_a of the phenol. Thus, succinates (**8.136**, X = CH_2, *Fig. 8.12*,) were *ca.* 150 times more reactive than glutarates (**8.136**, X = $(CH_2)_2$, *Fig. 8.12*). Methylation of the pro-moiety (*e.g.*, **8.136**, X = $CH(CH_3)CH_2$, *Fig. 8.12*) also increased reactivity. Esters of *paracetamol* (**8.136**, R = MeCONH, *Fig. 8.12*) were degraded approximately twice as fast as phenyl esters (**8.136**, R = H, *Fig. 8.12*).

Most of the hemiesters **8.136** underwent no or little enzymatic degradation in human plasma, in agreement with the known inertness of hemiesters toward cholinesterase (see *Chapt. 7*). In contrast, very rapid hydrolysis was usually seen in pig and rat liver preparations, indicating the involvement of carboxylesterases. The only inert compound was the 3,3-dimethylglutarate hemiester of paracetamol (**8.136**, X = $C(CH_3)_2CH_2$, *Fig. 8.12*). Data on the hydrolysis of such prodrugs by human hepatic enzymes will be welcome.

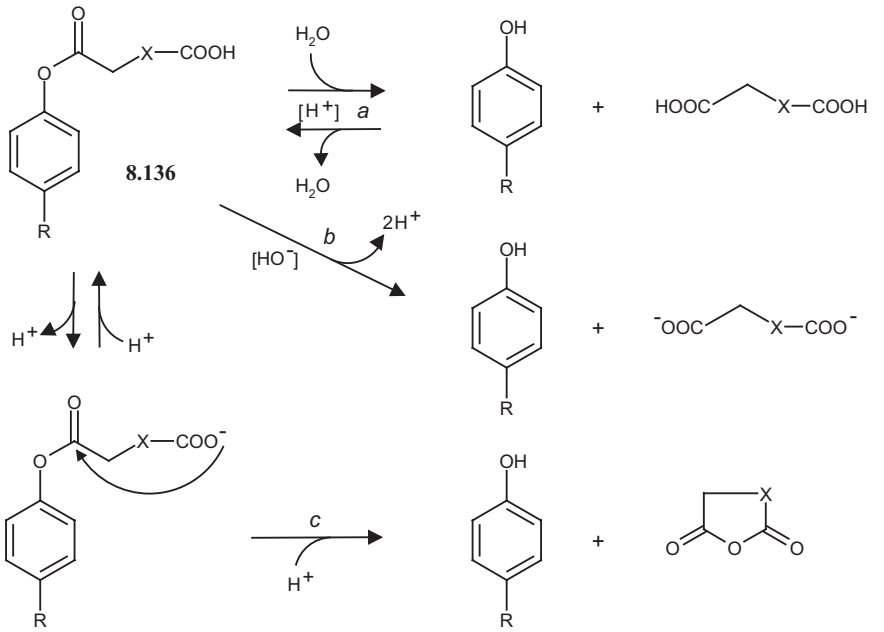


Fig. 8.12. *Activation of hemiester prodrugs of phenols by acid-catalyzed hydrolysis* (*Reaction a*), *base-catalyzed hydrolysis* (*Reaction b*), *and cyclization–elimination* (*Reaction c*). Enzymatic hydrolysis not shown (adapted from [174]).

8.5.7.4. *Intramolecular Attack by a Hydroxy Group*

Besides attack by amino, amido, or carboxylate groups, intramolecular attack by a alcoholic or phenolic OH group is also possible. Two particularly worthy examples of this type of activation are shown in *Fig. 8.13* [175]. N-*(2-Hydroxyphenyl)carbamates* such as **8.137** (*Fig. 8.13,a*) undergo intramolecular cyclization giving rise to quantitative liberation of a phenol or an alcohol (ROH in *Fig. 8.13,a*). This approach is interesting in that the cyclization product (*e.g.*, **8.138**, *Fig. 8.13,a*) is itself a drug, namely the skeletal muscle relaxant *chlorzoxazone*. A large variety of phenols and alcohols, most of which are model compounds but including paracetamol, have been investigated. In this case, a prodrug of the type **8.137** (*Fig. 8.13,a*) liberates not one but two drugs and can, thus, be termed a *mutual prodrug*.

The mechanism of activation of **8.137** in *Fig. 8.13,a* was shown to be an intramolecular nucleophilic attack by the phenolate ion at the carbonyl C-atom. Consequently, the rate of reaction increased linearly with pH, plateauing beyond 8 – 9. The major factor influencing the rate of reaction was the acidity of the leaving ROH, with $t_{1/2}$ values at pH 10 and 25° ranging from 290 d

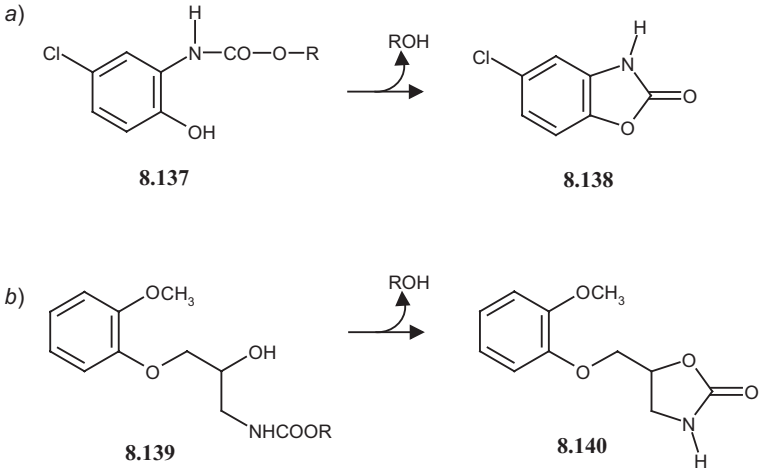


Fig. 8.13. *Cyclization* a) *of* N-*(2-hydroxyphenyl)carbamates as potential prodrugs of benzoxazoles and phenols, and* b) *of* N-*(2-hydroxypropyl)carbamates as potential prodrugs of oxazolidinones and phenols* [175]

for ROH = EtOH (pK_a 16.0) to 3 – 12 s for ROH = phenols of pK_a 9 – 10. The $t_{1/2}$ for ROH = paracetamol was 7.1 s at pH 7.4 and 37° [175].

N-*(2-Hydroxypropyl)carbamates* (**8.139**, *Fig. 8.13,b*) are prodrugs that resemble the *N*-(2-hydroxyphenyl)carbamates discussed above. Here, activation yielded the tranquilizer *mephenoxalone* (**8.140**, *Fig. 8.13,b*) and an alcohol or a phenol such as paracetamol. Other active oxazolidinones could be obtained by replacing the MeO group in **8.139** (*Fig. 8.13,b*) with another substituent. For this series, the mechanism of activation is not an intramolecular nucleophilic attack, but, rather, decomposition of the deprotonated carbamate group as shown in *Fig. 8.7,b*, *Reaction b*, with the intermediate isocyanate being trapped to form the oxazolidinone ring.

The stability of some prodrugs and mutual prodrugs (**8.137** and **8.139**, *Fig. 8.*13) in human and rat plasma was also examined [175]. An approximately tenfold acceleration was noted for the mutual prodrug **8.139** at pH 7.4 and 37° for human plasma ($t_{1/2}$ *ca.* 45 s) compared to buffer ($t_{1/2}$ *ca.* 400 s). This and other evidence indicated that cleavage of these carbamates is enzymatic. In contrast, the *N*-(2-hydroxyphenyl)carbamates (**8.137**, *Fig. 8.*13) showed two- to threefold increases in $t_{1/2}$ values in human and rat plasma compared to buffer, indicating the absence of an enzymatic hydrolysis, and modest stabilization due to binding to plasma proteins.

8.5.8. Prodrugs That Incorporate a 1,4-Dihydropyridine Pro-Pro-Moiety

The oxidation of dihydropyridine-based chemical delivery systems (CDSs) pioneered by *Bodor* and co-workers [176] has been discussed in a previous book (Chapt. 13 in [81]). There, we examined the principles by which such compounds function to deliver drugs to the brain. Here, we focus our attention to the last step in the activation of these double prodrugs, namely hydrolysis to release the drug.

The most frequently reported examples of dihydropyridine-based CDSs incorporate *1,4-dihydro*-N-*methylnicotinic acid* (dihydrotrigonelline) as the carrier. As shown in *Fig. 8.14*, the pro-prodrug first undergoes oxidation to a quaternary trigonellyl ester (*Reaction a*), followed by hydrolysis to release the drug and trigonelline (*Reaction b*). These reactions can occur anywhere in the body, but, for brain delivery, a number of conditions must be fulfilled, among which we emphasize *a*) good permeation of the pro-prodrug of the blood–brain barrier (BBB), followed by *b*) effective oxidation in the brain to the pyridinium prodrug, which may remain trapped there because of its polarity, and *c*) hydrolysis of the pyridinium intermediate in the brain. Oxidation of the dihydropyridine in the periphery is unavoidable but should be minimized since the pyridinium intermediate does not penetrate into the brain and may be readily eliminated.

Another potential limiting factor in the efficacy of dihydropyridine-type pro-prodrugs, one that appears until now to have escaped attention, is *d*) the role of *efflux pumps* at the BBB. Indeed, transporters such as P-glycoprotein

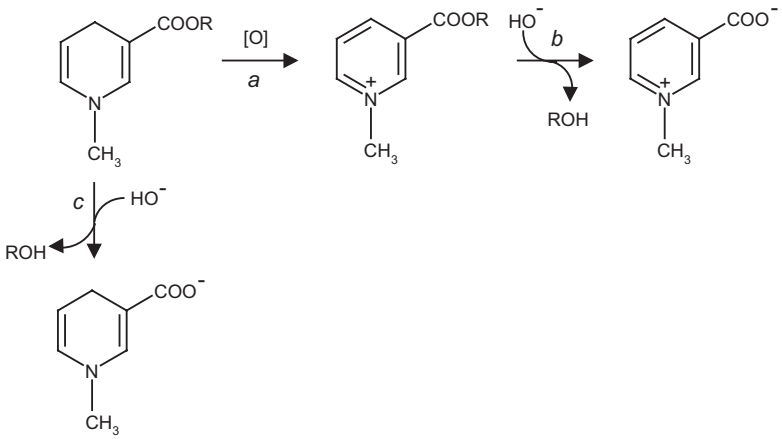


Fig. 8.14. *Stepwise activation of dihydrotrigonelline-based chemical delivery systems, first by oxidation to a pyridinium cation* (*Reaction a*), *and then by hydrolysis to trigonelline and the drug ROH* (*Reaction b*). Direct hydrolysis (*Reaction c*) is slow in comparison to the *Reactions a* and *b*.

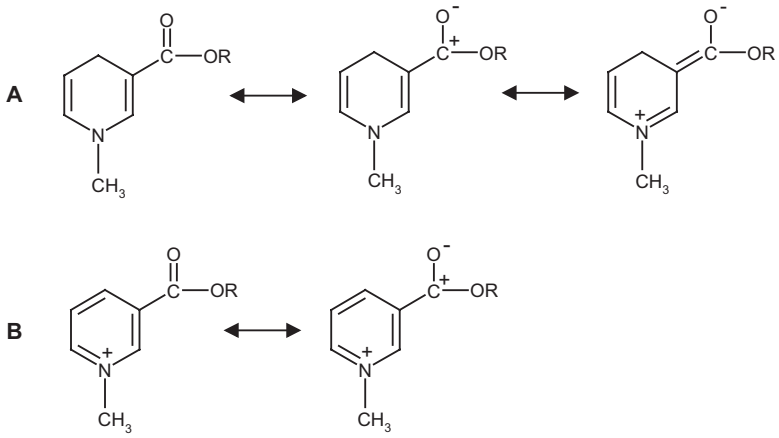
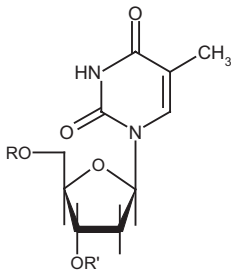


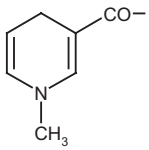
Fig. 8.15. *Resonance forms that show why the dihydropyridine pro-pro-moiety* (**A**) *is less susceptible to alkaline and enzymatic hydrolysis than the pyridinium pro-moiety* (**B**). Electron donation from the ring to decrease the electrophilicity of the carbonyl C-atom is possible for **A** but not for the pyridinium compound **B**.

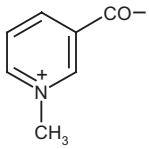
(P-gp) play an active role, and some of these function quite effectively as efflux pumps. One can, therefore, envisage a situation where brain efflux of the pyridinium intermediate competes with *in situ* activation by hydrolysis.

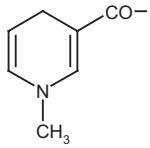
A further condition for good brain delivery, one that is particularly relevant in the present context, is that *e*) direct hydrolysis of the dihydropyridine pro-prodrug (*Fig. 8.14*, *Reaction c*) does not compete with oxidation, especially in the periphery, since this would decrease the amount of CDS available for brain delivery. In fact, the pyridinium metabolite is more susceptible than the dihydropyridine pro-prodrug to *alkaline and enzymatic hydrolysis*, since the carbonyl C-atom of the pyridinium compound (**B**, *Fig. 8.15*) is much more electrophilic than that of the dihydropyridine (**A**, *Fig. 8.15*).

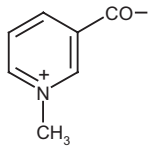
The faster base- and enzyme-catalyzed hydrolysis (but not acid-catalyzed hydrolysis, see below) of pyridinium *vs.* dihydropyridine compounds has been illustrated by a study of the stability of dihydrotrigonelline and trigonelline esters of *thymidine* (**8.141** – **8.144**) and *trifluorothymidine* in human plasma at 37° [177]. The observed $t_{1/2}$ values for hydrolysis (chemical plus enzymatic) of the 5′- and 3′-dihydrotrigonelline esters (**8.141** and **8.143**, respectively) were 210 and 220 min, respectively, while those of the corresponding trigonelline esters (**8.142** and **8.144**) were 15 and 14 min, respectively. Comparable $t_{1/2}$ values (15 and 22 min) were found for **8.144** in rat liver and brain homogenates, respectively. Thus, hydrolysis of the trigonelline esters was more than ten-times faster than that of the dihydrotrigonelline esters, confirming the enhanced electrophilicity of the pyridinium carbonyl C-atom.



8.141 R =  R' = −H

8.142 R =  R' = −H

8.143 R = −H R' = 

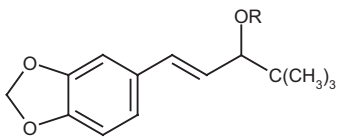
8.144 R = −H R' = 

Viral infections of the brain increasingly cause morbidity, hence the major current interest in the delivery of *antiviral agents* to the brain. For example, a variety of such agents have been coupled to dihydropyridine carriers for brain targeting. Successful preclinical investigations with the dihydrotrigonelline carrier include 2′,3′-dideoxynucleosides and zidovudine (AZT, **8.82**, R = H; see *Sect. 8.5.1*) [178][179]. In a series of zidovudine CDSs, the *N*-Me group of dihydrotrigonelline was changed to Et, Pr, i-Pr, $PhCH_2$, or cyclopropylmethyl [180]. Except for the benzyl derivative, administration of all other homologues produced markedly higher brain levels and lower blood levels of AZT than did administration of the drug itself. The *N*-Pr homologue proved to be the most effective CDS in the series, presumably because all the conditions above are best fulfilled by the *N*-Pr homologue. However, no data on individual reactions is currently available to us.

Brain delivery of *steroid hormones* is also of interest to medicinal chemists. Again, most data available on CDSs of steroids pertain to rates of oxidation of the dihydropyridine carrier, to blood and brain concentrations, and to pharmacological activities. The latter can then be taken as proof of efficient cerebral hydrolysis of the pyridinium metabolite. Thus, the dihydrotrigonelline carrier allowed good brain delivery of *estradiol* and some other estrogens [181][182].

Intriguing results were published on dihydropyridine-based CDS for stiripentol and analogous *anticonvulsants* [183]. Stiripentol (**8.145**, R = H)

has a relatively unfavorable brain-to-plasma AUC despite its lipophilicity. In rats, the dihydrotrigonelline ester (**8.145**, R = 1,4-dihydro-*N*-methylnicotinoyl) gave rise to a two- to threefold increase in this ratio. Under a variety of *in vitro* conditions, the pro-prodrug was readily oxidized, and the trigonelline underwent practically immediate hydrolysis ($t_{1/2}$ *ca.* 0.3 min). This extremely fast hydrolysis was due to an unexpected reaction mechanism: the trigonelline ester breaks down by heterolytic cleavage of the C–O bond rather than by nucleophilic attack at the carbonyl C-atom. The intermediate in the reaction appears to be a resonance-stabilized carbocation, which then binds HO^- to form stiripentol. This result may be of interest in prodrug design since it suggests that trigonelline esters of α,β-unsaturated alcohols may be very labile and liberate the drug almost immediately. In agreement with the proposed mechanism, the same study demonstrated that, with the saturated analogue of stiripentol (**8.146**, R = H), the dihydrotrigonelline carrier is not a viable CDS. Oxidation to the trigonelline intermediate occurred readily, but this ester was stable in all media and underwent neither heterolytic breakdown (which is theoretically impossible) nor enzymatic hydrolysis (a rather unexpected result). These findings were confirmed with a pair of analogues that are differently substituted on the phenyl ring.



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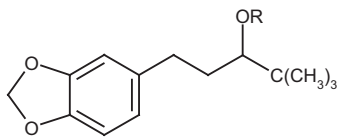
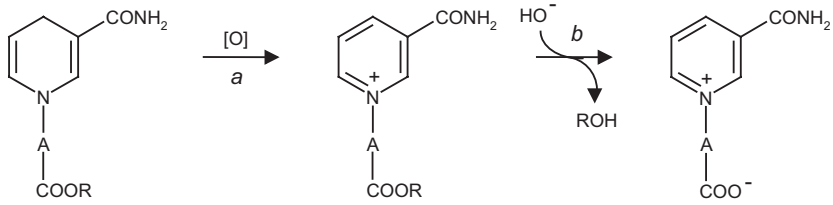


8.146

Bodor and co-workers have also proposed another type of 1,4-dihydropyridine-based CDS, *1-(carboxyalkyl)-1,4-dihydronicotinamide* carriers (**8.147**, *Fig. 8.16*). Here, the dihydronicotinamide moiety undergoes oxidative quaternization, and is linked to the drug by a carboxyalkyl spacer. Such a carrier can, thus, esterify an active alcohol or phenol. *Via* this carrier, *testosterone* was successfully released in a sustained manner in the brain in significantly higher concentrations than from the trigonelline–dihydrotrigonelline carrier [184]. When the spacer (A) in *Fig. 8.16* was CH_2, CH_2CH_2, or $CH(CH_3)$, the $t_{1/2}$ values for oxidation in rat brain homogenates were in the order of 10 – 80 min, much faster than in human blood or plasma (usually several hours). The quaternary metabolites were hydrolyzed in all these media with $t_{1/2}$ values of *ca.* 1 – 2 h. The slow oxidation in blood, the fast oxidation in brain, and the slow release of the drug by hydrolysis were all considered to be highly favorable features for sustained delivery to the brain with minimal peripheral effects.



8.147

Fig. 8.16. *Stepwise activation of 1-(carboxyalkyl)-1,4-dihydronicotinamide-based chemical delivery systems first by oxidation to a pyridinium cation* (*Reaction a*), *and then by hydrolysis to a 1-(carboxyalkyl)nicotinamide and the drug ROH* (*Reaction b*). The spacer –A– is $–CH_2–$, $–CH_2CH_2–$ or $–CH(CH_3)–$ [184].

The resonance mechanism shown in *Fig. 8.15* accounts for the greater stability of dihydropyridine pro-prodrugs *vs.* their pyridinium metabolites in base-catalyzed and enzymatic reactions of hydrolysis, but it also suggests a decreased stability of the former in *acid-catalyzed hydrolysis*. Indeed, the carbonyl O-atom is deduced from *Fig. 8.15* to be *more nucleophilic* in dihydropyridine (**A**) than in pyridinium derivatives (**B**). The stability of dihydropyridine pro-prodrugs under the acidic conditions of the stomach and small intestine should, therefore, be examined further.

8.6. Lactones as Prodrugs

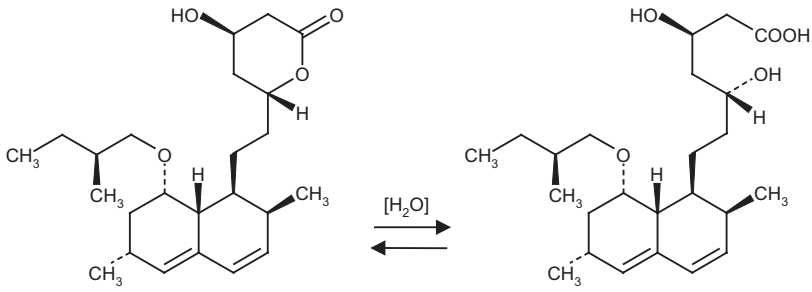
In *Sect. 7.6*, we examined the hydrolysis of lactones. Emphasis was placed on the reversibility of the reaction and the possibility of both chemical and enzymatic catalysis. Some enzymes active in lactone hydrolysis and the hydrolytic inactivation of a number of pharmacologically active lactones were discussed.

The present section is dedicated to the hydrolysis of lactone prodrugs. Here, hydrolytic ring opening, a reaction accompanied by a marked decrease in lipophilicity, unveils the active hydroxy acid. This simple argument would suggest that the value of lactone prodrugs lies in improvement of bioavailability and/or distribution, but, as discussed below, other factors that only complicate any evaluation of the relative pharmacokinetic merits of a lactone prodrug compared to a hydroxy acid drug must also be considered.

The therapeutic class that uniquely exemplifies lactone prodrugs are the *statins*, *i.e.*, the cholesterol-lowering agents that act by inhibiting 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34). This microsomal enzyme catalyzes conversion of HMG-CoA to mevalonate, an important rate-limiting step in cholesterol biosynthesis. Cholesterol synthesis occurs mainly

in the liver, thus, it is most desirable that *inhibitors of HMG-CoA reductase* exhibit high selectivity for distribution to the target organ [185 – 187].

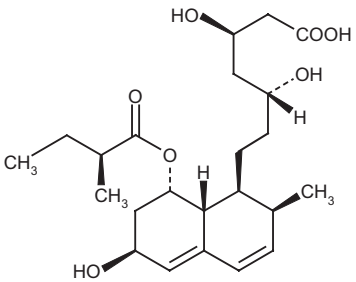
The reversibility of lactone hydrolysis in HMG-CoA reductase inhibitors has been demonstrated at gastric pH and temperature (pH 2.0, 37°) [186]. For the prodrug *lovastatin* (**8.148**), reversible lactone hydrolysis to its active hydroxy acid (**8.149**) occurred with a $t_{1/2}$ value of *ca.* 1 h and an equilibrium constant close to one. Similar results were obtained for some closely related compounds. In contrast, this reversible hydrolysis was much slower under the nearly neutral pH conditions of the intestine.



8.148 **8.149**

The oral bioavailability of lovastatin in humans and animals is *ca.* 25 – 30% compared to 80 – 100% for the hydroxy acid [188]. One may wonder, therefore, whether long gastric residence is not favorable for oral absorption. However, other pharmacokinetic factors are also of major importance in the disposition and bioactivation of lovastatin and analogues, such as hepatic extraction and enzymatic hydrolysis. A limited number of publications document the hydrolysis of lactone prodrugs, but the evidence is very clear. Thus, the rates of hydrolysis of lovastatin at 37° in undiluted plasma were highly species-dependent but seemingly always faster than nonenzymatic hydrolysis (*ca.* 0.4% per min in human plasma, *ca.* 0.8% for dogs and monkeys, and *ca.* 10 and 50% rats and mice, respectively) [188]. It is certainly of interest here that human serum paraoxonase (PON1, EC 3.1.8.1) is able to hydrolyze a wide variety of lactones [189].

The pharmacokinetic implications of these findings are not straightforward. One important factor that must also be considered is hepatic extraction, which is higher for lovastatin than for its hydroxy acid metabolite [188]. Some lactones are useful prodrugs of HMG-CoA reductase inhibitors due to this organ selectivity coupled with the efficiency of enzymatic hydrolysis. However, other factors may also influence the therapeutic response, in particular the extent and rate of metabolic reactions that compete with or follow hydrolysis, *e.g.*, cytochrome P450 catalyzed oxidations, β-oxidation, and tau-



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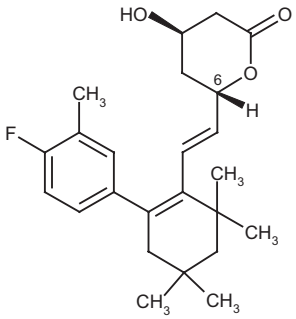


8.151

rine conjugation [190]. Another factor is active transport into the liver, as demonstrated for the hydroxy acid *pravastatin* (**8.150**) [191]. In such a case, little if any therapeutic gain can be expected from a lactone prodrug.

As a result of these and other factors, some HMG-CoA reductase inhibitors are administered as the lactone prodrug form (*e.g.*, lovastatin, simvastatin, and dalvastatin), while others are used as the active hydroxy acids (*e.g.*, pravastatin and fluvastatine) [185][187].

Another type of reaction was seen for *dalvastatin* (**8.151**), a prodrug that bears an unsaturated side chain. The hydrolysis of dalvastatin to the active acid competes with epimerization at C(6), the rate of the reaction being independent of pH above pH 2 [192]. The mechanism is believed to be one of heterolytic cleavage of the C(6)–O bond to generate a C-centered carbonium ion stabilized by the extended conjugated system characteristic of this compound. In the pH range 2 – 7, the rate of epimerization was found to be *ca.* 100 times faster than hydrolysis. Above pH 7, base catalysis accelerates hydrolysis, the rate of which increases *ca.* 100-fold between pH 7 and 9. These facts serve only to complicate the design of HMG-CoA reductase inhibitors and the interpretation of their pharmacokinetic behavior.

The occurrence of lactone prodrugs is not limited to the statins, but also includes some *prostaglandins*. Indeed, the 1,11-lactone prodrug of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been found to be superior to various $PGF_{2\alpha}$ 15-acyl esters in providing bioavailable $PGF_{2\alpha}$ for ocular hypotension while minimizing hyperemia [193].

8.7. Prodrugs of Active Amines and Amides

In addition to their presence as prodrugs of carboxylic acids, phenols, and alcohols, carboxylate moieties can also be used in the design of prodrugs of active amines and amides. The two major pro-moieties to be discussed here

are carbamates and *N*-[(acyloxy)methyl] derivatives. The first group can be further subdivided into alkyl and aryl carbamates on the one hand, and (acyloxy)alkyl carbamates on the other.

8.7.1. Alkyl and Aryl Carbamates

Carbamate prodrugs of active phenols and alcohols have been discussed in *Sect. 8.5*. The same principles operate when an active amine or amide is derivatized to a carbamate prodrug of the general formula RR′N–CO–OR″. Here again, the mechanisms of activation are those discussed in *Sect. 8.5.6*, namely enzymatic and nonenzymatic ones (see in particular *Fig. 8.7,b*). This and the subsequent section illustrate the importance of carbamates as prodrugs of active amines and amides. Here, we examine alkyl carbamates (*N*-[(alkyloxy)carbonyl] compounds) of general formula RR′N–CO–OR″, where R″ = alkyl, arylalkyl, or aryl. As will become apparent, this strategy can operate for cyclic amides as well as for amines of strong to weak basicity.

Turning our attention first to *alkyl carbamates of cyclic amides*, we find interesting attempts to improve the pharmaceutical and pharmacokinetic properties of *5-fluorouracil* (**8.152**, R = H) [194 – 196]. This antitumor agent, while clinically useful, suffers from poor water solubility, unsatisfactory delivery properties and low tissue selectivity. A variety of prodrug candidates were prepared, in particular the alkyl and aryl carbamates presented in *Table 8.12*. With the exception of the more-lipophilic derivatives, these compounds exhibited somewhat improved water solubility. More importantly, both rectal and oral bioavailability were markedly improved. The activation

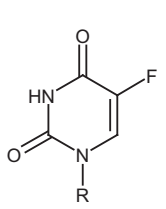
Table 8.12. *1-(Alkoxycarbonyl) Derivatives of 5-Fluorouracil* (**8.152**, R = H). Activation at pH 7.4 and 37° and bioavailability following rectal administration to rabbits [194].

R	Buffer $t_{1/2}$ [min]	80% Human plasma $t_{1/2}$ [min]	Bioavailability [%][a])
H			0
CH_3OCO	190	2	44
CH_3CH_2OCO	550	2	49
$CH_3(CH_2)_3OCO$	550	3	101
$CH_3(CH_2)_5OCO$	550	2	17
$(CH_3)_2CHOCO$	975	3	25
$(CH_3)_2CHCH_2OCO$	550	3	50
$ClCH_2CH_2OCO$	105	1	[b])
Cyclohexylcarbonyl	910	5	9
PhOCO	18	< 0.5	0
$PhCH_2OCO$	150	0.8	0

[a]) Mean of two values. [b]) Not reported.

was investigated in some detail, revealing that alkyl carbamates undergo relatively rapid chemical hydrolysis, the $t_{1/2}$ values under physiological conditions being *ca.* 3 – 15 h. The phenyl carbamate proved even more labile; enzymatic hydrolysis in human plasma was extremely rapid, with $t_{1/2}$ values of 0.5 – 5 min.

Phenytoin (**8.153**, R = H), an anti-epileptic drug, is poorly soluble in water and exhibits irregular bioavailability. This justifies the development of prodrug forms. Among the simplest prodrugs investigated are the alkyl carbamates: the pharmacokinetic behavior of ethyl and isopropyl carbamates (**8.153**, R = EtOCO and (i-Pr)OCO) was extensively investigated in rats [197]. The two compounds appeared stable in buffer at pH 2 and 7.4; in rat plasma at 37°, they yielded phenytoin with $t_{1/2}$ values of 2.5 and 4.3 min, respectively. The oral bioavailability of phenytoin increased 8.5- and 6-fold, respectively, by administration of these two prodrugs. Such results, together with the lack of toxicity of the fragments, render these carbamates quite promising.



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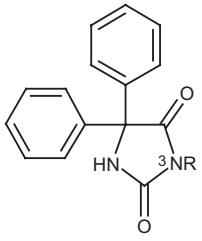

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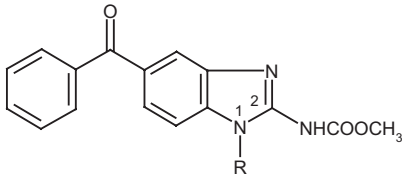


8.154

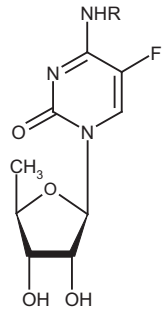
A comparable example involving a nonbasic endocyclic N-atom is provided by the antihelmintic drug *mebendazole* (**8.154**, R = H). This compound is notably water-insoluble, a shortcoming that results in poor oral absorption [198]. To overcome this problem, the solubility and rates of hydrolysis of various carbamates were examined [199]. The prodrugs **8.154** with R = MeOCO or PrOCO were approximately ten times more water-soluble than the parent compound. The $t_{1/2}$ values for hydrolysis in buffer at physiological pH and temperature were *ca.* 2.5 and 8 h, respectively, while corresponding $t_{1/2}$ values of few min were measured in human and rabbit plasma, and rabbit liver homogenates. Under all conditions, hydrolysis was reported to yield mebendazole quantitatively, which is rather intriguing, considering that the drug itself contains a pharmacophoric carbamate at C(2). When administered to experimental animals, only a modest fraction of a dose of mebendazole undergoes hydrolysis of the 2-carbamate group [198]. Thus, the *N*-

(methoxycarbonyl) group at C(2) of mebendazole appears to be much more resistant to chemical and enzymatic hydrolysis than the same group at C(1) or C(3). The reasons for these differences do not appear to have been elucidated.

Primary and secondary amino groups of different basicities have also been derivatized to yields carbamate prodrugs, often of antitumor agents. For example, *capecitabine* (**8.155**, R = $Me(CH_2)_4OCO$) is an innovative, recently marketed site-selective multistep prodrug of the antitumor drug *5-fluorouracil* (5-FU, **8.152**, R = H) [200][201]. The prodrug is well absorbed orally and is hydrolyzed by liver carboxylesterase. The resulting metabolite is a carbamic acid, which spontaneously decarboxylates to 5′-deoxy-5-fluorocytidine (**8.155**, R = H). The enzyme cytidine deaminase, which is present in the liver and tumors, transforms 5′-deoxy-5-fluorocytidine into 5′-deoxy-5-fluorouridine. Transformation to 5-FU is catalyzed by thymidine phosphorylase and occurs selectively in tumor cells. In animal tumor models with human cancer xenografts, capecitabine proved much safer and more effective than 5-FU. In particular, the prodrug delivered high levels of 5-FU selectively to tumors, in contrast to the parent 5-FU, which reached tumors nonselectively in only low concentrations.

Capecitabine was clinically first approved for the co-treatment of refractory metastatic breast cancer. Its therapeutic spectrum has been expanded to include metastatic colorectal cancer, and there are hopes that it might broaden further as positive results of new clinical trials become available. Capecitabine, thus, affords an impressive gain in therapeutic benefit compared to 5-FU due to improved oral bioavailability and relatively selective activation in and delivery to tumors.

The derivatization of a *basic function* is exemplified by the amidine peptidomimetic of structure **8.156** (R = R′ = H), an inhibitor of the platelet membrane glycoprotein $GPII_bIII_a$ [202]. The compound is a zwitterion at physiological pH and showed limited oral bioavailability. A double prodrug



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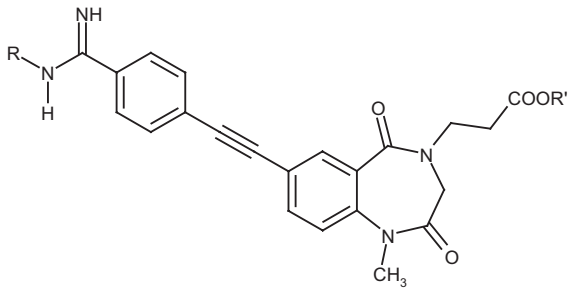
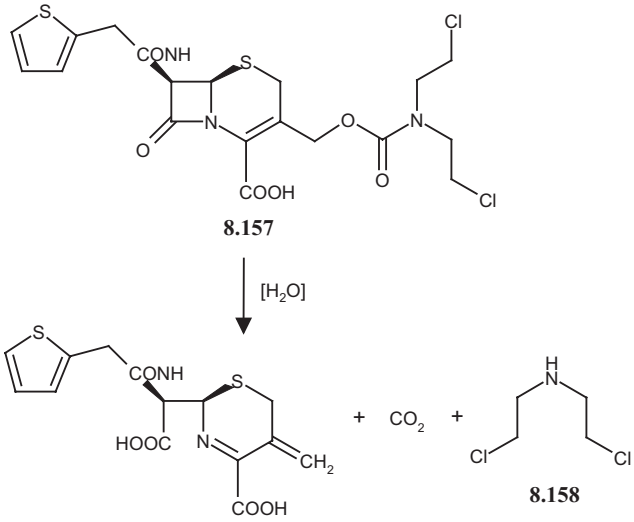


8.156

approach was examined, with R = alkoxycarbonyl and R′ = Et. The rate of hydrolysis of the carbamate function in buffer increased only slightly as a function of pH from pH 2 – 7, and then increased sharply in the alkaline range. In the small series of prodrugs investigated, the rate of hydrolysis increased sharply from R = EtOCO to R = EtSCO to R = PhOCO, *i.e.*, with increasing acidity of the leaving group (EtOH < EtSH < PhOH). These results are of interest since they suggest that the nonenzymatic activation of carbamate prodrugs of basic compounds may be tailored to desired values.

A promising concept in the design of tumor-targeted prodrugs is that of *ADEPT (antibody-directed enzyme prodrug therapy*) [203][204] (see also *Sect. 6.2*). In this approach, a tumor-specific antibody–enzyme conjugate is administered first, followed, after a given time interval, by a relatively nontoxic prodrug. The latter is a specific substrate of the antibody-bound enzyme, and its activation in the immediate vicinity of the tumor releases the cytotoxic agent. Interestingly, some prodrugs thus activated are carbamates, illustrated here with two examples. Nitrogen mustards coupled to cephalosporins by a carbamate bridge, *e.g.*, the prodrug **8.157** [205], are specific substrates of bacterial β-lactamases, which are of particular interest in the context of ADEPT due to their small size and absence of a mammalian counterpart. Incubation of prodrug **8.157** and analogues with a purified β-lactamase revealed high affinity and fast turnover for enzymatic hydrolysis, with concomitant liberation of the active agent, *e.g.*, the nitrogen mustard **8.158**. Nitrogen mustards such as **8.158** are unselective cytotoxic DNA alkylators. Only by selective delivery to tumor cells can their unwanted toxicity be minimized.



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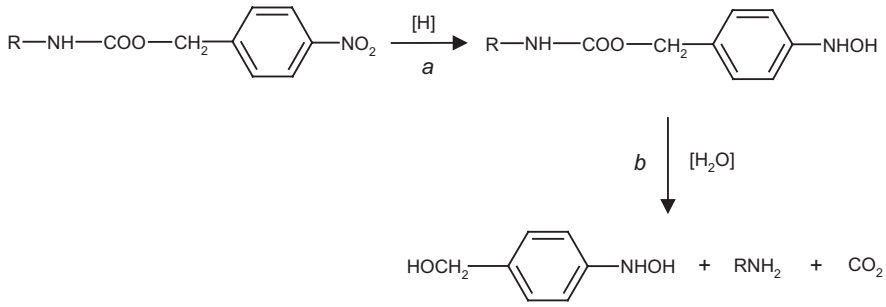


Fig. 8.17. *Mechanism of bioactivation of 'self-immolative' carbamate prodrugs of cytotoxic amines.* A bacterial nitroreductase coupled to a tumor-directed antibody reduces the prodrug to a hydroxylamine (*Reaction a*), which breaks down spontaneously to liberate the antitumor drug R–NH_2 (*Reaction b*) [206].

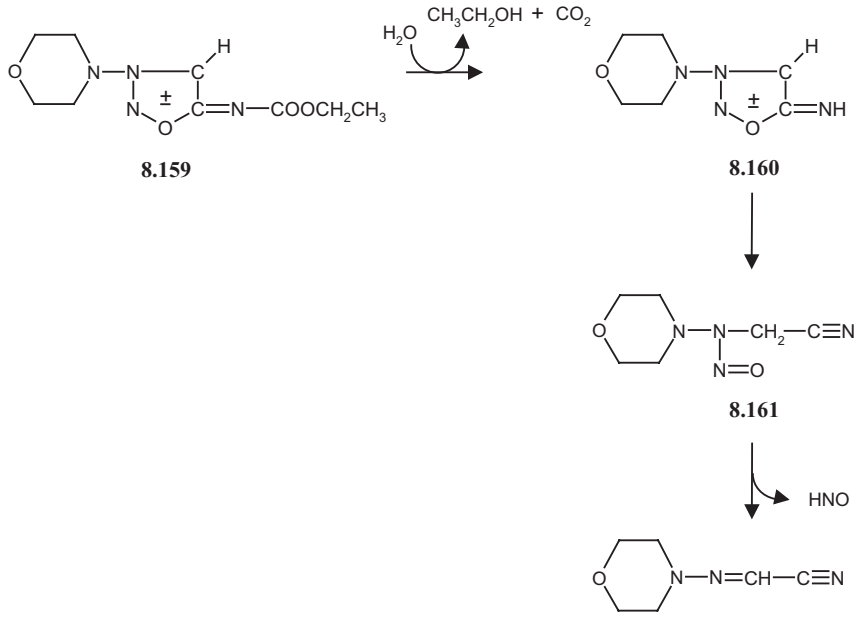


Fig. 8.18. *Metabolic activation of the prodrug molsidomine* (**8.159**), *first by enzymatic hydrolysis of the carbamic acid ester moiety to the inactive metabolite* **8.160**, *followed by spontaneous degradation to the active metabolite* **8.161**, *most likely an NO donor* [207][208]

The so-called *self-immolative prodrugs* are other relevant and intriguing examples as candidates for ADEPT (*Fig. 8.17*). Here, the primary bioactivation product is not the active agent, but an intermediate that breaks down spontaneously to liberate this active agent. Various cytotoxic drugs that bear an amino group were investigated, *i.e.*, 4-[bis(2-chloroethyl)amino]aniline, actinomycin D, doxorubicin, and mitomycin C [206]. These were trans-

formed into prodrugs by formation of the 4-nitrobenzyl carbamates. A bacterial nitroreductase was coupled to a tumor-directed antibody, and the prodrugs were, indeed, substrates for this enzyme. Reduction of the nitrobenzyl carbamate group produced a (hydroxylamino)benzyl carbamate (*Fig. 8.17*, *Reaction a*), which is unstable and breaks down to liberate the drug R–NH_2 (*Fig. 8.17*, *Reaction b*). Cytotoxicity to a tumor cell line was demonstrated in the presence of nitroreductase, while the prodrug of actinomycin D was at least 100 times less toxic than the drug itself in mice.

Molsidomine (**8.159**, *Fig. 8.18*), a very special example of a carbamate prodrug that acts by vascular smooth muscle relaxation, is an anti-angina agent effective mainly in the treatment of myocardial ischemia [207]. Molsidomine undergoes enzymatic hydrolysis in the liver to form the imine **8.160** (*Fig. 8.18*) [208]. This metabolite is inactive and unstable, breaking down spontaneously to the *N*-nitroso secondary metabolite known as Sin1A (**8.161**, *Fig. 8.18*). The latter was found to be active, but there are reasons to believe that it acts by releasing nitrogen monoxide in the form of nitroxyl (HNO), which dissociates to the nitroxide ion NO^-, *i.e.*, the reduced form of NO.

8.7.2. (Acyloxy)methyl Carbamates

(Acyloxy)methyl carbamates (and *N*-[1-(acyloxy)alkoxycarbonyl] compounds in general) have the formula RR′N–CO–O–CHR″–O–CO–R‴, where R″ = H or Me, and COR‴ = acyl. Acetoxymethyl carbamates of model amines have yielded important information on the possibilities and limitations of these potential prodrugs. For secondary amine derivatives, a clean mechanism of activation was characterized, shown in *Fig. 8.19* for one of the model compounds investigated [209]. Hydrolysis of the terminal ester moiety (*Fig. 8.19*, *Reaction a*) triggers the two subsequent breakdown reactions (*Fig. 8.19*, *Reactions b* and *c*). For compound **8.162** (*Fig. 8.19*), the $t_{1/2}$ value in buffer at pH 7.4 and 37° was 98 h, while it was 6.2 h in human plasma [210]. The parent secondary amine was hydrolyzed quantitatively.

Hydrolysis may be faster when R″ = H rather than Me in the general structure RR′N–CO–O–CHR″–O–CO–R‴. However, the toxicity of the formaldehyde released in this case may be a cause for concern.

Acetoxymethyl carbamates of primary amines behaved differently from the pathway depicted in *Fig. 8.19*, the predominant reaction being intramolecular acyl transfer to generate the *N*-acetylated amine as the major product [209]. This parasitic reaction was observed in buffer and proportionally less in plasma, disqualifying (acyloxy)methyl carbamates for use as prodrugs of primary amines. However, this type of derivative appears well suited for the preparation of prodrugs of secondary amines, as documented below.

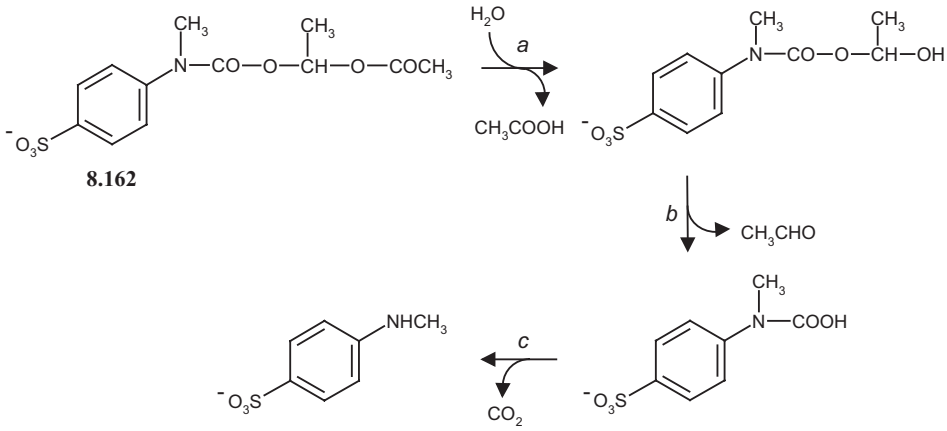
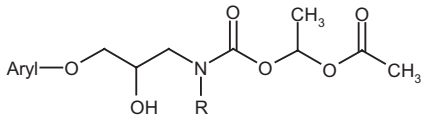


Fig. 8.19. *Sequential activation of acetoxymethyl carbamate derivatives of secondary amines, as exemplified with a model amine.* The sequence is initiated by chemical and enzymatic hydrolysis of the terminal ester group [209][210].

Attempts to prepare prodrugs of the quinolone antibacterial *norfloxacin* (**8.65**, R = R′ = H) included esterification of its carboxylate group (*Sect. 8.3.4*) and substitution of its secondary amino group. Thus, two acetoxymethyl carbamates were examined (**8.65**, R = H, R′ = Ac–O–CH_2–O–CO; R = H, R′ = Ac–O–$CH(CH_3)$–O–CO) [211]. In slightly acidic and neutral solutions, the prodrugs were less water-soluble than norfloxacin, presumably due to loss of basicity, an issue that should not be overlooked when substituting amino groups. The second derivative was examined in detail for its stability and pharmacokinetic behavior. Hydrolysis in a neutral buffer of pH 7.4 at 37° was very slow ($t_{1/2}$ *ca.* 130 h), but it was rapid in biological media ($t_{1/2}$ 1.9 h, 1.5 min, 6.0 min, and 2.6 min in human serum, rat serum, dog serum, and rat intestinal homogenates, respectively). Unfortunately, the oral bioavailability in monkeys was low, being approximately half that of norfloxacin.

A somewhat more promising study was published on acetoxymethyl carbamates of some *β-blockers* of the general formula **8.163** [212]. The rates of hydrolysis were comparable to those reported above for the norfloxacin prodrug, namely slow in buffer at pH 7.4 and 37° ($t_{1/2}$ 1 d or more), and very fast in rat and dog plasma ($t_{1/2}$ of a few min). Interesting results were reported for *in vitro* permeability studies (fuzzy rat skin and rabbit cornea), with the carbamates of hydrophilic *β*-blockers (atenolol and pindolol) showing severalfold better permeation than the parent drugs, although the carbamates of lipophilic *β*-blockers (propranolol and timolol) displayed no advantage.

Finally, we draw attention to the importance of carbamates, specifically of (acyloxy)methyl carbamates, as potential prodrugs of *guanidine deriva-*



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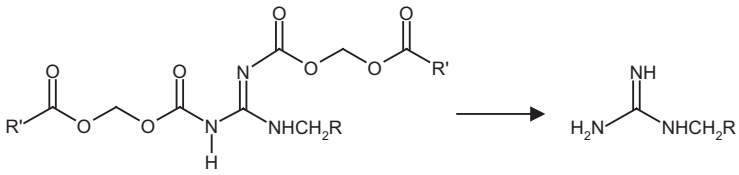


8.164 **8.165**

tives (compare also *Sect. 8.7.1*). In the absence of electron-withdrawing substituents, this moiety is very basic and polar, so much so that it may prevent good oral absorption in agents that contain it. A preliminary report indicates that *N*,*N*′-bis[(acyloxy)methyl carbamate] derivatives (**8. 164**) of guanidino-containing compounds (**8.165**) may serve as lipophilic, uncharged, esterase-activatable prodrugs [213].

8.7.3. *N*-[(Acyloxy)]methyl Derivatives

N-[(Acyloxy)methyl] derivatives of active amines and amides have the general formula RR′N–CHR″–O–COR‴ where R″ = H or Me (or an even larger substituent) and COR‴ is the acyl group. Activation of these derivatives occurs in two steps as depicted in *Fig. 8.20*, by analogy with the biotransformation of *O*-[(acyloxy)methyl] derivatives of phenols (*Sect. 8.5.6*) [62]. The first step is enzymatic or nonenzymatic cleavage of the ester bridge (*Fig. 8.20,a*), followed by chemical breakdown of the *N*-(hydroxymethyl) intermediate (see also Chapt. 5 in [81]). Here, again, liberation of toxic formaldehyde, *i.e.*, where R″ = H, should be avoided whenever possible by substitution of, *e.g.*, Me at R″.

8.7.3.1. *Cleavage of the* N-*(Hydroxymethyl) Intermediate*

Several reaction mechanisms are possible for the cleavage of *N*-(hydroxymethyl) intermediates [214]. In the case of derivatives of amides and imines, the reaction is *base-catalyzed*, with the rate-limiting step being deprotonation of the *N*-(hydroxymethyl) compound (which has a pK_a of *ca.* 13; *Fig.*

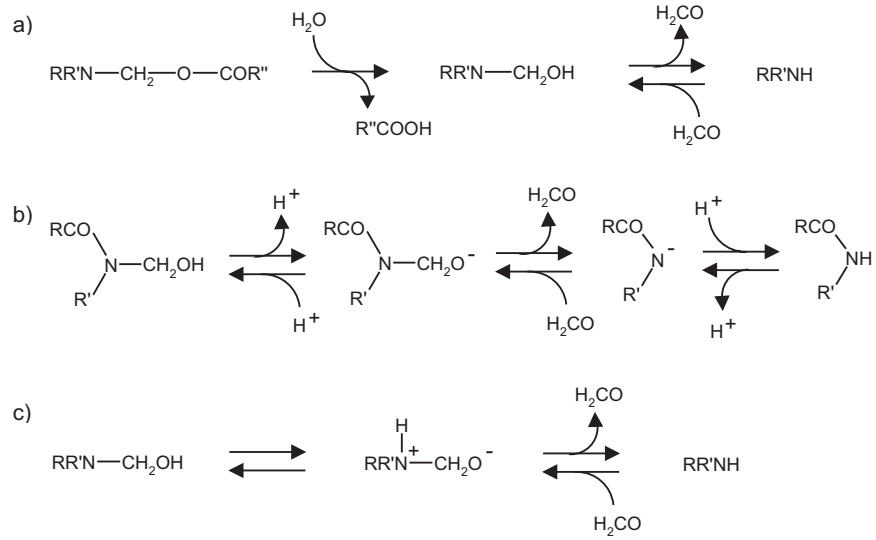


Fig. 8.20. *Two-step activation of* N-*[(acyloxy)methyl] prodrugs.* a) Cleavage of the ester bond, which may be enzymatic and/or nonenzymatic, is followed by decomposition of the *N*-(hydroxymethyl) intermediate. *b*) For *N*-(hydroxymethyl) derivatives of amides and imides, the decomposition is base-catalyzed. *c*) For *N*-(hydroxymethyl) derivatives of amines, the decomposition can be uncatalyzed or undergo acid or base catalysis (modified from [214]).

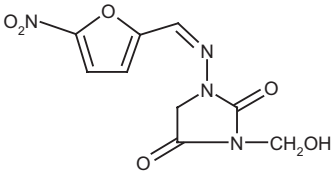
8.20,b). The decomposition rates of *N*-(hydroxymethyl) amides and imides are markedly influenced by the pK_a of the parent amide or imide such that the stronger the acidity, the faster the breakdown of the *N*-(hydroxymethyl) derivative. A linear correlation exists between the log of the second-order rate constant and the pK_a of parent amide or imide (range 7 – 14) [215]. For amides or imides with pK_a values < 13.1, the $t_{1/2}$ values are less than 1 h at pH 7.4 and 37°. For example, *N*-(hydroxymethyl)benzamide is rather stable ($t_{1/2}$ *ca.* 180 h under these conditions), whereas N^3-(hydroxymethyl)phenytoin is very unstable ($t_{1/2}$ 1 s under these conditions).

N-(Hydroxymethyl) derivatives of amines undergo *acid- and base-catalyzed* decomposition, plus an *uncatalyzed* reaction in the pH range of *ca.* 4 – 9. The postulated mechanism of this last reaction is shown in *Fig. 8.20,c* and involves intramolecular proton migration to form a zwitterion. It is estimated that the breakdown of *N*-(hydroxymethyl) derivatives of amines with pK_a > 2 should be < 3 min at pH 7 and 25° [214].

Note that *N*-(hydroxymethyl) derivatives are O-*Mannich* bases, and will be re-examined in this context in *Sect. 11.5*.

Interestingly, some *N*-(hydroxymethyl) derivatives (RR′N–CH_2OH) and, more generally, *N*-(1-hydroxyalkyl) derivatives (RR′N–CH(R″)OH), were examined as prodrug candidates. The *N*-(hydroxymethyl) derivative of *nitro-*

furantoin (**8.166**), an antibiotic used in the treatment of urinary-tract infections, is a useful prodrug with improved bioavailability [214]. Perhaps the liberation of formaldehyde contributes to the antibacterial effect of the prodrug, provided that it occurs in the organ of action, in this case the urinary tract.



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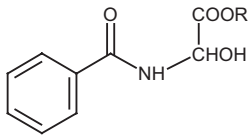


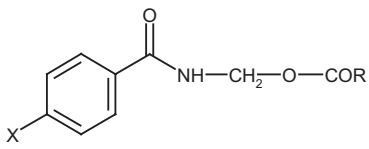
8.167

One approach to control the rate of decomposition of *N*-(hydroxymethyl) derivatives is to substitute the Me group with a COOH or COOR moiety, as investigated with some model compounds [216]. Thus, *N*-benzoyl-α-hydroxy glycine (**8.167**, R = H) broke down at pH 7.4 and 37° to benzamide with a $t_{1/2}$ value 24 times smaller than that of *N*-(hydroxymethyl)benzamide (see above), while decomposition of the esters (**8.167**, R = Me, $PhCH_2$, *etc.*) was even faster. Little enzymatic hydrolysis was seen in human plasma, except for the *N*,*N*-disubstituted carbamoylmethyl ester (**8.167**, R = Et_2NCOCH_2).

8.7.3.2. *Simple* N-*[(Acyloxy)methyl] Derivatives*

It is convenient to categorize *N*-[(acyloxy)methyl] derivatives according to the simple or complex (*i.e.*, functionalized) nature of the COR‴ acyl group. *Simple* N-*[(acyloxy)methyl] derivatives of amides* are treated first.

Model *primary amides* (**8.168**, R = Me or Ph, X = H, Cl, or NO_2) were used to investigate the mechanism of hydrolysis of their *N*-[(acyloxy)methyl] derivatives [217]. These compounds were hydrolyzed very rapidly, with $t_{1/2}$ values at pH 7.4 and 37° of *ca.* 1 min. This is much faster than predicted from structure–reactivity relationships, and led to the suggestion of an elimination–addition mechanism involving a reactive *N*-acylimine intermediate (acyl–N=CH_2). In contrast, *N*-[(acyloxy)methyl] derivatives of *imides*



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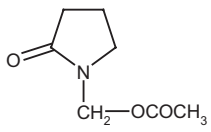


8.169

and secondary amides decomposed at the expected rates at physiological pH. As an example, the model compound **8.169** decomposed with a $t_{1/2}$ value of *ca.* 270 h at pH 7.4 and 37°. These findings led to the original suggestion that an *N*-acyliminium intermediate is *not* involved in the decomposition of *N*-[(acyloxy)methyl] derivatives of imides and secondary amides [217].

This view has been challenged with more recent evidence indicating that *N*-[(acyloxy)methyl] derivatives of both primary and secondary amides (**8.170**, *Fig. 8.21*) undergo decomposition by the *same mechanisms*, namely *a*) an acid-catalyzed process involving protonation followed by formation of an *N*-acyliminium species (*Fig. 8.21*, *Reaction a*); *b*) a pH-independent heterolytic cleavage forming the same *N*-acyliminium species (*Fig. 8.21*, *Reaction b*); and *c*) a base-catalyzed pathway, which for *N*-[(acyloxy)methyl] derivatives of *N*-methylamides is the normal mechanism (*Fig. 8.21*, *Reaction c*), but for *N*-[(acyloxy)methyl] derivatives of primary amides involves substrate deprotonation followed by *N*-acylimine formation (*Fig. 8.21*, *Reaction d*) [218].

What mainly differentiates *N*-[(acyloxy)methyl] derivatives of primary and secondary amides are the relative contributions of *Reactions a – d* as a function of pH, and, mainly the onset of the base-catalyzed *Reactions c* or *d*. Thus, the pathway for hydrolysis of derivatives of primary amides is pH-independent (*Reaction b*) at *ca.* pH 2 – 5, and is base-catalyzed (*Reaction d*) above pH 5, which explains the lability of primary amides at pH 7.4. In contrast, the base-catalyzed pathway for derivatives of secondary amides (*Reaction c*) is not important below pH 9 – 10, which is why these amides are relatively stable at pH 7.4 [218]. This specific behavior, thus, eliminates *N*-[(acyloxy)methyl] derivatives as potential prodrugs for primary amides.

Of course, the above considerations pertain only to chemical breakdown and give no information about possible enzymatic hydrolysis of the ester bridge to liberate the *N*-(hydroxymethyl) intermediate (*Fig. 8.20,a*). That such reactions do, indeed, occur is documented below as we examine some selected examples of *N*-[(acyloxy)methyl] prodrugs.

Four *N*-[(acyloxy)methyl] derivatives of 5-FU (**8.152**, R = $AcOCH_2$, Et-$COOCH_2$, $PrCOOCH_2$, and (*t*-Bu)$COOCH_2$) were very stable in buffer at pH 7.4 and 37° ($t_{1/2}$ 70, 90, 140, and 700 h, respectively) [219]. In 80% human plasma under the same conditions of pH and temperature, the $t_{1/2}$ values were 14, 9.6, 2.3, and 40 h, respectively, *i.e.*, a marked substrate-dependent acceleration. In rat liver homogenates, the $t_{1/2}$ values for decomposition were in the order of a few minutes or less. Clearly enzymatic hydrolysis is possible for such compounds and may even be quite significant. Additional data on *N*-[(acyloxy)methyl] prodrugs have confirmed their potential for topical delivery of 5-FU [220].

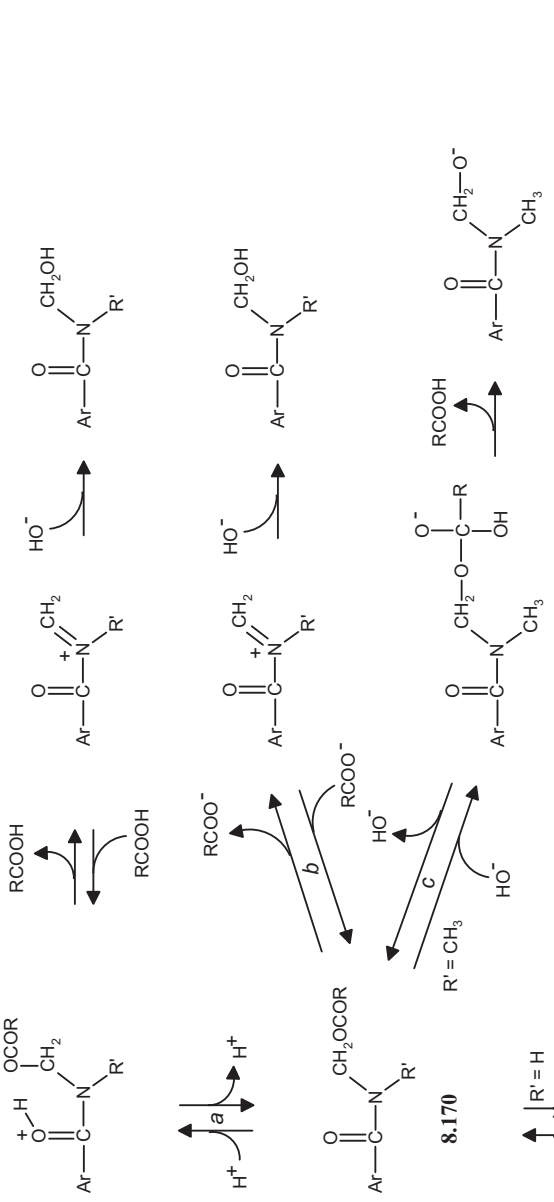
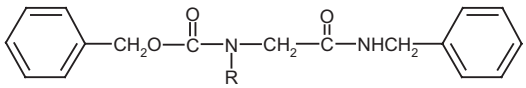


Fig. 8.21. *Mechanisms of cleavage of* N-*[(acyloxy)methyl] derivatives of primary amides* (**8.170**, R′ = H) *and secondary amides* (**8.170**, R′ = methyl) via *an acid-catalyzed pathway (Reaction a), a pH-independent pathway (Reaction b), and base-catalyzed pathways (Reactions c* and *d)* [218].

N-Derivatization of *phenytoin* has led to other prodrugs beside the carbamates discussed in *Sect. 8.7.1*. Thus, a homologous series of N^3-[(acyloxy)methyl] derivatives (**8.153**, R = $Me(CH_2)_nCOOCH_2$, $n = 0-8$) was examined [221]. In human plasma at 37°, the compounds released phenytoin with $t_{1/2}$ values that were shortest for $n = 1-4$ (1.6 – 2.6 min), and longer for the higher homologues, up to 33 min for the *N*-[(decanoyloxy)methyl] derivative. There was, thus, an apparent parabolic relationship between chain length and rate of decomposition. A comparable parabola also existed for the activation by rat intestinal homogenates, but the $t_{1/2}$ values were severalfold smaller. When the N^3-[(pentanoyloxy)methyl] derivative was administered orally to rats as a tributyrin solution, the blood AUC of phenytoin was four times greater than after a corresponding oral dose of sodium phenytoin in water. A more water-soluble derivative of phenytoin, namely the dihydroxyisobutanoate prodrug (**8.153**, R = $HC(CH_2OH)_2COOCH_2$) has been shown to greatly improve the oral bioavailability and activity of phenytoin in rats [222]. Improved activity has also been noted for the *N*-[(benzyloxy)carbonyl]glycine analogue (**8.153**, R = $PhCH_2OCONHCH_2COOCH_2$) [223]. Comparable results were obtained in the dog for two N^3-[(acyloxy)methyl] derivatives (**8.153**, R = $Me(CH_2)_nCOOCH_2$, $n = 3$ or 6) despite their low aqueous solubility compared to phenytoin [224].

N-[(Acyloxy)methyl] derivatization was also examined for its potential to improve the biological stability of *peptides*. For example, *the peptide-like model N*-[(benzyloxy)carbonyl]glycine benzylamide (**8.171**, R = H) was derivatized to a few N-*[(acyloxy)methyl] derivatives* whose chemical and enzymatic hydrolysis was investigated [225]. The results compiled in *Table 8.13* indicate a fast chemical hydrolysis, the mechanism of which is depicted as *Reaction b* in *Fig. 8.21*. Enzymatic hydrolysis also occurs in human plasma, resulting in short half-lives, with the exception of the pivaloyl analogue.



8.171

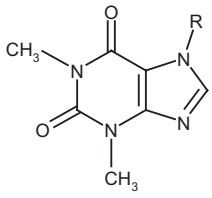
N-*[(Acyloxy)methyl] derivatives of cyclic amines* have received some attention as prodrug candidates with improved pharmacokinetic properties. Thus, *theophylline* (**8.172**, R = H) was derivatized to 7-[(acyloxy)methyl] prodrugs (**8.172**, R = $MeCOOCH_2$, $EtCOOCH_2$, $PrCOOCH_2$, *etc.*, (*t*-Bu)-$COOCH_2$) [226]. Diffusion through hairless mouse skin was found to be increased approximately three- to fivefold compared to the parent drug. As a

Table 8.13. N-*[(Acyloxy)methyl] Derivatives of the Peptide-like Model* **8.171**. Activation at pH 7.4 and 37° [225].

R	Buffer $t_{1/2}$ [min]	80% Human plasma $t_{1/2}$ [min]
$CH_3CO–OCH_2$	260	36
$CH_3CH_2CO–OCH_2$	530	10
$(CH_3)_2CHCO–OCH_2$	670	17
$(CH_3)_3CCO–OCH_2$	400	240
$PhCO–OCH_2$	58	9

result of this improved delivery, several of these theophylline derivatives were effective antiproliferative agents when applied topically to UV-irradiated mice.

Among functional groups with an endocyclic N-atom, basic NH functionalities are not the only ones that can yield *N*-[(acyloxy)methyl] derivatives. The *tetrazole ring* is of particular interest in drug design due to its remarkable acidity, which has led to its use as an isostere of the carboxy group. For example, 5-phenyltetrazole has a pK_a of 4.5 and exists as the two tautomers (**8.173a** and **b**, R = H). To gain preliminary insight into the hydrolytic behavior of acyloxymethyl derivatives of 5-phenyltetrazole taken as a model compound, its two non-interconverting isomeric *N*-(pivaloylmethyl) derivatives (**8.173a** and **b**, R = (*t*-Bu)$COOCH_2$) were examined [227]. Hydrolysis to the parent compound was quantitative with second-order rate constants (81 and 71 M^{-1} min^{-1}, respectively) not substantially different from that of the corresponding 5-FU prodrug (**8.152**, R = (*t*-Bu)$COOCH_2$; 12 M^{-1} min^{-1}; see above). In 10% rat plasma at pH 7.4 and 37°, the two pivaloylmethyl derivatives of 5-phenyltetrazole decomposed rapidly (50% hydrolysis in *ca.* 3.5 and 1 min, respectively), but the kinetics deviated from first order.



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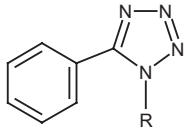

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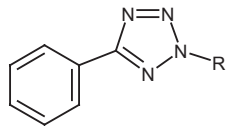
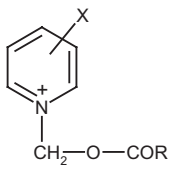


8.173b

A peculiar type of *N*-[(acyloxy)methyl] derivatives are those that contain *basic N-atoms of the pyridine type* (–N=). Here, derivatization yields a quaternary N-atom, *e.g.*, **8.174** and **8.175**. These prodrugs often demonstrate good water solubility, and their rates of activation (chemical and enzymatic)



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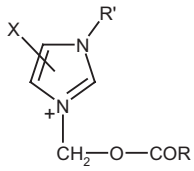

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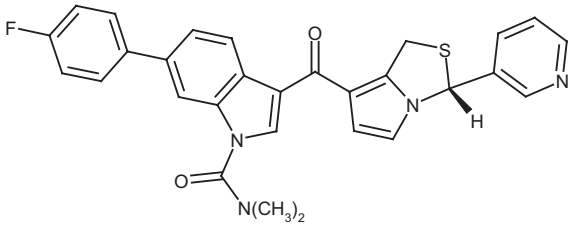


8.176

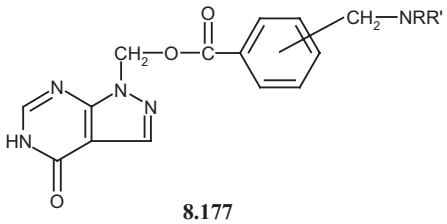
are highly dependent on the nature of the acyl moiety. A number of such *N*-[(acyloxy)methyl] derivatives have been reported for *pilocarpine* (**8.87**, *Fig. 8.6*) [214].

An interesting example is that of the *pyrrolothiazole compound* **8.176**, a potent antagonist of effects mediated by the platelet-activating factor (PAF). Since this agent, despite being active in a variety of pharmacological models, lacks sufficient water solubility, *N*-[(acyloxy)alkyl]pyridinium prodrugs were thus prepared, all of which exhibited good water solubility [228]. The rates of activation were determined in buffer at pH 7 and room temperature, and in buffered human plasma at pH 7 and 37°. Thus, the $t_{1/2}$ values in buffer and plasma, respectively, were 540 and 2.6 min when the pro-moiety was $MeCOOCH_2$, 2700 and 21 min when the pro-moiety was (*t*-Bu)$COOCH_2$, and 1050 and 31 min when the pro-moiety was (*t*-Bu)$COOCH(CH_3)$. The comparison between these derivatives is interesting since it shows that both the acyl and alkyl groups influence breakdown. However, the differences between the last two derivatives are not easy to understand.

8.7.3.3. *Complex* N-*[(Acyloxy)methyl] Derivatives*

Among *N*-[(acyloxy)methyl] derivatives that contain a complex acyl group, a variety of promising pro-moieties that either contain a basic group or are lipid-like have been reported in the literature.

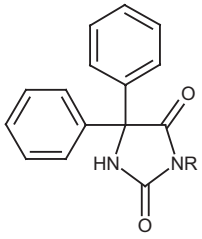
One interesting group of *N*-[(acyloxy)methyl] derivatives has a *basic acyl group* that ensures good water solubility and, usually, fast activation. This is exemplified for endocyclic NH groups by N-*[({[(aminomethyl)phenyl]carbonyl}oxy)methyl] derivatives of allopurinol* (**8.177**) [148]. With such prodrug candidates, the acyl moiety was a 4- or 3-(aminomethyl)benzoyl group, with the amino function being disubstituted, *e.g.*, dimethylamino, morpholino, *N′*-methylpiperazino. Analogous derivatives of *phenytoin* and *theophylline* (**8.153** and **8.172**, respectively, R = H_2NCH_2–C_6H_4–$COOCH_2$) were also prepared. Hydrolysis in human plasma proved to be fast, with $t_{1/2}$ values of 0.5 – 20 min at pH 7.4 and 37° (see also *Sect. 8.5.5.2*, **8.109** – **8.115**).



8.177

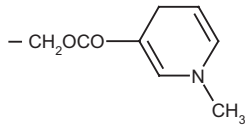
Amides and imides as well as endocyclic amines can form this type of basic *N*-[(acyloxy)methyl] derivative. The 3-[{(*N*,*N*-dimethylglycyl)oxy]-methyl} and 3-{[(*N*,*N*-diethyl-*β*-alanyl)oxy]methyl} derivatives (**8.178** and **8.179**, respectively) of the major antiepileptic drug *phenytoin* (**8.153**, R = H), discussed above, are interest and important examples. The solubilities of these derivatives at neutral pH were *ca.* 4000 and 40 times higher, respectively, than that of phenytoin. The $t_{1/2}$ values for conversion to phenytoin in human plasma at pH 7.4 and 37° were 23 s and 6.8 min, respectively. Oral administration of these prodrugs to dogs gave plasma levels of phenytoin severalfold higher than by administration of the drug itself [229].

Other basic *N*-[(acyloxy)methyl] pro-moieties include *dihydropyridine-based redox brain delivery systems* (see also *Sect. 8.5.8*). Here again, phenytoin has been the object of active investigations. Although phenytoin enters the CNS well, it is not always evidently efficacious in epilepsy [230]. A number of dihydropyridine-based chemical delivery systems were, therefore, examined, in which 3-(hydroxymethyl)phenytoin is esterified with dihydro-*N*-methylnicotinic acid (yielding **8.180**), *N*-(carboxymethyl)dihydronicotinamide (yielding **8.181**), and analogues thereof [230 – 232]. Increased lipophilicity, quantitative oxidation in rat brain homogenates, and ready hydrolysis of the pyridinium metabolites in various tissue preparations were evident. Compound **8.181** was examined for its anticonvulsant properties in rodents and found to be distinctly more-potent than phenytoin, indicating that it delivered the drug efficiently to the brain.

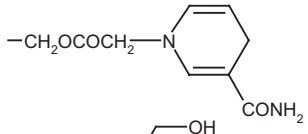


8.178 R = $-CH_2OCOCH_2N(CH_3)_2$

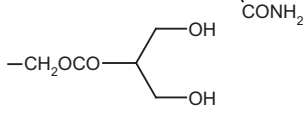
8.179 R = $-CH_2OCOCH_2CH_2N(CH_2CH_3)_2$



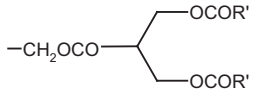
8.180 R =



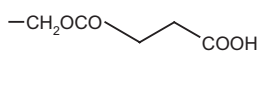
8.181 R =



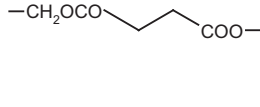
8.182 R =



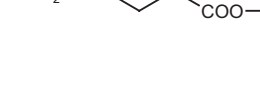
8.183 R =



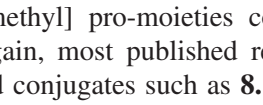
8.184 R =



8.185 R =



8.186 R =



A further group of *N*-[(acyloxy)methyl] pro-moieties contains *acidic and/or lipid-like substituents*. Here again, most published results concern phenytoin. Thus, some phenytoin–lipid conjugates such as **8.183** and **8.186** (with R′ = various fatty acyl moieties) were reported [233]. Such prodrugs are, of course, insoluble in water but formed dispersions when briefly sonicated in EtOH/water mixtures containing sodium taurodeoxycholate. No significant hydrolysis was seen in buffer or plasma. In contrast, incubation with pancreatic lipase yielded the bis-deacyl derivatives (*i.e.*, **8.182** and **8.185**, respectively), with subsequent liberation of phenytoin; the time for 50% liberation of phenytoin varied from 20 to 200 min under the conditions of the studies [233][234]. The intermediates **8.182**, **8.184**, and **8.185** were also substrates for human and rat plasma hydrolases.

After intraperitoneal administration, the phenytoin–lipid conjugates were at best equipotent with phenytoin in protection mice against seizures

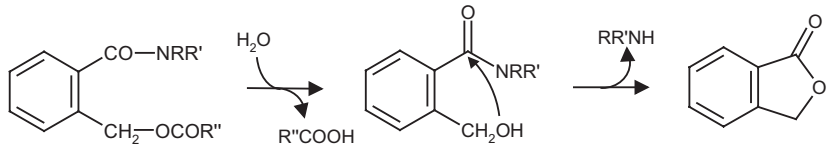
[234][235]. The anticonvulsant activity was related to the rate of lipase-catalyzed hydrolysis, confirming that these derivatives acted as prodrugs. One compound, however, proved of interest, namely the 2-monoglyceride derivative (**8.185**). The bioavailability of phenytoin in rats after oral administration was approximately four times higher with **8.185** than with phenytoin itself [236]. Furthermore, the prodrug was approximately three times more effective in antagonizing seizures than the parent drug. It was concluded that monoglyceride-derived prodrugs such as **8.185** could be useful systems for the oral delivery of poorly water-soluble drugs.

8.7.4. Cyclization-Activated Double Prodrugs of Amines and Peptides

The prodrugs examined here undergo a common, two-step mechanism of activation (hence their designation as double prodrugs); first, hydrolysis of the carboxylate group occurs, followed by intramolecular nucleophilic substitution to liberate the active amine (for reviews see [168][169][237][238]). Such *reactions of cyclization–elimination* are analogous to those discussed in *Sect. 8.5.7*.

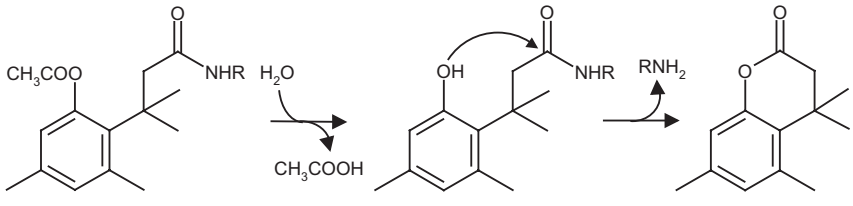
The first case presented is that of *2-[(acyloxy)methyl]benzamides* of the general structure **8.187** (*Fig. 8.22*) [239]. Two model compounds were examined (NRR′ = MeNH or morpholino, R″ = Me); they reacted, as expected, to give the secondary amine and phthalide in quantitative yields. At pH 9.3 and 60°, chemical hydrolysis was 2 – 10 times faster than the subsequent cyclization–elimination. At pH 7.4 and 37°, the chemical hydrolysis was slow ($t_{1/2}$ *ca.* 400 h), while hydrolysis in human plasma was fast ($t_{1/2}$ 3.2 and 1.4 h, respectively).

The lactonization–elimination step was investigated independently with a broad array of *N*,*N*-disubstituted 2-(hydroxymethyl)benzamides. The rate of this reaction was highly sensitive to the nature of the *N*-substituents and to pH (minimum at pH 5 – 6). Above pH 6, general base catalysis occurred.



8.187

Fig. 8.22. *2-[(Acyloxy)methyl]benzamides* (**8.187**) *as double prodrugs of active amines.* Activation is by cyclization–elimination in a two-step sequence, namely hydrolase-catalyzed hydrolysis of the carboxylate moiety followed by an intramolecular nucleophilic substitution with liberation of the amine [239]



8.188

Fig. 8.23. *3-(2-Acetoxy-4,6-dimethylphenyl)-3-methylbutanamides* (**8.188**) *as potential prodrugs of amines and peptides.* Again, hydrolysis is followed by lactonization [240].

Under physiological conditions of temperature and pH, $t_{1/2}$ values for lactonization of tens to hundreds of hours were seen. Thus, prodrugs of this type are useful only when the nature of the substituents allows lactonization to be sufficiently fast.

Another type of two-step prodrug of potential value for the delivery of amines and peptides are the *3-(2-acetoxy-4,6-dimethylphenyl)-3-methylbutanamides* (**8.188**, *Fig. 8.23*) [240]. Here, a relatively large pro-moiety is attached to the amino group of the active compound. Again, and as shown in *Fig. 8.23*, hydrolysis is followed by lactonization. To examine this delivery system, 6-methoxyaniline was used as a model compound coupled to the pro-moiety. In buffer at physiological pH and temperature, no intermediate phenol was detected; the disappearance of the prodrug and the appearance of the lactone occurred with a $t_{1/2}$ value of *ca.* 4000 min. In the presence of porcine liver esterase or human plasma, the $t_{1/2}$ value was *ca.* 12 and 54 min, respectively, with very little intermediate phenol being detected. Compared with the 2-[(acyloxy)methyl]benzamides discussed above, this prodrug system is characterized by greater susceptibility to hydrolysis and essentially instantaneous lactonization–elimination. The disadvantage, however, is that the larger pro-moiety adds significantly to the molecular weight of the protected amine.

The 3-(2-hydroxy-4,6-dimethylphenyl)-3-methylbutanoic acid shown in *Fig. 8.23*, as well as another phenylpropanoic derivative presented below, have been used as pro-moieties to prepare a number of prodrugs of therapeutic peptides [169][238]. Of interest here is that the pro-moiety is linked to the peptide by both amide and ester bonds to form a *cyclic*, *double prodrug*, the two-step activation of which is schematized in *Fig. 8.24*. Briefly, enzymatic hydrolysis of the ester bond unmasks a nucleophile (in this case, a phenol) that carries out an intramolecular attack on the amide bond, resulting in cyclization of the pro-moiety and elimination of the peptide. *[Leu^5]enkephalin* was one of the therapeutic peptides used to validate the concept, as illustrated in *Fig. 8.25* [241].

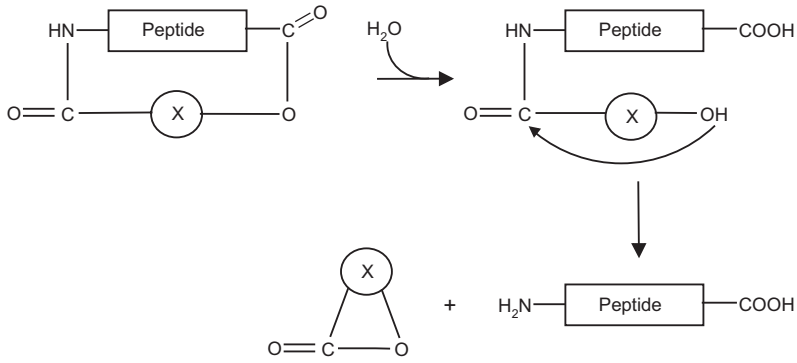


Fig. 8.24. *Schematic representation of cyclic, double prodrugs of peptides and their mechanism of activation by enzymatic ester cleavage, followed by cyclization–elimination* [168][169][238]

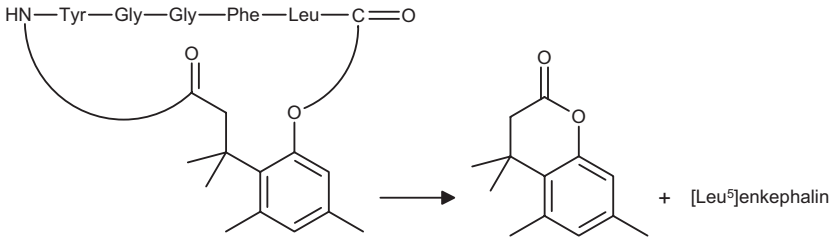
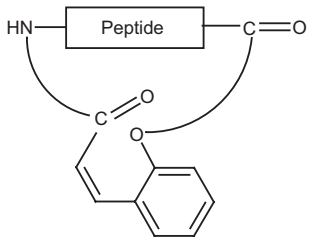


Fig. 8.25. *3-[2-(Acyloxy)-4,6-dimethylphenyl)-3-methylbutanamide as double pro-moiety of [Leu^5]enkephalin* [241]. The activation mechanism is identical to that shown in *Fig. 8.24.*

Coumarinic acid is another phenylpropanoic acid pro-moiety used to prepare double prodrugs of amines (in analogy with the phenylpropanamides shown in *Fig. 8.23*), as well as cyclic prodrugs of a number of peptides (**8.189**) [242 – 246]. These studies confirm the considerable potential of the cyclic prodrug strategy, such compounds being characterized by metabolic stability to peptidases and good cellular permeation.



8.189

8.7.5. Special Cases

A few additional prodrug types are of interest in the present context. In common with the examples discussed earlier, these are prodrugs of active amines or amines that contain a carboxylate moiety, but would be difficult to classify with the prodrugs discussed in the above sections.

In *Sect. 8.7.3*, we discussed *N*-(hydroxymethyl) (or *N*-(1-hydroxyalkyl) derivatives, showing how they break down more or less rapidly with liberation of the amine or amide drug, and formaldehyde or another aldehyde (see *Fig. 8.20*). *N*-(Hydroxymethyl) derivatives are usually 'tamed' by esterification. Interestingly, there is another type of precursor of *N*-(1-hydroxyalkyl) derivatives for peptides and *N*-acyl amino acids, namely *3-acyloxazolidin-5-ones* (**8.190** in *Fig. 8.26*), obtained by allowing an aldehyde to react with both the free (terminal) carboxylate group and the vicinal amido N-atom. Such derivatives were subjected to preliminary investigations for their suitability as prodrugs (or rather pro-prodrugs), and the available results suggest that they might be worth further exploration [247 – 249]. Indeed, the delivery of peptide drugs is never easy, and any promising prodrug approach is welcome [237].

The two-step activation of oxazolidinones is depicted in *Fig. 8.26*. Hydrolysis yields an *N*-(1-hydroxyalkyl) derivative, which breaks down to liberate the peptide or *N*-acylamino acid (*Fig. 8.26*, *Reactions a* and *b*). Since oxazolidinones are prepared by the condensation of a peptide and an aldehyde (*Fig. 8.26*, *Reaction c*), the reverse reaction (*i.e.*, one-step activation) cannot be excluded. Examples of this type of prodrug are provided by a series of oxazolidinones of the general structure **8.190** (R = $PhCH_2O$; R′ = H, Me, i-Pr, or $PhCH_2$; R″ = H, Me, or Ph; *Fig. 8.26*) [248]. In phosphate buf-

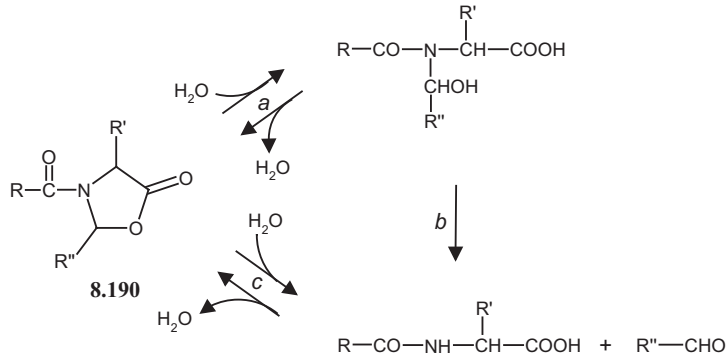
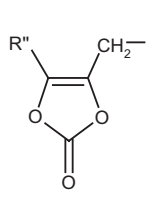


Fig. 8.26. *The two-step activation of oxazolidin-5-one derivatives of peptides and* N*-acyl amino acids* (**8.190**). Hydrolysis (*Reaction a*) yields an *N*-(1-hydroxyalkyl) derivative that breaks down to liberate the peptide or *N*-acyl amino acid (*Reaction b*) [247][248].

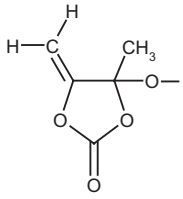
fer at pH 7.4 and 37°, these derivatives were hydrolyzed with $t_{1/2}$ values of *ca.* 3 – 8 min for R′ = H, and *ca.* 20 – 80 min for R′ = alkyl. Hydrolysis in 80% human plasma was 4 – 40 times faster. These results indicate that the rates of activation are influenced by structural factors, and that these compounds are substrates for plasma hydrolases.

Oxodioxolyl derivatives offer another possible route for the design of prodrugs of active amines. First, *(oxodioxolyl)methyl carbamates* can be envisaged, in analogy with the prodrugs of active carboxylic acids discussed in *Sect. 8.3.4* [250]. A recent example is that of the cyclic depsinonapeptide pseudomycins, which are highly active and promising antifungal agents. Prodrugs of pseudomycin B and C′ were prepared by derivatizing one, two, or all three primary amino groups to give an (oxodioxolyl)methyl carbamate [251]. These prodrugs exhibited *in vivo* efficacy comparable to that of the parent compounds, yet were free of some of their toxic side effects.

Another possibility is exemplified by *N*-(5-substituted 2-oxo-1,3-dioxol-4-yl)methyl derivatives of norfloxacin (**8.65**, R = H, R′ = **8.191**) [75b]. Modest differences were reported for the stability of the 5′-unsubstituted and 5′-Me analogues (**8.191**, R″ = H or Me) in artificial gastric juice (pH 1.2) and artificial intestinal juice (pH 6.8), with $t_{1/2}$ values at 37° in the order of 1 and 10 h, respectively. The other analogues tested (R″ = *t*-Bu or Ph) were more stable ($t_{1/2}$ 20 h). In 40% mouse blood, activation proceeded much faster, hydrolysis being complete in 10, 15, 120, and 150 min for the four respective analogues. In mice, oral administration of the *N*-(5-methyl-2-oxo-1,3-dioxol-4-yl)methyl derivative of norfloxacin (**8.65**, R = H, R′ = **8.191** with R″ = Me) produced approximately fivefold higher blood levels of norfloxacin than those seen after administration of the parent drug. These promising results were postulated to be due to improved absorption and rapid activation.



8.191



8.192

Upon chemical oxidation, the *N*-(5-methyl-2-oxo-1,3-dioxol-4-yl)methyl derivative of norfloxacin (**8.65**, R = H, R′ = **8.191** with R″ = Me) discussed above yielded an *oxodioxolane derivative*, *i.e.*, *N*-[(4-methyl-5-methylidene-2-oxo-1,3-dioxolan-4-yl)oxy]norfloxacin (**8.65**, R = H, R′ = **8.192**) [252]. This compound had modest activity *in vitro* as an antibacterial agent.

However, its *in vivo* activity in infected mice was a few-fold higher than that of norfloxacin, and even slightly better than that of its oxodioxole precursor. Bioavailability studies showed that, after oral administration in mice, the AUC of norfloxacin was three times higher for the oxodioxole or oxodioxolane prodrugs than after administration of the drug itself. Oxodioxolane derivatives appear, thus, to be a novel and interesting type of prodrug for secondary amine drugs.

8.8. Macromolecular Prodrugs

To conclude this long chapter, we give here a brief and schematic survey of prodrugs that have a macromolecule as the pro-moiety [253]. There is considerable interest in some of these systems for selective delivery and improved therapy [254]. From the pharmacochemical perspective, macromolecular prodrugs can be approached from three angles:

a) *Macromolecules* being evaluated as pro-moieties include:
- Polysaccharides (*e.g.*, dextran, inulin, hyaluronic acid, modified starch) [255 – 259];
- Proteins (*e.g.*, modified plasma proteins, lysozyme, synthetic peptides, or peptide analogues) [260 – 262];
- Antibodies (*e.g.*, monoclonal antibodies) [263 – 267];
- A large variety of synthetic polymers [254][268 – 271].

b) *Drugs* linked to such macromolecular carriers include:
- Anti-inflammatory agents (*e.g.*, colon-, kidney-, or liver-targeted delivery) [255a][261][272];
- Glucocorticoids (*e.g.*, colon-targeted delivery) [256][257];
- Antiviral agents [267];
- Chemotherapeutic agents (tumor-targeted delivery) [253][254][260] [262a][263 – 265][268][269][271][273];
- Neuroactive agents and neuropeptides (brain-targeted delivery) [266];
- Diagnostic agents (*e.g.*, tumor- or brain-targeted delivery).

c) The *link* between drug and macromolecule may be direct or indirect (*i.e.*, *via* a spacer). The two major types of chemical bonds used to link the pro-moiety and the drug, or the spacer to pro-moiety and drug, are:
- Amide groups, as relevant to *Chapt. 4* and *6*;
- Carboxylic acid ester groups, as relevant to the present chapter.

An example is provided by the renal delivery of naproxen coupled to lysozyme *via* an L-lactic acid spacer [261b]. Here, the terminal amino group

of a lysyl residue in lysozyme is acylated with L-lactic acid, while the OH group of the spacer forms an ester linkage with the drug. The kidney is the main site of uptake of such conjugates, from which the drug is released locally.

A second example is the colon-specific delivery of glucocorticoids linked to dextran *via* a succinic acid or glutaric acid spacer [256a] . Such conjugates resist hydrolysis in the upper gastrointestinal tract, but are rapidly degraded by bacteria in the colon and caecum where little drug absorption occurs.

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Chapter 9

The Cleavage of Esters of Inorganic Acids

Contents

9.1. Introduction

This chapter discusses pharmacologically and toxicologically relevant esters of inorganic acids, namely:

- organic nitrites (R–O–N=O);
- organic nitrates (R–O–NO_2);

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- organic phosphates ((RO–)(R′O–)(R″O–)P(=O)) and phosphonates (R–P(=O)(–OR′)(–OR″));
- organic sulfuric acid monoesters (R–O–S(=O)$_2$(–OH)) and sulfamates (R–O–SO_2NH_2).

These compounds contain the fragment R as an alkyl or aryl moiety. In other words, they result from the esterification of an alcohol or a phenol with nitrous acid, nitric acid, phosphoric acid, sulfuric acid, or sulfamic acid, respectively. Many of the esters to be examined in this chapter must be *activated* prior to eliciting their effects, *e.g.*, the organic nitrites and nitrates, which act as donors of nitric oxide or an analogous molecule, and phosphates, which are activated by hydrolysis or even by phosphorylation (antiviral agents). Sulfates are very seldom active or used as prodrugs, but they have significance as metabolites and as industrial xenobiotics.

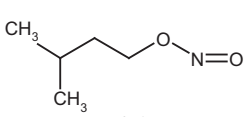
In this chapter, the words 'cleavage' and 'de-esterification' are often used to make it clear that reactions other than hydrolysis may be involved in the breakdown of some inorganic esters. This is particularly true for nitrates, and various phosphates and phosphonates, as discussed in the relevant sections.

9.2. De-esterification of Organic Nitrates and Nitrites

9.2.1. Introduction

Organic nitrates and nitrites are coronary vasodilators of great value for the treatment of angina pectoris. The discovery of glyceryl trinitrate and amyl nitrite dates back to the middle of the 19th century, and their introduction into therapy was quick to follow. The commercial drug amyl nitrite is a mixture of nitrites containing mainly isopentyl nitrite (**9.1**) and 2-methylbutyl nitrite (**9.2**). Besides glyceryl trinitrate (nitroglycerin, GTN, **9.3**), more recent nitrates include erythritol tetranitrate (**9.8**), mannitol hexanitrate (**9.9**), pentaerythritol tetranitrate (**9.10**), isosorbide dinitrate (**9.11**) and trolnitrate (**9.14**) [1][2]. These drugs are known collectively as *nitrovasodilators*, and some of them are discussed below.

Despite the many decades amyl nitrite and glyceryl trinitrate have been used in therapy, it is only in recent years that the molecular mechanism of action of the nitrovasodilators has begun to be understood [3–5]. The drugs act by releasing *nitric oxide* (NO$^{\bullet}$, a neutral radical usually written simply as NO), which produces smooth muscle relaxation in blood vessels and exhibits a range of other biological effects [6]. Thus, bioactivation to yield NO precedes the main therapeutic effect of nitrovasodilators and would justify their classifica-



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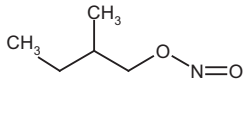

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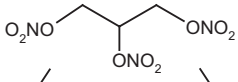

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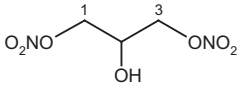

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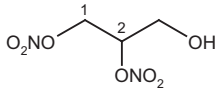

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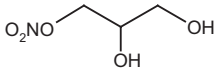

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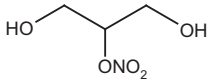

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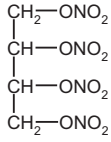

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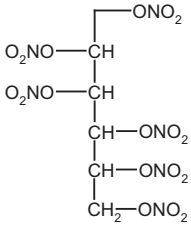

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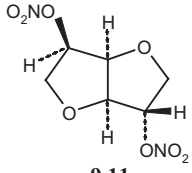

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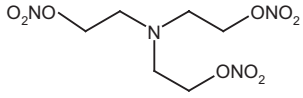

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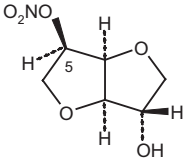

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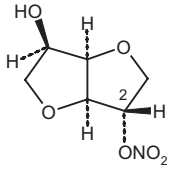


9.13

tion as *prodrugs* and more precisely as *bioprecursors* [7]. However, some old habits never die, and the nitrovasodilators are usually referred to as drugs.

Despite the apparent simplicity of their molecular structure, the metabolism of these agents is chemically so complex, and their routes of bioactivation and inactivation so intimately intertwined, that a detailed and coherent picture of their behavior in the body is not available. The presentation to follow considers first their *in vivo* de-esterification, and only then the nonenzymatic and enzymatic mechanisms postulated to be involved.

9.2.2. *In vivo* Metabolism

Amyl nitrite (**9.1**) has a very short duration of action (*ca.* 1 min), indicating an extremely rapid breakdown. Nitroglycerin (**9.3**) also undergoes fast degradation (duration of action *ca.* 0.5 h); it is administered transdermally, sublingually, or orally in slow release form. The other agents listed above are given orally and have a duration of action of *ca.* 3 – 6 h, indicating greater *in vivo* stability [8][9].

The metabolism of *nitroglycerin* (**9.3**) has been comprehensively reviewed and compared with that of other nitrovasodilators [10]. Plasmatic half-lives of elimination after intravenous or intracardial administration to rats were in the order of a few minutes. Extensive first-pass metabolism in the liver was seen [11]. After administration of [^{14}C]nitroglycerin to rats, the urinary metabolites were the two dinitrates (*i.e.*, glycerol-1,3-dinitrate (G13DN, **9.4**)) and glycerol-1,2-dinitrate (G12DN (**9.5**)), the two mononitrates (*i.e.*, glycerol-1-nitrate (G1N, **9.6**) and glycerol-2-nitrate (G2N, **9.7**)), glycerol, and other unidentified products including acids. These metabolites together represented a small fraction of the dose after oral administration (*ca.* 20% in 4 h), and no intact drug was detected. In contrast, subcutaneous administration led to the recovery in urine of *ca.* 1% of the dose as nitroglycerin, and *ca.* 40% as nitrate metabolites.

A comparable pattern of metabolism is seen in humans. In one study, of a slow-release capsule containing 20 mg of the drug was administered to healthy subjects [12]. No intact drug was detected in any subject, while the four nitrate metabolites were found in the plasma of all volunteers. The AUC decreased in the order G2N > G1N > G12DN > G13DN, the ratios being 64:19:4:1. This indicates a clear regioselective cleavage of the terminal ester group, as also found in dogs.

Due to its symmetrical structure, *pentaerythritol tetranitrate* (PETN, **9.10**) can yield only one metabolite at each de-esterification step. All successive steps occur in humans receiving therapeutic doses of the drug [13]. The only metabolites found in blood were, in decreasing order of importance, penta-

erythritol (PE), pentaerythritol mononitrate (PEMN), and pentaerythritol dinitrate (PEDN). The same metabolites were also found in urine, and the last de-esterification from PEMN to PE was dose-dependent. The overall results were interpreted as indicating rapid denitration of PETN in humans to PEMN after oral ingestion, but a limited capacity for the conversion of PEMN to PE. Relatively large quantities of the unchanged drug were excreted in the feces, suggesting incomplete absorption and some intestinal or fecal de-esterification. Metabolism of PETN by gut flora has indeed been confirmed [14].

The fact that *pentaerythritol trinitrate* (PETriN) was never seen following administration of PETN to humans was one of the reasons that led to its metabolism being investigated under comparable conditions [15]. Rapid de-esterification was confirmed, the unchanged PETriN and PEDN being minor plasmatic and urinary metabolites; urinary recovery was *ca.* 0.1 and 1% of a dose, respectively. Interestingly, their *O*-glucuronides were characterized for the first time, and each of these two conjugates accounted for *ca.* 10% of a dose in urine. The major urinary metabolites were the mononitrate (more than 50% of a dose) and PE (*ca.* 15% of a dose). These results are in complete agreement with rapid de-esterification to the mononitrate followed by slow formation of PE.

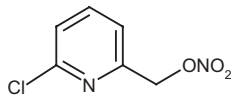
Another nitrovasodilator, the metabolism of which has been extensively investigated, is *isosorbide dinitrate* (ISDN, **9.11**; *e.g.*, [16][17]). The first de-esterification step to the two isomeric isosorbide mononitrates is fast (comparable to the first denitration of nitroglycerin), whereas the second step to isosorbide is slower. In dogs, for example, the plasmatic concentrations of isosorbide 5-mononitrate (IS-5-MN, **9.12**) were severalfold higher than those of isosorbide 2-mononitrate (IS-2-MN, **9.13**). These, in turn, were comparable to the concentrations of isosorbide [17]. The regioselective de-esterification of isosorbide dinitrate at C(2) is understandable in terms of the 2-NO_2 group (*exo*-configuration) being more accessible than the 5-NO_2 group (*endo*-configuration).

Interestingly, isosorbide dinitrate is also metabolized in the skin when administered for transdermal delivery. During *in vitro* investigations with porcine skin, *ca.* 14% of the amount permeated was recovered as IS-2-MN and 10% as IS-5-MN [18]. Because isosorbide was not reported, the relative importance of 2- and 5-denitration cannot be deduced.

A few modern nitrovasodilators have been designed and developed to improve pharmacokinetic properties. Two examples discussed below are (6-chloropyridin-2-yl)methyl nitrate and *trans*-2-amino-2-methyl-*N*-[4-(nitrooxy)cyclohexyl]propanamide.

Following administration to rats or dogs, the metabolism of *(6-chloropyridin-2-yl)methyl nitrate* (**9.15**) was complete, no unchanged drug being excreted [19]. Its plasmatic half-life for the β-phase was 42 min in rats and 66

min in dogs following *i.v.* administration. The primary metabolite resulting from denitration, *i.e.*, 6-chloropyridine-2-methanol, was not observed directly. However, its formation was ascertained by the fact that all characterized metabolites were derived from it. These metabolites, which together accounted for 80 – 90% of a dose, were the *O*-glucuronide of 6-chloropyridine-2-methanol, the carboxylic acid resulting from dehydrogenation (6-chloropyridine-2-carboxylic acid), the glycine conjugate of the latter, and *N*-acetyl-*S*-[(6-chloropyridin-2-yl)methyl]-L-cysteine. Thus, compound **9.15** is rapidly and completely de-esterified in rats and dogs.



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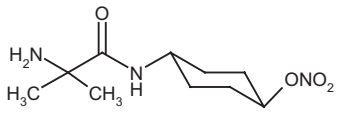

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The anti-anginal agent trans-*2-amino-2-methyl*-N-*[4-(nitrooxy)cyclohexyl]propanamide* (BM 12.1179, **9.16**), when examined in dogs, displayed a remarkable pharmacokinetic behavior uncharacteristic for nitrovasodilators [20]. Most unusual is the fact that the unchanged drug was the major compound found in plasma and urine (40 – 70% of dose). The absolute bioavailability was 80 – 100%. The only metabolites were the secondary alcohol resulting from de-esterification, and trace amounts of the ketone produced by dehydrogenation of the secondary alcohol. Another noteworthy property of BM 12.1179 is its long half-life (*ca.* 8 – 14 h) following *i.v.* or oral administration. High polarity due to the presence of a basic group was postulated to account for the low degree of denitration.

9.2.3. Mechanisms of Cleavage

The *in vivo* metabolism of nitrovasodilators, as exemplified above, can result from a number of pathways and mechanisms. The obvious pathways that come to mind first are enzymatic and nonenzymatic hydrolysis. As discussed below, the former pathway does not appear to occur, while the latter should play a limited role *in vivo*. The two other pathways to be discussed are hemoprotein-catalyzed and thiol-mediated reductive denitrations.

9.2.3.1. *Chemical Hydrolysis of Organic Nitrates*

The possibility of *enzymatic hydrolysis* of nitrates has been mentioned [10]. However, we are not aware of any reliable proof for the existence in eu-

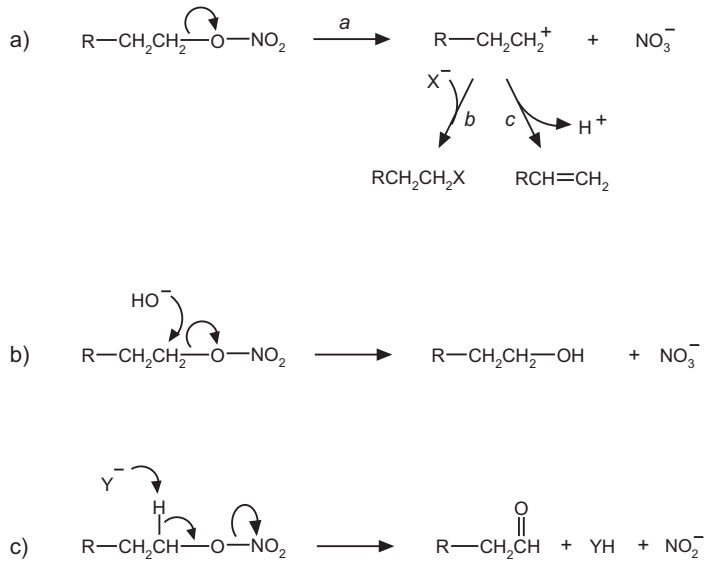


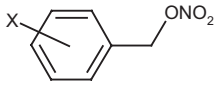
Fig. 9.1. *Simplified reaction mechanisms in the hydrolytic decomposition of organic nitrates. Pathway a:* Solvolytic reaction (*Reaction a*) with formation of a carbonium ion, which subsequently undergoes S_N1 addition of a nucleophile (*e.g.*, HO^-) (*Reaction b*) or proton E_1 elimination to form an olefin (*Reaction c*). *Pathway b:* HO^--catalyzed hydrolysis (S_N2). *Pathway c:* The bimolecular carbonyl-elimination reaction, as catalyzed by a strong base (*e.g.*, HO^- or RO^-), which forms a carbonyl derivative and nitrite.

karyotes of a hydrolase acting on nitrates, nor does the Enzyme Nomenclature make reference to such an activity [21]. In contrast, *nonenzymatic hydrolysis* is documented in a number of publications, but only a few of these have real pharmacological relevance. Thus, *isosorbide dinitrate* (**9.11**) was stable for 48 h at 37° in the pH range of 1.2 – 10, but was unstable above pH 12 [22]. In 0.1N HCl at 100°, the 2- and 5-denitration reactions proceeded with first-order rate constants of *ca.* 0.05 h^{-1} ($t_{1/2}$ *ca.* 14 h), whereas the denitration of the two mononitrates had a first-order rate constant of *ca.* 0.04 h^{-1} ($t_{1/2}$ *ca.* 17 h). In 0.1N NaOH at 100°, isosorbide dinitrate was denitrated with a first-order rate constant of 1.5 h^{-1} ($t_{1/2}$ *ca.* 0.5 h); the corresponding values for isosorbide 2- and 5-mononitrate were *ca.* 1.0 h^{-1} ($t_{1/2}$ *ca.* 0.7 h) and 0.1 h^{-1} ($t_{1/2}$ *ca.* 7 h). From these results, it is clear that nonenzymatic hydrolysis does not play any role in the biotransformation of isosorbide nitrates in the stomach, blood, or tissues.

There are a number of *reaction mechanisms* by which nitrates can undergo hydrolytic decomposition, as summarized in simplified form in *Fig. 9.1* [23]. *Pathway a* is the *unimolecular solvolytic route*, which forms nitrate and a carbonium ion (*Pathway a*, *Reaction a*). The latter can add a nucleophile,

and particularly HO^- to form the alcohol (*Pathway a*, *Reaction b*). Alternatively, a strong base may catalyze proton removal to form an olefin (*Pathway a*, *Reaction c*).

Pathway b is the *specific base catalyzed* (*HO^--catalyzed*) *hydrolysis*. This bimolecular S_N2 reaction leads to the alcohol and nitrate. A peculiar pathway is carbonyl elimination (*Fig. 9.1,c*). This bimolecular reaction is catalyzed by strong bases and produces a dismutation of the two moieties, the organic group being oxidized to a carbonyl compound and nitrate being reduced to nitrite. Note that *proton-catalyzed hydrolysis* does not appear in *Fig. 9.1* since this mechanism either does not occur or is negligible.



9.17

From the above, it appears that the only route that might have some pharmacokinetic significance is the S_N1 hydrolysis, *i.e.*, *Reactions a* and *b* in *Fig. 9.1,a*. However, estimating the hydrolytic reactivity of organic nitrates under physiological conditions is rendered difficult not only by the paucity of experimental evidence, but also by the very large differences in reaction rates shown by even closely related compounds. *Benzyl nitrates* (**9.17**, X = Me) taken as model compounds will serve to illustrate this point. In pure (*i.e.*, unbuffered) water, the compounds underwent clean solvolysis with liberation of the corresponding benzyl alcohol and HNO_3 (*i.e.*, S_N1 hydrolysis), with <0.01% of nitrite formed by the carbonyl-elimination reaction [24]. The most stable compound was benzyl nitrate ($t_{1/2}$ 4.5 h at 40°) and the least stable 2,4-dimethylbenzyl nitrate ($t_{1/2}$ 0.005 h), *i.e.*, a difference of more than 800-fold [25]. All other monomethyl and dimethyl analogues examined had intermediate stabilities depending on the position and number of the Me groups. Benzyl nitrates with electron-withdrawing ring substituents (*e.g.*, 4-Cl, 4-CF_3, 4-NO_2) also showed large variations in their reactivity [24]. The differences in reactivity were explained by charge development during the course of the reaction, charge separation in the transition state, and C–O bond fission to form the carbonium ion (see *Reaction a* in *Fig. 9.1,a*).

9.2.3.2. *Chemical Hydrolysis of Organic Nitrites*

The reactivity of organic nitrites toward hydrolysis differs markedly from that of organic nitrates. The *reaction mechanisms* are summarized in *Fig. 9.2*. The *base-catalyzed hydrolysis* of organic nitrites (*Fig. 9.2,a*) is quite slow, in

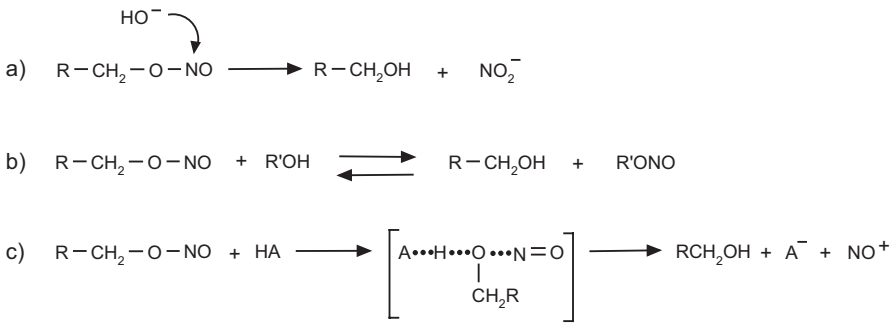


Fig. 9.2. *Simplified reaction mechanisms in the hydrolytic decomposition of organic nitrites. Pathway a:* Base-catalyzed hydrolysis with liberation of nitrite. *Pathway b:* Reversible nitrosyl exchange between organic nitrites and alcohols. *Pathway c:* General acid catalysis with concerted mechanism in the acid hydrolysis of organic nitrites.

fact several orders of magnitude slower than that of the corresponding formates (R′–O–N=O *vs.* R′–O–C(H)=O) [26]. Alkaline hydrolysis occurs by nucleophilic attack at the N-atom of nitrites, in analogy with carboxylates, where the nucleophilic attack is on the carbonyl C-atom. This mechanism distinguishes the nitrites from the nitrates, where nucleophilic attack is believed to be at C(α) (*Fig. 9.1,b*) [23a].

As a result of their very low reactivity with the HO^- anion, the organic nitrates show a practically pH-independent (and very slow) hydrolysis at pH 7 and above [27]. In contrast, they will react rapidly with alcohols in nonpolar media by *nitrosyl exchange* (*Fig. 9.2,b*) [28]. Formally, H^+ and NO^+ (nitrosyl cation) are exchanged, but the actual reaction mechanism is quite complex. The reaction leads to an equilibrium, which depends on the partners and presumably also on conditions. However, the biological relevance of this reaction remains to be understood.

In contrast to their relative inertness toward alkaline hydrolysis, the organic nitrites are particularly sensitive to *acid hydrolysis*, much more so than the corresponding carboxylates [26]. For example, rate constants of hydrolysis were found to increase approximately tenfold between pH 7 and 5 [27]. The reaction has been shown to be subject to general acid catalysis and results in the liberation of NO^+. The postulated mechanism is a concerted one (*Fig. 9.2,c*) [29]. The fact that the reaction is subject to general acid catalysis rather than simple proton catalysis should be of metabolic relevance since a number of endogenous acidic groups (*e.g.*, phenols) are potential catalysts. The same reasoning might apply to SH groups (see *Sect. 9.2.3.3*).

A novel mechanism of hydrolysis, and one of distinct pharmacological significance, has recently been uncovered in the biotransformation of *glyceryl trinitrate* [27]. In a series of primary alkyl nitrites at pH 7.6 and 28°, $t_{1/2}$

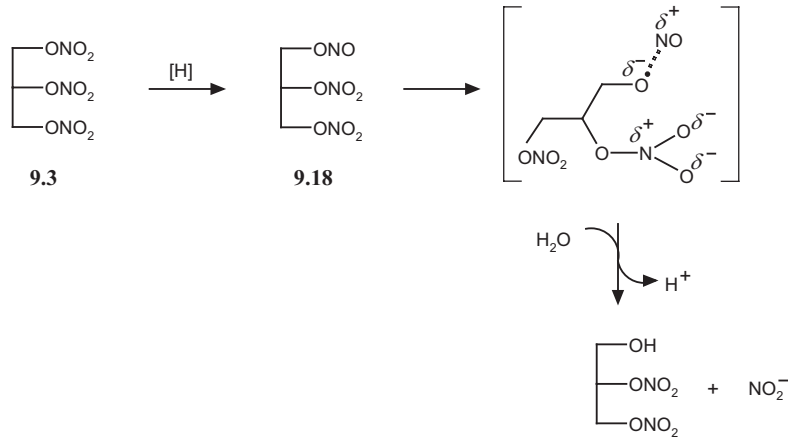


Fig. 9.3. *Intramolecular catalysis in the hydrolysis of 3-nitrosoglycerol-1,2-dinitrate* (**9.18**)*, a postulated intermediate in the biotransformation of glycerol-1,2,3-trinitrate* [27]

values of a few tens of minutes were seen (*e.g.*, *ca.* 40 min for Cl_2CHCH_2ONO), from which it was calculated that the corresponding value for 3-nitrosoglycerol-1,2-dinitrate (**9.18**, *Fig. 9.3*) should be 24 min. Experiments showed complete hydrolysis in less than 2 min, an unexpected result that could be explained by a mechanism of *intramolecular catalysis* in which charge transfer from the leaving-group O-atom to the vicinal NO_2 group assists N–O bond cleavage (*Fig. 9.3*). Such a mechanism appears to be of great biological significance, since 3-nitrosoglycerol-1,2-dinitrate has never been detected as a metabolite of glyceryl trinitrate despite the great likelihood of its formation by a reductive reaction (see *Sect. 9.2.3.3*). This lack of detection is now explained by poor stability under physiological conditions, rather than by absence of formation.

The above mechanism of intramolecularly catalyzed cleavage is possible only for compounds with a NO_2 group close to the departing NO group. In other words, it can be postulated to follow nitro reduction in erythritol tetranitrate (**9.8**), mannitol hexanitrate (**9.9**), pentaerythritol tetranitrate (**9.10**), and perhaps trolnitrate (**9.14**), but not in isosorbide dinitrate (**9.11**).

9.2.3.3. *Thiol-Dependent Cleavage of Organic Nitrates and Nitrites*

Two major pathways are recognized to be of great pharmacological significance in the nonhydrolytic cleavage of nitrates, namely the thiol-dependent pathway and the hemoprotein-dependent pathway. A thiol-dependent pathway is also apparent for organic nitrites [5][30][31]. Before discussing

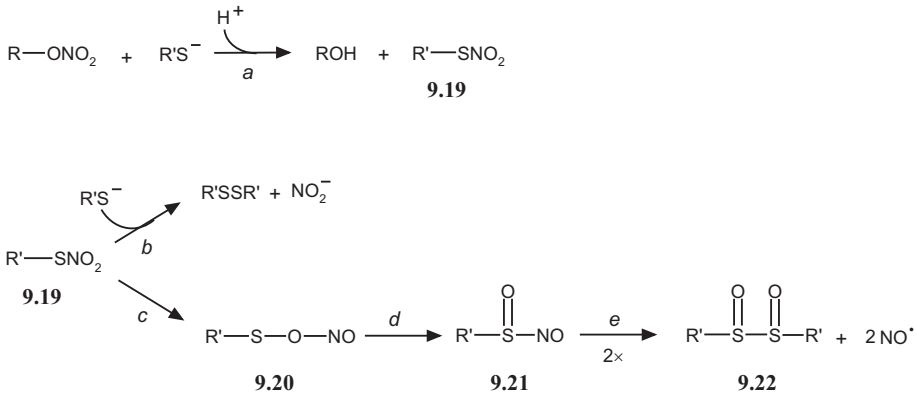


Fig. 9.4. *Postulated mechanisms in the thiol-dependent denitration of organic nitrates* [31][33]

these pathways, we note here that many reactions examined above or below yield nitrite ions (NO_2^-). According to various sources, this type of reaction is an irreversible inactivation, since nitrite does not share the many biological activities of $NO^\bullet$. However, this view may not always be valid given that NO_2^- can be reduced nonenzymatically to $NO^\bullet$ in tissues under conditions of acidosis and very low O_2 concentrations [32].

The *thiol-dependent pathway* for the denitration of organic nitrates is summarized in *Fig. 9.4.* Attack by a thiolate anion forms a nitrothioate (**9.19**, *Fig. 9.4*) and liberates the alcohol (*Fig. 9.4*, *Reaction a*). The nitrothioate can then be transformed in two ways, either by reacting with a second molecule of thiolate to form a disulfide and liberate nitrite (*Fig. 9.4*, *Reaction b*), or by rearranging to a sulfanyl nitrite (*Fig. 9.4*, *Reaction c* and **9.20**) and then to a *N*-oxosulfinamide (*Fig. 9.4*, *Reaction d* and **9.21**). *N*-Oxosulfinamides are unstable and decompose to a disulfinyl and nitric oxide (*Fig. 9.4*, *Reaction e* and **9.22**) [31][33 – 35].

The major contributors to the thiol-dependent pathway appear to be the cytosolic and microsomal enzymes *glutathione* S-*transferases* (GSTs) and their cofactor *glutathione* (GSH) [36 – 41]. This enzyme system has been found to act as a low-affinity, high-capacity pathway in the denitration of nitroglycerin (**9.3**), with low or no selectivity in the formation of glycerol-1,3-dinitrate (**9.4**) *vs.* glycerol-1,2-dinitrate (**9.5**) [30]. The involvement of different GST isozymes in nitroglycerin denitration is a proven fact [38]. There are also a few reports pointing to the existence of another thiol-dependent cytosolic enzyme able to cleave nitrates. This enzyme appears distinct from glutathione *S*-transferase and does not use glutathione as a cofactor. Under *in vitro* conditions, its cofactor was dithiothreitol, but the endogenous cofactor is unknown.

The first step in the glutathione *S*-transferase catalyzed reaction can be summarized in *Fig. 9.4*, *Reaction a*. The enzyme is known to maintain its cofactor glutathione in the anionic state, which is the attacking reactant, and this increased acidity is certainly one component of the catalytic mechanism. As far as the second step is concerned, the situation is complex. Formation of nitrite (*i.e.*, *Fig. 9.4*, *Reaction b*) is often cited and indeed well-documented [33][42]. In contrast, there are conclusive reports that nitric oxide (*i.e.*, formed by *Reactions c – e* in *Fig. 9.4*) is not detectable as a metabolite of nitroglycerin formed by GSTs [42][43]. This appears to contradict the fact that GST-dependent metabolism of nitroglycerin in vascular tissues is correlated with its pharmacological activity [33][41]. A likely explanation is that nitroglycerin is activated to a nitric oxide adduct, presumably an *S*-nitroso thiol species [43].

The *formation of* S-*nitroso thiols* (R′–S–N=O) is partly understood. A hypothetical mechanism is the reduction of *N*-oxosulfinamide derivatives (**9.21**, *Fig. 9.4*), but nothing appears to be known about such a possibility. What has been demonstrated is that nitric oxide by itself does not react with thiols to form *S*-nitroso thiols, but does so in the presence of O_2. Detailed kinetic analyses led to the mechanism summarized by *Eqns. 9.1 – 9.3* [44][45]. In these and the following reactions, thiols are written as R′–SH in consistency with *Figs. 9.4* and *9.5*.

$$2\ NO + O_2 \rightarrow 2\ NO_2 \tag{9.1}$$

$$NO_2 + NO \rightarrow N_2O_3 \tag{9.2}$$

$$N_2O_3 + R'\text{–}SH \rightarrow R'\text{–}SNO + NO_2^- + H^+ \tag{9.3}$$

$$N_2O_3 + H_2O \rightarrow 2\ NO_2^- + 2\ H^+ \tag{9.4}$$

In these reactions, the nitrosating species is N_2O_3, which is formed by oxidation of NO (*Eqns. 9.1* and *9.2*) and acts as a donor of NO^+ (*Eqn. 9.3*), while also undergoing competitive hydrolysis (*Eqn. 9.4*). Thus, effective nitrosation requires sufficiently high concentration of the thiol to compete efficiently with hydrolysis of N_2O_3. Note that nitric oxide can also be oxidized by other oxidants (*e.g.*, ferric hemoproteins, see below) to form the nitrosonium ion (NO^+), which then reacts rapidly with thiolates (*Eqns. 9.5* and *9.6*):

$$NO - e^- \rightarrow NO^+ \tag{9.5}$$

$$NO^+ + R'\text{–}S^- \rightarrow R'\text{–}SNO \tag{9.6}$$

Organic nitrites also react with thiols to form *S*-nitroso thiols, but, in contrast to organic nitrates, can do so in a single step (*Fig. 9.5*, *Reaction a*) [31].

The *release of NO from* S-*nitroso thiols* is a complex process involving a number of factors, in particular cellular factors [46 – 48]. In a schematic

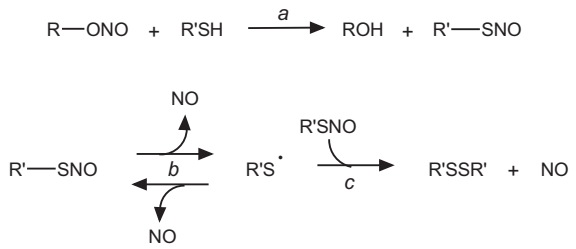


Fig. 9.5. *The reaction of organic nitrites with thiols* (*Reaction a*), *and a general mechanism for the release of nitric oxide from* S-*nitroso thiols* (*Reactions b* and *c*) [31]

manner, this release is described in *Reactions b* and *c* in *Fig. 9.5*. Thus, the overall reaction can be written as follows (*Eqn. 9.7*):

$$2\ R'\text{–SNO} \rightarrow R'\text{–SS–}R' + 2\ NO \qquad (9.7)$$

In addition, *S*-nitroso thiols can act as NO^+ donors in *S*-transnitrosation reactions, which are of great physiological significance, or they can release NO^- when reacting with thiols [49][50]. A further mechanism exists for *S*-nitrosoglutathione, which is a substrate of glutathione peroxidase. In this case, the enzyme catalyzes the release of NO or a reaction of transnitrosation [51].

9.2.3.4. *Hemoprotein-Dependent Reductive Cleavage of Organic Nitrates and Nitrites*

The second major pathway of metabolic cleavage of organic nitrates is catalyzed by hemoproteins, mainly cytochrome P450, hemoglobin, and myoglobin. In human blood, for example, *hemoglobin* plays a major role in the formation of glycerol dinitrates and mononitrates from nitroglycerin, and in the transduction of NO-related activities [52 – 54]. No detectable degradation of nitroglycerin occurred at pH 7.4 and 37° during incubation with buffer for 21 h. In human plasma, the half-life of degradation was *ca.* 200 min; in blood, the $t_{1/2}$ value varied between 15 and 30 min depending on substrate concentration and the source of blood. Clearly, red blood cells have a high capacity for cleaving nitroglycerin. *Myoglobin* is also able to metabolize nitroglycerin [55].

Cytochrome P450 (CYP) is well-known for its capacity to catalyze the denitration of nitrovasodilators. Thus, experiments in a variety of cultured cell lines have confirmed the role of cytochrome P450 in the activation of nitroglycerin (**9.3**) and isosorbide dinitrate (**9.11**) [56]. Such studies also suggested that the hemoprotein-mediated denitration of nitroglycerin is a high-affinity–low-capacity pathway with clear selectivity for the formation of

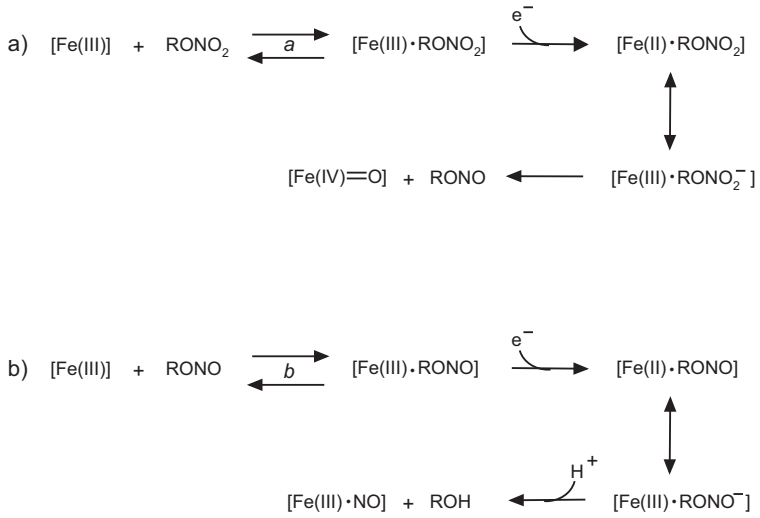


Fig. 9.6. *Postulated mechanisms in the cytochrome P450 catalyzed reductive denitration of organic nitrates. Pathway a* shows the hypothetical mechanism for the reduction of organic nitrates to organic nitrites, whereas *Pathway b* presents the same reaction for organic nitrites.

glycerol-1,2-dinitrate (**9.5**) [30]. It has also been found that CYP3A activity in aorta is correlated with nitroglycerin activation *in vivo*, although the contribution of this enzyme to the overall bioactivation of the drug is limited [57].

The *mechanism of cytochrome P450 catalyzed denitration* of nitrovasodilators is partly but not fully understood. The reaction requires NADPH as electron donor and is sensitive to inhibition by O_2 as well as by some usual inhibitors of cytochrome P450 [5]. Based on existing evidence and on the known reductive mechanism of cytochrome P450, a reasonable hypothesis can be formulated for the denitration of nitrovasodilators (*Fig. 9.6*) [40][58] (see also Chapt. 12 in [59]). As shown in *Pathway a*, binding to the oxidized form of P450 (*Fig. 9.6*, *Reaction a*) is followed by one-electron reduction and electron migration. Cleavage of the organic nitrate anion would then liberate the organic nitrite metabolite, perhaps leaving the enzyme in the ferryl state as shown.

In the case of nitroglycerin, we have seen above that the reduced metabolite 3-nitrosoglycerol-1,2-dinitrate (**9.18**, *Fig. 9.3*) is remarkably labile and undergoes very fast intramolecularly catalyzed hydrolysis. *Pathway a* in *Fig. 9.6* leading to the reduced metabolite 3-nitrosoglycerol-1,2-dinitrate is, thus, sufficient to explain the reduction of nitroglycerin to glycerol-1,2-dinitrate (**9.5**). For organic nitrates unable to undergo the same intramolecular catalysis, the organic nitrite metabolite must undergo further P450-catalyzed reduc-

tion, the mechanism of which is hypothesized in *Pathway b* (*Fig. 9.6*). Again, binding to cytochrome P450 (*Fig. 9.6*, *Reaction b*) is followed by reduction, electron migration, and cleavage. Here, NO is released and can form a metal–nitrosyl complex with cytochrome P450. It is, indeed, recognized that NO has a strong affinity for hemoproteins [3][31][60][61]. Both ferric and ferrous porphyrins can bind NO (*Eqns. 9.8* and *9.9*, resp.) to form complexes, which can be described by a number of resonance forms:

$$[\mathrm{Fe(II)} \cdot \mathrm{NO^+}] \leftrightarrow [\mathrm{Fe(III)} \cdot \mathrm{NO}] \leftrightarrow [\mathrm{Fe(IV)} \cdot \mathrm{NO^-}] \qquad (9.8)$$

$$[\mathrm{Fe(II)} \cdot \mathrm{NO}] \leftrightarrow [\mathrm{Fe(III)} \cdot \mathrm{NO^-}] \qquad (9.9)$$

Such metal–nitrosyl complexes have great physiological significance and are involved for example in enzyme regulation and protection against oxidation [62].

To conclude, the story of nitrovasodilators is a fascinating one. This ‘old’ class of drugs (in the sense that its first members have been in therapeutic use since a number of decades) has in recent years witnessed a profound rejuvenation and an exponential growth in interest. The discovery of their molecular mechanisms of action has had a major impact on pharmacology and physiology. Seldom if at all have molecular pharmacology, molecular physiology, and drug biotransformation been so intertwined.

9.3. Hydrolysis and Oxidative Cleavage of Organophosphates

9.3.1. Introduction

Organic phosphates, phosphonates, and analogues are of interest in medicinal chemistry as drugs or prodrugs, and in toxicology mainly as plasticizers, insecticides, or warfare agents. Their breakdown by enzymatic routes has received marked attention since many years, but it is most unfortunate for the advancement of science that an unknown number of highly relevant results remain classified information.

The *chemistry of organophosphorus compounds* is rather complex. Derivatives of interest include:

- aryl phosphates ((aryl–O–)(RO–)(R′O–)P(=O)), which can be mono-, di-, or triesters (R, R′ = alkyl, aryl, or H);
- alkyl phosphates ((RCH_2O–)(R′O–)(R″O–)P(=O)), which can also be mono-, di-, and triesters (R′, R″ = alkyl or H);
- phosphonates (R–P(=O)(–OR′)(–OR″); R = alkyl or aryl; R′, R″ = alkyl, aryl, or H);
- phosphinates (RR′P(=O)(–OR″); R, R′, R″ = alkyl or aryl);

- phosphorothioates ((RO–)(R′O–)P(=O)(–SR″) or (RO–)(R′O–)P(=S)(–OR″); R, R′ = alkyl, aryl, or H; R″ = alkyl or aryl);
- phosphorodithioates ((RO–)(R′O–)P(=S)(–SR″); R, R′ = alkyl, aryl, or H; R″ = alkyl or aryl);
- phosphonothioates (*e.g.*, R–P(=O)(–OR′)(–SR″); R, R′ = alkyl or aryl; R″ = alkyl or aryl);
- phosphoramidates ((RO–)(R′O–)P(=O)(–NHR″); R, R′, R″ = alkyl, aryl, or H) and analogues such as phosphoramidothioates;
- *P*-halide compounds (*e.g.*, X–P(=O)RR′; X = halogen; R, R′ = alkyl, aryl, alkoxy, or aryloxy) and analogues.

Phosphatases are numerous and important enzymes (see also *Chapt. 2*). They are classified as phosphoric monoester hydrolases (phosphatases, EC 3.1.3), phosphoric diester hydrolases (phosphodiesterases, EC 3.1.4), triphosphoric monoester hydrolases (EC 3.1.5), diphosphoric monoester hydrolases (pyrophosphatases, EC 3.1.7), and phosphoric triester hydrolases (EC 3.1.8) [21][63]. Most of these enzymes have a narrow substrate specificity restricted to endogenous compounds. However, some of these enzymes are active toward xenobiotic organophosphorus compounds, *e.g.*, alkaline phosphatase (EC 3.1.3.1), acid phosphatase (EC 3.1.3.2), aryldialkylphosphatase (paraoxonase (PON1), EC 3.1.8.1) and diisopropyl-fluorophosphatase (tabunase, somanase, EC 3.1.8.2) [64 – 70]. However, such a classification is far from definitive and will evolve with further biochemical findings. Thus, a good correlation has been found in human blood samples between somanase and sarinase activities on the one hand, and paraoxonase (PON1) type Q isozyme concentrations on the other [71].

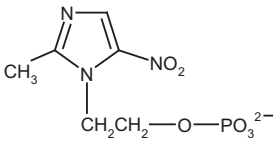
In addition to these various phosphatases, *carboxylic ester hydrolases* interact with phosphates and/or phosphonates. The carboxylesterases (EC 3.1.1.1) and the cholinesterases (EC 3.1.1.7 and EC 3.1.1.8) are sensitive to inhibition by organophosphorus esters, whereas the arylesterases (EC 3.1.1.2) are not inhibited by these compounds and readily hydrolyze them [72]. As explained in *Chapt. 2* and *3*, esters that react rapidly and allow high regeneration rates of the phosphorylated enzyme are substrates, whereas slow regeneration rates result in enzyme inactivation.

The above enzymes are not the only ones able to cleave phosphoric acid esters. Indeed, the cleavage of some organophosphates can be catalyzed by *monooxygenases*, as illustrated below. Furthermore, *glutathione* S-*transferases* can also act on some methyl and ethyl phosphates [73], but these reactions will not be covered here.

This section is concerned mostly with the enzymatic hydrolysis of substrates classified as phosphoric acid mono-, di-, and triesters, phosphonates, phosphoro(di)thioates, phosphonodithioates, and *P*-halide compounds.

9.3.2. Hydrolysis of Medicinal Phosphoric Acid Alkyl and Aryl Monoesters

A number of phosphoric acid monoesters have been examined as potential *prodrugs of active alcohols or phenols*, the added phosphate pro-moiety contributing water-solubility and being cleaved with relative ease by enzyme reactions. A telling example of a *phosphoric acid alkyl monoester* developed as a potential prodrug is provided by *metronidazole phosphate* (**9.23**) [74]. The antibacterial drug metronidazole is a weak base (pK_a 2.6) characterized by a very low aqueous solubility except at very acidic pH values. In contrast, its phosphate exhibited a 50-fold higher aqueous solubility at neutral pH and appeared suitable for the preparation of parenteral solutions. In human serum, the hydrolysis of metronidazole phosphate followed zero-order kinetics (*ca.* 1×10^{-4} M h^{-1} at 37°) at an initial concentration of 0.25 mg ml^{-1} or higher, presumably due to enzyme saturation. Subcutaneous administration of **9.23** to rats produced blood levels of metronidazole that were comparable to those observed after administration of the drug itself. Thus, quantitative and fast hydrolysis was verified.



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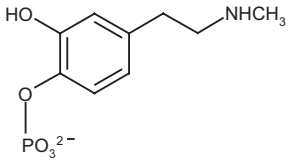

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Phosphoric acid aryl monoesters can also be found in the literature, as exemplified by epinine (*N*-methyldopamine). The pharmacodynamic and pharmacokinetic properties of epinine were markedly improved when it was administered as *epinine 4*-O-*phosphate* (**9.24**) [75]. Not only was the prodrug more effective than the drug as a renal vasodilator in animal experiments, it was also more selective, being devoid of cardiostimulant and hypertensive properties at comparable doses. In humans, the prodrug again displayed favorable pharmacodynamic and pharmacokinetic properties. Following oral administration, it was rapidly absorbed, quickly and extensively hydrolyzed to epinine, and then further metabolized and excreted [76]. The total plasma concentration of epinine (*i.e.*, epinine plus its sulfoconjugate) peaked 2 – 3 h after oral administration of the prodrug and decreased relatively slowly. These data suggested epinine 4-*O*-phosphate to be a promising prodrug of epinine.

Another promising prodrug undergoing clinical evaluation is *etoposide phosphate*. Here, the phenolic group of this chemically complex antitumor

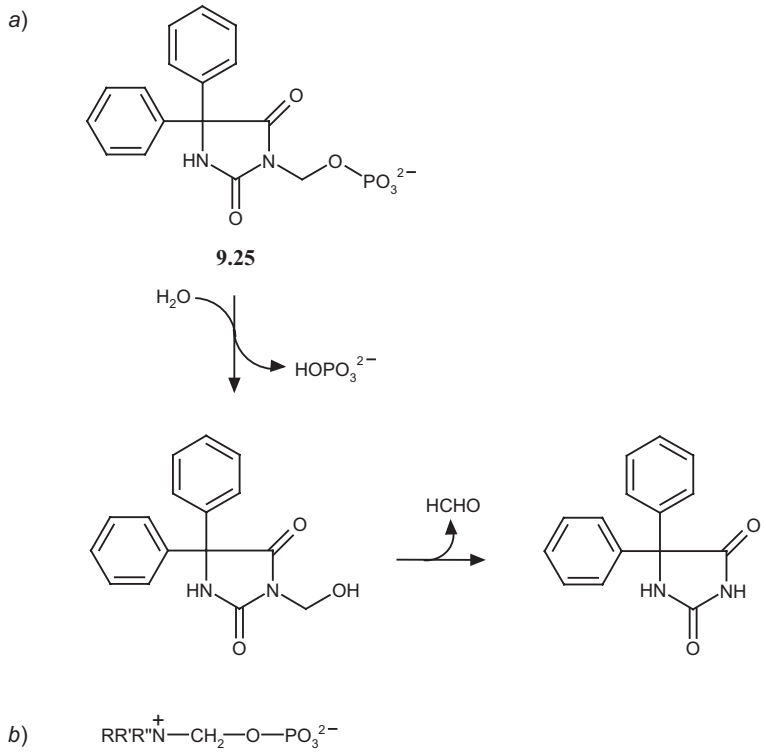


Fig. 9.7. a) *Mechanism of activation of (phenytoin-3-yl)methyl phosphate* (**9.25**) *to release phenytoin.* Phosphoric acid ester hydrolysis is mediated by alkaline phosphatase. *b*) *(Phosphoryloxy)methyl prodrugs of tertiary amines, whose activation occurs by the same two-step mechanism shown in* a [79].

agent was monophosphorylated to increase water solubility and chemical stability [77]. After parenteral administration to patients, rapid and quantitative conversion to the parent drug was observed. Besides its pharmaceutical advantages, the prodrug proved to be bioequivalent to etoposide and its administration resulted in a comparable efficacy. The phosphorylation of a phenolic group to produce potential prodrugs appears to be a strategy of continued interest in medicinal chemistry [78].

In a few cases, the activation of phosphate prodrugs occurs in more than one step, phosphate hydrolysis producing an unstable metabolite, which breaks down to the active compound. An example of such *multistep phosphate prodrugs* is provided by *(phenytoin-3-yl)methyl phosphate* (**9.25** in *Fig. 9.7,a*). This water-soluble derivative was developed to overcome the poor solubility of phenytoin, an unfavorable physicochemical property preventing the ready parenteral use of the drug. The phosphate prodrug proved to have a high water solubility and a good chemical stability in solution [80].

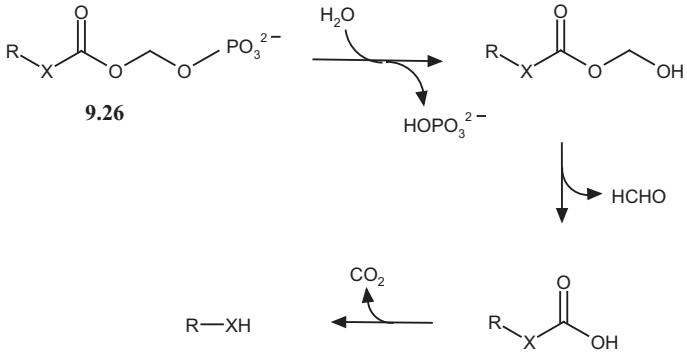


Fig. 9.8. *Reaction cascade in the activation of (phosphoryloxy)methyl carbonates and carbamates* (X = O or NH, respectively) *as potential prodrugs of alcohols, phenols, and amines* [84]

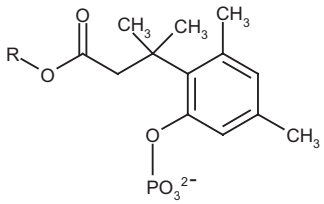
Its mechanism of activation is shown in *Fig. 9.7,a*; enzymatic hydrolysis produced 3-(hydroxymethyl)phenytoin, which then broke down to phenytoin and formaldehyde. The potential for unwanted effects of formaldehyde should not be forgotten.

Parenteral administration of **9.25** (*Fig. 9.7,a*) to rats afforded quantitative release of phenytoin. Furthermore, the blood levels of phenytoin following intramuscular administration of the prodrug were far superior to those generated from similarly administered phenytoin sodium [81]. In epileptic patients, *i.m.* and *i.v.* administration of the prodrug led to its rapid disappearance from blood, with high levels of phenytoin persisting for several hours [82]. Enzymatic studies confirmed the high activity of alkaline phosphatases toward the prodrug [83]. The same strategy has been extended to *tertiary amines*, which were derivatized to (phosphoryloxy)methyl prodrugs (*Fig. 9.7,b*) [79]. Again, alkaline phosphatase cleaved the phosphoric acid monoester bond, and spontaneous breakdown liberated the tertiary amine RR′R″N and formaldehyde.

Fig. 9.8 presents another, more complex type of phosphate prodrugs, namely *(phosphoryloxy)methyl carbonates and carbamates* (**9.26**, X = O or NH, resp.) [84]. Here, the [(phosphoryloxy)methyl]carbonyl carrier appears quite versatile and of potential interest to prepare prodrugs of alcohols, phenols, and amines. The cascade of reactions leading from prodrug to drug as shown in *Fig. 9.8* involves three steps, namely ester hydrolysis, release of formaldehyde, and a final step of carbonate hydrolysis (X = O) or *N*-decarboxylation (X = NH). Three model compounds, a secondary alcohol, a primary aliphatic amine, and a primary aromatic amine, were derivatized with the carrier moiety and examined for their rates of breakdown [84]. The alcohol, indan-2-ol, yielded a carrier-linked derivative that proved relatively

stable at low pH values, but hydrolyzed with a $t_{1/2}$ value of 1 h at 25° and pH 7.4, *i.e.*, too rapidly to appear of interest. This was due to the carbonate moiety being hydrolyzed under intramolecular catalysis. The aliphatic amine, [2-(3,4-dimethoxyphenyl)ethyl]amine, led to a stable derivative ($t_{1/2}$ 48 d at 25° and pH 7.4). As for the aromatic amine (the drug benzocaine), its prodrug had an intermediate stability ($t_{1/2}$ 115 h under the same conditions), and it proved to be a very good substrate of alkaline phosphatase. Thus, the [(phosphoryloxy)methyl]carbonyl carrier appears as a promising moiety in the design of prodrugs of aromatic amines. However, here, again, caution is warranted due to the release of formaldehyde.

In the above example, intramolecular hydrolysis of the carboxylic acid ester following the hydrolysis of the phosphate was not retained as a promising model. In contrast, a comparable cascade occurring with a *[(phosphoryloxy)phenyl]propanoate carrier* (**9.27**) was seen as favorable and, indeed, led to interesting prodrugs. The drug being derivatized was *paclitaxel* (taxol), an antitumor agent, the poor water solubility of which represents a serious problem in its clinical use. Simple phosphate esterification of HO–C(2′) or HO–C(7) led to derivatives that were not activated by alkaline phosphatase [85]. However, the 2′- and 7-[(phosphoryloxy)phenyl]propanoate prodrugs were readily hydrolyzed by alkaline phosphatase. In a second step, the unmasked phenolic group attacks the C=O group to split the carboxylic acid ester bond, taxol is released and a lactone is formed [86]. Such reactions of prodrug activation by cyclization–elimination have been extensively discussed in *Chapt. 8*.



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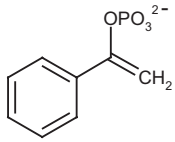

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There are comparatively few studies addressing the *structure–metabolism relationships* of phosphoric acid monoester hydrolysis. For example, kinetics of decomposition in rat whole blood were examined for the phosphoric acid monoesters of estrone, 17α- and 17β-testosterone, 3-(hydroxymethyl)phenytoin (see *Fig. 9.7,a*), and 1-phenylvinyl alcohol (**9.28**, the enolic form of acetophenone) [87]. As a general trend, the rate of hydrolysis increased with the acidity of the leaving hydroxylated compound. In other words, hydrolysis was the fastest for the phosphoric acid aryl monoester (estrone 3-phosphate), and slowest for the two testosterone phosphoric acid

monoesters. The only exception to this trend was 1-phenylvinyl 1-phosphate, which underwent very fast hydrolysis.

In whole blood, phosphatase activity is associated primarily with red blood cells, but it is recognized that blood has a low phosphatase activity compared to other tissues such as the kidney, brain, liver, lung, and heart ([87][88] and refs. cit. therein). Thus, studies with blood preparations should underestimate the *in vivo* rate of hydrolysis of phosphoric acid esters, a point of significance in the development of phosphate prodrugs.

9.3.3. Hydrolysis of Medicinal Phosphoric Acid Diesters and Triesters

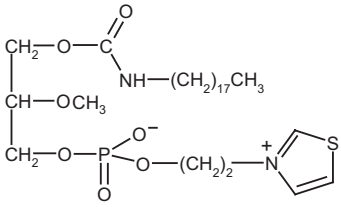
The prodrugs discussed in the previous section were obtained by esterifying a OH group in an active agent with phosphoric acid. A few comparable cases exist where the esterifying agent is not phosphoric acid, but a phosphoric acid monoester or diester. However, most examples presented in this section are either :

- *prodrugs* resulting from the esterification of drugs containing a phosphate moiety, or
- *drugs* having a phosphoric acid diester or triester motif.

An example of a drug having a *phosphoric acid diester moiety* is that of (*RS*)-2-methoxy-3-[(octadecylcarbamoyl)oxy]propyl 2-(3-thiazolio)ethyl phosphate (MOTP, **9.29**), a platelet-aggregating factor (PAF) antagonist endowed with promising pharmacological properties. When administered to rats and dogs, this compound was found to be extensively hydrolyzed with loss of the (thiazolio)ethyl phosphate moiety, yielding urinary metabolites that had undergone ω- and β-oxidation [88].

Another relevant example is provided by *benzylpenicillin methyl phosphate* (**9.30**), a compound designed to be both a prodrug of benzylpenicillin and a potential inactivator of bacterial β-lactamase [90]. In strict chemical terms, this compound is an *acyl phosphate* and, as such, undergoes slow chemical hydrolysis to liberate benzylpenicillin ($t_{1/2}$ 90 h at 25° in a buffer at pH 7.5). But, besides being a prodrug of benzylpenicillin, it is also an irreversible inactivator of β-lactamase, the activity of which it reduces by half within 2.5 min. Thus, benzylpenicillin methyl phosphate appears to confirm its potential as both a prodrug and a synergetic agent.

In an attempt to obtain *prodrugs of the antiviral agents* 2′,3′-didehydro-2′,3′-dideoxyadenosine (**9.33**, Base = adeninyl) and 2′,3′-didehydro-2′,3′-dideoxycytosine (**9.33**, Base = cytosinyl), a variety of phosphorylated derivatives were prepared and tested [91]. Whereas phosphonates (**9.31**, R = Me or Ph) were inactive, diesters of structure **9.31** (R = MeO or PhO, Base =



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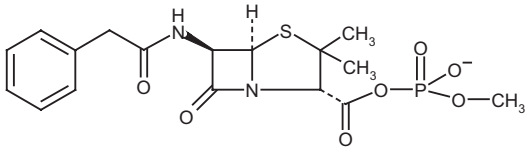

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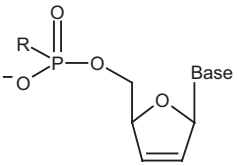

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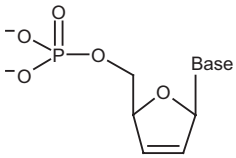
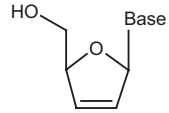


9.32

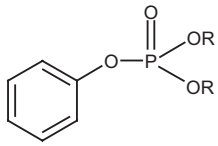


9.33

adeninyl or cytosinyl) demonstrated inhibition of the cytophatic effects of HIV-1 and HIV-2 and cytotoxicities at concentrations comparable to those of their parent compound. This suggested facile hydrolysis to the parent compounds and/or their active 5′-monophosphates (**9.32**). Indeed, incubations with fetal calf serum resulted in marked hydrolysis to **9.32** and then to **9.33**, confirming the potential of these diesters as effective prodrugs.

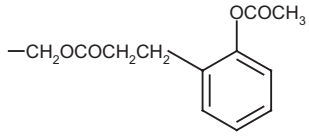
Whereas there are relatively few phosphoric acid diesters of medicinal or toxicological interest, *phosphoric acid triesters* are of greater significance. Such compounds occur mainly as prodrugs, as discussed in the present section, or plasticizers and insecticides (see next section).

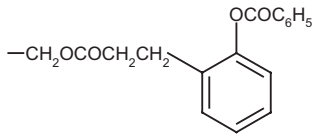
A number of compounds containing a phosphate or phosphonate group are of medicinal interest, *e.g.*, nucleotides and bisphosphonates, respectively. However, the high polarity of these compounds often result in poor membrane permeability and unsatisfactory bioavailability. There is, thus, some interest in developing prodrugs of decreased polarity and increased lipophilicity. In an informative study, novel lipophilic *bis[(acyloxy)methyl] esters of phenyl phosphate* (**9.34**) were prepared as model compounds and examined for their hydrolysis in buffers and biological media [92]. What renders these compounds particularly interesting is the fact that they contain several sites of hydrolytic attack. The kinetics of hydrolysis of compound **9.35** was investigated in great detail, leading to the proposed degradation route shown in *Fig. 9.9*. In a buffer solution of pH 7.4 at 37°, compound **9.35** was hydrolyzed rapidly with loss of an acetoxymethyl moiety (*Fig. 9.9*, *Reaction a*; $t_{1/2}$ *ca.* 4 h), whereas the resulting product underwent much slower hydroly-



9.34

9.35 R = $-CH_2OCOCH_3$

9.36 R = 

9.37 R = 

9.38 R = $-CH_2OCO(CH_2)_3OCOCH_3$

9.39 R = $-CH_2OCO(CH_2)_3OCOCH_2CH_3$

9.40 R = $-CH_2OCO(CH_2)_3OCO(CH_2)_3CH_3$

sis (*Reaction b*; $t_{1/2}$ 150 h) probably due to polar effects; hydrolysis of the aryl ester moiety was not observed (*Reactions c* and *d*).

The hydrolysis of compound **9.35** in biological media resulted in different regioselectivities and reaction rates compared to hydrolysis in buffers. Thus, in 80% human plasma, the hydrolysis of the parent compound was markedly accelerated (in *Fig. 9.9*, *Reaction a*; $t_{1/2}$ 1.5 min), but hydrolysis of the second acetoxymethyl moiety was not catalyzed (*Reaction b*). In contrast to the reaction in buffers, hydrolysis at the aryl phosphate moiety was relatively fast (*Reactions c* and *d*; $t_{1/2}$ 20 and 4.5 h, resp.). In rat liver homogenates, both acetoxymethyl moieties were hydrolyzed in a few minutes. When the other derivatives (**9.36** – **9.40**) were examined in human plasma, they all underwent a very fast *Reaction a* ($t_{1/2}$ *ca.* 1 min), but the amount of phenyl phosphate produced varied from 10% (**9.35** and **9.37**) to 100% (**9.36**). Such a study shows the difficulty of predicting the regioselectivity and rate of activation of prodrug derivatives of phosphate-containing drugs.

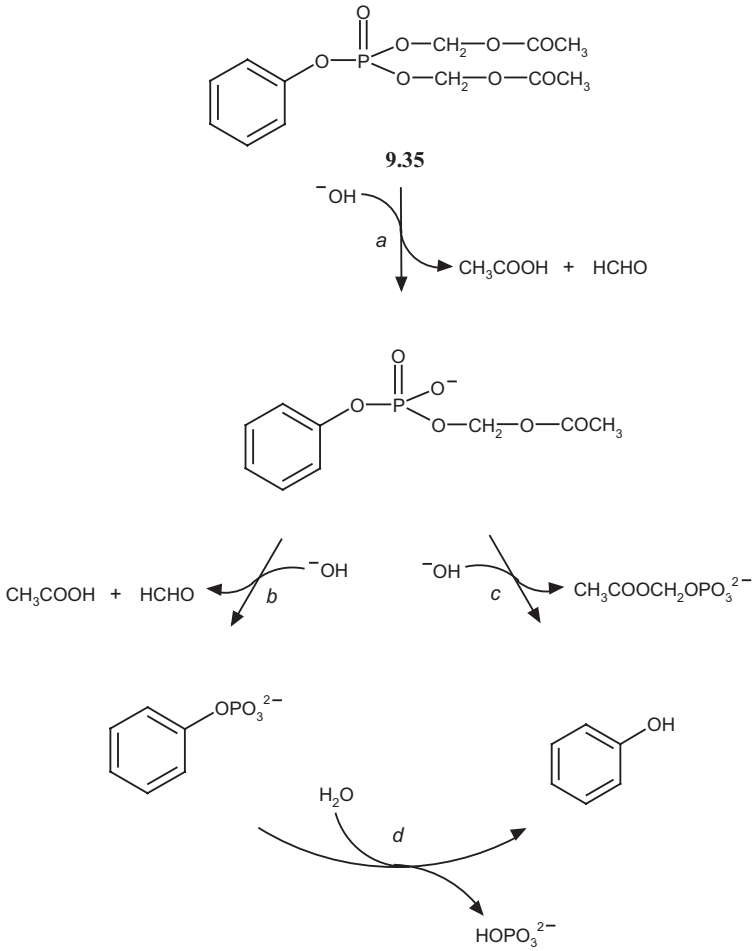
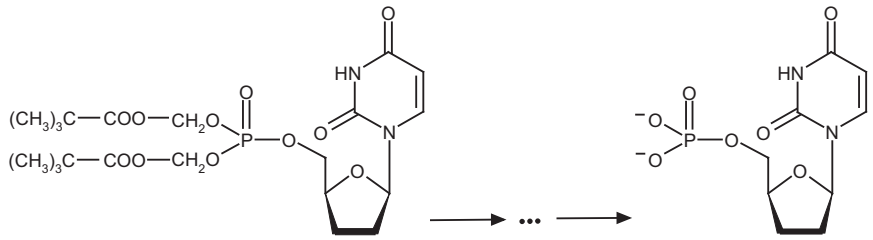
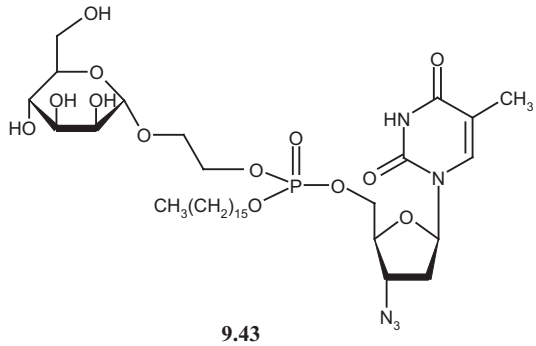


Fig. 9.9. *Proposed route of degradation of bis(acetoxymethyl) phenyl phosphate* (**9.35**) *and presumably other bis[(acyloxy)methyl] phenyl phosphates* [92]

2′,3′-Dideoxyuridine (ddU) is an antiviral agent that proved ineffective at controlling human immunodeficiency virus type 1 (HIV-1) infection in human T-cells. This ineffectiveness was ascribed to a lack of substrate affinity of ddU for cellular nucleoside kinases, which prevent it from being metabolized to the active 5′-triphosphate. To overcome this problem, bis[(pivaloyloxy)methyl] 2′,3′-dideoxyuridine 5′-monophosphate (**9.41**) was prepared and shown to be a membrane-permeable prodrug of 2′,3′-dideoxyuridine 5′-monophosphate (ddUMP, **9.42**) [93]. Indeed, human T-cell lines exposed to **9.41** rapidly formed the mono-, di-, and triphosphate of ddU, and antiviral activity was observed. This example again documents



9.41 **9.42**



9.43

the biological hydrolysis of (acyloxy)methyl esters of medicinal phosphates.

A comparable, yet chemically more-complex example can be found in a prodrug of the anti-HIV drug AZT (*zidovudine*, 3′-azido-3′-deoxythymidine). The investigated prodrug was a *glycosyl phosphoric acid triester derivative* (**9.43**) [94]. Its main metabolite in mouse plasma was AZT 5′-monophosphate, with total concentrations of AZT derivatives in brain being *ca.* 50-fold higher than after administration of AZT. *In vitro* penetration into synaptosomes was also demonstrated. Thus, **9.43** appears to be a promising brain delivery system for AZT, with ready hydrolysis of the hexadecyl and (mannopyranosidyl)ethyl moieties. More recent attempts have used *S*-acyl-2-thioethyl pro-moieties (acyl–S–CH_2CH_2–O–) to mask the two acidic O-atoms of AZT 5′-monophosphate [95][96]. Intracellular delivery of AZT 5′-monophosphate and inhibition of HIV replication were indeed achieved with a number of such prodrugs.

These examples are but a few of the many reports on the use of *pronucleotides* as delivery systems of antiviral and anticancer nucleotides. The interested reader is referred to an extensive review on this subject [97].

The interest of glycosyl phosphoric acid triester moieties (**9.43**) as brain transport vectors is seemingly not limited to AZT and analogues. Indeed,

moieties of this type were used to derivatize *N*-acetyl-5-hydroxytryptamine (the immediate bioprecursor of *melatonin*) and successfully elicit central effects in mice following peripheral administration [98]. Under these conditions, the underivatized agent was inactive.

9.3.4. Cleavage of Industrial Phosphoric Acid Triesters

A variety of industrial compounds are also phosphoric acid triesters of interest in xenobiotic metabolism and molecular toxicology. First, we examine here a few triesters used for example as *plasticizers*, *hydraulic fluids*, and *flame retardants*. In the second part of the section, insecticides of the phosphoric acid triester class will be discussed.

Tris(2-methylphenyl) phosphate (**9.44**, *Fig. 9.10*) has been the object of innumerable investigations to understand its biotransformation and its connection with a toxic condition known as organophosphorus-compound-induced delayed neurotoxicity [99]. This compound, which has found industrial use as plasticizer, flame retardant, and heat exchanger, undergoes a complex metabolic fate involving reactions of hydrolysis and oxidation. The reactions of interest in the present context are shown in *Fig. 9.10*, with the *ortho*-cresyl moieties undergoing cleavage to bis(2-methylphenyl) phosphate (**9.45**, *Fig. 9.10*), then to 2-methylphenyl phosphate and presumably also to free phosphate. According to some investigations, these reactions may also be catalyzed by cytochrome P450 [100][101]. However, the relative contributions of hydrolases and monooxygenases to the dearylation of tris(2-methylphenyl) phosphate might be highly variable; this aspect does not appear to have been extensively investigated.

Another mechanism of dearylation has been proven for tris(2-methylphenyl) phosphate, and it is a pathway whose first step must be cytochrome P450 dependent. As shown in *Fig. 9.10*, the compound is hydroxylated at a Me group to yield the hydroxymethyl analogue **9.46**. The latter then breaks down by intramolecular nucleophilic attack to form 2-(2-methylphenoxy)-4*H*-1,3,2λ^5-benzodioxaphosphinin-2-one (**9.47**, *Fig. 9.10*). This reaction of cyclization occurs with loss of a *ortho*-cresyl moiety and is catalyzed by serum albumin [102]. The cyclized product is a more potent inhibitor of esterases than tris(2-methylphenyl) phosphate, and it is also more toxic [100][101].

A comparable metabolic fate is documented for the hydraulic fluid *tributyl phosphate*. Following administration to rats, the Bu groups were oxidized to alcoholic, ketonic, and acidic metabolites. The oxidized Bu groups were then cleaved by enzymatic hydrolysis [103]. With *2-ethylhexyl diphenyl phosphate* (**9.48**), an interesting case of regioselectivity was noted during its *in vivo* metabolism in rats. Indeed, this flame retardant and plasticizer was

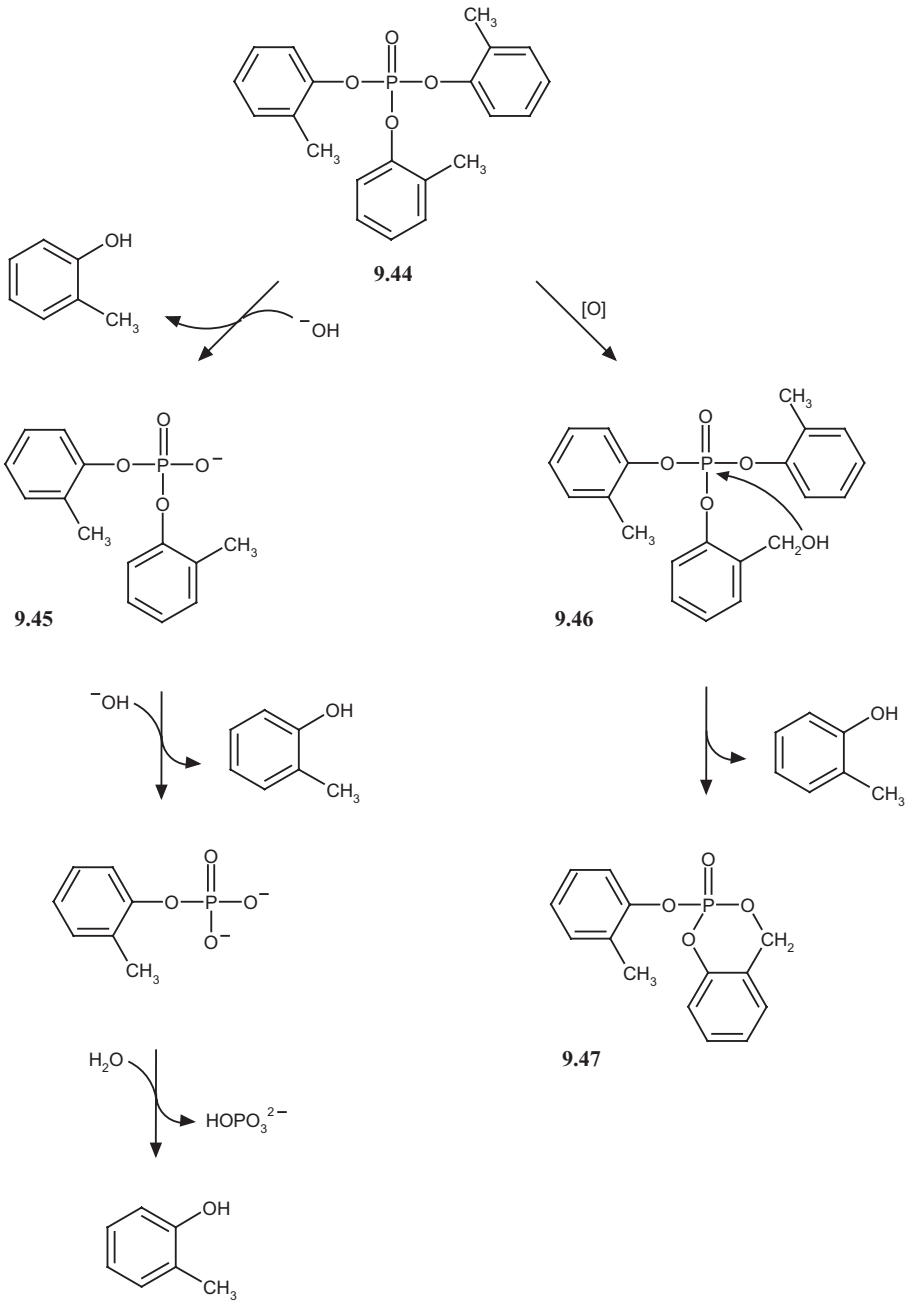
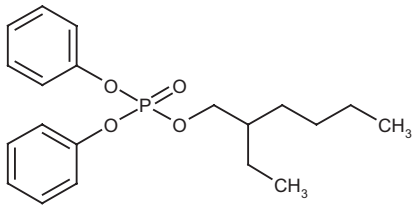


Fig. 9.10. *Partial metabolic scheme of tris(2-methylphenyl) phosphate* (**9.44**) *showing hydrolysis to the diester* **9.45***, the monoester, and phosphate.* Also shown is a reaction of oxidation to the hydroxymethyl analogue **9.46**, followed by cyclization to the toxic cyclic phosphate **9.47** of saligenin.

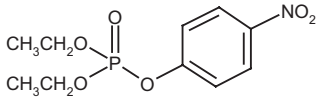


9.48

metabolized for the totality of the dose, and all metabolites were *O*-dealkylated ones [104]. Diphenyl phosphate was the major metabolite, further confirming that rapid cleavage of the 2-ethylhexyl moiety was the fastest route of biotransformation. Subsequent steps were the slow hydrolysis of one Ph moiety to yield phenyl phosphate as a minor metabolite, and faster hydrolysis of the second Ph moiety to yield PhOH as a major metabolite. Minor oxidation of a Ph ring was also seen.

The metabolism of *organophosphorus insecticides* is of great toxicological significance and has been the object of innumerable studies. Here, we examine insecticides of the phosphoric acid triester class. Insecticides belonging to the phosphorothioate (*e.g.*, parathion) and phosphorodithioate (*e.g.*, malathion) classes will be discussed in *Sect. 9.3.6*. Another important group of organophosphates is that of *P*-halide compounds (the nerve gases used as warfare agents) will be discussed in *Sect. 9.3.7*.

Insecticides of the phosphoric acid triester class include paraoxon (**9.49**) and dichlorvos (**9.50**). The phosphorothioate derivative parathion is a relatively non-toxic insecticide that undergoes monooxygenase-catalyzed oxidative desulfuration to paraoxon [105] (see also Chapt. 7 in [59]; see *Sect. 9.3.6*). Paraoxon itself, like its congeners and the *P*-halide nerve gases, is highly toxic through its potent inactivation of acetylcholinesterase [69].



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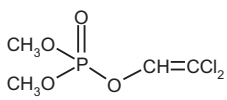


9.50

The inactivation and detoxification of *paraoxon* and congeners are catalyzed by the so-called A-esterases, which, as discussed, comprise *arylesterase* (sometimes still called paraoxonase, EC 3.1.1.2) and phosphoric triester hydrolases (*phosphotriesterases*, EC 3.1.8) subdivided into aryldialkylphosphatase (organophosphate hydrolase, paraoxonase, EC 3.1.8.1) and organophosphorus acid anhydrolases (EC 3.1.8.2; see *Sect. 9.3.7*) [65][69][106–108]. These activities, which occur mostly in the mammalian liver and

serum, were found to range from *ca.* 50 to 300 nmol paraoxon (ml plasma)$^{-1}$ min^{-1} in human subjects and involve genetic polymorphism [106][109 – 111]. Affinities in human liver microsomes and human plasma were identical (K_m *ca.* 250 μM), whereas V_{max} values were approximately fourfold higher in plasma (*ca.* 200 *vs.* 50 nmol (g tissue)$^{-1}$ min^{-1}) [112]. A-Esterases hydrolyze paraoxon by *O*-dearylation to form 4-nitrophenol and diethyl phosphate. A detailed kinetic study in human serum showed that, following substrate binding and transesterification in the catalytic site, 4-nitrophenol was the first product to leave the enzyme, followed by diethyl phosphate (ordered Uni Bi kinetic mechanism) [113].

Dichlorvos (**9.50**) is an insecticide of reportedly wide use, the metabolites of which in humans include dichloroethanol and dimethyl phosphate. Like paraoxon, dichlorvos is hydrolyzed by human serum. However, the enzyme activities hydrolyzing the two substrates were shown to differ by a number of criteria [114]. Clearly large gaps remain in our understanding of the human metabolism of organophosphorus insecticides and other toxins. A bacterial phosphotriesterase appears as a promising tool to understand the catalytic mechanisms of organophosphoric acid triester detoxification [115 – 117].

9.3.5. Cleavage of Phosphonates

Of interest in this section are *phosphonates* (R–P(=O)(–OR′)(–OR″)), including *bisphosphonates* (R–CH(PO_3H_2)$_2$) and *phosphonoformates* ((RO–)(R′O–)P(=O)(COOR″)); *phosphinates* (RR′P(=O)(–OR″)) will also be mentioned briefly. In contrast to phosphates, alkyl phosphonates are not likely to be hydrolyzed by esterases due to lower acidity, a different shape, and, in some cases, the inability to undergo pseudorotation [118]. This increases the probability of alternative cleavage pathways, in particular oxidative ones, as documented below.

Bisphosphonates are drugs of great interest in a number of metabolic bone diseases [119]. This therapeutic class comprises bis(phosphonic acids) and, more recently, bis(phosphonic acid) esters. The former are hydrophilic and poorly bioavailable, and they are generally not metabolized. In contrast, *bis(phosphonic acid) esters* may be more prone to biotransformation, as exemplified with the lead compound known as *U-91502* (**9.51** in *Fig. 9.11*).

When administered orally to rats at various doses, U-91592 was completely absorbed and extensively metabolized [118]. Two metabolites (a phenol glucuronide and a glutathione adduct) were formed following initial cytochrome P450 mediated epoxidation of the heterocycle. The third metabolite was the triester (**9.52**, *Fig. 9.11*), which was also a major product of *in vitro* metabolism in rat liver microsomes. Interestingly, the formation of the tries-

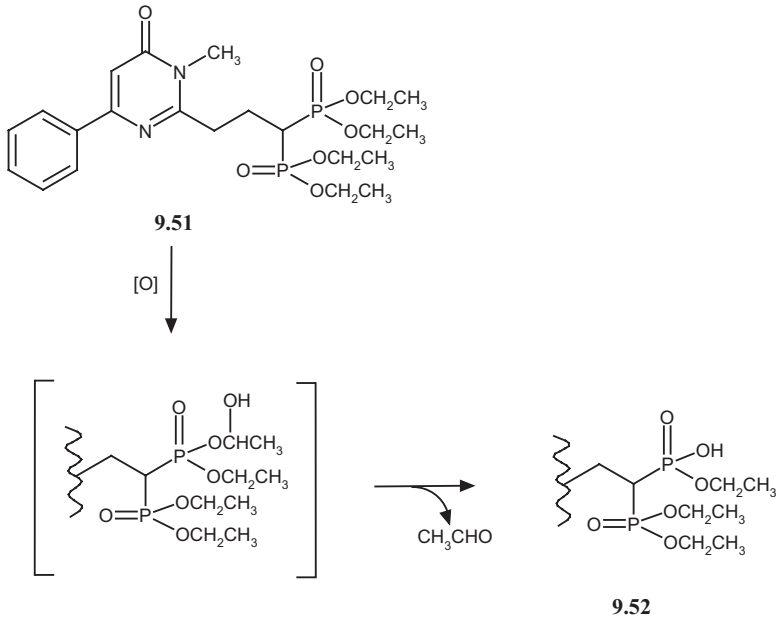
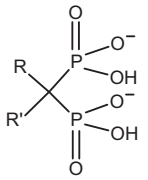


Fig. 9.11. *Postulated mechanism of cytochrome P450 catalyzed phosphoric acid ester cleavage of the bisphophonate U-91502* (**9.51**) *to produce a triester metabolite* **9.52** [118]

ter was completely NADPH-dependent and catalyzed by cytochrome P450, as also revealed by inhibition studies. The postulated mechanism involves hydroxylation at C(α), followed by spontaneous hydrolysis and release of acetaldehyde (*Fig. 9.11*), in other words, a typical reaction of oxidative *O*-dealkylation (see Chapt. 7 in [59]).

The highly polar *clodronic acid* (**9.53**, R, R′ = Cl), *etidronic acid* (**9.53**, R = Me, R′ = OH), and a number of analogues have demonstrated their clinical value in the treatment of osteoporosis, but they must often be administered by slow intravenous infusion due to their poor oral bioavailability. Esterification to orally available prodrugs is, thus, an actively investigated strategy. Promising results have, for example, been obtained with (pivaloyloxy)methyl esters of clodronic acid [120].

There are quite a number of other cases in the literature of active but poorly bioavailable phosphonic acids that have been gainfully derivatized to prodrugs. A case in point is that of inhibitors of neprilysin (EC 3.4.24.11) developed as potential antihypertensive agents. Thus, the agent *CGS 24592* (**9.54**, R = H) is a highly active inhibitor, but it lacks satisfactory oral bioavailability. Among a number of prodrugs investigated, the diphenyl phosphonate (**9.54**, R = Ph) proved quite interesting [121]. This prodrug underwent ready chemical hydrolysis. At high pH values (12 – 13), a single hydro-



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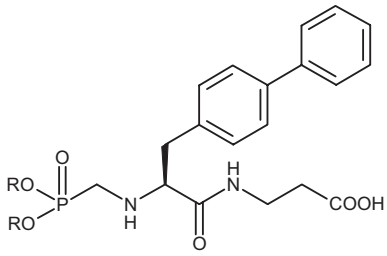

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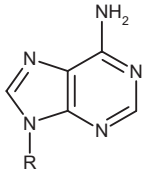
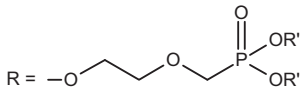
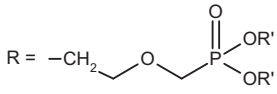
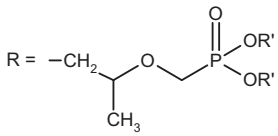


9.55 R = 

9.56 R = 

9.57 R = 

lytic step occurred very rapidly to yield the monoester. At pH values close to 8.5, hydrolysis was very slow in phosphate buffers, but proceeded to completion in a few hours in carbonate buffers, suggesting a general base catalysis. In human plasma at 37°, the $t_{1/2}$ values of the first and second hydrolytic steps were 1.4 and 4.2 h, respectively. Thus, all evidence points to this prodrug being a substrate of plasma hydrolases [121a].

When administered orally to rats, (acyloxy)alkyl prodrugs of CGS 24592 gave long-lasting and high plasma concentrations of the active agent (300–800 times the IC_{50} value). However, the hydrolysis of such derivatives can liberate formaldehyde, and it was found remarkable and promising that the structurally simpler diphenyl phosphonate (**9.54**, R = Ph) should behave practically as well [121b].

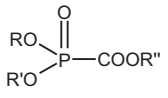
There are also some interesting reports in the literature of phosphonic acids active as antiviral agents, as exemplified by *9-[2-(phosphonomethoxy)ethoxy]adenine* (**9.55**, R′ = H). This compound has potent and selective activity against HIV-1 and HIV-2, with less toxicity than AZT. However, the high hydrophilicity of the phosphonic acid moiety has again been postulated to account for poor oral bioavailability (*ca.* 2% in mice). In a systematic effort to correct this pharmacokinetic defect, a large number of prodrugs were prepared and administered orally to mice [122]. Blood concentrations of the diester, monoester, and free acid were monitored. Dialkyl esters (**9.55**, R′ = Me, Et, i-Pr, or Bu) gave mostly the monoester with little or no parent drug

(**9.55**, R′ = H). The same was true for a few bis(substituted ethyl) esters (**9.55**, R′ = 2-bromoethyl, 2-chloroethyl, or 2-ethoxyethyl). In contrast, bis[(acyloxy)alkyl] esters proved promising; thus, the bis[(pivaloyloxy)methyl] ester (**9.55**, R′ = Me_3C–CO–OCH_2) gave 30% bioavailability of the parent drug, with the first hydrolytic step proceeding very rapidly (<1 min) and the second much more slowly. A few diaryl esters were also interesting, and the best prodrug was in fact the diphenyl ester (**9.55**, R′ = Ph), which, when used as the recrystallized hydrochloride, gave 50% bioavailability of the parent drug. This is another example of a diphenyl phosphonate proving to be a very good prodrug candidate.

A close analog of 9-[2-(phosphonomethoxy)ethoxy]adenine, namely *9-[2-(phosphonomethoxy)ethyl]adenine* (PMEA, **9.56**, R′ = H), is another promising antiviral agent active against HIV and related viruses. Here again, a number of diesters were prepared and examined as potential prodrugs [123 – 126]. Derivatives such as the bis[(pivaloyloxy)methyl] ester (**9.56**, R′ = Me_3C–CO–O–CH_2) had $t_{1/2}$ values of a few minutes for the first hydrolytic step, whereas the introduction of a S-atom markedly slowed this reaction, *e.g.*, the bis[2-(pivaloylthio)ethyl] ester (**9.56**, R′ = Me_3C–CO–S–CH_2CH_2–). When tested in infected cell lines, the prodrugs demonstrated an antiviral efficiency markedly greater than that of the parent drug, indicating their improved cell penetration and their intracellular bioactivation.

Further studies seem to have focused mainly on various prodrugs of a higher homologue of PMEA, namely 9-[2-(phosphonomethoxy)propyl] adenine (PMPA, *tenofovir*, **9.57**, R′ = H). Tenofovir disoproxil (**9.57**, R′ = i-PrO–CO–O–CH_2) proved of particular interest and has been selected for further studies [127][128].

Among *phosphonoformates*, the drug of major interest is phosphonoformic acid (*foscarnet*, **9.58**), a potent anti-HIV agent also active against a variety of other viruses (*e.g.*, human cytomegalovirus, HCMV) associated with opportunistic infections in AIDS. The poor bioavailability and cell permeation of foscarnet arise from its highly polar nature; it is mostly a tri-anion at physiological pH. Many attempts have been made to obtain a prodrug with good pharmacokinetic behavior and characteristics. The design of triesters appears difficult, since they have a tendency to undergo C–P bond cleavage. This is exemplified by dibenzyl methoxycarbonylphosphonates (**9.59**), which were found to have a marked tendency for C–P bond cleavage when X = H, but not when X was an electron-withdrawing substituent (*e.g.*, X = NO_2 or CF_3) [129]. An additional problem is that hydrolysis of the three ester groups is difficult or practically impossible to achieve for some triesters, even in the presence of esterases. This was seen with (acyloxy)alkyl esters (**9.60**) [130]. Only with compound **9.60** having R″′ = 2,4-$Cl_2C_6H_3$ and R″′ = Me was a slow formation of foscarnet observed, as well as a dose-dependent inhibition of HIV



9.58 R = R' = R" = H

9.59 R = R' = $-CH_2-C_6H_4-X$

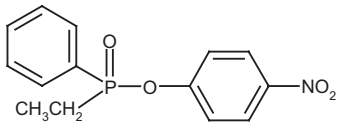
R" = $-CH_3$

9.60 R = R' = $-CH_2OCOR'''$

R" = alkyl or aryl

9.61 R = $-CH_2CHOHCH_2O(CH_2)_{17}CH_3$

R' = R" = H



9.62

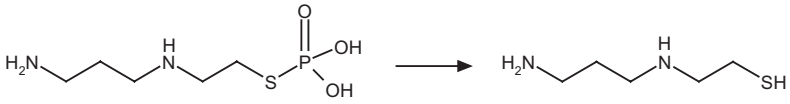
in infected cells. Another example is that of bis[2-(*S*-acylthio)ethyl] ester prodrugs of foscarnet, which showed good *in vitro* activity and slow release of foscarnet in rat liver microsomes. However, no foscarnet was detected in rat plasma following oral administration of these prodrugs [131].

Recently, highly lipophilic monoesters of foscarnet have been reported, *e.g.*, 1-*O*-octadecyl-*sn*-glycero-3-phosphonoformate (**9.61**) and propane-1,3-diol analogues [132]. Some prodrugs had much higher activities against HIV and HCMV than foscarnet itself, indicating a good cell permeation and intracellular liberation of the drug.

Phosphinates are a class of organophosphorus compounds, the metabolism of which has received less attention than that of phosphates (see above) or phosphorothioates and *P*-halide compounds (see below). Many phosphinates are rapid but transient inhibitors of acetylcholinesterase and carboxylesterases. And like organophosphates and phosphonates, phosphinates are substrates of arylesterases (EC 3.1.1.2). This is exemplified by 4-nitrophenyl ethyl(phenyl)phosphinate (**9.62**), whose (–)-enantiomer was hydrolyzed by rabbit serum arylesterase almost 10 times faster than the (+)-enantiomer [133].

9.3.6. Cleavage of Phosphorothioates and Phosphonothioates

An informative case exists in the literature of a *phosphorothioic* S-*acid ester*. Indeed, *amifostine* (ethyol, WR-2721, **9.63**) is a phosphorothioate first



9.63 **9.64**

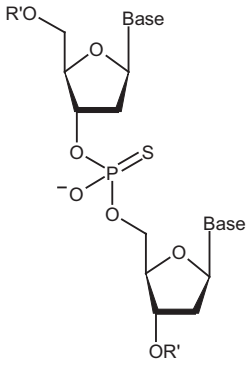
developed as a radioprotective agent. In addition, the compound is also useful as a chemoprotector of healthy tissues during chemotherapy, and it has mucolytic properties of value in cystic fibrosis [134][135].

Amifostine is hydrolyzed rapidly to 2-[(3-aminopropyl)amino]ethanethiol (**9.64**) at the acidic pH of the stomach, and by alkaline phosphatases in various tissues. After intravenous administration to cancer patients, the plasma half-lives were found to be in the order of minutes. This is highly relevant, since most or all pharmacological effects of amifostine can be ascribed to its thiol metabolite.

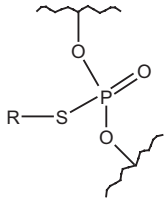
Considerable efforts are devoted to *anti-gene* and mainly *antisense therapeutics*, namely agents that will bind to and inactivate either a gene (antigene) in double-stranded DNA, or a m-RNA (antisense) [136]. An essential condition for the successful design of such agents is an extreme selectivity toward the coding sequence to be targeted, as best achieved with a complementary oligonucleotide or an oligonucleotide analogue. Statistically, a complementary sequence of a 17-mer oligonucleotide (*i.e.*, consisting of 17 nucleotides) occurs only once in the human genome, hence, this length should be enough to guarantee selective inhibition of the synthesis of the product(s) of a single gene. In practice, *20-mer oligonucleotides* (eicosanucleotides) or analogues are often prepared.

Whereas it appears straightforward to design an antisense oligonucleotide, once the target sequence is known, pharmacokinetic problems are bound to arise. Thus, the rapid *in vitro* degradation of the phosphodiester oligonucleotides by exo- and endonucleases severely undermines their potential as therapeutic agents [137]. Solutions to solve these problems include structural modifications of the backbone, sugars, or bases. One particularly promising modification of the backbone is the replacement of all or some phosphodiester by phosphorothioic *O*,*O*-acid ester bridges (**9.65**) [136]. Such *phosphorothioate oligonucleotides* have a number of advantages over their phosphodiester congeners, *e.g.*, good water solubility, increased stability toward nucleases, relatively efficient uptake by cells, and high specificity toward target RNA [138].

A variety of such phosphorothioate oligonucleotides have, thus, been reported in the literature [138 – 142]. In one particularly illustrative study, the 20-mer phosphodiester oligonucleotide 5′-TCATGCTCATGCGCTCATGC-3′ and its phosphorothioate congener were compared for their distribution



9.65



9.66

and degradation in mice [143]. The phosphodiester oligonucleotide reached peak levels in organs within a few minutes, but its concentration in blood and organs except the spleen decreased comparatively rapidly, indicating fast degradation. In contrast, the phosphorothioate congener demonstrated slow metabolism and marked organ extraction, resulting in a rapid disappearance from the blood but high and stable organ concentrations. It was concluded that such phosphorothioate oligonucleotides may have therapeutic potential.

Interestingly, the pharmacokinetic properties of phosphorothioate oligonucleotides may be further improved by a careful *prodrug* strategy. Thus, short phosphorothioate dinucleotides were derivatized to prodrugs of general structure **9.66** [144]. *S*-[(Acyloxy)alkyl] derivatives (**9.66**, R = R′–CO–O –CH_2–) underwent ready esterase-mediated hydrolysis to the parent compound, but desulfuration also occurred to a small extent. This problem was overcome by *S*-[(acyloxy)aryl] derivatives (**9.66**, R = R′–CO–O–C_6H_4 –CH_2–). These prodrugs were stable in acidic and neutral buffers but underwent clean esterase-mediated hydrolysis to the parent phosphorothioate dinucleotide with only minimal desulfuration.

Phosphorothioates and phosphonothioates are of particular significance as *insecticides*. Schematically, it can be stated that these xenobiotics undergo activation by oxidative desulfuration, and detoxification by hydrolytic cleavage. Oxidative desulfuration transforms phosphorothioates and phosphonothioates to the corresponding oxon derivatives (see Chapt. 7 in [59]), which are highly toxic as potent inactivators of acetylcholinesterase [69]. This route of toxification can be competitive with and/or followed by cleavage reactions, which can be either hydrolytic or oxidative.

The formal mechanism of *oxidative desulfuration* has been discussed in detail in an earlier book (Chapt. 7 in [59]). As shown in schematic and simplified form in *Fig. 9.12*, monooxygenase-catalyzed *S*-oxygenation of phosphorothioates (**9.67**, X = O) and phosphorodithioates (**9.67**, X = S) yields an

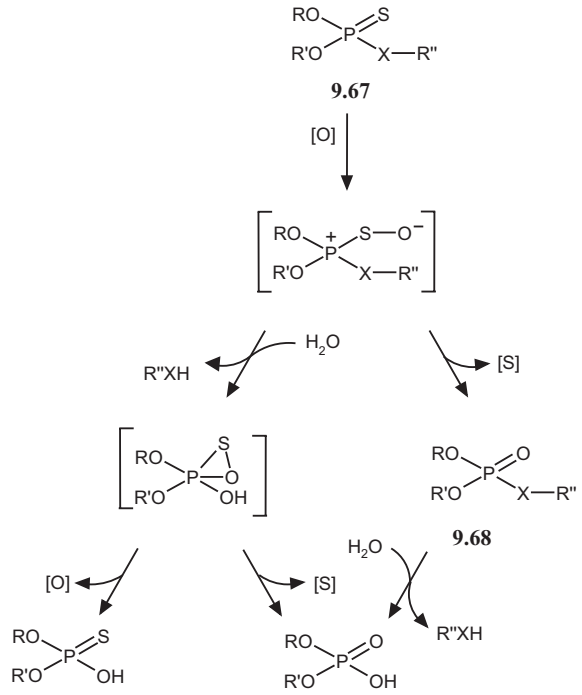


Fig. 9.12. *Mechanism of monooxygenase-catalyzed oxidative desulfuration and dephosphorylation of phosphorothioates* (**9.67**, X = O) *and phosphorodithioates* (**9.67**, X = S). The first step is believed to be an *S*-oxygenation followed by rearrangement with sulfur expulsion (oxidative desulfuration) or hydrolysis to form phosphate and phosphorothioic *O*,*O*-acid diesters.

intermediate, which can react in two ways. Rearrangement with sulfur expulsion is an oxidative desulfuration, which produces the oxon analogue (**9.68**, *Fig. 9.12*). However, the *S*-oxygenated intermediate can also undergo hydrolysis followed by loss of a S- or O-atom to yield phosphoric acid diester and phosphorothioic *O*,*O*-acid diester. Thus, toxification (desulfuration) and detoxification (hydrolysis) follow from the same metabolic intermediate. However, the latter reaction may be of limited biological significance.

In contrast, an effective detoxification of the oxon analogue is catalyzed by *serum paraoxonase* (PON1, EC 3.1.8.1). Experiments with PON1-knockout mice showed that these animals were extremely sensitive to the toxic effects of chlorpyrifos oxon, an analogue of paraoxon [145]. This and other evidence prove that paraoxonase plays a major role in the detoxification of organophosphorus insecticides and nerve gases processed through the CYP/PON1 pathway. Serum paraoxonase is also endowed with another protective role in that it hydrolyzes lipid peroxides in low-density lipoproteins (LDL), thereby helping to prevent oxidative modifications of LDL, which

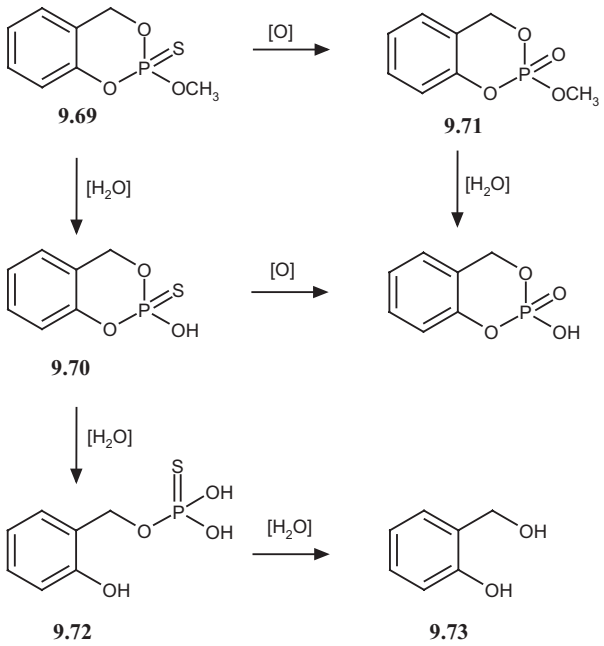
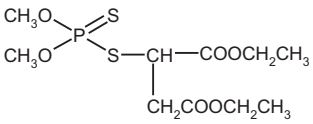


Fig. 9.13. *Hypothetical metabolic scheme of salithion* (**9.69**)*, based on its metabolism in rats* [147]

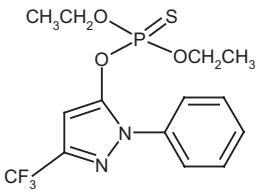
lead to atherosclerosis. This dual protective role appears markedly affected by the polymorphisms of the human PON1 gene [70][146].

Reactions of this type have been characterized for numerous phosphoro(di)thioate and phosphono(di)thioate insecticides [148]. The example of *parathion* (**9.67**, R = R′ = Et, X = O, R″ = 4-$NO_2C_6H_4$; *Fig. 9.12*) has been presented elsewhere (Chapt. 7 in [59]). However, the diversity in chemical structures and functional groups in compounds of this type is such that several other metabolic reactions may take place, rendering any generalization difficult and making each insecticide a case of its own. For instance, the metabolism of *salithion* (**9.69**, *Fig. 9.13*) in rats led to the product of *O*-demethylation (**9.70**, *Fig. 9.13*) as the major product (*ca.* 1/2 of an oral dose) [147]. The ring-opened phosphorothioic *O*-acid ester (**9.72**, *Fig. 9.13*) was probably formed from **9.70** and may be the progenitor of saligenin (**9.73**, *Fig. 9.13*). It is likely that *O*-demethylation and opening of the 4*H*-dioxaphosphinin ring occur by a hydrolytic mechanism, given in particular that oxidative desulfuration to form the oxon metabolite (**9.71**, *Fig. 9.13*) was a very minor route.

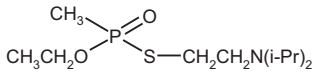
In the case of *malathion* (**9.74**), oxidative desulfuration is again a very minor route in mammals, but for another reason. Here indeed, the two carboxylate functions make the compound such a good substrate of carboxyles-



9.74



9.75



9.76

terases that it is sometimes used to monitor their activity [65][148a][149]. The reaction is one of facile and fast detoxification.

Flupyrazofos (**9.75**) is an insecticide whose relatively simple metabolic scheme appears quite informative. Indeed, rat liver microsomes formed the oxon analogue as the only detectable primary metabolite [150]. The latter then underwent chemical hydrolysis to the dephosphorylated phenol with a $t_{1/2}$ value of *ca.* 50 min at pH 7.4 and 37°. In rat liver microsomes, the phenol appeared to be produced exclusively from flupyrazofos oxon, indicating the resistance of flupyrazofos toward chemical hydrolysis.

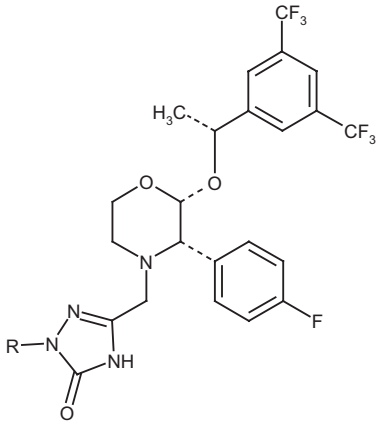
A number of fundamental studies have been conducted with various phosphonothioates to understand their *mechanisms and kinetics of esterase inhibition*. For example, human butyrylcholinesterase was irreversibly inhibited by the nerve agent *VX* (**9.76**). Site-directed mutagenesis experiments were designed to replace with histidine some residues located close to the active site [151]. One of the mutants, G117H, was found to behave as an active esterase, having acquired the capability to catalyze its own dephosphonylation, and to do so rapidly. Indeed, the mutated butyrylcholinesterase enhanced VX hydrolysis *ca.* 2000-fold above the uncatalyzed reaction at pH 6 and 25°. Studies of this type offer a deep insight into individual interactions and reactions occurring in the catalytic site.

9.3.7. Hydrolysis of Phosphoramidates and *P*-Halide Compounds

Phosphoramidates are organophosphorus compounds containing a *P–N bond*. In recent years, innovative prodrug approaches have been developed based on the cleavage of the P–N bond, two examples of which are presented here.

A direct reaction of activation is exemplified by the NK_1 receptor antagonist *L-754,030* (**9.78**) developed as an *i.v.* drug against emesis, migraine, and chronic pain. Because the compound has a low water solubility unfavorable for *i.v.* administration, a phosphoramidate prodrug (**9.77**) has been examined [152]. This compound hydrolyzed rapidly under acidic conditions. More importantly, it was rapidly converted to L-754,030 in rat blood but was

stable in dog and human blood, perhaps suggesting the involvement of carboxylesterases. Indeed, the conversion was fast in dog and human liver microsomes, and so it was also *in vivo* after *i.v.* administration to rats and dogs. Thus, available evidence confirms the interest in a prodrug such as **9.77**.



9.77 R = $-PO_3H_2$

9.78 R = $-H$

A more complex pathway of activation is seen in N-*amino acid derivative of phosphoramidic acid diesters of antiviral nucleosides*, as exemplified by prodrugs of stavudine (**9.79**, *Fig. 9.14*) [153 – 155]. The activation begins with a carboxylesterase-mediated hydrolysis of the terminal carboxylate. This is followed by a spontaneous nucleophilic cyclization–elimination, which forms a mixed-anhydride pentacycle (**9.80**, *Fig. 9.14*). The latter hydrolyzes spontaneously and rapidly to the corresponding phosphoramidic acid monoester (**9.81**, *Fig. 9.14*), which can then be processed by phosphodiesterase to the nucleoside 5′-monophosphate, and by possible further hydrolysis to the nucleoside.

These reactions were demonstrated with pure enzymes or various biological media such as human peripheral blood mononuclear cells uninfected or infected with HIV. In the latter case, highly promising antiviral results were obtained. Furthermore, a variety of amino acyl residues, carboxylic acid ester moieties, and aryl substituents X have been reported.

A few *organophosphorus insecticides* are also phosphoramidates, hydrolysis of the P–N bond being considered a route of detoxification. This is exemplified by the metabolism of *acephate* (**9.82**, *Fig. 9.15*), whose mechanisms of activation and detoxification have recently been re-examined in mice to better understand the relative innocuity of the compound in mammals and its selective toxicity in insects [156].

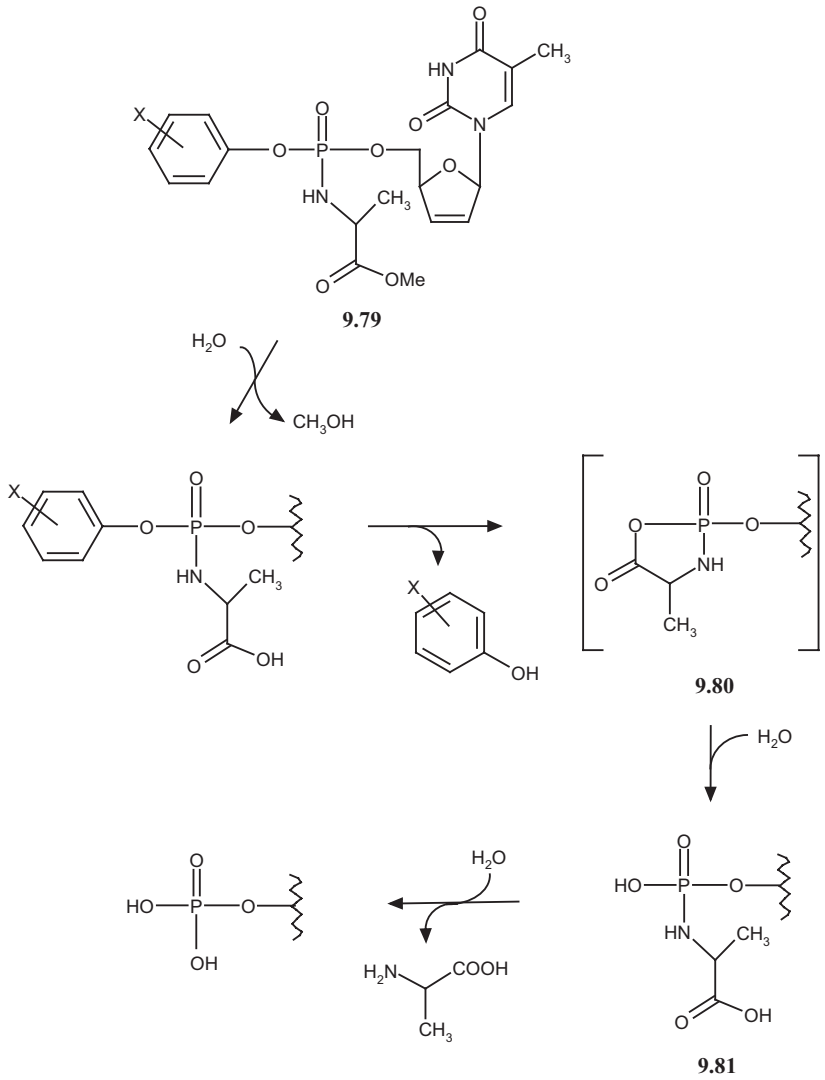


Fig. 9.14. *Metabolic activation of phosphoramidic acid diester prodrugs* **9.79** *of stavudine* (and analogous nucleosides). Carboxylesterase-mediated hydrolysis of the terminal carboxylate is followed by spontaneous cyclization–elimination with formation of a pentacyclic mixed-anhydride species. The latter hydrolyzes rapidly to the corresponding phosphoramidic acid monoester, which can then be processed to stavudine 5′-monophosphate.

Pathway a in *Fig. 9.15* is one of amide hydrolysis mediated by a carboxyamidase. The metabolite thus produced is methamidophos, the toxic species formed predominantly in insects and far less in mammals. *Pathways b* and *c* lead to an *O*-demethyl and a demethylthio metabolite, respectively. The re-

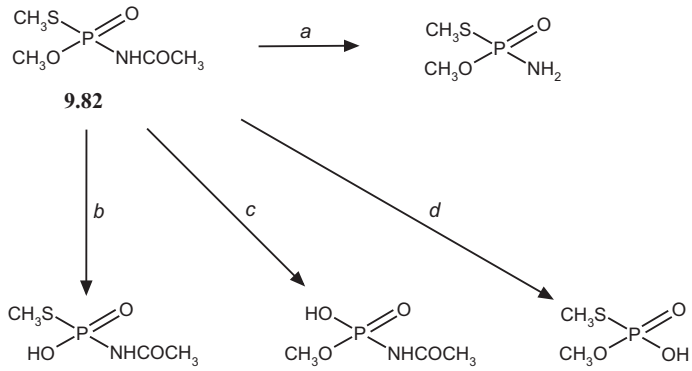
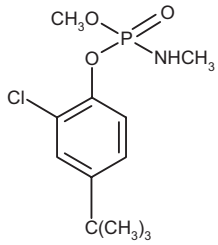


Fig. 9.15. *Metabolism of acephate* (**9.82**) *in mammals* [156]. *Pathway a* leads to toxification by producing methamidophos. *Pathways b – d* are reactions of detoxification that lead to an *O*-demethyl, a demethylthio, and a deacetylamino metabolite, respectively.

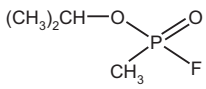
action of relevance here is a P–N bond cleavage (*Pathway d*), which, although of minor quantitative significance, is carefully documented *in vivo* [156].

Another relevant example is that of the insecticide *crufomate* (**9.83**), which, in sheep, underwent various reactions of C-oxidation, as well as hydrolysis of the C–N bond to yield phosphate analogues as urinary metabolites [148a]. These phosphates accounted for almost 15% of a dose in urine, indicating that the reaction of hydrolysis was a significant one in this case.



9.83

Organophosphorus acid anhydrides of the P-*halide type* are xenobiotics of particular significance, being highly toxic nerve agents used as large-scale chemical weapons. These agents include the phosphono- or phosphorofluoridates *sarin* (**9.84**), *soman* (**9.85**), and *DFP* (**9.86**, diisopropyl phosphorofluoridate), and the phosphoramidocyanidate analogue *tabun* (**9.87**). The P–halogen or P–CN bond in these compounds is an anhydride bond, the hydrolysis of which liberates a phosphate functionality and F^- or CN^-.



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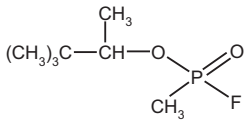

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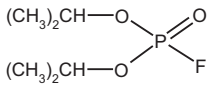

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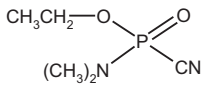


9.87

The *toxicity* of the *P*-halide anhydrides, like that of phosphoroxychloride ($POCl_3$) and of other organophosphorus compounds discussed earlier in this section, is due to their high efficiency as irreversible inactivators of acetylcholinesterase [157]. The main target organs for the lethal effects of these chemical weapons are the brain and diaphragm. As for the *detoxification* of the *P*-halide anhydrides, it can occur by a number of biochemical mechanisms, namely chemical hydrolysis, enzymatic hydrolysis, and binding to hydrolases such as carboxylesterases, cholinesterases, and albumin [68][158][159].

As anhydrides, such compounds are subject to *spontaneous hydrolysis*, which may contribute to detoxification [160]. Thus, soman hydrolysis at pH 7.5 and 37° occurs with a rate constant of 0.003 – 0.004 min^{-1} and an activation energy of *ca.* 55 kJ mol^{-1} [161]. However, most of the published data refer to enzymatic hydrolysis. Enzymes hydrolyzing P–X anhydride bonds are now known as *organophosphorus acid anhydrolases* (OPA anhydrolases) classified as EC 3.1.8.2 (also known as diisopropyl-fluorophosphatase, DFPase, tabunase, somanase), an activity related to EC 3.1.8.1 (aryldialkylphosphatase, paraoxonase, A-esterase) and formerly classified as EC 3.8.2.1 [64][65][69]. Much public information on these enzymes can be found in [106].

The P-atom in sarin (**9.84**), soman (**9.85**), and tabun (**9.87**) is a stereogenic center, allowing for *stereoselective enzymatic hydrolysis* [162]. This aspect has been extensively investigated for soman, which exists as four stereoisomers by virtue of the presence of a second stereogenic center (C-atom). These stereoisomers are usually designated as *C*(+)*P*(–), *C*(–)*P*(+), *C*(+)*P*(+), and *C*(–)*P*(–), where *C*(+/–) refers to the 1,2,2-trimethylpropyl moiety and *P*(+/–) to the P-atom. Such a nomenclature may be convenient but has no implication for the absolute configuration. The *C*(+)*P*(–) and *C*(–)*P*(–) epimers are the more active toward acetylcholinesterase and, hence, the more toxic ones. In contrast, the *C*(+)*P*(+) and *C*(–)*P*(+) epimers are preferentially hydrolyzed

in liver and plasma preparations from various animal species and in human plasma [106][160][161][163].

Perhaps a word of caution is appropriate here, since the metabolism of the *P*-halide anhydrides is often investigated by monitoring the disappearance of the substrate by a chromatographic technique. Given that the ester bonds in these compounds may also undergo enzymatic hydrolysis, many results in the literature cannot be considered as specific for hydrolysis of the P–halogen anhydride bond. This problem has been overcome in investigations where reaction rates were determined by monitoring fluoride formation with a fluoride-specific electrode [164].

9.4. Hydrolysis of Sulfuric Acid Esters and Related Compounds

9.4.1. Introduction

In this section, we examine the fate of hydrolyzable derivatives of sulfuric acid, namely:

- alkyl and aryl sulfates ($R–O–SO_3^-$, R = alkyl or aryl),
- *O*-alkyl and -aryl sulfamates ($R–O–SO_2NH_2$, R = alkyl or aryl);
- *N*-sulfamates ($R–NH–SO_3^-$).

These compounds have very little importance as drugs and prodrugs, but find some industrial applications. The greatest interest of sulfuric acid esters in the present context is their reversible formation as conjugates of endogenous and exogenous alcohols and phenols. Indeed, hydrolysis of sulfate conjugates in the body (*i.e.*, deconjugation) is a reaction of distinct physiological and pharmacological significance [165][166].

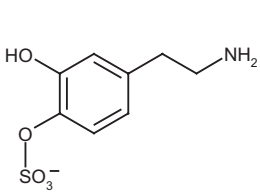
A number of *enzymes* known as sulfuric ester hydrolases (EC 3.1.6) are able to hydrolyze *sulfuric acid esters*. They comprise arylsulfatase (sulfatase, EC 3.1.6.1), steryl-sulfatase (steroid sulfatase, steryl-sulfate sulfohydrolase, arylsulfatase C, EC 3.1.6.2), choline-sulfatase (choline-sulfate sulfohydrolase, EC 3.1.6.6), and monomethyl-sulfatase (EC 3.1.6.16). Whereas monomethyl-sulfatase is highly specific and does not act on higher homologues, arylsulfatase has a broad substrate specificity and is of particular significance in the hydrolysis of sulfate conjugates of phenols, be they endogenous compounds, drugs, or their metabolites [167 – 169].

Sulfuric acid esters also undergo *chemical hydrolysis*, which can be acid- or base-catalyzed [170]. As a rule, the former reaction is faster than the latter, the slowest rates being found around neutrality. High dependence on chemical structure is another characteristic of sulfuric acid ester hydrolysis.

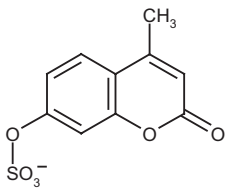
Sulfamates (R–NH–SO_3^-) behave differently in that they are stable in alkaline conditions but are readily hydrolyzed in acidic media, with *N*-alkyl sulfamates being more stable than *N*-aryl sulfamates. The reaction is initiated by protonation of the N-atom followed by a nucleophilic attack of H_2O at the S-atom [170].

9.4.2. Sulfuric Acid Esters

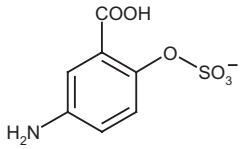
Studies on the fate of *dopamine 4-sulfate* (**9.88**) will serve to illustrate the reaction of deconjugation mentioned above. With this substrate, arylsulfatase activity in the dog was found to be highest in the kidney and liver, low in the intestine and heart, and almost nil in the brain and skeletal muscle [171]. Since this conjugate exists in high amounts in the plasma of humans, monkeys and dogs, the possibility was raised that it might be looked upon as a precursor or reservoir of free dopamine.



9.88



9.89



9.90

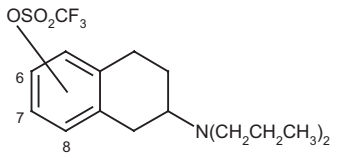
A convenient substrate, *4-methylumbelliferyl sulfate* (**9.89**), was used to investigate some characteristics of sulfate hydrolysis in the perfused rat liver [172]. Hydrolysis proceeded with maximal calculated rates of 41 and 29 μmol g^{-1} h^{-1} in pericentral and periportal regions, respectively. However, this was not due to differences in enzyme concentrations, since these regions showed similar activities in homogenates of microdissected liver samples. Rather, the regional differences in the perfused liver corresponded to differences in substrate concentrations, which were 1.5- to 2-fold higher in pericentral than in periportal regions presumably due to differential uptake. Studies such as this one have served to suggest that a futile cycle regulates sulfate conjugation within hepatocytes.

A medicinal example is found with *5-aminosalicylic acid* O-*sulfate* (5-ASA sulfate, **9.90**). 5-ASA is an agent for the treatment of ulcerative colitis and *Crohn*'s disease of the large intestine, but it is unstable in the gastric juice. 5-ASA sulfate was, therefore, developed as a prodrug able to reach its site of action (the colon) following oral application [173]. In healthy human

subjects, the prodrug was almost completely metabolized in the colon to the active agent. A high fecal and a low urinary excretion of the active metabolite 5-ASA were observed.

9.4.3. Related Compounds

An intriguing analog of sulfuric acid is trifluoromethanesulfonic acid (CF_3SO_2OH), commonly known as *triflic acid*. Esters of this acid have been reported to have promising properties in drug design. This is the case for 2-(dipropylamino)tetralins whose phenolic group in the 5-, 6-, 7-, or 8-position was esterified with trifluoromethanesulfonic acid (**9.91**) [174]. Most relevant in the present context is the fact that the trifluoromethanesulfonates were stable toward chemical and enzymatic hydrolysis *in vitro* and *in vivo*. In addition, some of them showed improved oral bioavailability presumably due to higher lipophilicity and/or decreased ring hydroxylation. Furthermore, direct binding affinity to dopamine and serotonin receptors was seen. Clearly, the trifluoromethanesulfonate group, as long as it does not prevent receptor affinity, is of interest to improve the pharmacokinetic behavior of drugs having a phenolic group.



9.91

9.92

Coming now to *sulfamic acid esters*, we note their use as therapeutic agents and prodrugs. The former case is illustrated by 1,7-heptanediyl bis(sulfamate) (**9.92**), an anticancer agent [175]. Metabolism *in vitro* (rat liver microsomes) and *in vivo* (mice) was mostly by oxidation, with seemingly very little hydrolysis since only traces of the monoester were found. This is a rather intriguing observation given that *estrogen 3-sulfamates*, which are prodrugs with little if any intrinsic activity, underwent very effective activation in rats and had systemic estrogen activity 10- to 90-fold higher than that of the parent estrogen [176]. Too little is known at present about the enzymes involved and their substrate specificity toward sulfamates. Presumably steroid sulfatase (EC 3.1.6.2) is not involved in the hydrolytic activation of estrogen 3-sulfamates since these are site-directed inhibitors of the enzyme [177].

Up to this point, we have examined the hydrolysis of the S–O bond in sulfates and sulfamates. In *sulfamates* ($R–NH–SO_3^-$), cleavage occurs at the

N–S bond, as exemplified by the sweetening agent *cyclamate* (**9.93**). This compound sometimes undergoes metabolic cleavage to yield cyclohexylamine, a compound of toxicological significance. For example, the urine of a group of diabetic patients receiving 1 g of cyclamate per day was examined for cyclamate and cyclohexylamine [178]. There was no significant urinary excretion of cyclohexylamine in the majority of cases (*ca.* 80%), but a small percentage of the subjects (4%) were found to excrete over 20% of the daily dose as cyclohexylamine. The reaction of cleavage, which is mediated by the gut flora, is presumably one of hydrolysis although reduction cannot be excluded [179].

9.93

In conclusion, we note the paucity of metabolic data on sulfuric acid esters, compared to the wealth of information on esters of N- and P-containing acids. One of the reasons for this state of affairs is presumably a lack of interest of medicinal chemists in preparing esters of S-containing acids. But because of the numerous applications of such compounds, is it hoped that more metabolic data will become available in a near future.

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Chapter 10

The Hydration of Epoxides

Contents

10.1. Introduction

Epoxides, also known to chemists as *oxiranes*, result from the monooxygenation of a C=C bond in olefins or aromatic rings. Such reactions of monooxygenation to produce epoxides as metabolites or metabolic intermediates

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are of utmost importance in drug and xenobiotic metabolism, being catalyzed by cytochromes P450 as explained in great detail in [1]. In addition, epoxides are also found as endogenous metabolites (*e.g.*, epoxides of squalene, some steroids, and vitamin K), as natural products (*e.g.*, scopolamine), and as industrial chemicals (*e.g.*, dieldrin).

The *chemical reactivity* of epoxides varies widely depending on chemical structure and conditions, and that reactivity is often of toxicological significance [2]. From a metabolic and toxicological viewpoint, it is customary to distinguish three classes of epoxides, namely:

- *Alkene oxides*, *i.e.*, epoxides of C=C bonds, be they isolated or conjugated;
- *Arene oxides*, *i.e.*, epoxides of aromatic rings;
- *Diol epoxides*, a very special and highly reactive subclass of alkene oxides encountered in the metabolism of polycyclic aromatic hydrocarbons.

This chapter begins, thus, with a short introduction to the chemical reactivity of epoxides. We continue with a description of the epoxides hydrolases and their biochemistry, and devote most of its length to a systematic discussion of the substrates hydrated by these enzymes. Arene oxides and diol epoxides will be presented first, followed by a large variety of alkene and cycloalkene oxides.

10.2. Chemical Reactivity of Epoxides

Due to conjugation, epoxides are less basic than linear ethers or larger saturated oxaheterocycles. Also, ring strain and conjugation render the two adjacent C-atoms markedly electrophilic, resulting in the characteristic alkylating activity of epoxides [3].

Schematically, the two major types of reactions undergone by epoxides are rearrangements and addition of nucleophiles. Rearrangements can lead to toxic intermediates, precursors, or metabolites, whereas nucleophilic additions can lead to alkylation of biomacromolecules, *i.e.*, formation of covalent adducts.

10.2.1. Rearrangement Reactions

In *rearrangement reactions that lead to isomerization*, an important discrimination must be made between epoxides of aromatic compounds, *e.g.*, benzene oxide (**10.1**, *Fig. 10.1*), and epoxides of alkenes. As a class, epoxides of aromatic compounds (also known as *arene oxides*) are markedly un-

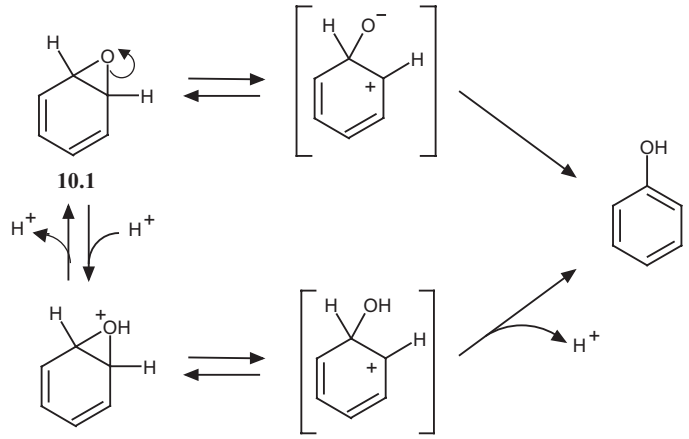


Fig. 10.1. *Simplified mechanisms of the spontaneous and acid-catalyzed isomerization of arene oxides to phenols* via *a carbonium ion intermediate* (benzene oxide (**10.1**) as the model)

stable and undergo spontaneous or acid-catalyzed *isomerization to phenols* (*Fig. 10.1*) [4 – 9] (see also Chapt. 4 in [1a]). The rate of reaction depends heavily on structure and conditions. Thus, benzene oxide (**10.1**, *Fig. 10.1*) has a $t_{1/2}$ value of *ca.* 2 min in water at pH 7, and, under these conditions, the $t_{1/2}$ value of naphthalene 1,2-oxide (**10.2**) is *ca.* 3 min [10]. On the other hand, stability is increased in polycyclic aromatic hydrocarbons.

Another isomerization reaction of arene oxides is *equilibrium with oxepins* [5]. Here, the fused six-membered carbocycle and three-membered oxirane merge to form a seven-membered heterocycle, as shown in *Fig. 10.2*. An extensive computational and experimental study involving 75 epoxides of monocyclic, bicyclic, and polycyclic aromatic hydrocarbons has revealed much information on the structural factors that influence the reaction rate and position of equilibrium [11]. Thus, some compounds were stable as oxepins (*e.g.*, naphthalene 2,3-oxide), while others exhibited a balanced equilibrium

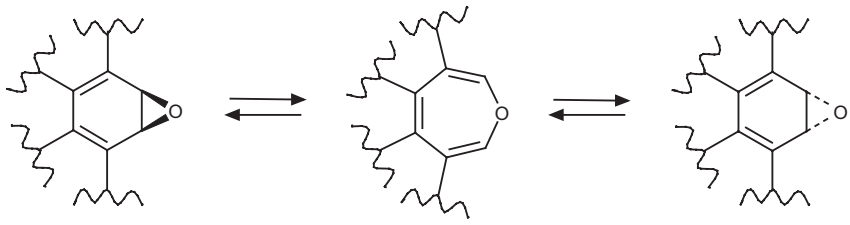
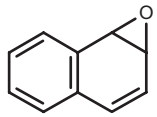


Fig. 10.2. *The arene oxide–oxepin isomerization, which may lead to racemization in certain substrates*



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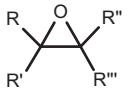

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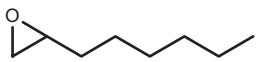

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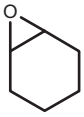

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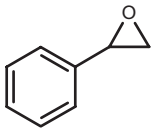

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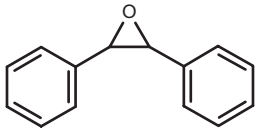

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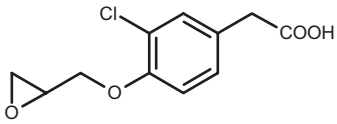

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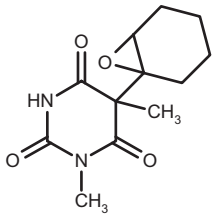
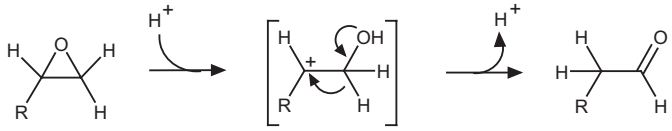


10.9

(*e.g.*, benzene oxide); others, still, were stable as arene oxides but were rapidly racemized (*e.g.*, benz[*a*]anthracene 3,4-oxide), whereas other compounds racemized more slowly (*e.g.*, phenanthrene 1,2-oxide) or not at all (*e.g.*, naphthalene 1,2-oxide).

In contrast, a number of *alkene epoxides* (**10.3**) are chemically quite stable, *i.e.*, intrinsically less reactive than arene oxides. Examples of epoxide metabolites that have proven to be stable enough to be isolated in the absence of degrading enzymes include 1,2-epoxyoctane (**10.4**), 1,2-epoxycyclohexane (**10.5**), 1-phenyl-1,2-epoxyethane (styrene oxide, **10.6**), and *cis*-1,2-diphenyl-1,2-epoxyethane (*cis*-stilbene oxide, **10.7**) [12]. The same is true of alclofenac epoxide (**10.8**), hexobarbital epoxide (**10.9**), and a few other epoxides of bioactive compounds.

Such stability is only relative, however, given the possibility of the acid-catalyzed 1,2-shift of a proton observed in some olefin epoxides of general structure **10.10** (*Fig. 10.3*) [12]. Such a reaction occurs in the *in vivo* metabolism of *styrene* to phenylacetic acid; the first metabolite formed is styrene oxide (**10.10**, R = Ph, *Fig. 10.3*; also **10.6**), whose isomerization to phenylacetaldehyde (**10.11**, R = Ph, *Fig. 10.3*) and further dehydrogenation to phenylacetic acid has been demonstrated by deuterium-labeling studies. A com-



10.10 **10.11**

Fig. 10.3. *Mechanism of the acid-catalyzed proton shift in the rearrangement of some alkene epoxides* (**10.10**) *to aldehydes* (**10.11**)

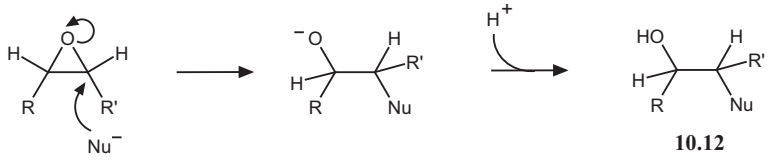
parable mechanism (the halogen shift) operates in the rearrangement of some haloalkene oxides, as discussed in *Sect. 10.5.4* [13].

The various rearrangement reactions discussed above do not involve hydration and would seem, thus, to fall outside the scope of this work. However, they are of relevance, being competitive with the addition of nucleophiles and, particularly, with enzymatic hydration. As such, they should be taken into account in the interpretation of metabolic and toxicological results.

10.2.2. Addition of Nucleophiles

The second class of major reactions involving epoxides is the *addition of a nucleophile*. The reaction mechanism as shown in *Fig. 10.4* often leads to a product of *trans*-configuration (**10.12**, *Fig. 10.4*) [14]. Such reactions are toxicologically highly significant when the nucleophile is a critical biomacromolecule such as a nucleic acid [9][10][12][13][15][16]. In contrast, addition is a major mechanism of cellular protection when the nucleophile is a protective thiol, particularly *glutathione* (L-γ-glutamyl-L-cysteinylglycine) [17]. Depending on the reactivity of the epoxide, glutathione addition can occur nonenzymatically, but the efficiency of the reaction is usually greatly increased in biological systems by the catalytic action of glutathione *S*-transferases (EC 2.5.1.18) [18].

A priori, H_2O would also be expected to act as protective nucleophile against reactive epoxides. Because H_2O is a very weak nucleophile [17], the *nonenzymatic hydration of epoxides* to vicinal diols (**10.12**, Nu = OH, *Fig.*



10.12

Fig. 10.4. *General mechanism of nucleophilic addition to epoxides.* The site of attack (*i.e.*, C(1) *vs.* C(2)) depends on the steric and electronic properties of the substituents.

10.4) is not a documented metabolic reaction. Only in the alkaline pH range, when the nucleophilic HO^- ion is present at sufficiently high concentration, can the hydration of epoxides become significant. However, such conditions are not biologically relevant.

Yet, despite the relative inertness of H_2O and the very low physiological concentrations of the HO^- ion, hydration of epoxides is known to be a metabolic reaction of both qualitative and quantitative significance. This hydrolysis is mediated physiologically by the *epoxide hydrolases*, a group of enzymes whose remarkable efficiency and versatility are the main topic of this chapter.

10.3. Epoxide Hydrolases

10.3.1. Enzymology

Epoxide hydrolases (EC 3.3.2.3), a subset of the ether hydrolases (EC 3.3.2), were formerly referred to as epoxide hydrases or epoxide hydratases. They are a group of related enzymes sharing a similar catalytic mechanism but differing in substrate specificity and biochemical characteristics [3][19 – 23]. These enzymes, which are located in many organs and tissues [24 – 29], play essential physiological roles, *e.g.*, vitamin K_1 oxide reductase [30], and are major modifiers of biological activity in the metabolism of xenobiotics [20][22][31][32]. In this context, the relationship between epoxide hydrolase genotype (genetic polymorphism) and susceptibility to various cancer types is now the focus of steadily increasing attention [33 – 37]. Epoxide hydrolases are also of interest as tools in the synthesis of fine chemicals [38 – 40].

According to biochemical separation, location, and substrate specificity, epoxide hydrolases (EH) have been divided into a number of groups. In mammals, the insoluble microsomal epoxide hydrolases and the soluble cytosolic epoxide hydrolases are enzymes of broad and complementary substrate specificity.

The *microsomal epoxide hydrolases* (*microsomal EH*, *mEH*), predominantly found in the endoplasmic reticulum, regio- and stereoselectively catalyze the hydration of both alkene and arene oxides, including oxides of polycyclic aromatic hydrocarbons. These enzymes have been purified to homogeneity from various species and tissues [22][41 – 46]. The human microsomal EH contains 455 amino acids (M_r 52.5 kDa) and is the product of the *EPHX1* gene [47] (also known as *HYL1* [48]).

The human *cytosolic epoxide hydrolase* (*cytosolic EH*, *cEH*, also known as soluble EH) has 554 amino acids (M_r 62.3 kDa) and is the product of the *EPHX2* gene [49]. Its specific substrate is *trans*-stilbene oxide, and it appears

unable to hydrate epoxides of bulky steroids or polycyclic aromatic hydrocarbons [22][50 – 52]. This gene has also been called *HYL2* [48].

In addition to these broadly acting enzymes, there exist epoxide hydrolases with narrow substrate specificities [20][48][53], namely:

- *Cholesterol epoxide hydrolase*, which is expressed in the endoplasmic reticulum and catalyzes the *trans*-addition of H_2O to cholesterol 5,6α-oxide and cholesterol 5,6β-oxide, as well as to a number of other steroid 5,6-oxides. The products are the corresponding vicinal diols [54][55].
- The cytosolic enzyme *leukotriene A_4 hydrolase* (EC 3.3.2.6), which stereoselectively converts leukotriene A_4 (LTA_4) to leukotriene B_4 [56]. This enzyme catalyzes the hydrolytic cleavage of the 5,6-epoxide ring in LTA_4, but, in contrast to what happens with other EHs, the product is not a vicinal diol but a 5,12-diol. As a zinc metalloenzyme, LTA_4 hydrolase does not appear to be related to any other epoxide hydrolase.
- *Hepoxilin hydrolase* (EC 3.3.2.7), which converts hepoxilin A_3 to trioxilin A_3.

Amino acid sequence relationships have suggested a number of HYL families based on percent identity, enzymes with >40% identity belonging to the same family [48]. Families so identified include the mammalian microsomal EH (HYL1), the mammalian cytosolic EH (HYL2), the plant cytosolic EH (HYL3), and bacterial C–X bond hydrolases (haloacid dehydrogenases, HAD, and haloalkane dehalogenases, HLD).

10.3.2. Catalytic Mechanism

From the above, it is clear that the epoxide hydrolases of greatest significance in drug and xenobiotic metabolism are the microsomal and soluble ones. Their catalytic mechanism, which we now examine, is different from that of cholesterol epoxide hydrolase and LTA_4 hydrolase (*e.g.*, [57][58]).

The overall reaction catalyzed by epoxide hydrolases is the addition of a H_2O molecule to an epoxide. Alkene oxides, thus, yield *diols* (*Fig. 10.5*), whereas arene oxides yield *dihydrodiols* (*cf. Fig. 10.8*). In earlier studies, it had been postulated that epoxide hydrolases act by enhancing the nucleophilicity of a H_2O molecule and directing it to attack an epoxide, as pictured in *Fig. 10.5,a* [59][60]. Further evidence such as the lack of incorporation of ^{18}O from $H_2{}^{18}O$ into the substrate, the isolation of an *ester intermediate*, and the effects of group-selective reagents and carefully designed inhibitors led to a more-elaborate model [59][61 – 67]. As pictured in *Fig. 10.5,b*, nucleophilic attack of the substrate is mediated by a carboxylate group in the catalytic site to form an ester intermediate. In a second step, an activated H_2O

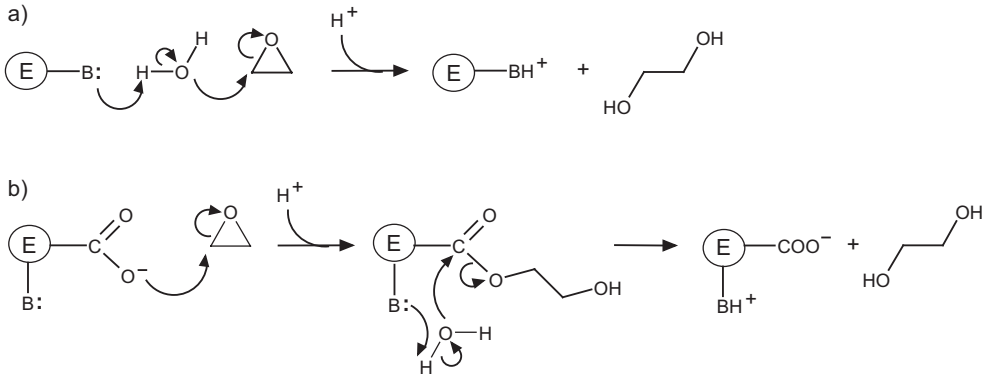


Fig. 10.5. *Catalytic models of epoxide hydrolase* (modified from [59]). *a*) In an earlier model, a basic group in the enzyme activates a H_2O molecule during nucleophilic attack on the epoxide. *b*) A more-elaborate model showing a carboxylate group in the catalytic site that carries out the nucleophilic attack on the substrate to form an ester intermediate. Only in the second step is the intermediate hydrolyzed by an activated H_2O molecule, leading to enzyme reactivation and product liberation.

molecule carries out hydrolysis of the intermediate, leading to enzyme reactivation and product liberation.

A critical input in unraveling the catalytic mechanism of epoxide hydrolases has come from the identification of essential residues by a variety of techniques such as analysis of amino acid sequence relationships with other hydrolases, functional studies of site-directed mutated enzymes, and X-ray protein crystallography (*e.g.*, [48][53][68 – 74]). As schematized in *Fig. 10.6*, the reaction mechanism of microsomal EH and cytosolic EH involves a *catalytic triad* consisting of a nucleophile, a general base, and a charge relay acid, in close analogy to many other hydrolases (see *Chapt. 3*).

The *nucleophile* (Asp^{333} in cytosolic EH; Asp^{226} in microsomal EH) is potentiated by the electrophilic assistance of a number of auxiliary residues (presumably Phe^{265} plus Trp^{334} in cytosolic EH; Trp^{150} plus Trp^{227} in microsomal EH). In addition, activation of the epoxide substrate appears mediated by a general acid (Tyr^{465} in cytosolic EH) (*Fig. 10.6,a*). Attack of the epoxide by the nucleophile cleaves the oxirane ring and creates an oxy anion intermediate (*e.g.*, Asp–CO–O–CH_2CH_2–O^-), a transition state stabilized by an *oxy anion hole*. It is believed that the terminal amino group of Lys^{328} may be involved in stabilization or protonation of the oxy anion in microsomal EH. In addition, Phe^{265} (cytosolic EH) or Trp^{150} (microsomal EH) may also be part of the oxy anion hole.

Following protonation of the oxy anion, the former nucleophile now exists as a β-hydroxyalkyl ester whose hydrolysis marks the second catalytic step of epoxide hydrolases. As shown in *Fig. 10.6,b*, this hydrolysis in-

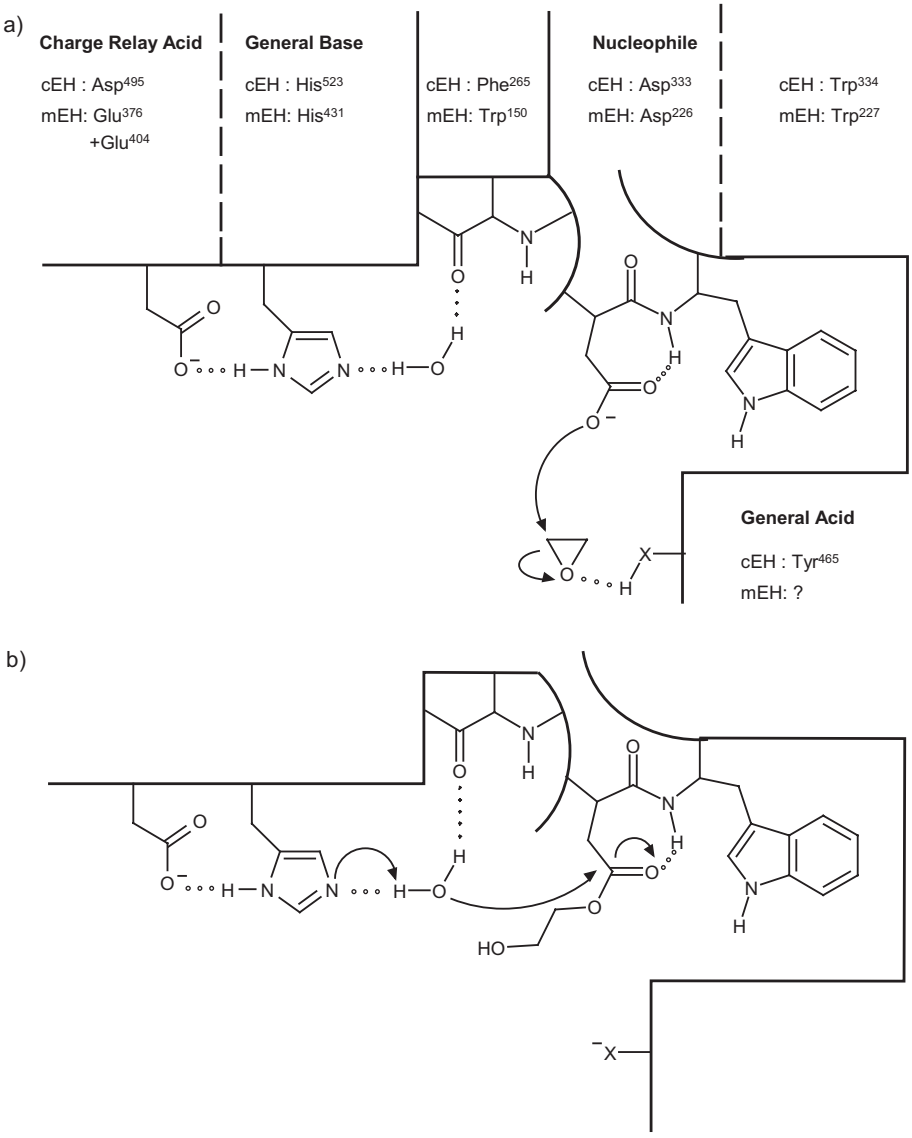


Fig. 10.6. *Simplified representation of the postulated catalytic cycle of microsomal and cytosolic epoxide hydrolases, showing the roles played by the catalytic triad* (*i.e.*, nucleophile, general base, and charge relay acid) *and some other residues. a*) Nucleophilic attack of the substrate to form a β-hydroxyalkyl ester intermediate. *b*) Nucleophilic attack of the β-hydroxyalkyl ester by an activated H_2O molecule. *c*) Tetrahedral transition state in the hydrolysis of the β-hydroxyalkyl ester. *d*) Product liberation, with the enzyme poised for a further catalytic cycle.

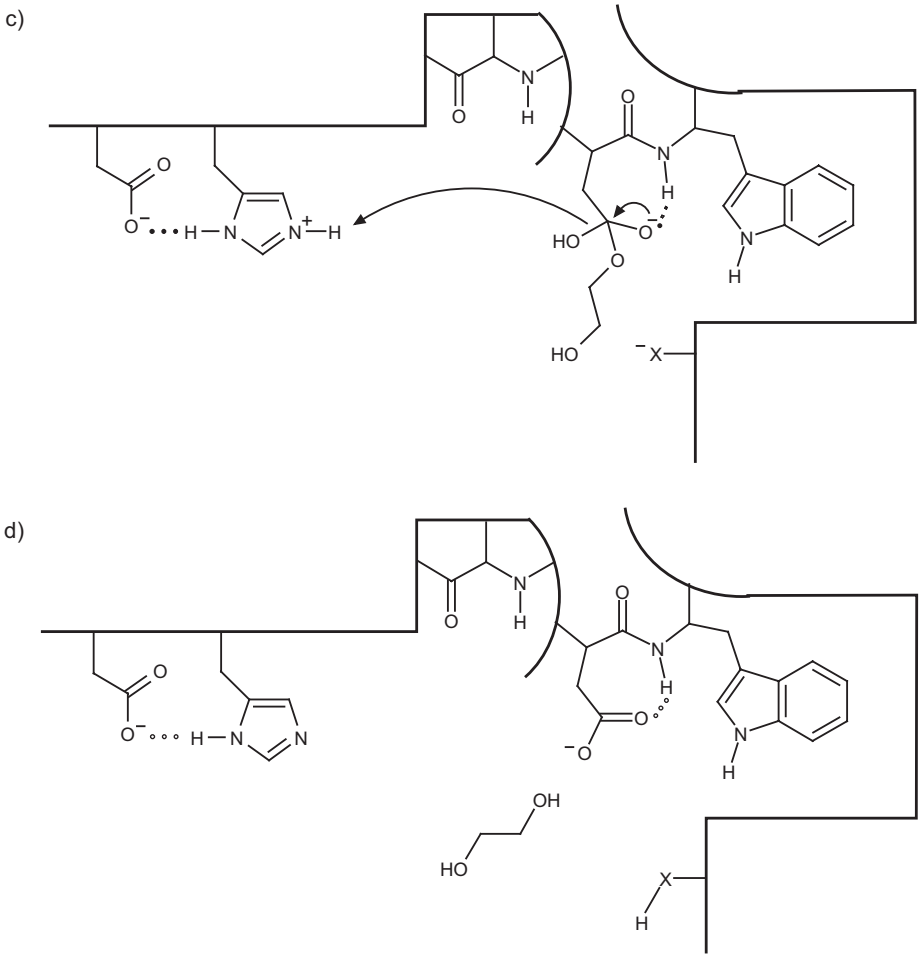


Fig. 10.6 (cont.)

volves the activation of a H_2O molecule by the *general base* (His^{523} in cytosolic EH; His^{431} in microsomal EH), with the assistance of the *charge relay acid* (Asp^{495} in cytosolic EH; Glu^{376} plus Glu^{404} in microsomal EH). In the transition state (*Fig. 10.6,c*), neighboring residues presumably stabilize the tetrahedral intermediate, whereas the protonated general base contributes to the final cleavage.

Product liberation is shown in *Fig. 10.6,d*, with the enzyme regenerated for a further catalytic cycle. Despite the clarity and attractiveness of this catalytic mechanism, a number of unanswered questions remain, in particular some kinetics aspects and the role of allosteric reorganizations to stabilize the transition states.

10.4. Arene Oxides

10.4.1. Introduction

Together with glutathione conjugation, hydration is a major pathway in the inactivation and detoxification of arene oxides. Exceptions to this rule will be treated when discussing polycyclic aromatic hydrocarbons.

Arene oxides are good substrates for microsomal EH, as evidenced in *Table 10.1*, where hydration of selected arene oxides, alkene oxides, and cycloalkene oxides by purified rat liver epoxide hydrolase is compared. The hy-

Table 10.1. *Relative Activity of Rat Liver Microsomal Epoxide Hydrolase toward Some Xenobiotic Substrates*

Substrate	Relative activity	
	Purified enzyme[a]) [%]	Liver microsomes[b]) [%]
Arene oxides		
Benzene oxide (**10.1**)	–[c])	78
Naphthalene 1,2-oxide (**10.2**)	100	100
Phenanthrene 9,10-oxide (**10.29**)	280	430
Benz[*a*]anthracene 5,6-oxide	107	135
Benzo[*a*]pyrene 4,5-oxide	47	80
Benzo[*a*]pyrene 7,8-oxide	34	86
Benzo[*a*]pyrene 9,10-oxide	41	70
Benzo[*a*]pyrene 11,12-oxide	–	8.8
3-Methylcholanthrene 11,12-oxide	4.4	13
Dibenz[*a*,*h*]anthracene 5,6-oxide	1.4	4.4
Alkene and haloalkene oxides		
Propylene oxide	0.42	–[c])
1,2-Epoxybutane	2.8	–[c])
Butadiene monoxide	1.6	–[c])
1,2-Epoxyoctane (**10.4**)	88	145
2-Bromooxirane	140	–[c])
3,3,3-Trichloropropene oxide (**10.75**)	0.95	–[c])
Styrene 7,8-oxide (**10.6**)	73	36 / 140
4-Nitrostyrene 7,8-oxide	255	–[c])
cis-Stilbene oxide (**10.7**)	–[c])	200
Cycloalkene oxides		
1,2-Epoxycyclohexane (**10.5**)	4.5	–[c])
1,2-Epoxycyclododecane	4.3	–[c])
exo-2,3-Epoxynorbornane	12	–[c])
Estroxide[d])	–[c])	140
Androstene oxide[e])	–[c])	780

[a]) Calculated from data compiled by *Guengerich* [19]; Naphthalene 1,2-oxide = 942 nmol (mg protein)$^{-1}$ min^{-1}. [b]) Calculated from data compiled by *Seidegard* and *DePierre* [44]; Naphthalene 1,2-oxide = 9.1 nmol (mg protein)$^{-1}$ min^{-1}. [c]) Not reported. [d]) 16α,17α-Epoxyestra-1,3,5(10)-trien-3-ol. [e]) 16α,17α -Epoxyandrost-4-en-3-one.

Table 10.2. *Epoxide Hydrolase Activity in Human Liver Microsomes* [119]

Substrate	Activity [nmol product (mg protein)$^{-1}$ min^{-1} ± SD (n = 9)]
Naphthalene 1,2-oxide (**10.2**)	9 ± 3
Phenanthrene 9,10-oxide (**10.29**)	60 ± 11
Benz[*a*]anthracene 5,6-oxide	30 ± 5
Benzo[*a*]pyrene 4,5-oxide	28 ± 4
Benzo[*a*]pyrene 7,8-oxide	32 ± 4
Benzo[*a*]pyrene 9,10-oxide	21 ± 4
Benzo[*a*]pyrene 11,12-oxide	0.3 ± 0.04
3-Methylcholanthrene 11,12-oxide	1 ± 0.1
Dibenz[*a*,*h*]anthracene 5,6-oxide	0.3 ± 0.04
1,2-Epoxyoctane (**10.4**)	49 ± 9
Styrene 7,8-oxide (**10.6**)	19 ± 4

dration of some of these substrates was also measured in human liver microsomes (*Table 10.2*), showing comparable relative activities. Clearly, most arene oxides examined are good substrates, *e.g.*, naphthalene 1,2-oxide and phenanthrene 9,10-oxide. The same is true of some alkene oxides to be discussed in *Sect. 10.5*, particularly the most lipophilic ones such as octane 1,2-oxide and the 2-haloethylene oxides. In contrast, modest activities are characteristic for the reported cycloalkene oxides (*Sect. 10.5*).

In this section, we examine first how drugs and other xenobiotics containing a phenyl ring are metabolized to a dihydrodiol metabolite *via* the epoxide. The next subsection discusses the epoxides of bi- and polycyclic aromatic hydrocarbons and their toxicological significance. As this chapter shows, the variety of intertwined metabolic pathways in which arene oxides play an integral role is not always easy to grasp. The general scheme presented in *Fig. 10.7* should help clarify the metabolic context of arene oxides and facilitate the discussion.

10.4.2. The Epoxide–Dihydrodiol Pathway of Benzene and Phenyl-Containing Drugs

A large number of studies have investigated the metabolism of *benzene per se* or in relation to toxification and, particularly, myelotoxicity. Most evidence shows that benzene oxide (**10.1**, *Fig. 10.8*) is not the ultimate toxic species, as was initially believed. Indeed, phenol and quinone metabolites of benzene are more active in forming adducts with macromolecular nucleophiles and eliciting cellular toxicity. For example, the efficacy of benzene metabolites (see *Fig. 10.8*) to inhibit DNA synthesis in a mouse lymphoma cell line decreased in the order benzoquinone (**10.17**) > hydroquinone (**10.16**)

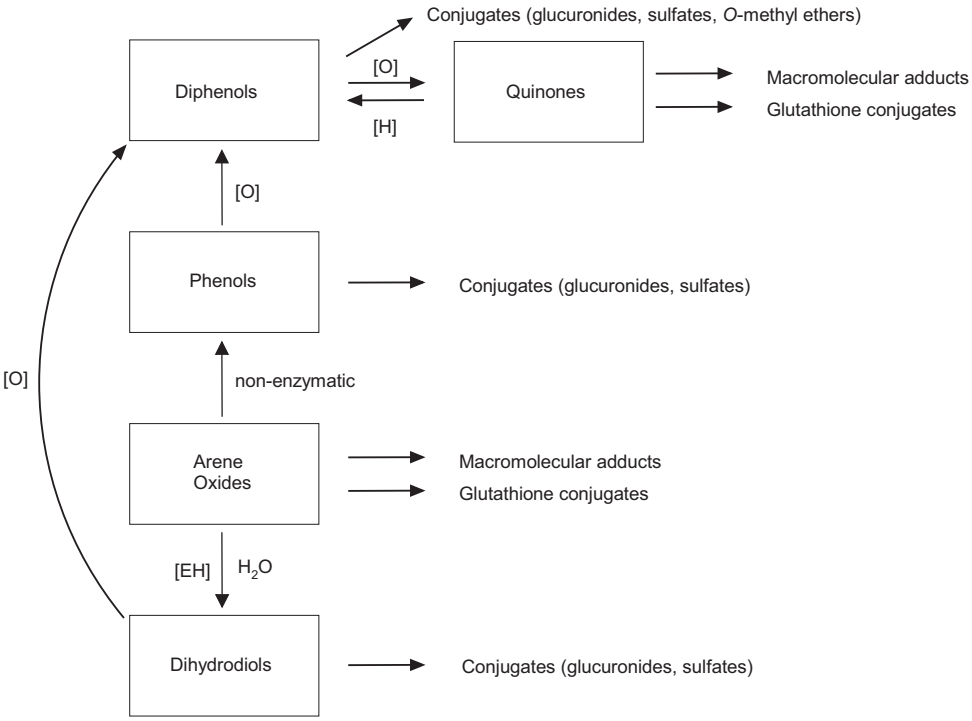


Fig. 10.7. *Metabolic reactions centered on arene oxides* (adapted from [44])

> 1,2,4-trihydroxybenzene > catechol (**10.15**) > phenol (**10.14**), and the ease of electrochemical oxidation of these metabolites was correlated with the IC_{50} value in this test [75]. Several factors explain the apparent lack of toxicity of benzene oxide. First, benzene oxide is not the only intermediate in the formation of phenol (Chapt. 4 in [1a]). More importantly, benzene oxide isomerizes so rapidly to phenol that its intrinsic toxicity cannot be evaluated. And, finally, it is also a good substrate for epoxide hydrolase (*Table 10.1*), producing 1,2-dihydro-1,2-dihydroxybenzene (**10.13**) in the *epoxide–dihydrodiol pathway* of significance in the metabolism of aromatic compounds.

1,2-Dihydro-1,2-dihydroxybenzene (**10.13**) is oxidized by dihydrodiol dehydrogenase (EC 1.3.1.20) to *catechol* (**10.15**) (Chapt. 4 in [1a]) [76]. In a typical experiment in which **10.13** is incubated with phenobarbital-induced rabbit liver microsomes, phenol (**10.14**), catechol (**10.15**), and hydroquinone (**10.16**) represent 54, 39, and 7%, respectively, of the total metabolites detected [75]. In other words, neither benzene oxide (**10.1**) nor its hydration product 1,2-dihydro-1,2-dihydroxybenzene (**10.13**) was detected.

In contrast to benzene, isolation of dihydrodiol metabolites has been achieved for a number of *phenyl-containing drugs*, and, particularly, for neu-

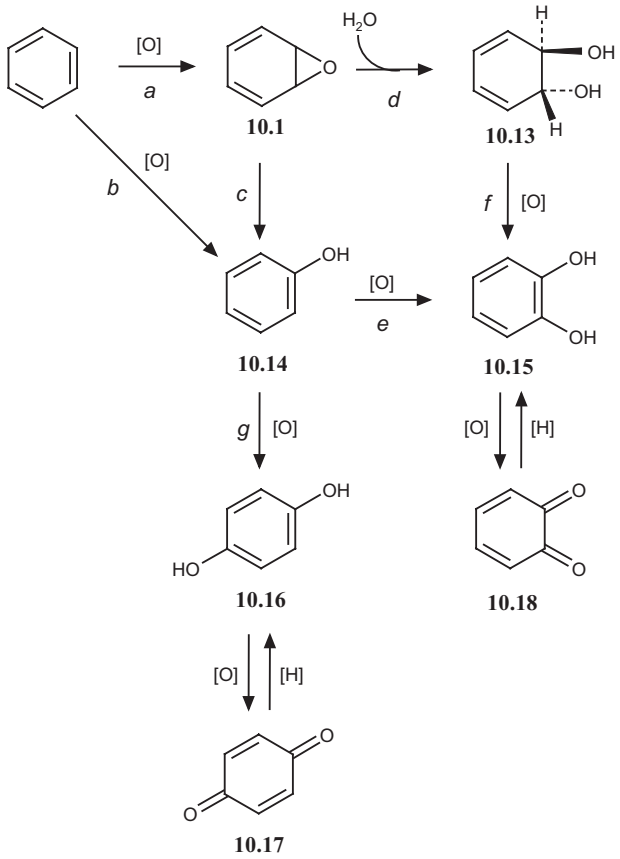
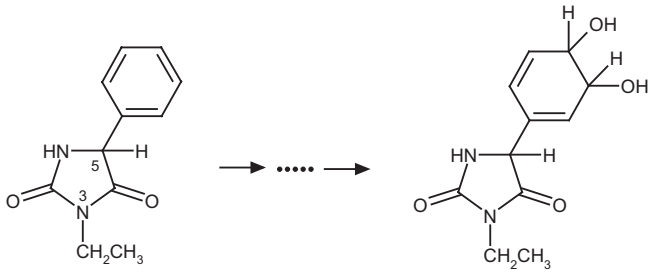
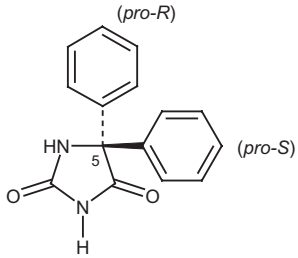


Fig. 10.8. *Simplified and partial metabolic scheme of benzene.* Cytochrome P450 mediated oxidation (*Reaction a*) yields benzene oxide (**10.1**), which produces phenol (**10.14**) by isomerization (*Reaction c*) and 1,2-dihydro-1,2-dihydroxybenzene (**10.13**) by epoxide hydrolase catalyzed hydration (*Reaction d*). Direct formation of phenol also occurs (*Reaction b*). Phenol (**10.14**) and 1,2-dihydro-1,2-dihydroxybenzene (**10.13**) can both be metabolized to catechol (**10.15**), the former by cytochrome P450 (*Reaction e*) and the latter by dihydrodiol dehydrogenase (*Reaction f*). Phenol can also form hydroquinone (**10.16**) (*Reaction g*). The two diphenols (*i.e.*, catechol and hydroquinone) can be oxidized to *ortho*-quinone (**10.18**) and benzoquinone (**10.17**), respectively, either catalyzed by peroxidases or by autoxidation (Chapt. 4, 7, and 12 in [1a]).

rodepressant drugs such as hypnotics (*e.g.*, glutethimide) and anti-epileptics (*e.g.*, ethotoin and phenytoin). Thus, a metabolic study of *ethotoin* (**10.19**) in humans revealed a variety of oxidative routes, namely hydroxylation at C(5), *N*-de-ethylation, and oxidation at various positions of the phenyl ring [77]. Phenyl oxidation yielded the *para*-phenol as the major metabolite, with the *ortho*-phenol, the *meta*-phenol, and the 3′,4′-catechol as minor metabolites. Interestingly, 3′,4′-dihydrodiolethotoin (**10.20**) was also a minor urinary me-



10.19 **10.20**



10.21

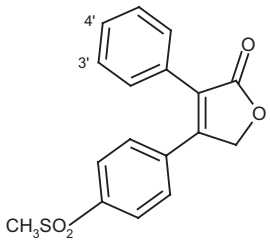
tabolite, thus proving the occurrence of the epoxide–dihydrodiol pathway in the human metabolism of ethotoin.

Valuable metabolic insights have been gained from in-depth studies of *phenytoin* (diphenylhydantoin, **10.21**), whose main biotransformation pathway is by cytochrome P450 catalyzed phenyl oxidation. Incubations with rat liver 9000 *g* supernatant produced the *para*-phenol (4′-hydroxyphenytoin) as the major metabolite, and the dihydrodiol in smaller proportions. Minute amounts of other metabolites were also detected, *e.g.*, the *meta*-phenol and the 3-*O*-methylcatechol. Studies in rats confirmed the urinary excretion, in decreasing order of importance, of the *para*-phenol, the dihydrodiol, and the 3-*O*-methylcatechol metabolites [78][79].

^{18}O-Incorporation studies have clarified the relative importance of the two routes that lead to the catechol metabolite of phenytoin, namely phenol oxidation and dihydrodiol dehydrogenation (*Fig. 10.8*, *Reactions e* and *f*, respectively) [80]. Indeed, upon incubation of phenytoin in an $^{18}O_2$ atmosphere, two atoms of ^{18}O are incorporated into the catechol when formed *via* phenol oxidation, but only one when formed *via* dihydrodiol dehydrogenation. This elegant study showed quite clearly that most of the catechol (75 ± 10%) came from the phenol, indicating a comparatively slow dehydrogenation of the dihydrodiol.

Another aspect in phenytoin oxidation and subsequent hydration is the *product stereoselectivity* of the reactions [81]. Phenytoin is a prochiral compound, the two phenyl groups being enantiotopic. Interestingly, aromatic oxidation in humans and other mammals except the dog occurs almost exclusively at the (*pro-S*) phenyl ring (**10.21**), the *para*-phenol having the (*S*)-configuration at C(5), *i.e.*, (5*S*)-5-(4-hydroxyphenyl)-5-phenylhydantoin. The dihydrodiol similarly has the (*S*)-configuration at C(5). In addition, the C(3′)- and C(4′)-atoms in the oxidized phenyl ring have the *trans*-(*R,R*)-configuration, *i.e.*, (5*S*)-5-[(3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dien-1-yl]-5-phenylhydantoin. Different stereoselectivity was exhibited by the dog, for which the major urinary phenol excreted is the (*R*)-*meta*-phenol, *i.e.*, (5*R*)-5-(3-hydroxyphenyl)-5-phenylhydantoin. Also, the dihydrodiol excreted by the dog was a mixture of two diastereoisomers, with the major epimer being (5*R*)-5-[(3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dien-1-yl]-5-phenylhydantoin and the minor epimer having the (5*S*)-configuration, in other words being identical with the dihydrodiol excreted in other species. These differences indicate the high product enantiospecificity of the cytochromes P450 involved in phenytoin ring oxidation, and also that epoxide hydrolase is active toward both the (5*S*)- and (5*R*)-phenytoin epoxides.

While comparatively few dihydrodiols have been observed in the metabolism of phenyl-containing drugs, the examples above are far from unique. Thus, *oxazepam* incubated in rat, mouse, and human microsomes did yield a dihydrodiol besides the *para*-phenol [82]. A more-recent example is that of *rofecoxib* (**10.22**), a potent and selective cyclooxygenase-2 (COX-2) inhibitor. In rats and dogs, phenyl oxidation produced 4′-hydroxyrofecoxib and rofecoxib-3′,4′-dihydrodiol as urinary metabolites of intermediate quantitative importance [83].



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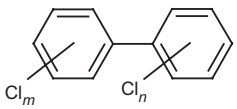


10.23

Besides the drugs discussed above, other phenyl-containing xenobiotics are also substrates of the epoxide–dihydrodiol pathway. Thus, a number of isomeric *dichlorobiphenyls* (**10.23**, $m = 2$, $n = 0$, or $m = n = 1$) were metabolized by rat liver microsomes to stable monophenols and dihydrodiols [84]. Like for benzene, the second step in this pathway can be assumed to be a de-

toxification reaction when it prevents the formation of phenols leading to quinones, and when the dihydrodiols undergo only limited dehydrogenation to catechols and then quinones.

10.4.3. The Epoxide–Dihydrodiol Pathway of Bicyclic and Tricyclic Aromatic Hydrocarbons

This section is dedicated to arene oxides of bicyclic and tricyclic arenes (*e.g.*, naphthalene, anthracene, and phenanthrene oxides), whereas higher arenes and particularly benzo[*a*]pyrene (one of the most toxic and intensively investigated PAHs) are examined in *Sect. 10.4.4*.

Like for benzene, the cytotoxicity of *naphthalene* is not due to the epoxide but to the quinone metabolites, namely 1,2-naphthoquinone and 1,4-naphthoquinone [85]. As shown in *Table 10.1*, *naphthalene 1,2-oxide* (**10.2**) is a better substrate than benzene oxide for epoxide hydrolase. Its rapid isomerization to naphthalen-1-ol, facile enzymatic hydration to the dihydrodiol and lack of reactivity toward nucleophiles such as glutathione may explain its absence of direct toxicity [85].

The enzymatic epoxide–dihydrodiol pathway is characterized by *high stereoselectivity*, as observed with naphthalene and numerous PAHs. *Fig. 10.9* summarizes important results obtained by *van Bladeren et al.* [86]. Depending on the cytochrome P450 used, *naphthalene* and *anthracene* yielded different ratios of the (+)-(1*R,*2*S*)- and (–)-(1*S,*2*R*)-oxides. Of relevance here is the subsequent EH-catalyzed hydration, which, in three cases out of the four in *Fig. 10.9*, proceeded with high stereoselectivity. Only with (–)-(1*S,*2*R*)-naphthalene 1,2-oxide was a mixture of two enantiomeric dihydrodiols formed. A number of factors influenced the results, *e.g.*, the type of cytochrome P450 used, the concentration of EH, the relative K_m and V_{max} values of the epoxides for EH, and their rate of spontaneous isomerization to phenols ($t_{1/2}$ 2.8 and 1.2 min for **10.2** and **10.24**, respectively, at pH 7.4 and 37°). However, the determining factor that controls the absolute configuration of the dihydrodiols was the regioselectivity of nucleophilic hydration, which proceeded with inversion of configuration. Indeed, attack at the *allylic position* (*i.e.*, C(2)) inverted the absolute configuration at this position, whereas attack at the *benzylic position* inverted C(1). The mechanistic implication of these findings is that hydration of the two (+)-(1*R,*2*S*)-epoxides occurred at the allylic position, as did hydration of (–)-(1*S,*2*R*)-anthracene 1,2-oxide. In contrast, hydration of (–)-(1*S,*2*R*)-naphthalene 1,2-oxide occurred at both the benzylic and allylic positions.

It is interesting to note that the oxides of a few hetetocyclic aromatic compounds undergo specific intramolecular rearrangements that lead to un-

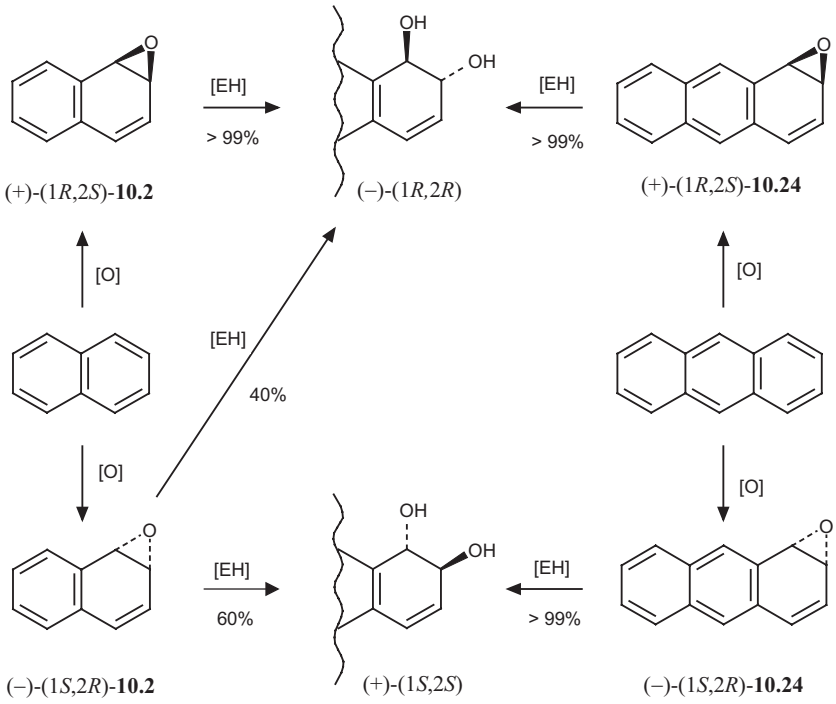
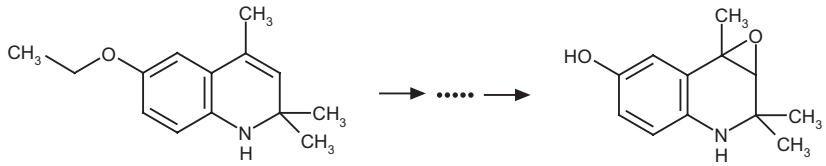


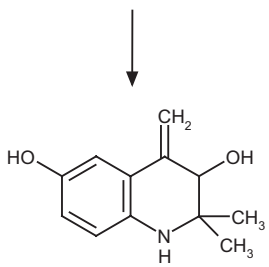
Fig. 10.9. *Stereoselective formation of the 1,2-dihydrodiols of naphthalene and anthracene from their precursor 1,2-oxides,* **10.2** *and* **10.24***, respectively* (modified from [86])

expected metabolites. To take but one example, the industrial anti-oxidant *ethoxyquin* (**10.25**) is oxidized and conjugated in the rat to a number of urinary metabolites, including the sulfate ester of compound **10.27** [87]. Preliminary evidence suggested that this metabolite was produced by rearrangement of the 3,4-oxide (**10.26**). Although the exact mechanism of its formation was not elucidated, metabolite **10.27** can be viewed as being the result of epoxide hydration followed by vicinal H_2O elimination to create the exocyclic C=C bond.

Phenanthrene (**10.28**, *Fig. 10.10*) is the positional isomer of anthracene, yet the differences in reactivity and metabolism between the two compounds are marked. Whereas epoxidation of anthracene in mammals occurs only at the 1,2-position (*Fig. 10.9*), phenanthrene is epoxidized at the 9,10- (major), 1,2- (minor), and 3,4-positions (trace). The reason for preferential oxygenation at the 9,10-position is due at least in part to its higher reactivity. This position within a phenanthrene-like topography, known as the *K region*, is found in a number of PAHs with four or more cycles. Phenanthrene is also representative of higher PAHs since it contains a so-called '*bay region*' (*Fig.*



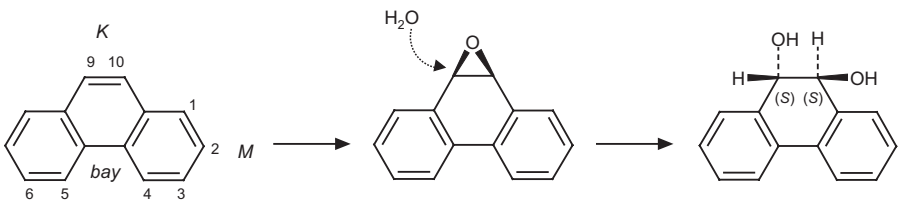
10.25 **10.26**



10.27

10.10). As discussed below for higher PAHs, their epoxidation and hydration at the neighboring *M region* (C(1)–C(2) in phenanthrene) is of major toxicological significance when followed by epoxidation near the bay region (C(3)–C(4) in phenanthrene) [10][88].

Detailed kinetic studies comparing the *chemical reactivity of K-region* vs. *non-K-region arene oxides* have yielded important information. In aqueous solution, the *non-K-region epoxides* of phenanthrene (the 1,2-oxide and 3,4-oxides) yielded exclusively phenols (the 1-phenol and 4-phenol, respectively, as major products) in an acid-catalyzed reaction, as do epoxides of lower arenes (*Fig. 10.1*). In contrast, the *K-region epoxide* (*i.e.*, phenanthrene 9,10-oxide **10.29**) gave at pH < 7 the 9-phenol and the 9,10-dihydro-9,10-diol (predominantly *trans*) in a ratio of *ca.* 3:1. Under these conditions, the formation of this dihydrodiol was found to result from trapping of the carbonium ion by H_2O (*Fig. 10.11*, *Pathway a*). At pH > 9, the product formed was nearly ex-



10.28 **10.29** **10.30**

Fig. 10.10. *Metabolic route to the formation of (–)-(9*S*,10*S*)-9,10-dihydrophenanthrene-9,10-diol* (**10.30**). The arrow indicates the direction of nucleophilic attack by epoxide hydrolase.

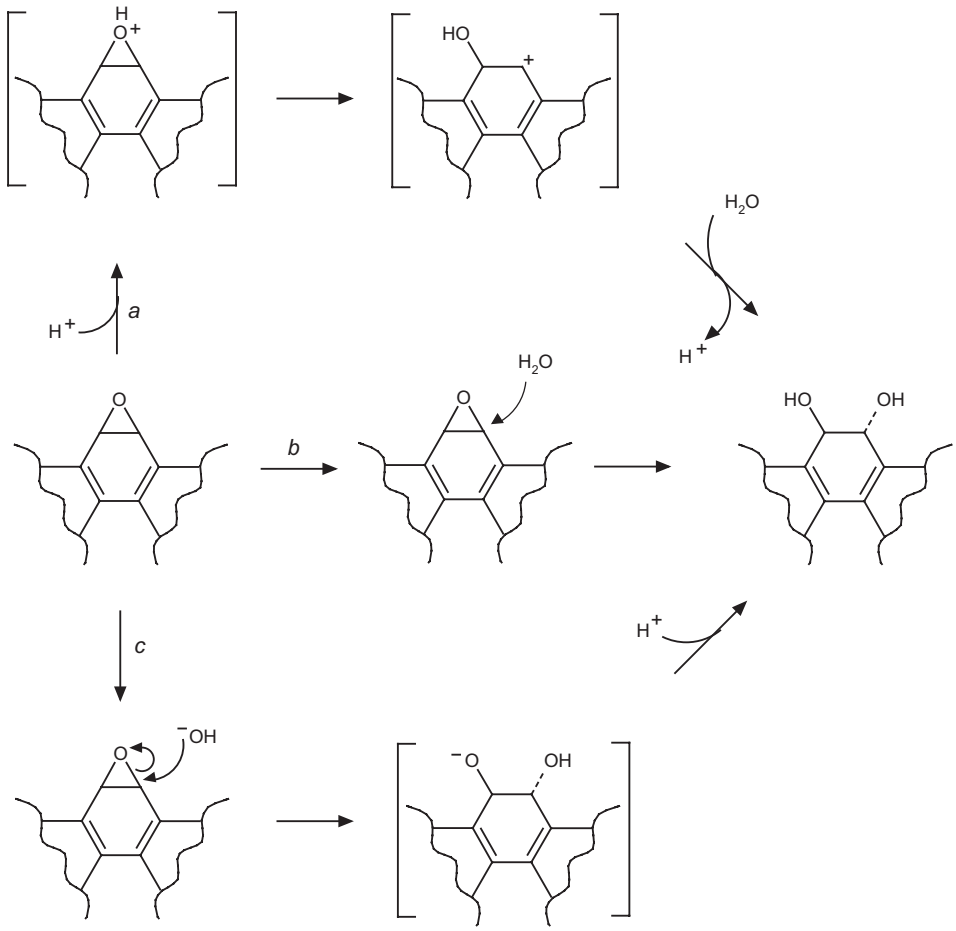


Fig. 10.11. *Chemical mechanisms in the hydrolysis of K-region epoxides*. *Pathway a*: characteristic proton-catalyzed hydrolysis under acidic conditions; *Pathway b*: nucleophilic hydrolysis by H_2O; *Pathway c*: HO^--catalyzed hydrolysis under basic conditions. *Pathways b* and *c* form the *trans*-diol. In the case of *Pathway a*, partial configurational inversion may occur at the carbonium ion, resulting in a mixture of the *trans*- and *cis*-diols.

clusively the dihydrodiol, the mechanism being similar to that found for olefin epoxides (*Fig. 10.4*), namely direct nucleophilic attack of the oxirane ring by H_2O and/or HO^- (*Fig. 10.11*, *Pathways b* and *c*, respectively).

In other words, the non-K-region epoxides of phenanthrene react like epoxides of lower arenes (*Fig. 10.1*). In contrast, the K-region epoxide of phenanthrene, under alkaline conditions, hydrolyzes as does an olefin epoxide (*i.e.*, as in *Fig. 10.4*), but seemingly faster. Under acidic conditions, however, it exhibits dual behavior, isomerizing mainly like an arene oxide (*i.e.*,

as in *Fig. 10.1*), while, at the same time, undergoing characteristic proton-catalyzed hydrolysis [89].

Turning to enzymatic hydration, we see from the data in *Table 10.1* that *phenanthrene 9,10-oxide* (*Fig. 10.10*, **10.29**) is an excellent substrate for epoxide hydrolase. Comparison of enzymatic hydration of the three isomeric phenanthrene oxides shows that the V_{max} with the 9,10-oxide is greater than with the 1,2- or the 3,4-oxide; the affinity was higher as well, as assessed by the tenfold lower K_m value [90]. Furthermore, phenanthrene 9,10-oxide has a plane of symmetry and is, thus, an achiral molecule, but hydration gives rise to a chiral metabolite with high product enantioselectivity. Indeed, nucleophilic attack by epoxide hydrolase occurs at C(9) with inversion of configuration (*i.e.*, from below the oxirane ring as shown in *Fig. 10.10*) to yield the (–)-(9*S*,10*S*)-9,10-dihydro-9,10-diol (**10.30**) [91].

10.4.4. The Epoxide–Dihydrodiol Pathway of Polycyclic Aromatic Hydrocarbons

As discussed below for benzo[*a*]pyrene and a few other PAHs, stereoselectivity plays an important role in the metabolism and toxification of these compounds. Examination of three isomeric tetracyclic aromatic hydrocarbons reveals further insights into this aspect of catalysis by epoxide hydrolase. *Fig. 10.12* shows that *benz[*a*]anthracene* (BaA, **10.31**) contains one M region, one K region, and one bay region, whereas *chrysene* (CR, **10.32**) contains two of each. *Benzo[*c*]phenanthrene* (BcPh, **10.33**) contains two K regions and two M regions, plus another topological feature we encounter here for the first time, namely a *fjord region*, which is, in fact, a deeper bay region. A noteworthy feature of BcPh due to the presence of a fjord region, one that is not apparent in 2D-representations, is the nonplanarity of the compound caused by the steric hindrance/repulsion of the H-atoms C(1) and C(12). This nonplanarity creates helicity and, hence, chirality in the molecule. BcPh shares the property of nonplanarity with other PAHs that contain a fjord region, as well as with methylated analogues such as the 12-methyl- and 7,12-dimethylbenzo[*a*]phenanthrenes.

The EH-catalyzed hydration of the enantiomers of the *K-region epoxides* of BaA, CR, and BcPh allows informative comparisons to be made [92 – 94]. With four among the six substrates, nucleophilic attack is selective for the oxirane C-atom with (*S*)-configuration (*Fig. 10.12*). This is, for example, true for the two enantiomers of chrysene 5,6-oxide. Looking at the data in another way, it is also apparent that, irrespective of the enantiomer, nucleophilic attack occurs preferentially at C(5) for benz[*a*]anthracene 5,6-oxide, but at C(6) for benzo[*c*]phenanthrene 5,6-oxide. In other words, the *regio-* and

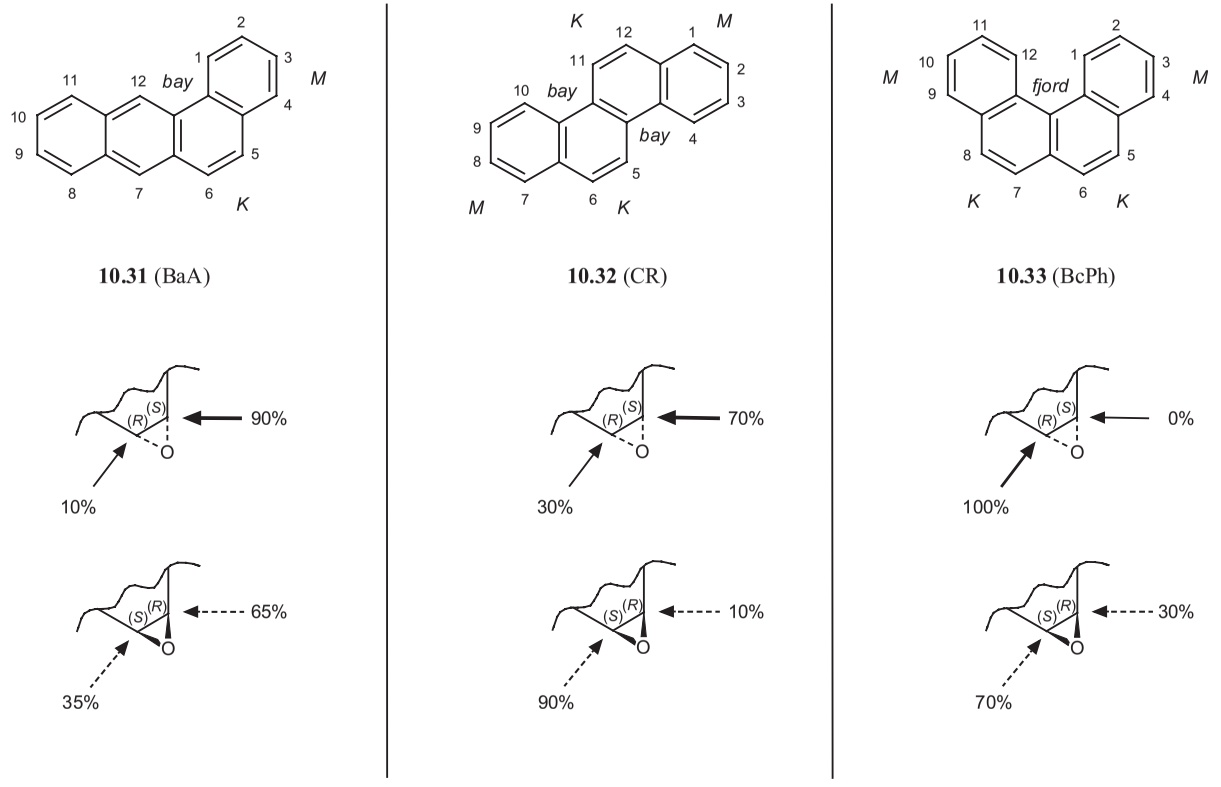


Fig. 10.12. *Upper part: The K, M, bay, and fjord regions of three isomeric tetracyclic aromatic hydrocarbons* (benz[*a*]anthracene (BaA, **10.31**), chrysene (CR, **10.32**), and benzo[*c*]phenanthrene (BcPh, **10.33**)). *Lower part*: *The three pairs of enantiomeric* (S,R)- *and* (R,S)-*K-region epoxides and regioselective attack by epoxide hydrolase* [92][93a].

stereoselectivity of EH-catalyzed hydration appears to depend not only on local structural factors (*i.e.*, absolute configuration of the site of attack), but also on molecular factors such as substrate topology.

Here, K-region epoxides are discussed first since they are often the predominant metabolites in the oxidation of PAHs catalyzed by cytochromes P450 [93a]. Furthermore, from a toxicology point of view, the *epoxide–dihydrodiol pathway of K-region epoxides* is considered a route of detoxification [95][96].

In contrast, the pathway best known to yield adduct-forming metabolites (the ultimate carcinogens) is the formation of *dihydrodiol epoxides*, usually referred to as '*diol epoxides*'. This pathway involves three steps: *a*) formation of an M-region epoxide, *b*) its hydration to the M-region dihydrodiol, and *c*) epoxidation of the latter at the vicinal C=C bond bordering the bay or fjord region.

The formation and reactivity of dihydrodiol epoxides is now illustrated for *benzo[*a*]pyrene* (BaP, **10.34**, *Fig. 10.13*), one of the most extensively investigated PAHs and a highly active carcinogen. However, far from being exclusive to BaP, this toxification pathway is known to occur for a number of PAHs containing a bay or fjord region.

Three major metabolites formed from BaP are its K-region (4*S,*5*R*)-, its M-region (7*R,*8*S*)-, and its (9*S,*10*R*)-epoxides (*Fig. 10.13*). The formation of these epoxides occurs with good to high enantioselectivity. Of relevance here is the hydration to the corresponding (+)-(4*R,*5*R*)- (**10.35**), (–)-(7*R,*8*R*)- (**10.36**), and (–)-(9*R,*10*R*)- (**10.37**) dihydrodiols, which result from attack at C(4), C(8), and C(9), respectively (*Fig. 10.13*) [93a][97 – 102].

The formation of BaP 9,10-epoxide is considered a reaction of detoxification of BaP, as is the formation of its K-region epoxide. However, this last statement must now be qualified by the recent finding that the K-region dihydrodiol of benzo[*a*]pyrene, *i.e.*, *trans*-4,5-dihydro-4,5-dihydroxy-BaP (**10.35**, *Fig. 10.13*), can ultimately form DNA adducts. This is due to its further metabolism to *bis[dihydrodiols]* such as 1,2,4,5-, 4,5,7,8-, and 4,5,9,10-bis[dihydrodiol]. The *trans-trans*-BaP-4,5,7,8-bis[dihydrodiol] has been shown to be a metabolic intermediate in the formation of DNA-adducts [103].

Hydration of the M-region epoxide to the *M-region dihydrodiol* of BaP (**10.36**, *Fig. 10.13*) and of other carcinogenic PAHs is a major step in their toxification. As explained above, M-region dihydrodiols can undergo a specific and final metabolic reaction of epoxidation to the *ultimate carcinogen*, namely the corresponding bay-region diol epoxide. As exemplified for BaP, the formation of the bay-region diol epoxide (**10.38**, *Fig. 10.13*) occurs stereoselectively, the (–)-*anti*-(7*R,*8*S,*9*S,*10*R*)-diol epoxide being formed with large predominance over the (+)-*syn*-(7*R,*8*S,*9*R,*10*S*)-diol epoxide.

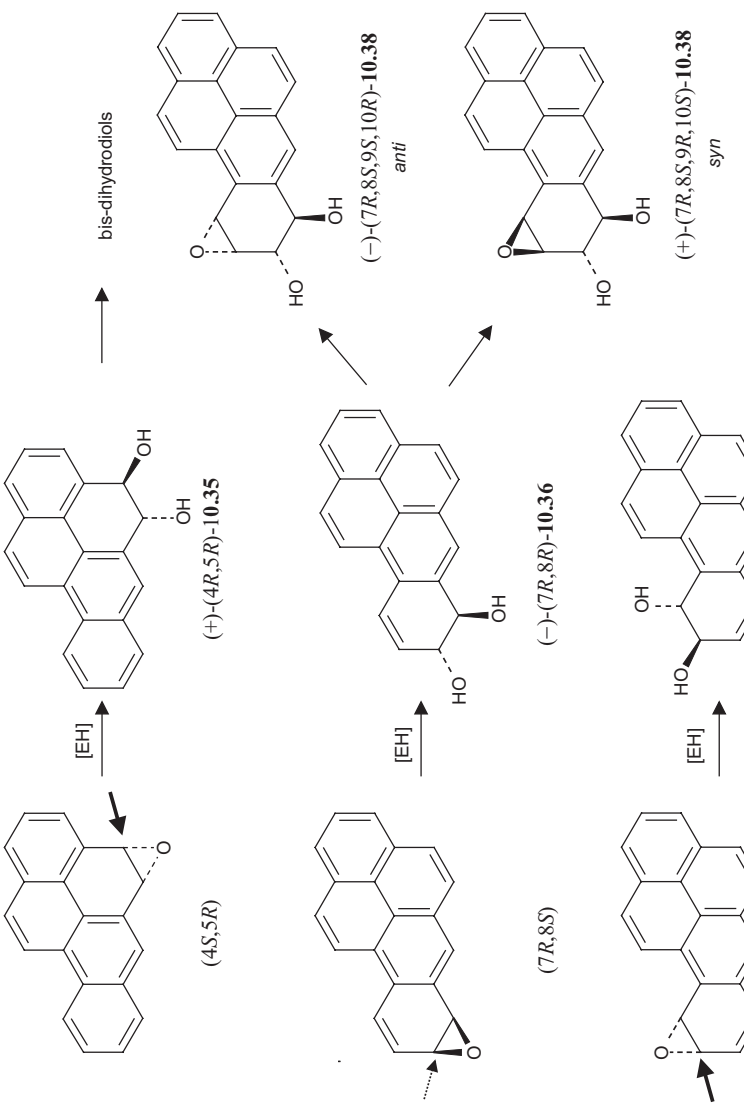


Fig. 10.13. *Metabolism of benzo[a]pyrene* (**10.34**). Shown are stereoselective formation of three isomeric epoxides, EH-catalyzed, stereoselective hydration to the dihydrodiols **10.35**, **10.36**, and **10.37**, and, finally, 9,10-epoxidation of **10.36** to the bay-region diol epoxide **10.38**. The latter exists as the *syn*- and *anti*-stereoisomer (adapted from [93a][97]).

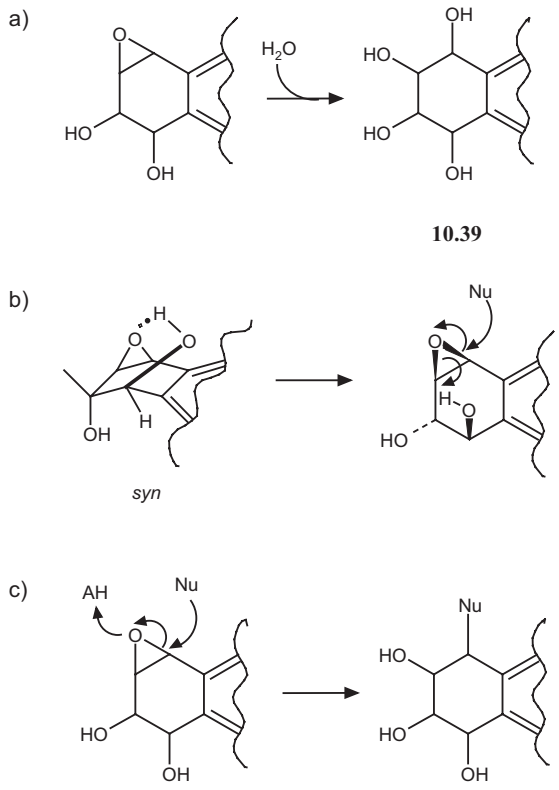


Fig. 10.14. *Reactivity of diol epoxides* (Nu = H_2O, HO^-, or another nucleophile). *a*) Hydrolytic reaction of diol epoxides to tetrols. *b*) Internal H-bonding in diol epoxides with *syn*-configuration and rendering the distal C-atom more electrophilic (modified from [104]). *c*) General representation of proton-catalyzed (A–H = H^+), general acid catalyzed (A–H = acid), or intramolecularly catalyzed (A–H = *syn*-OH group) activation of the distal C-atom toward nucleophilic attack.

The *high reactivity of bay-region (and fjord-region) diol epoxides* has intrigued chemists for years. Numerous experimental and computational studies have been carried out, affording a wealth of information on the mechanisms by which bay-region diol epoxides form adducts with nucleic acids and are deactivated by reaction with protective nucleophiles or by hydrolysis. Indeed, the hydration of diol epoxides forms unreactive tetrahydroxy metabolites known as *tetrols* (**10.39**, *Fig. 10.14,a*).

Hulbert published a landmark paper [104], in which he reasoned that diol epoxides should react with nucleophiles as *carbonium ions*. Although bay-region diol epoxides were not specifically considered in this hypothesis paper, the essence of the argument was that diol epoxides with *syn*-configuration (see *Fig. 10.13*) should be more reactive than their *anti*-stereoisomers since

only the former can form internal H-bonds between the epoxide O-atom and the distal OH group (*Fig. 10.14,b*). Experimental studies have verified *Hulbert*'s hypothesis at neutral pH, since *spontaneous* (in fact**,** *intramolecularly catalyzed*) hydration of bay-region epoxides was, indeed, markedly faster for the *syn*-isomers due to internal H-bonding [105]. The toxicological relevance of this intramolecular catalysis is comparatively modest because the bay-region diol epoxide of BaP formed metabolically is mostly the *anti*-isomer (see above).

Other mechanisms must be evoked to account for the high reactivity of the bay-region diol epoxides with *anti*-configuration that are formed metabolically. At acidic pH, these diol epoxides are more reactive toward *proton-catalyzed hydration* than their *syn*-isomers. Proton-catalyzed hydration (*i.e.*, specific acid catalysis) involves protonation of the epoxide O-atom followed by nucleophilic attack of the distal C-atom by H_2O. In other words, bay-region diol epoxides are comparable to K-region epoxides in terms of proton-catalyzed hydration (*Fig. 10.11*, *Pathway a*). The configuration of the resulting tetrol depends on the starting diol epoxide and on the experimental conditions [106 – 108].

Further studies that demonstrate that hydration of bay-region diol epoxides under acidic conditions can occur by *general acid catalysis* in addition to proton catalysis have expanded our understanding of their reactivity. General acid catalyzed hydration involves H-bonding of the epoxide O-atom by the acid catalyst, followed by nucleophilic attack of the distal C-atom by H_2O/HO^- [108][109].

Thus, at least three mechanisms facilitate the hydration (and, hence, detoxification) of diol epoxides, namely proton catalysis, general acid catalysis, and internal H-bonding (*Fig. 10.14,c*). Significantly, the same catalytic mechanisms that facilitate hydration by activating the distal epoxide C-atom can also activate it toward endogenous nucleophiles (*Fig. 10.14,c*). This would be a reaction of detoxification in the case of protective nucleophiles such as glutathione or noncritical cellular agents, but a cytotoxic one in the case of adduct formation. Indeed, numerous studies have shown that a high proportion of bay-region and fjord-region diol epoxides form *adducts* with critical macromolecules such as nucleic acids or constituents thereof [110 – 114].

In conclusion, it is clear that a variety of stereoelectronic (internal) factors and external conditions favor a substantial positive charge in the transition state of diol epoxides as they undergo hydration or react with nucleophiles [115 – 118]. Interpreting the reactivity of diol epoxides (or of numerous other electrophilic metabolites) in terms of toxification *vs.* detoxification is particularly difficult since toxicity depends as much on the nature of the endogenous nucleophile as on the intrinsic reactivity of the metabolites.

10.5. Alkene Oxides

10.5.1. Introduction

As explained in the *Introduction*, alkene oxides (**10.3**) are generally chemically quite stable, indicating *reduced reactivity* compared to arene oxides. Under physiologically relevant conditions, they have little capacity to undergo rearrangement reactions, one exception being the acid-catalyzed 1,2-shift of a proton observed in some olefin epoxides (see *Sect. 10.2.1* and *Fig. 10.3*). Alkene oxides are also resistant to uncatalyzed hydration, thus, in the absence of hydrolases enzymes, many alkene oxides that are formed as metabolites are stable enough to be isolated.

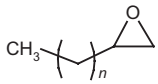
The data in *Table 10.1* suggest that the reactivity of epoxide hydrolase toward alkene oxides is highly variable and appears to depend, among other things, on the size of the substrate (compare epoxybutane to epoxyoctane), steric features (compare epoxyoctane to cycloalkene oxides), and electronic factors (see the chlorinated epoxides). In fact, comprehensive structure–metabolism relationships have not been reported for substrates of EH, in contrast to some narrow relationships that are valid for closely related series of substrates. A group of arene oxides, along with two alkene oxides to be discussed below (epoxyoctane and styrene oxide), are compared as substrates of human liver EH in *Table 10.2* [119]. Clearly, the two alkene oxides are among the better substrates for the human enzyme, as they are for the rat enzyme (*Table 10.1*).

In the present section are discussed, in turn, the most important subclasses of alkene oxides that are known to be substrates of EH. The sequence begins with epoxides of unconjugated alkenes and ends with epoxides of complex, conjugated cycloalkenes of biochemical and pharmacological interest.

10.5.2. Epoxides of Unconjugated Alkenes

Maynert et al. [120] provided evidence that epoxides are obligatory intermediates in the metabolism of olefins to glycols. When incubated with rat liver microsomes, selected substrates (oct-1-ene, oct-4-ene, and 3-ethylpent-2-ene) were converted to the corresponding glycols with no trace of the epoxide being detected. However, in the presence of another epoxide acting as competitive substrate, detection of the epoxide metabolite was possible due to inhibition of the hydrating enzyme. To the best of our knowledge, this study afforded the first proof that alkene oxides can be hydrated enzymatically to the corresponding glycols.

Many features of the hydration of alkene oxides have since been elucidated, in particular the various *substrate selectivities* of the reaction. In what was perhaps one of the first *structure–hydration relationship* studies, homologous *monoalkyloxiranes* (**10.40**) were compared as substrates for hydration by rabbit liver microsomes [121]. Reactivity was highest (30 – 35 nmol (mg protein)$^{-1}$ min^{-1}) for the homologues with n = 4, 11, and 13, intermediate (*ca.* 20 nmol (mg protein)$^{-1}$ min^{-1}) for n = 5 and 9, and lowest (*ca.* 10 nmol (mg protein)$^{-1}$ min^{-1}) for n = 7. In other words, the rate of microsomal EH catalyzed hydration was fastest for the lowest and highest homologues, and slowest for the medium-length alkyloxiranes. Interestingly, the rate of enzymatic hydration was inversely proportional to the alkyloxirane's capacity to inhibit the enzyme.



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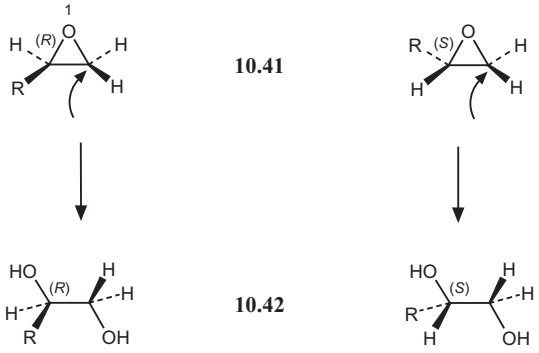


10.41

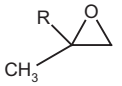
10.42

Monoalkyloxiranes are chiral compounds, C(2) being a stereogenic center (**10.41**). When incubated as racemates with rabbit liver microsomal or cytosolic EH, the compounds showed a *substrate enantioselectivity* that was markedly influenced by the branching of the alkyl group [122]. For R = Bu (1,2-epoxyhexane = 2-butyloxirane) and R = octyl (1,2-epoxydecane = 2-octyloxirane), there was only a modest excess of the (*R*)-diol produced (**10.42**). In contrast, for R = $(CH_3)_3CCH_2$ (4,4-dimethyl-1,2-epoxypentane = 2-neopentyloxirane) and R = *t*-Bu (3,3-dimethyl-1,2-epoxybutane = 2-(*tert*-butyl)oxirane), the (*R*)-diol was obtained in, respectively, marked and nearly complete excess. These results were extended by incubations of separate enantiomers with rat liver microsomal EH [123]. Thus, 2-methyloxirane (1,2-epoxypropane, **10.41**, R = Me) was hydrolyzed with slight preference for the

(*S*)-enantiomer to form the (*S*)-diol, in contrast to the behavior of higher alkyloxiranes (see above). However, 2-(*tert*-butyl)oxirane (**10.41**, R = *t*-Bu) confirmed a faster hydrolysis of the (*R*)-enantiomer, but this was shown to be due to complex inhibitory effects.

Substrate regioselectivity was also involved since nucleophilic attack occurred mostly (>95%) at the unsubstituted C(3)-atom of oxiranes. Complete or almost complete C(3)-regioselectivity in the hydration of 2-alkyloxiranes (alkene 1,2-oxides) has often been reported. This rule is significant since nucleophilic attack at C(3) results in retention of configuration at the stereogenic center C(2), whereas attack at C(2) involves inversion of this center (see also *Sect. 10.4.3* and *Fig. 10.10*). With both enantiomers of 2-methyloxirane (**10.41**, R = Me) and analogues as substrates for rat liver microsomal EH, practically complete retention of configuration was observed in the diols produced [123].

The patterns of regio- and stereoselectivities become more complex in *disubstituted oxiranes*. Beginning with *2,2-disubstituted oxiranes*, attack is always at the accessible C-atom. In terms of substrate enantioselectivity, it was found that 2-butyl-2-methyloxirane (2-methyl-1,2-epoxyhexane, **10.43**, R = Bu) was hydrolyzed with a preference for the (*S*)-enantiomer. This substrate enantioselectivity was lost for branched analogues, namely 2-(*tert*-butyl)-2-methyloxirane (**10.43**, R = *t*-Bu) and 2-(2,2-dimethylpropyl)-2-methyloxirane (**10.43**, R = $(CH_3)_3CCH_2$) [124]. Thus, it appears that the introduction of a geminal Me group suppresses the enantioselectivity seen with branched monoalkyloxiranes, and reverses it for straight-chain alkyloxiranes.



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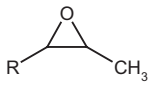


10.44

We also note that some 2,2-disubstituted oxiranes have toxicological significance, as exemplified by 2,2-dimethyloxirane (2-methyl-1,2-epoxypropane, **10.43**, R = Me). This compound is the toxic metabolite of *2-methylprop-1-ene* (isobutene), a gaseous alkene widely used as a monomer in the industrial production of adhesives, plastics, and other polymers. Interestingly, detoxification of this epoxide catalyzed by liver epoxide hydrolase was high in the human, intermediate in the rat, and low in the mouse [125]. These activities were inversely correlated with the epoxide levels measured *in vitro* in liver tissue of these species.

2,3-Disubstituted oxiranes of general structure **10.44**, namely *2-alkyl-3-methyloxiranes*, are another case in point. Again, nucleophilic attack is exclusively or predominantly at the more accessible C(3), with substrate

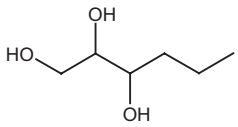
stereoselectivity being a complicating factor. With a variety of model compounds, it has been shown that the (3*S*)-configuration is a critical feature of good substrates for microsomal EH [126]. As shown in *Fig. 10.15,a*, attack at C(3) is from below with inversion of configuration. Thus, *cis*-substrates yield *threo*-diol metabolites. The general model in *Fig. 10.15,b* summarizes substrate stereoselectivity in the microsomal EH catalyzed hydration of monoalkyloxiranes (**10.41**), *cis*- and *trans*-2-alkyl-3-methyloxiranes (**10.44**), and 2,2,3-trimethyloxiranes (**10.45**).

10.5.3. Epoxides of Functionalized Alkenes

In addition to the unfunctionalized alkene epoxides discussed in the previous subsection, various other types of epoxides exist that are also derived from unconjugated alkenes but that share two additional features, *i.e.*, being characterized by the presence of one or more functional group(s) and having biological significance. Thus, the present subsection examines epoxy alcohols, epoxy fatty acids, allylbenzenes 2′,3′-oxides, as well as alkene oxide metabolites of a few selected drugs.

A simple *epoxy alkanol* to begin with is *1,2-epoxyhexan-3-ol* (**10.47**), which has been postulated as a metabolite of the air pollutant hex-1-ene (**10.46**). This compound was found to be a good substrate for rat liver microsomal EH, yielding hexane-1,2,3-triol (**10.48**) [127]. 1,2-Epoxyhexan-3-ol contains two stereogenic centers and exists as four stereoisomers that were hydrated at different rates, in the order (2*S,*3*R*)-*erythro* > (2*S,*3*S*)-*threo* > (2*R,*3*S*)-*erythro* > (2*R,*3*R*)-*threo*. In other words, the metabolic hydrolysis of this substrate is not influenced by the configuration at C(3), but clearly by that at C(2), with the (2*S*)-epimers being better substrates than the (2*R*)-epi-

10.46 **10.47**



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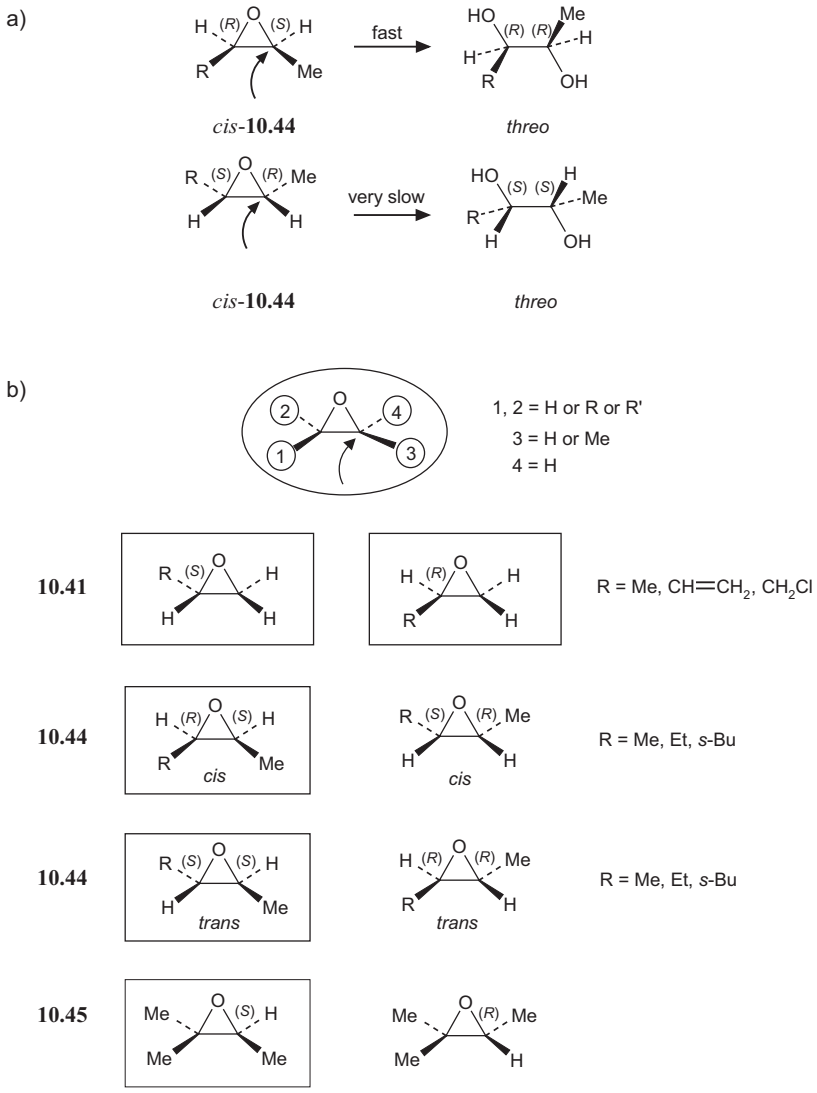


Fig. 10.15. *Summary of regio- and enantioselectivities in the microsomal epoxide hydrolase catalyzed hydration of di- and trisubstituted oxiranes* (modified from [126]). *a*) The case of chiral 2-alkyl-3-methyloxiranes showing substrate enantioselectivity for the (3*S*)-configured oxirane and regioselective attack at C(3) resulting in inversion of configuration at this center. *b*) General model (in oval), showing structural features of good substrates, namely one H in position 4 and one H or one Me in position 3 (resulting in an (*S*)-configuration). Attack occurs from below C(3) with inversion of configuration. Underneath the general model, good substrates are shown in boxes, namely (2*R*)- and (2*S*)-monoalkyloxiranes, *cis*-(2*R*,3*S*)-2-alkyl-3-methyloxiranes, *trans*-(2*S*,3*S*)-2-alkyl-3-methyloxiranes, and (3*S*)-2,2,3-trimethyloxiranes.

Compounds not in boxes are poor substrates, namely the (3*R*)-configured oxiranes.

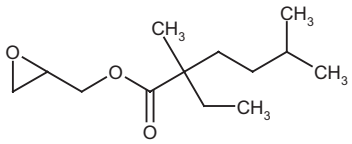
mers. Such substrate stereoselectivity is comparable to that noted above for 2-methyloxirane ((*S*) > (*R*)), but opposite to that exhibited by straight-chain alkyloxiranes ((*R*) > (*S*)) and branched alkyloxiranes ((*R*) ⩾ (*S*)). Thus, the presence of the OH group, but not the configuration of the adjacent C-atom, does appear to influence the substrate selectivity of 1,2-epoxyhexan-3-ol (**10.47**).

In epoxy alkanols that carry two functional groups separated by a further C-atom, the OH group did not interfere significantly with EH-catalyzed hydrolysis. This was seen with 1,2-epoxyheptan-4-ol and 3,4-epoxyhexan-1-ol [128]. The OH group, however, did enhance the bioalkylating potential of even aliphatic epoxides.

An unusual case of intramolecular competition (*chemoselectivity*, see Chapt. 1 in [1a]) between ester and oxirane occurs in the detoxification of *(oxiran-2-yl)methyl 2-ethyl-2,5-dimethylhexanoate* (**10.49**), one of the most abundant isomers of an epoxy resin. The compound is chemically very stable, *i.e.*, resistant to aqueous hydrolysis, but is rapidly hydrolyzed in cytosolic and microsomal preparations by epoxide hydrolase and carboxylesterase, which attack the epoxide and ester groups, respectively [129]. The rate of overall enzymatic hydrolysis was species dependent, decreasing in the order mouse > rat > human, but was relatively fast in all tissues examined (lung and skin as portals of entry, and liver as a further barrier). In mouse and rat lung microsomes, ester hydrolysis was 3 – 4 times faster than epoxide hydration, whereas the opposite was true in human lung microsomes.

The hydrolysis of *epoxy fatty acids* is a topic of rapidly growing interest, their biological and toxicological implications being far-reaching [130]. As a rule, epoxy fatty acids are good substrates for soluble and/or microsomal EH. Thus, *cis*-9,10-epoxystearic acid (**10.50**), the epoxy derivative of oleic acid, is a highly selective substrate of cytosolic EH from mouse, rat, and human [131]. Polyunsaturated fatty acids in general and arachidonic acid in particular are especially of interest. With its four C=C bonds, *arachidonic acid* (**10.51**) can be oxidized by cytochrome P450 to four regioisomeric monoepoxides, namely the epoxyeicosatrienoic acids (EETs). The four epoxides, although chemically stable, were shown to be hydrolyzed to the corresponding vicinal diols by mouse liver cytosolic EH but not by microsomal EH [132]. Furthermore, marked substrate selectivity was noted, such that the rate of reaction decreased the series in the order 14,15-epoxy > 11,12-epoxy > 8,9-epoxy ⩾ 5,6-epoxy. In other words, the more removed the oxirane ring was from the carboxy group, the faster its enzymatic hydration. It was suggested that the low reactivity of 5,6-EET is related to its possible physiological functions.

Further studies have revealed some aspects of the stereoselectivity of these reactions [133]. (14*R*,15*S*)-, (11*S*,12*R*)-, and (8*S*,9*R*)-EET, the predom-



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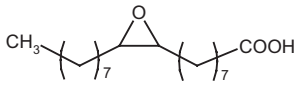

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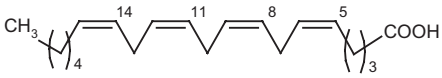

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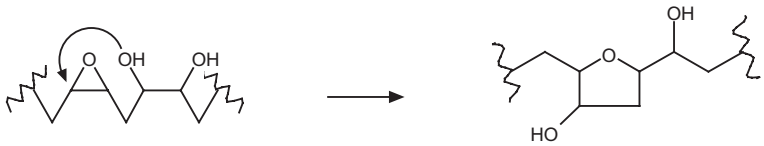


10.52 **10.53**

inant enantiomers present endogenously in rat tissues, were hydrated at markedly higher rates than their antipodes. The regio- and/or stereoselectivity of hydration depended on the positional isomer. With both enantiomers of 11,12-EET, addition of H_2O showed no preference for C(11) or C(12). In contrast, hydration was selective for C(9) in both enantiomers of 8,9-EET, but it was selective for C(15) only in (14*R*,15*S*)-EET. These results suggested that the active site of cytosolic EH is highly structured and capable of recognizing substrate features in a regio- and stereoselective manner.

In contrast to the relative chemical stability of mono-epoxides, *diol epoxides of fatty acids* (**10.52**), which are formed from di-epoxides by EH, are subject to a different fate. In such metabolites, intramolecular nucleophilic substitution may occur, such that oxirane opening is accompanied by formation of a tetrahydrofuran ring [134]. Such reactions of *intramolecular nucleophilic substitution* are discussed in detail in *Sect. 11.9*. In the case of diol epoxides of fatty acids, the resulting *tetrahydrofuran-diols* (**10.53**) are part of a much larger ensemble of oxygenated metabolites of fatty acids, the potential cytotoxicities of which are being evaluated [135].

There is an informative parallel to be drawn between the metabolism of fatty acids and that of *valproic acid* (VA), a well-known anti-epileptic drug. One of the metabolites of VA is *Δ^4-valproic acid* (**10.54**, *Fig. 10.16*), which undergoes a number of metabolic transformations, including oxygenation to epoxide **10.55**. Rather than being a substrate for EH, this epoxide reacts by intramolecular nucleophilic attack of the carboxylate anion at the internal C-

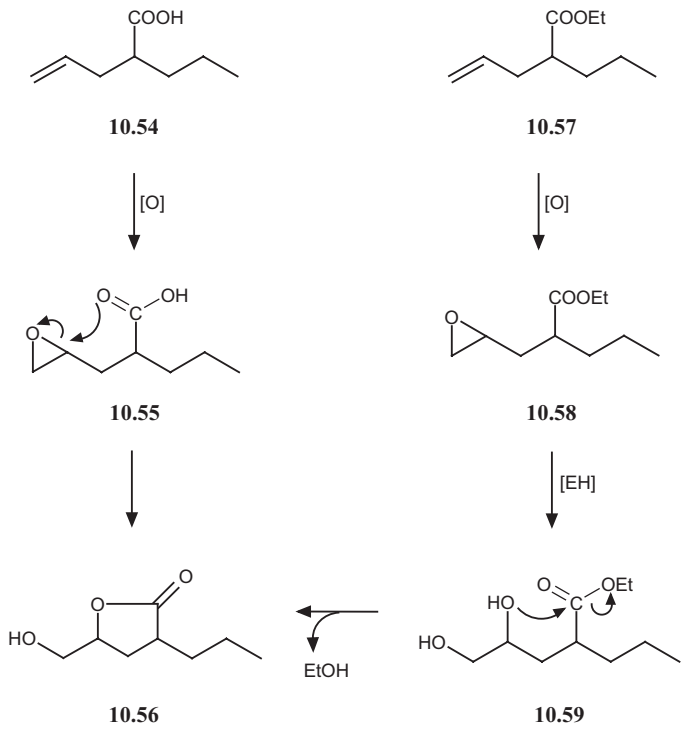
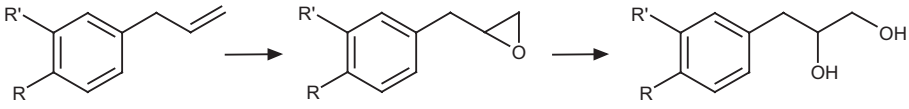


Fig. 10.16. *Comparison of the metabolism of Δ^4-valproic acid* (**10.54**)*, a metabolite of valproic acid, with that of ethyl Δ^4-valproate* (**10.57**)*, a synthetic analogue.* Both compounds undergo cytochrome P450 catalyzed oxygenation to form the corresponding epoxides (**10.55** and **10.58**, respectively). The former reacts intramolecularly to form the lactone **10.56** and is not detectably a substrate for epoxide hydrolase. Epoxide **10.58**, in contrast, is a substrate for epoxide hydrolase, forming the diol **10.59**, which, in turn, carries out an intramolecular nucleophilic attack to form lactone **10.56** [136].

atom of the oxirane ring, forming the lactone **10.56** as shown [136]. Ethyl Δ^4-valproate (**10.57**, *Fig. 10.16*), a synthetic analogue of Δ^4-VA, also formed the lactone **10.56** but by a longer route. Indeed, ethyl Δ^4-valproate (**10.57**) underwent cytochrome P450 catalyzed oxygenation to the corresponding epoxide (**10.58**, *Fig. 10.16*), but this metabolite proved to be a substrate for EH, forming the diol **10.59**. It was this diol, and not the parent epoxide, that reacted intramolecularly by nucleophilic attack of the proximal OH group to form the lactone **10.56**. Thus, fast intramolecular trapping, and perhaps much lower lipophilicity due to the ionized carboxylate group, prevented the epoxide **10.55** to being hydrated by EH, in contrast to the ethyl ester **10.58**.

Another EH substrate of interest is the 2′,3′-oxide of *allylbenzene* (**10.60**, R = R′ = H) as well as analogues such as *estragole* (**10.60**, R = MeO, R′ = H), *eugenol* (**10.60**, R = OH, R′ = MeO), and *safrole* (**10.60**, R–R′ = –OCH_2O–).

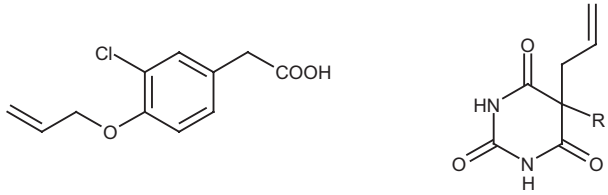
These three analogues are natural products found in some foods and are suspected of being cytotoxic, if not carcinogenic, under certain circumstances. The mechanism underlying these toxic effects is believed to result from benzylic (C(1′)) hydroxylation, followed by conjugation of the resulting OH group to an electrophilic, adduct-forming *O*-sulfate [137][138] (see also Chapt. 4 in [1a]). In addition to this route of toxification, allylbenzenes are also substrates of the epoxide–diol pathway. Their 2′,3′-epoxide metabolites (**10.61**) are seldom detected in greater than trace amounts due to rapid inactivation *via* glutathione conjugation as well as hydration to the diol (**10.62**) [139]. Indeed, allylbenzene 2′,3′-oxide and its analogues (**10.61**) are good substrates for both microsomal and cytosolic EH [137]. In fact, experiments in the isolated perfused rat liver have shown that EH-catalyzed hydration of allylbenzene 2′,3′-oxide and estragole 2′,3′-oxide is markedly faster than conjugation with glutathione [140]. The epoxide–diol pathway is, thus, a detoxification route for allylbenzenes since it yields inactive metabolites and competes efficiently with the hydroxylation–sulfation route.



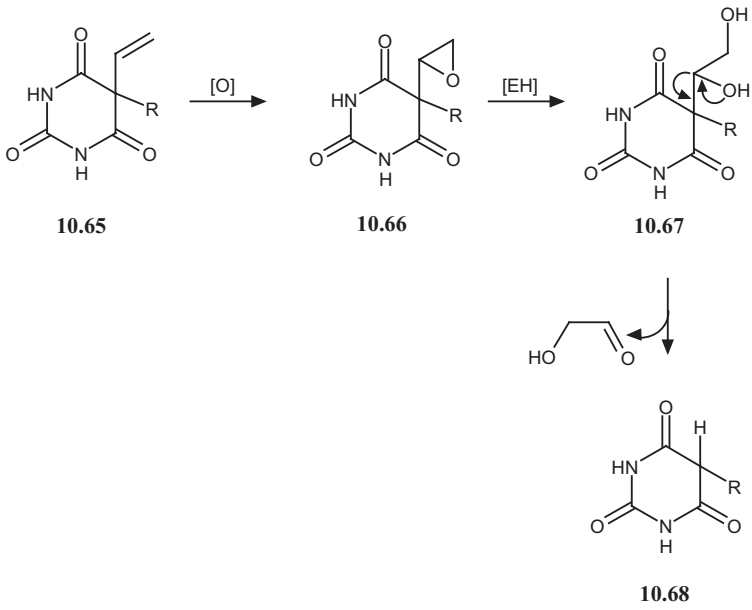
10.60 **10.61** **10.62**

Some drugs fall within the class of functionalized alkenes discussed here. Thus, the anti-inflammatory agent *alclophenac* (**10.63**) contains an *O*-allyl group. Its epoxide was found as a stable metabolite in the urine of mice and humans, and so was the diol, proving the involvement of the epoxide–diol pathway in the metabolism of this drug. The epoxide proved mutagenic, but only in the absence of a rat liver S-9 suspension (which contains EH) [141].

A few barbiturates also contain an allylic group at C(5), *e.g.*, *allobarbital* (**10.64**, R = allyl), *alphenal* (**10.64**, R = Ph), *aprobarbital* (**10.64**, R = i-Pr), and *secobarbital* (**10.64**, R = 1-methylbutyl). These compounds were substrates of the epoxide–diol pathway in rats and guinea pigs. The relative



10.63 **10.64**



10.65 **10.66** **10.67**

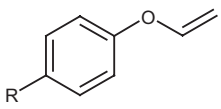
10.68

Fig. 10.17. *The epoxide–diol pathway in 5-vinylbarbiturates such as vinylbital* (**10.65**, R = 1-methylbutyl). The difference with 5-allylbarbiturates is that the diol metabolite of 5-vinylbarbiturates cannot be isolated due to its immediate decomposition *via* a *retro*-aldol-type reaction [142].

importance of this metabolic route was species-dependent, but was also markedly influenced by the nature of the other substituent at C(5) [143].

5-Vinylbarbiturates (**10.65**, *Fig. 10.17*) bear a close resemblance to 5-allylbarbiturates and are also substrates of the epoxide–diol pathway, yet the metabolic endpoint of the reaction is not the diol but a devinylated barbiturate. As shown for *vinylbital* (**10.65**, R = 1-methylbutyl, *Fig. 10.17*), the diol metabolite of 5-vinylbarbiturates (**10.67**, R = 1-methylbutyl) decomposes immediately *via* a *retro*-aldol-type reaction to form glycolaldehyde and 5-(1-methylbutyl)barbiturate (**10.68**, R = 1-methylbutyl) [142]. The latter metabolite accounted for 6 – 11% of a dose of vinylbital in the urine of rats and humans. In these studies, 1′,2′-epoxyvinylbital (**10.66**, R = 1-methylbutyl) was also prepared and found to be relatively unstable under acidic or basic conditions; at pH 7.4, however, its hydration was almost entirely enzymatic.

The relative stability of 1′,2′-epoxyvinylbital, and the great instability of its diol, can be gainfully compared with the fate of the corresponding metabolites of *phenyl vinyl ether* (**10.69**, R = H) and *4-nitrophenyl vinyl ether* (**10.69**, R = NO_2), two mutagenic and tumorigenic compounds [144][145]. Their primary metabolic route is by vinyl epoxidation to the corresponding



10.69

epoxide, two highly reactive metabolites that – in contrast to 1′,2′-epoxyvinylbital – are unstable in water and are hydrated. The intermediate diols were not isolated but decomposed immediately *via* the same *retro*-aldol-type reaction shown in *Fig. 10.17*, forming the corresponding phenol and glycolaldehyde. Under physiological conditions of pH and temperature, hydration of the oxiranyl phenyl ether and 4-nitrophenyl oxiranyl ether occurred with $t_{1/2}$ values of 2.7 and 4.5 min (activation energy 15 kcal mol^{-1}), respectively. This hydration was accelerated up to twofold by microsomal EH. Thus, whereas the reactivity of these vinyl epoxides is markedly greater with phenyl vinyl ethers than with vinylbital, their diols are similarly unstable and liberate an acidic functionality (O–H *vs.* C–H, pK_a values of 8 – 10) with loss of glycolaldehyde.

The high reactivity of the two oxiranyl phenyl ethers accounts for the observed cytotoxicity, since they readily formed adducts with DNA; further, the $t_{1/2}$ values corresponding to mutagenicity matched the chemical half-lives [145].

10.5.4. Haloalkene Oxides

Haloalkene oxides form a particular group of xenobiotics generally known for being reactive and toxic. Here, we discuss two classes of compounds, separated according to the vicinal or geminal position of their halogen atom(s) relative to the oxirane ring.

The simplest *oxirane with a vicinal halogen* is *1-chloro-2,3-epoxypropane* (2-(chloromethyl)oxirane, epichlorohydrin, **10.70**, *Fig. 10.18*), a mutagenic and carcinogenic compound [13]. The glutathione pathway is a major route of detoxification, yielding a variety of conjugates and metabolites thereof [146]. Evidence for epoxide hydration also exists, with 3-chloropropane-1,2-diol (α-chlorohydrin, **10.71**) as the primary product. Administration of 1-chloro-2,3-epoxypropane and 3-chloropropane-1,2-diol to rats gave cogent results, showing that the latter metabolite is further oxidized to 3-chlorolactic acid and, ultimately, to oxalic acid [146][147]. In addition, 3-chloropropane-1,2-diol reacts intramolecularly by nucleophilic substitution and Cl^- elimination to form 2,3-epoxypropan-1-ol (glycidol, **10.72**). This secondary metabolite is also a reactive epoxide, which presumably plays a role in the

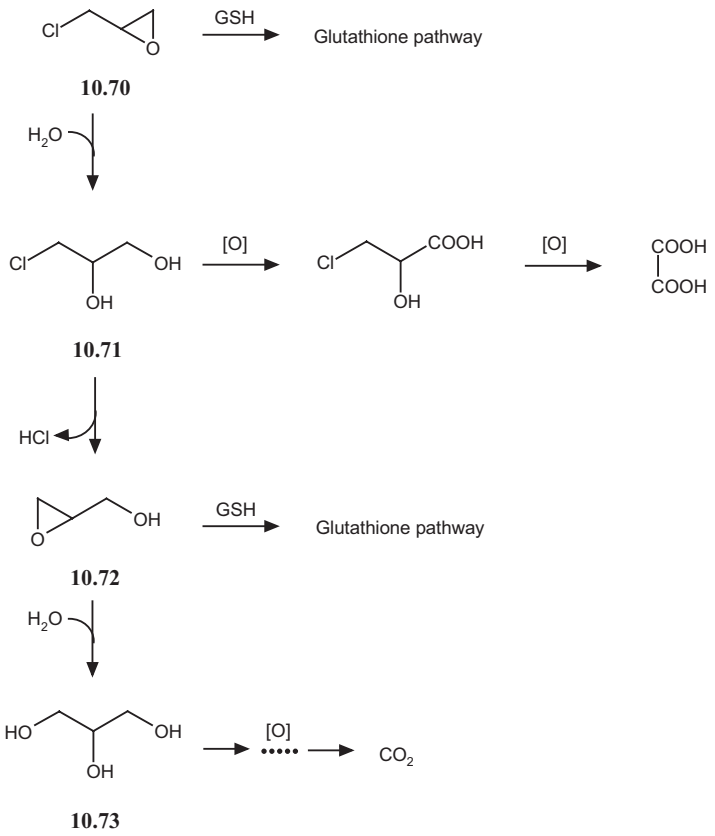


Fig. 10.18. *Metabolism of epichlorohydrin* (**10.70**)*, showing the sequential steps of hydration to α-chlorohydrin* (**10.71**)*, cyclization with loss of HCl to form glycidol* (**10.72**)*, and hydration to glycerol* (**10.73**) [146][147]

toxic effects of epichlorohydrin, and is detoxified by glutathione conjugation and hydration to form glycerol (**10.73**). Indeed, incubation of 2,3-epoxypropan-1-ol in rat liver preparations led to the formation of metabolites (50 – 60% derived from glutathione, and 30 – 35% derived from glycerol, the ultimate metabolite of which is CO_2) [147].

1,1-Dichloro-2,3-epoxypropane (2-(dichloromethyl)oxirane, **10.74**) and *1,1,1-trichloro-2,3-epoxypropane* (3,3,3-trichloropropene oxide, 2-(trichloromethyl)oxirane, **10.75**) are also substrates for epoxide hydrolase, with the

10.74 **10.75**

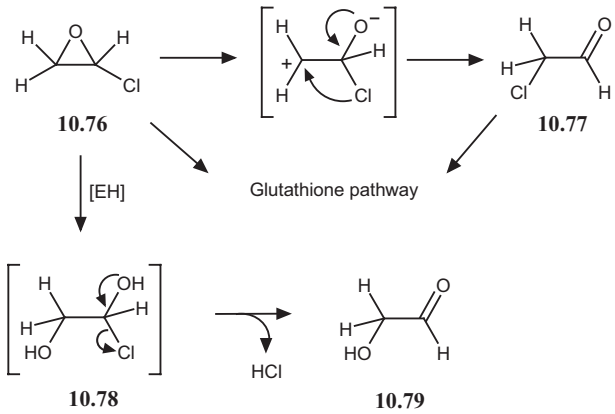


Fig. 10.19. *Simplified reactivity and metabolism of monochlorooxirane* (**10.76**)

dichloro compound being a seemingly better substrate than either the monochloro or trichloro analogue [148]. This is easily understandable for trichloropropene oxide, a well-known and potent inhibitor of microsomal and cytosolic EH. Kinetic studies suggest that trichloropropene oxide acts as an inhibitor of the cytosolic EH, probably by an uncompetitive inhibition mechanism [25]. Trichloropropene oxide also appears to be a substrate, which may explain the observation that prolonging the duration of pre-incubation markedly decreased the IC_{50} value toward rat and human microsomal EH [149]. However, whatever the reactivity of the three chloropropene oxides toward epoxide hydrolase, it is a far less efficient route of detoxification than glutathione conjugation [148].

Halogenated oxiranes (*i.e.*, oxiranes that contain one or more geminal halogen) show even more complex patterns of reactivity and rearrangement than the vicinal halo-oxiranes discussed above [13]. Here, we examine monochloroethylene oxide and the dichloroethylene oxides before focusing on trichloroethylene oxide. All these epoxides are biologically significant, mainly as metabolites of the corresponding chloroethylenes.

Monochlorooxirane (2-chlorooxirane, **10.76**, *Fig. 10.19*) rapidly rearranges by Cl migration to 2-chloroacetaldehyde (**10.77**) as shown in *Fig. 10.19*. Monochlorooxirane and 2-chloroacetaldehyde, which are both relatively reactive, form adducts with proteins and nucleic acids and are detoxified by glutathione conjugation. However, monochlorooxirane remains sufficiently stable compared to other halogenated oxiranes to be a substrate for epoxide hydrolase. Monochloroglycol (**10.78**), formed by enzymatic hydration, undergoes fast dehydrochlorination to glycolaldehyde (**10.79**); this aldehyde in turn is reduced to ethyleneglycol and oxidized to oxalic acid.

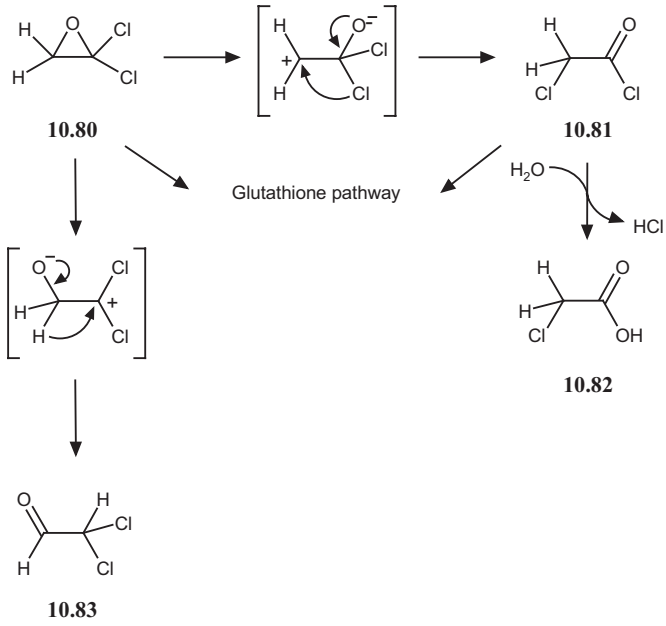


Fig. 10.20. *Simplified reactivity and metabolism of 2,2-dichlorooxirane* (**10.80**)

2,2-Dichlorooxirane (**10.80**, *Fig. 10.20*), the most reactive chlorooxirane of those discussed, cannot be isolated. Its major product of rearrangement is monochloroacetyl chloride (**10.81**), which, like its precursor, can be detoxified by glutathione or react very aggressively toward proteins, nucleic acids, and membrane phospholipids, forming adducts that are cytotoxic and carcinogenic. Furthermore, monochloroacetyl chloride reacts with H_2O like other acyl chlorides to be detoxified to the corresponding carboxylic acid (**10.82**). A second rearrangement mechanism has been characterized for 2,2-dichlorooxirane formed from 1,1-dichloroethylene in liver microsomes, namely a hydride shift to yield 2,2-dichloroacetaldehyde (**10.83**). In addition to the reactions shown in *Fig. 10.20*, 2,2-dichlorooxirane undergoes C–C bond scission [13][150][151].

cis- and trans-*2,3-dichlorooxirane* (**10.84** and **10.85**, respectively, *Fig. 10.21*) react by Cl migration as shown to form 2,2-dichloroacetaldehyde (**10.86**) as the sole primary product. The latter is then reduced to 2,2-dichloroethanol and oxidized to 2,2-dichloroacetic acid. Interestingly, both isomers of 1,2-dichloroethylene have been found to be nonmutagenic whether in the presence or absence of metabolizing enzymes [13].

Trichloroethylene oxide (trichlorooxirane, **10.89**, *Fig. 10.22*) has received particular attention due to its toxicological significance and the widespread use of its parent compound trichloroethylene (**10.87**) [153][155]. Chloral

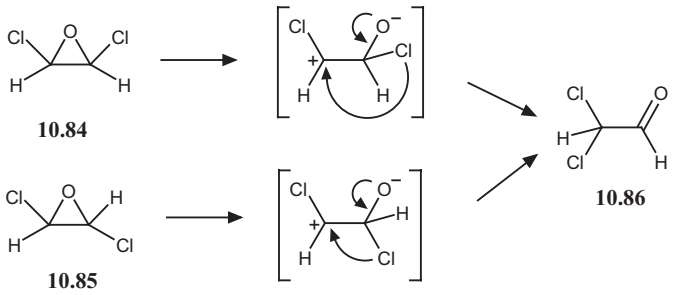


Fig. 10.21. *Simplified reactivity of* cis- *and* trans-*2,3-dichlorooxirane* (**10.84** and **10.85**, respectively)

(2,2,2-trichloroacetaldehyde, **10.88**) and mainly its redox metabolites 2,2,2-trichloroethanol and 2,2,2-trichloroacetic acid are the major *in vivo* metabolites of trichloroethylene, and it was thought for many years that their formation was *via* trichloroethylene oxide [13]. However, careful work showed quite conclusively that both chloral and the epoxide arise at the cytochrome P450 active site from a common metabolic intermediate, as shown in *Fig. 10.22* [156]. Trichloroethylene oxide itself is unstable in water, but the mechanisms of its decomposition in water have only recently been clarified [152].

Cleavage of the oxirane C–O bond produces a zwitterionic intermediate (*Fig. 10.22*), which that can undergo chloride shift (*Pathway a*) to 2,2-dichloroacetyl chloride (**10.90**) followed by hydrolysis to 2,2-dichloroacetic acid (**10.91**). Furthermore, the zwitterionic intermediate reacts with H_2O or H_3O^+ (*Pathway b*) by pH-independent or a H_3O^+-dependent hydrolysis, respectively. The pH-independent pathway only is shown in *Fig. 10.22*, *Pathway b*, but the mechanism of the H_3O^+-dependent hydrolysis is comparable. Hydration and loss of Cl^-, thus, leads to glyoxylyl chloride (**10.92**), a reactive acyl chloride that is detoxified by H_2O to glyoxylic acid (**10.93**), breaks down to formic acid and carbon monoxide, or reacts with lysine residues to form adducts with proteins and cytochrome P450 [157]. There is also evidence for reaction with phosphatidylethanolamine in the membrane.

Two further compounds are briefly discussed here. *Tetrachloroethylene* administered to animals yielded 2,2,2-trichloroacetic acid (**10.95**, *Fig. 10.23*) as the only chlorinated metabolite [13]. These findings provided the first evidence that tetrachloroethylene is oxidized by cytochrome P450 to its epoxide (**10.94**), which rearranges by Cl migration to 2,2,2-trichloroacetyl chloride (*Fig. 10.23*). The latter hydrolyzes to 2,2,2-trichloroacetic acid (**10.95**), but also acylates tissue proteins, a reaction of unclear toxicological significance. *In vitro* investigations of tetrachloroethylene oxide (2,2,3,3-tetrachlorooxirane, **10.94**) further showed that it hydrolyzes to the vicinal diol (**10.96**

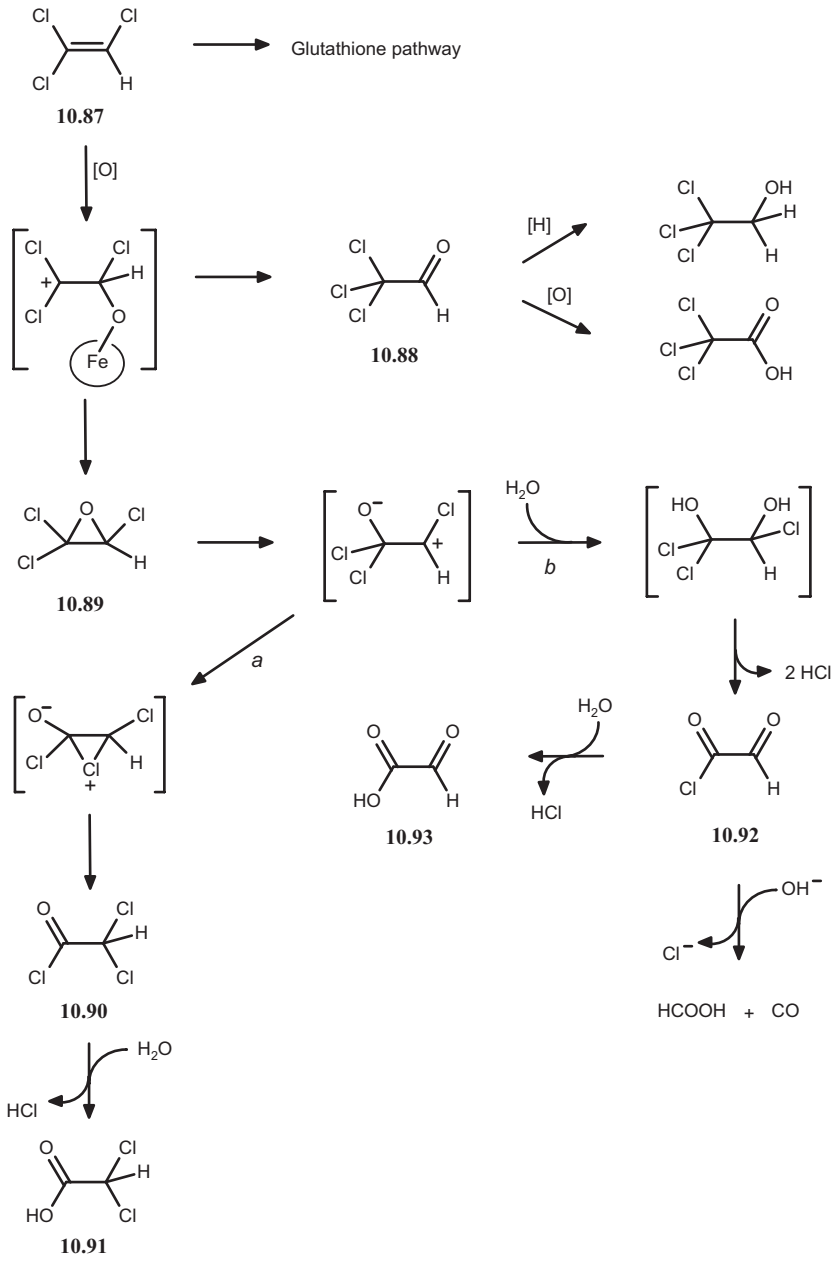


Fig. 10.22. *Simplified reactivity and metabolism of trichloroethylene* (**10.87**) *to chloral* (**10.88**) *and to trichloroethylene oxide* (**10.89**)*, followed by rearrangement reactions and/or hydrolysis of the latter* [13][152 – 154]

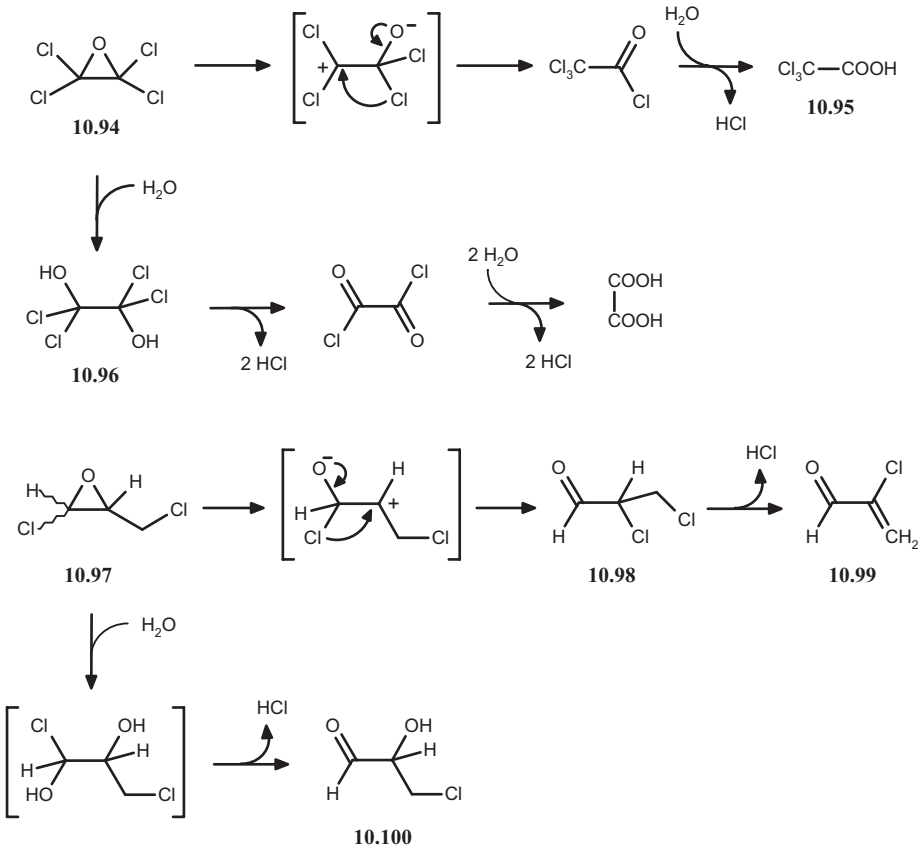


Fig. 10.23. *Simplified reactivity and metabolism of tetrachloroethylene oxide* (**10.94**) *and 1,3-dichloropropene oxide* (**10.97**). In both cases, the conditions dictate the relative contributions of heterolytic oxirane opening with Cl migration and oxirane hydration followed by loss of HCl [13][158].

in *Fig. 10.23*), which, in turn, yields oxalic acid by dechlorination to oxalyl chloride followed by hydrolysis. That this pathway occurs *in vivo* is evidenced by the presence of oxalic acid as a urinary metabolite in rodents administered tetrachloroethylene.

1,3-Dichloropropene, a fumigant used as a nematocide, is possibly a human carcinogen. A *cis/trans* mixture of the compound was oxidized to the corresponding *cis*- and *trans*-diastereoisomeric forms of 1,3-dichloropropene oxide (**10.97**, *Fig. 10.23*), both *in vivo* in mice and *in vitro* in mice liver microsomes [158]. This epoxide, which reacts directly with glutathione, is stable in acetone or $CHCl_3$, but decomposes rapidly in dimethylsulfoxide to 2-chloroacrolein (**10.99**) *via* 2,3-dichloropropanal (**10.98**). Slower decomposition occurred in water at physiological pH, with almost exclusive formation

of 3-chloro-2-hydroxypropanal (**10.100**) *via* the vicinal diol, as shown in *Fig. 10.23*. 1,3-Dichloropropene oxide (**10.97**) was shown to be a substrate of microsomal EH, indicating that the diol and, hence, also 3-chloro-2-hydroxypropanal must be formed *in vivo*. Mutagenicity studies indicate that the genotoxicity of the epoxide is either *via* direct action or, more probably, *via* the product 3-chloro-2-hydroxypropanal (**10.100**) [158].

In conclusion, this subsection has offered insights into the complex chemical and metabolic reactivity of haloalkene oxides. Contributing factors include:

- competitive metabolic pathways of the parent haloalkene, in particular its enzymatic and nonenzymatic conjugation with glutathione [154];
- enzymatic and nonenzymatic reaction with glutathione and other thiols;
- enzymatic (epoxide hydrolase) and nonenzymatic hydration to the corresponding diols; and
- a variety of rearrangement reactions that follow heterolytic oxirane ring opening.

An *a priori* classification of these various reactions as either toxification or detoxification is simply impossible, since each product from these various pathways may be toxic or not depending on its chemical properties and own products. Furthermore, the biological context plays a critical role [154], yet this role, best viewed as the influence of biological factors on the relative importance of competitive routes of metabolism, is often underplayed by those who venture to make predictions of metabolic outcome. Indeed, in the cascade of intertwined metabolic routes exemplified by haloalkenes, a small difference in pathway selectivity at an early metabolic crossroad may be amplified downstream, giving rise to major differences in relative levels of metabolites and overall toxicity.

Such essential limitations may markedly decrease the reliability and predictive capacity of quantitative structure–toxicity relationships (STRs) in haloalkenes and all other classes of toxic xenobiotics, but recognition of limitations does not suppress the need for predictive tools. In fact, any approach, empirical or mechanistic, that is able to uncover qualitative STR trends and to assign *a priori* labels of potential toxicity is certainly welcome.

A fitting illustration of such qualitative predictions exists for haloalkene oxides. The energy of the C–O bond has been calculated for a variety of haloalkene oxides and other alkene oxides by means of a semi-empirical quantum mechanical method [159]. The stability of the oxirane ring allows for a qualitative (yes/no) prediction of the mutagenicity/oncogenicity, such that the most-stable and the least-stable oxides are predicted to be inactive, whereas the oxiranes with intermediate stability are thought to be cytotoxic. In other words, there is a range of C–O bond energies that corresponded to cytotox-

icity. A test of a set of further analogues led to correct classifications that could be explained in terms of a balance between DNA binding and enzymatic detoxification. Whereas this view is too simplistic to remain compatible with the metabolic behavior of alkene oxides as detailed above, the model remains of interest as a virtual screen of cytoxicity.

10.5.5. Epoxides of Conjugated Dienes

The alkene oxides examined in the previous subsections were all derived from isolated (unconjugated) C=C bonds. The presence of conjugated C=C bonds may change the metabolism of the derived epoxides, as now discussed. Our presentation begins with simple conjugated dienes in this subsection, before considering in the next subsection the case of styrenes and stilbenes where the C=C bond is conjugated to one or two aromatic rings, respectively.

Buta-1,3-diene (**10.101**, *Fig. 10.24*) is a gaseous chemical used heavily in the rubber and plastics industry, the presence of which in the atmosphere is also a concern. Butadiene is suspected of increasing the risks of hematopoietic cancers, and it is classified as a probable human carcinogen. Butadiene must undergo metabolic activation to become toxic; the metabolites *butadiene monoepoxide* (**10.102**, a chiral compound) and *diepoxybutane* (**10.103**, which exists in two enantiomeric and one *meso*-form) react with nucleic acids and glutathione [160 – 163], as does a further metabolite, 3,4-epoxybutane-1,2-diol (**10.105**). Interestingly, butadiene monoepoxide is at least tenfold more reactive than diepoxybutane toward nucleic acids or H_2O. Conjugation between the C=C bond and the oxirane may account for this enhanced reactivity.

Both the mono- and diepoxides of butadiene are substrates for epoxide hydrolase [163]. In rat liver microsomes, (*R*)- and (*S*)-butadiene monoepoxides were hydrolyzed to but-3-ene-1,2-diol (**10.104**, *Fig. 10.24*) with complete retention of configuration at C(2), indicating attack at C(1) [164]. In mouse liver microsomes, in contrast, 15 – 25% inversion of configuration was observed, suggesting partial attack at C(2). Preliminary results indicate that human liver microsomes are more efficient than mouse or rat liver microsomes in hydrolyzing butadiene monoepoxide [165]. The hydrolysis of diepoxybutane (**10.103**) yields 3,4-epoxybutan-1,2-diol (**10.105**), which can be further hydrated to erytritol (**10.106**) [163].

The metabolite but-3-ene-1,2-diol (**10.104**, *Fig. 10.24*) is of particular interest since further oxidation by alcohol dehydrogenase yields reactive products such as α,β-unsaturated ketones [166][167]. Dehydrogenation of the primary alcoholic group to the α-hydroxyaldehyde followed by fast rearrange-

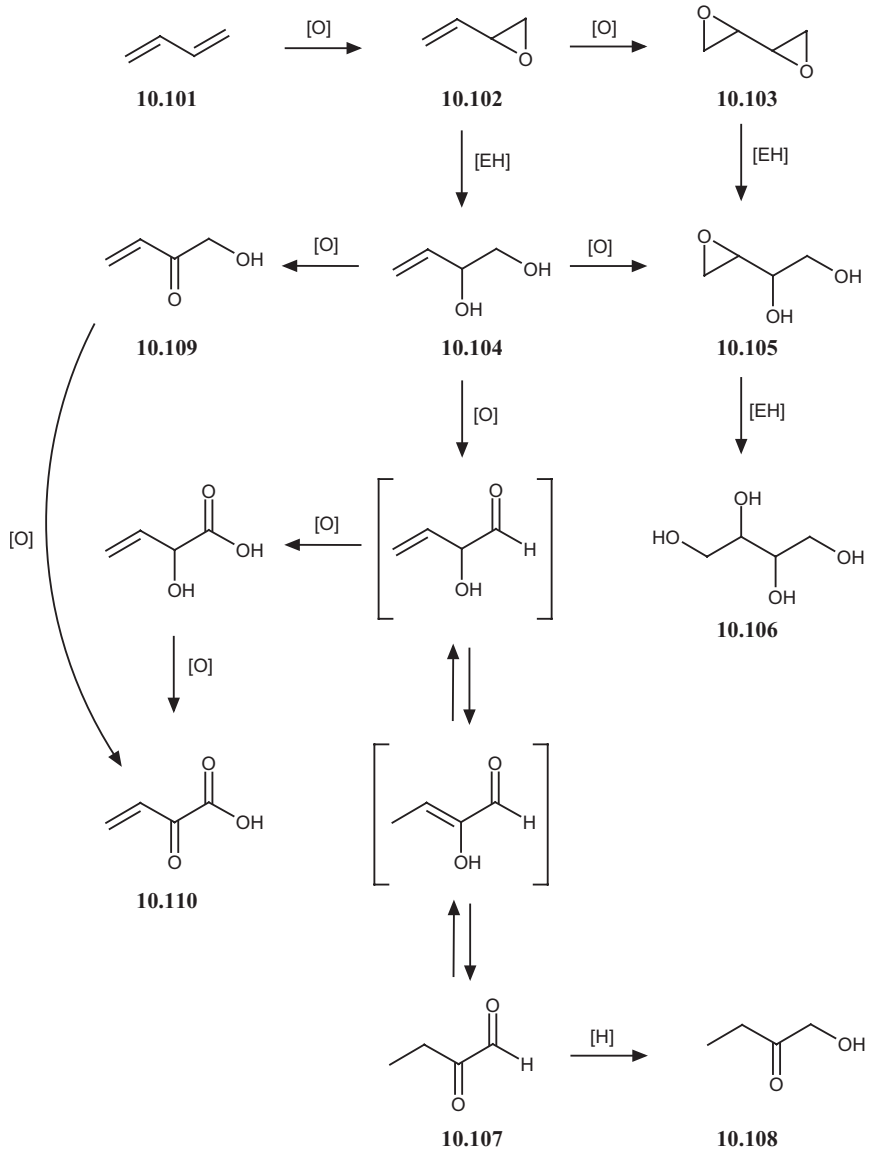
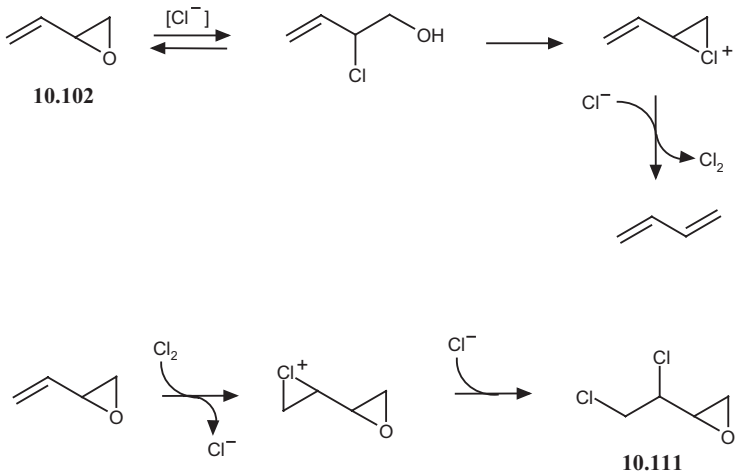


Fig. 10.24. *Simplified metabolism of buta-1,3-diene* (**10.101**)*, showing the formation of reactive metabolites bearing an epoxy group* (*i.e.*, **10.102**, **10.103**, and **10.105**) *or an* α,β*-unsaturated carbonyl moiety* (*e.g.*, **10.109** and **10.110**)

ment to 2-oxobutanal (**10.107**) and subsequent reduction was assumed to be the pathway of formation of minute amounts of 1-hydroxybutan-2-one (**10.108**). These postulated intermediates appear to be quite reactive electrophiles that may be trapped by glutathione and other endogenous nucleophiles.



10.102 **10.111**

Fig. 10.25. *Reaction pathway of butadiene monoxide* (**10.102**) *in aqueous NaCl solution to yield 1,2-dichloro-3,4-epoxybutane* (**10.111**) [168]. The sequence involves the intermediate formation of Cl_2.

The formation of another metabolite, 1-hydroxybut-3-ene-2-one (**10.109**), postulated to arise by dehydrogenation of the secondary alcoholic group, could be demonstrated only indirectly. 1-Hydroxybut-3-ene-2-one is believed to react with glutathione and other endogenous nucleophiles, to be oxidized to 2-oxobut-3-enoic acid (**10.110**), and to break down to form unidentified products.

In summary, a growing body of evidence suggests that at least part of the chronic toxicity of buta-1,3-diene is caused by reactive metabolites that bear an epoxy or α,β-unsaturated carbonyl group. These metabolites form adducts with protective (glutathione) or critical (*e.g.*, nucleic acids) endogenous nucleophiles [163][166][167].

Solvolysis of butadiene monoxide (**10.102**) in saline solution is a rather unexpected reaction that further documents this compound's reactivity [168]. In aqueous NaCl solution at physiological pH and temperature, butadiene monoxide disappeared rapidly to form *1,2-dichloro-3,4-epoxybutane* (**10.111**, *Fig. 10.25*). There was a linear dependence of the rate of reaction on the Cl^- concentration (in the range investigated (34 – 135 mM)). The reaction pathway was described as slow solvolytic formation of the butenylchloronium ion, followed by Cl^- attack to yield Cl_2 and butadiene. Cl_2 is then rapidly trapped by a second molecule of butadiene monoxide to form a different chloronium ion that also reacts with Cl^- to yield the final, stable dichloro product (**10.111**). The formation of 1,2-dichloro-3,4-epoxybutane under physiological conditions is believed to be toxicologically significant.

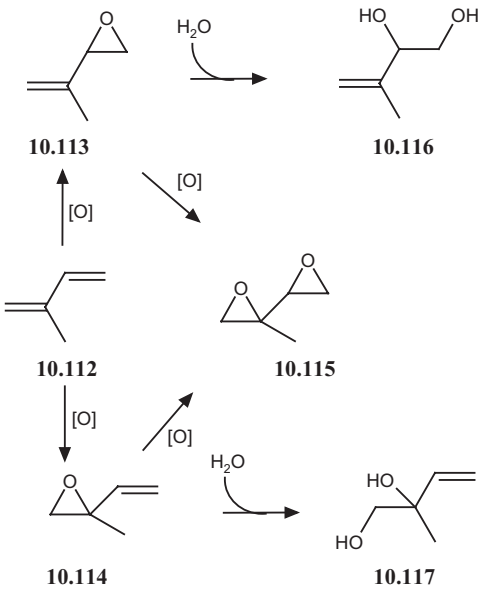


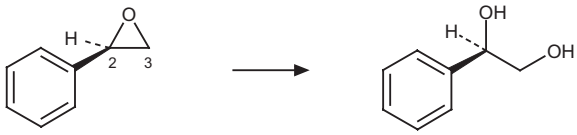
Fig. 10.26. *Partial outline of isoprene* (**10.112**) *metabolism to three epoxide* (**10.113** – **10.115**) *and two diol* (**10.116** and **10.117**) *products* [169][170].

Isoprene (2-methylbuta-1,3-diene, **10.112**, *Fig. 10.26*) is another conjugated diene of major industrial importance. Whereas its metabolism does not appear to have been investigated as comprehensively as that of butadiene, important findings have nevertheless been reported, particularly regarding regio- and stereochemical aspects of its reactivity [169][170]. Thus, cytochrome P450 catalyzes the oxidation of isoprene to the two chiral monoepoxides, 2-(isopropenyl)oxirane (2-(1-methylethenyl)oxirane, **10.113**) and 2-methyl-2-vinyloxirane (**10.114**). A second epoxidation follows to give 2-methyl-2,2′-bioxirane (**10.115**), which contains two stereogenic centers. Mice liver microsomes formed more **10.114** than **10.113**, whereas the ratio was closer to unity in rat liver microsomes. The reactions of mono-oxygenation also displayed complex aspects of substrate and product stereoselectivity.

2-(Isopropenyl)oxirane (**10.113**, *Fig. 10.26*) and 2-methyl-2-vinyloxirane (**10.114**) were hydrolyzed by EH to the corresponding diols (**10.116** and **10.117**, respectively). Nucleophilic ring opening took place at the less-hindered, unsubstituted C-atom, with retention of configuration at C(2). (2*R*)-2-(Isopropenyl)oxirane was a better substrate than the (2*S*)-enantiomer. Substrate enantioselectivity was more modest in the hydration of 2-methyl-2-vinyloxirane (**10.114**), since this compound is chemically more reactive and undergoes partly nonenzymatic hydrolysis.

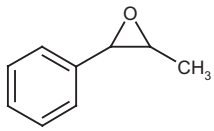
10.5.6. Styrene and Stilbene Oxides

Styrene (vinylbenzene), which is used extensively in industrial chemistry settings, is of serious concern as a pollutant and as a toxin in animals and humans. One of its major routes of metabolism is by epoxidation of the vinyl group to *styrene 7,8-oxide* (2-phenyloxirane, **10.6**). This metabolite, also an industrial chemical in its own right, is detoxified by conjugation with glutathione or may initiate physiological insult by reacting with nucleophilic sites in biomacromolecules [171]. Another route of detoxification is by hydration to 1-phenylethane-1,2-diol (**10.118**) (see *Table 10.1*), followed by oxidation of the side chain to mandelic acid and other aromatic acids. The hydration of 2-phenyloxirane is catalyzed by microsomal and cytosolic EH and has been characterized in various tissues and organs and in a variety of animal species including humans [172 – 174].



(*R*)-**10.6** (*R*)-**10.118**

(*S*)-**10.6** (*S*)-**10.118**



10.119

The base-catalyzed hydration of 2-phenyloxirane involves nucleophilic attack preferentially at C(3) (O–C(3) cleavage), but with only partial regioselectivity. Acid-catalyzed hydration is mainly by O–C(2) cleavage. The hydration of 2-phenyloxirane catalyzed by epoxide hydrolase is characterized by its very high regioselectivity for the less-hindered, unsubstituted C(3) [175][176], involving retention of configuration at C(2). In other words, (*R*)- and (*S*)-2-phenyloxirane are metabolized to (*R*)- and (*S*)-1-phenylethane-1,2-diol (**10.118**), respectively. Substrate enantioselectivity was also character-

ized in rat liver microsomes, since (*S*)-2-phenyloxirane had a lower affinity but a higher velocity (K_m = 155 μM, V_{max} = 44 nmol (mg protein)$^{-1}$ min^{-1}) compared to its (*R*)-enantiomer (K_m = 29 μM, V_{max} = 12 nmol (mg protein)$^{-1}$ min^{-1}). This suggests that, in racemic 2-phenyloxirane, the (*R*)-epoxide partly inhibited the hydrolysis of the (*S*)-enantiomer [177].

A postulated mechanism for nucleophilic attack in enzymatic hydrolysis suggests that electron-withdrawing substituents on the phenyl ring must accelerate the reaction. This is seen in *Table 10.1* when comparing 2-phenyloxirane with 2-(4-nitrophenyl)oxirane. A systematic study of a few aryl-substituted phenyloxiranes confirmed this inference [175].

2-Methyl-3-phenyloxirane (**10.119**) has two stereogenic centers and exists as diastereoisomeric pairs of enantiomers. The racemic *trans*-2-methyl-3-phenyloxirane was found to be a much better substrate for human EH than 2-phenyloxirane and *cis*-2-methyl-3-phenyloxirane. Indeed, the V_{max} value in human liver microsomes was higher by three and four orders of magnitude, respectively, and in human liver cytosol it was higher by two and three orders of magnitude, respectively [52a]. Further stereochemical and mechanistic insights have been reported, with the nucleophilic attack of epoxide hydrolase occurring at the less-hindered C(2) with inversion of configuration at C(2) and retention of configuration at C(3), in agreement with the mechanism discussed in *Sect. 10.4.3* and *10.5.2* [178].

An important epoxide is the 4′-methoxy analogue of **10.119**, namely *trans*-anethole epoxide (*trans*-2-(4-methoxyphenyl)-3-methyloxirane, **10.120**, *Fig. 10.27*). trans-*Anethole* is a volatile substance responsible for the characteristic flavor of aniseed. It is somewhat cytotoxic, but not genotoxic, in rat hepatocytes. The cytotoxicity is due to the formation of anethole epoxide, which is detoxified by conjugation with glutathione and by EH-catalyzed hydration. Hydrolytic ring opening also occurs in buffer, although no comparison between the chemical and enzymatic reaction appears to have been carried out under physiologically relevant conditions. Both acid- and base-catalyzed hydrolyses occurred with nucleophilic substitution at the benzylic position, H_2O/OH^- entering from above or below (*Fig. 10.27*, *Reaction a* or *b*, respectively) with an ratio of *ca.* 4:1 in the formation of *threo*- and *erythro*-diols, respectively [178][179]. In contrast, the enzymatic reaction involved preferential attack at the less-hindered C-atom with inversion of configuration at that position and retention of configuration at the benzylic position.

Stilbene oxide (2,3-diphenyloxirane) is another compound of interest. It exists as two diastereoisomers, namely *cis*-stilbene oxide (**10.7**, *Fig. 10.28*) and the *trans*-(*R*,*R*)- and *trans*-(*S*,*S*)-stilbene oxides (**10.121**, *Fig. 10.28*), which exhibit significant but condition-dependent substrate selectivity. Cytosolic EH purified from mouse liver metabolized racemic *trans*-stilbene oxide with high affinity and high velocity, and *cis*-stilbene oxide with lower affinity and lower

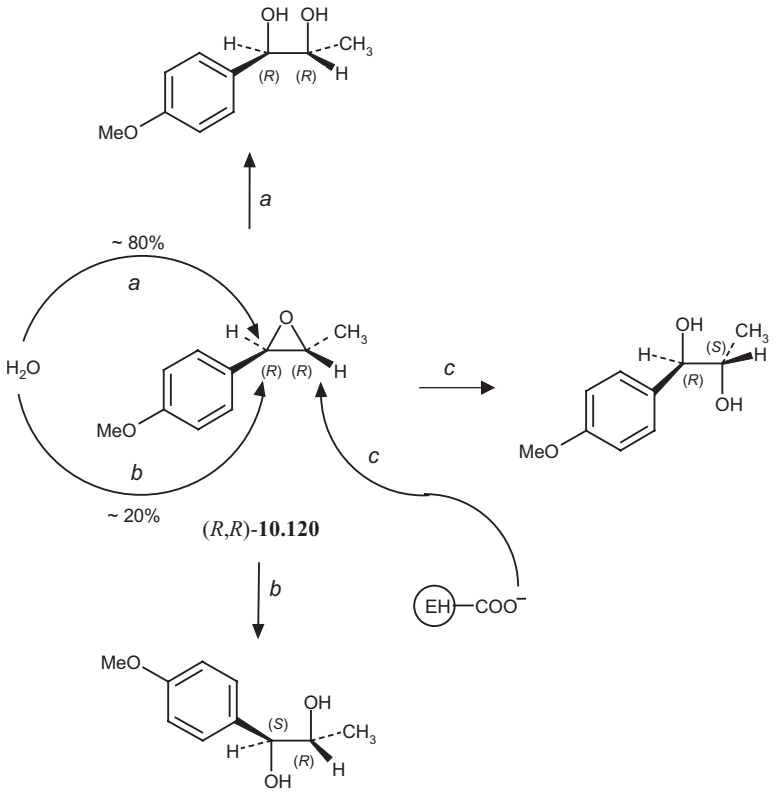


Fig. 10.27. *Mechanistic and stereochemical aspects of the hydration of* trans-*(*R,R*)-anethole epoxide in the absence* (*Reactions a* and *b*) *and presence of epoxide hydrolase* (*Reaction c*) [178][179]. The ratio of *Reaction a* to *b* is *ca.* 4:1 in acid- and base-catalyzed hydration to yield the *threo*- and *erythro*-diols, respectively. The enzymatic *Reaction c* also yields preferentially the *erythro*-diol.

velocity [180]. Under these conditions, styrene oxide exhibited very low affinity and high velocity. Different results were found with human liver EH [52a]. Thus, the human microsomal EH was 1000-fold, and the cytosolic EH 10-fold, more active toward *cis*-stilbene oxide than *trans*-stilbene oxide. When the microsomal and cytosolic enzymes were compared, it was found that microsomal EH was 20-fold more active toward *cis*-stilbene oxide, whereas cytosolic EH was 10-fold more active toward *trans*-stilbene oxide. A study across animal species with *cis*-stilbene oxide as substrate and microsomal EH as enzyme showed large interspecies differences, with the order of specific activity being human > rabbit > dog > rat > hamster > mouse [181].

The hydration of stilbene oxide also demonstrates unusual stereochemical characteristics [175][182][183]. Indeed, stilbene oxide exists as two *trans*-stilbene oxide enantiomers ((*R*,*R*)- and (*S*,*S*)-**10.121**) and as the *meso*,

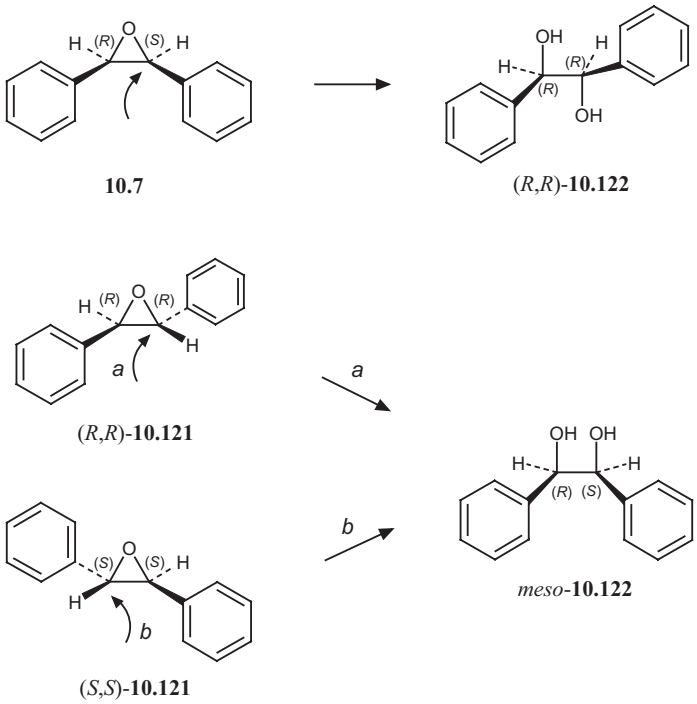


Fig. 10.28. *The stereochemistry of enzymatic hydrolysis of stilbene oxide.* The achiral (*meso*) *cis*-stilbene oxide (**10.7**) yields only one of the two enantiomers of *threo*-1,2-diphenylethane-1,2-diol (**10.122**). In contrast, the chiral *trans*-stilbene oxide (**10.121**) is hydrated exclusively to *meso*-1,2-diphenylethane-1,2-diol [182][183].

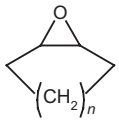
symmetric *cis*-stilbene oxide (**10.7**). The 1,2-diphenylethane-1,2-diol metabolite (**10.122**, *Fig. 10.28*) of stilbene oxide also exists as three stereoisomers, namely the *threo*-(*R*,*R*)- and *threo*-(*S*,*S*)-enantiomers and the *meso*-(*R*,*S*)-form. The hydrolysis of *cis*-stilbene oxide by rabbit liver microsomal EH was found to yield exclusively one of the two enantiomeric products, namely the *threo*-(*R*,*R*)-1,2-diphenylethane-1,2-diol ((*R*,*R*)-**10.122**, *Fig. 10.28*) [182]. This implies that enzymatic attack with inversion of configuration occurs with high selectivity at the (*S*)-configured C-atom. Identical results were obtained with 4,4′-disubstituted *cis*-stilbene oxides.

The chiral substrate *trans*-stilbene oxide (**10.121**) behaved differently, yielding *meso*-1,2-diphenylethane-1,2-diol (*meso*-**10.122**) [183]. This means that, in both enantiomeric substrates, the enzyme does not discriminate between the two oxirane C-atoms, bringing about inversion of configuration at the C-atom attacked. Interestingly, the various stereoisomers of 1,2-diphenylethane-1,2-diol can be interconverted metabolically by alcohol/ketone equilibria catalyzed by alcohol dehydrogenases.

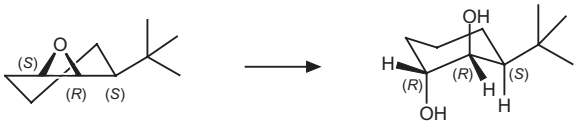
10.5.7. Simple Cycloalkene Oxides

The reaction of *cycloalkene oxides* with EH should be *a priori* mechanistically similar to that of acyclic analogues, except that the presence of a cycle may impose characteristic stereochemical constraints on the products. A look at *Table 10.1*, however, indicates that cycloalkene oxides are poorer substrates for EH than their acyclic analogues.

A systematic study has confirmed the low activity of EHs toward cycloalkene oxides (1,2-epoxycycloalkanes, **10.123**) [184]. In the presence of mouse liver microsomal EH, activity was very low for cyclopentene oxide and cyclohexene oxide (**10.123**, n = 1 and 2, respectively), highest for cycloheptene oxide (**10.123**, n = 3), and decreased sharply for cyclooctene oxide (**10.123**, n = 4) and higher homologues. Mouse liver cytosolic EH showed a different structure–activity relationship in that the highest activity involved cyclodecene oxide (**10.123**, n = 6). With the exception of cyclohexene oxide, which exhibited an IC_{50} value toward microsomal EH in the μM range, cycloalkene oxides were also very weak inhibitors of both microsomal and cytosolic EH.



10.123



10.125 **10.126**

Cyclohexene oxide (1,2-epoxycyclohexane, **10.5**, *Fig. 10.29*) has received particular attention as a substrate for EH (see *Table 10.1*). The compound has a *meso-cis* geometry like *cis*-stilbene oxide (**10.7**), and, like the latter, is hydrated preferentially to the chiral (*R*,*R*)-*trans*-cyclohexane-1,2-diol (**10.124**) [185]. There was a difference, however, between the activities of the rabbit liver microsomal and cytosolic EHs. The former was *ca.* 20-fold more active than the latter toward this substrate; also, it formed the (*R*,*R*)-diol with 94% enantiomeric excess (ee) compared to only 22% ee for the cytosolic EH.

With a variety of substituted cyclohexene oxides, *e.g.*, the 1-methyl and 3-bromo derivatives, *Bellucci et al.* demonstrated that the substrates in the catalytic site adopted the conformation shown in *Fig. 10.29* for **10.5** (3,4*M*

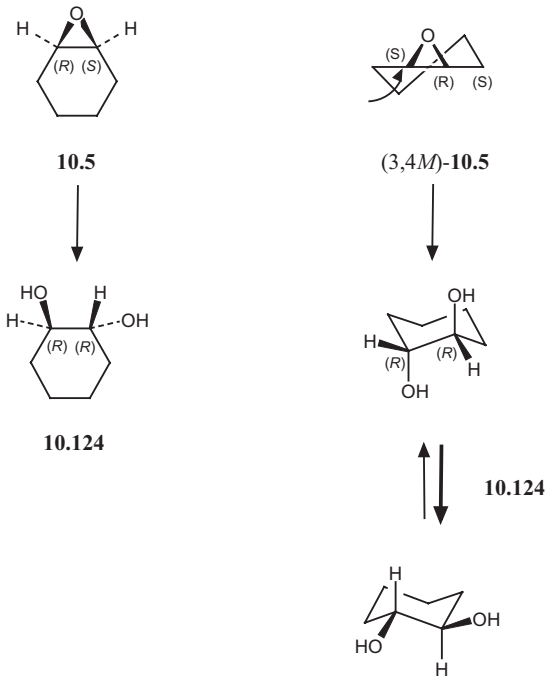


Fig. 10.29. *Stereochemistry of the EH-catalyzed hydration of 1,2-epoxycyclohexane* (**10.5**) *to the chiral* trans-*(*R,R*)-cyclohexane-1,2-diol* (**10.124**). The substrate in the catalytic site adopts the conformation shown (3,4*M* helicity), and enzymatic attack occurs at the (*S*)-configured C-atom with *trans*-diaxial opening [185][186].

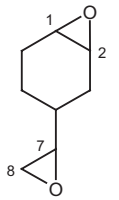
helicity), and that the enzymatic attack occurred at the (*S*)-configured C-atom [185][186]. This resulted in *trans*-diaxial opening of the oxirane ring, followed by conversion of the diaxial conformer to the more stable diequatorial form, as shown in *Fig. 10.29* for **10.124**.

Such a conformational rearrangement to the diequatorial conformer is also influenced by other ring substituents, as seen in the hydrolysis of 3-(*tert*-butyl)-1,2-epoxycyclohexane. This substrate is particularly informative since its bulky *t*-Bu group has such an overpowering preference for an equatorial position that it can freeze other substituents in an axial position. Furthermore, the compound has three stereogenic centers and exists as four stereoisomers, of which the *cis*-(1*S,*2*R,*3*S*)-isomer (**10.125**) was by far the best substrate for rabbit liver microsomal EH [187], probably due to the 3,4*M* helicity of the preferred conformation that presents the (*S*)-configured C-atom as the target for catalytic attack. The enzymatic reaction yielded almost exclusively the (1*R*,2*R*,3*S*)-diol (**10.126**) following *trans*-diaxial opening. In this case, however, the diaxial conformation of the two OH groups

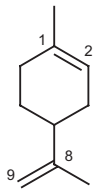
remained frozen by the equatorial *t*-Bu group. In contrast to the highly substrate-selective enzymatic hydration, the rates of acid-catalyzed hydration of the four stereoisomers of 3-(*tert*-butyl)-1,2-epoxycyclohexane were comparable.

Thus, all results with cyclohexene oxides are compatible with a configurational and conformational selection by the enzyme, preferential attack at an (*S*)-configured C-atom, and *trans*-diaxial opening.

An interesting regioselectivity was noted in the hydration of the diepoxide *4-vinylcyclohex-1-ene dioxide* (4-(oxiran-2-yl)-1,2-epoxycyclohexane, **10.127**). Its precursor, 4-vinylcyclohex-1-ene, which is employed as an intermediate in the production of various industrial chemicals, is an ovarian toxin and carcinogen in mice. This toxicity is ascribed to the diepoxide metabolite (**10.127**) [188], which is a substrate for EH, but a poor one compared to mono-epoxy alkanes because it also acts to inhibit the enzyme. Enzymatic hydration of the two epoxy groups occurs at unequal rates, there being an approximately twofold preference for the 7,8-epoxy group [189]. Both monoepoxy diols so formed were further hydrated to the tetrol with a substrate selectivity that again favored the 4-vinyl oxide group. These reactions are some among the many factors that influence the toxification and detoxification of 4-vinylcyclohex-1-ene [190]. In fact, the dioxides of 4-vinylcyclohex-1-ene, buta-1,3-diene (**10.101**, *Fig. 10.24*), and analogues have consistently demonstrated a tenfold higher reactivity toward nucleophiles compared to related monoepoxides, leading to the conclusion that the diepoxides are ultimate ovarian toxins [191].



10.127



10.128

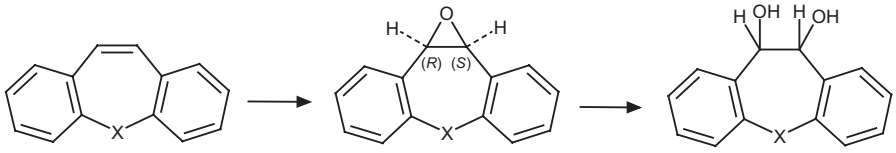
Limonene (**10.128**) is an analogue of 4-vinylcyclohexene, and, like the latter, it undergoes epoxidation of both the C(1)=C(2) and C(8)=C(9) bonds. Like in the dioxide **10.127**, the two epoxide groups are hydrated at different rates by EH. Indeed, incubations in rat liver microsomes showed that hydrolysis of limonene 1,2-epoxide was 70 times slower than that of the 8,9-epoxide, a much larger difference than that observed for the dioxide **10.127** [192]. Comparison of EH-catalyzed hydration of the four epoxy groups in 4-vinylcyclohexene and limonene confirmed that the relative rates decreased with increasing steric hindrance at these groups.

10.5.8. Cycloalkene Oxides of Medicinal or Toxicological Interest

Not unexpectedly, cycloalkene oxides are equally important as alkene oxides in medicinal chemistry and drug metabolism, as illustrated below with a few selected examples. Other compounds of interest that will not be discussed here include epoxytetrahydrocannabinols and endogenous 16,17-epoxy steroids.

The peculiar reactivity of epoxides of 5-vinylbarbiturates (**10.65**, *Fig. 10.17*) has been discussed in *Sect. 10.5.3*. Here, we note that *hexobarbital epoxide* (**10.9**) shows the same reactivity and decomposes *via* the same *retro*-aldol reaction to form 5-(1-methylbutyl)barbiturate [193].

A number of neurotropic agents contain a stilbene motif incorporated in an *iminostilbene* (5*H*-dibenzo[*b*,*f*]azepine, **10.129**, X=RN) or *dibenzosuberene* (5*H*-dibenzo[*a*,*d*]cycloheptene, **10.129**, X=RCH or RCH=C) ring system. Examples include the anticonvulsant *carbamazepine* (**10.129**, X = H_2NCON) and the antidepressants *protriptyline* (**10.129**, X = $MeNHCH_2CH_2CH_2CH$) and *cyclobenzaprine* (**10.129**, X = $Me_2NCH_2CH_2CH$=C). As a rule, these drugs are oxidized by cytochrome P450 to the corresponding epoxides (**10.130**), and hydration to the dihydrodiols (**10.131**) is usually low, as illustrated below [194].



10.129 **10.130** **10.131**

Resistance to hydration was elucidated with tricyclic model compounds that lack the side chain and, hence, pharmacological activity. In this context, a useful comparison has been made between two *meso* compounds, namely *5*H-*dibenzo[*a,d*]cycloheptene 10,11-oxide* (**10.130**, X = CH_2) and *cis*-stilbene oxide (**10.7**) [195]. The former compound proved to be a very poor substrate for rabbit liver microsomal EH, with a K_m value comparable to that of *cis*-stilbene oxide, but V_{max} *ca.* 100-fold lower. This indicates that the two compounds have a comparable affinity for the enzyme, but that nucleophilic attack in the catalytic step is much less efficient for dibenzo[*a*,*d*]cycloheptene 10,11-oxide than for *cis*-stilbene oxide. This implies that the former compound acts better as an inhibitor than as a substrate of microsomal EH. Furthermore, there was also a fundamental steric difference in the reaction course of the two substrates, since the predominant stereoisomer formed from dibenzo[*a*,*d*]cycloheptene 10,11-oxide had the (10*S*,11*S*)-configuration,

whereas *cis*-stilbene oxide yielded the (*R*,*R*)-diol (**10.122**, *Fig. 10.28*; see *Sect. 10.5.6*). In other words, the enzymatic attack of dibenzo[*a*,*d*]cycloheptene 10,11-oxide is at the (*R*)-configured rather than at the (*S*)-configured C-atom (as for *cis*-stilbene oxide).

The information obtained with the model substrate dibenzo[*a*,*d*]cycloheptene 10,11-oxide has helped us understand why related tricyclic drugs yield modest or very low proportions of dihydrodiols, despite formation of the 10,11-oxides. For example, both the epoxide and the dihydrodiol were characterized in the urine of rats given *protriptyline* (**10.129**, X = MeNH-$CH_2CH_2CH_2CH$), but *cyclobenzaprine* (**10.129**, X = $Me_2NCH_2CH_2CH$=C) did not yield the dihydrodiol despite the epoxide and other oxygenated metabolites being formed *in vivo* and *in vitro* [194].

Carbamazepine (**10.129**, X = H_2NCON) is a major anti-epileptic drug, the metabolism of which has long been of interest to biochemists and clinical pharmacologists [181][196 – 198]. Well over 30 metabolites of this drug have been characterized *in vivo* and/or *in vitro* [199][200]. Thus, the 10,11-epoxide and the 10,11-dihydrodiol are urinary metabolites in humans and rats given the drug. In epileptic patients, the range of plasma concentrations of the epoxide and the diol was *ca.* 0.8 – 17 and 0.8 – 36 μM, respectively, *i.e.*, a predominance of the latter [201]. A number of studies have also addressed the origin of toxic reactions seen in some patients, *e.g.*, CNS symptoms, gastrointestinal and hepatic disturbances, and hypersensitivity. Whereas no single factor seems to account for such toxic effects, the pharmacologically active 10,11-epoxide appears to contribute to clinical toxicity [202][203]. In this perspective, the EH-catalyzed hydration of the epoxide appears as a reaction of detoxification.

Interestingly, there is a marked species difference in the *in vitro* hydrolysis of carbamazepine 10,11-epoxide, such that the reaction was observed only in liver microsomes from humans but not in liver microsomal or cytosolic preparations from dogs, rabbits, hamsters, rats, or mice [181][196]. Thus, carbamazepine appears to be a very poor substrate for EH, in analogy with the simpler analogues **10.129** (X = RN, RCH, or RCH=C). The human enzyme is exceptional in this respect, but not, however, in the steric course of the reaction. The diol formed (**10.131**, X = H_2NCON) is mostly the *trans*-(10*S*,11*S*)-enantiomer [196]. In other words, the product enantioselectivity of the hydration of carbamazepine epoxide catalyzed by human EH is the same as that of dibenzo[*a*,*d*]cycloheptene 10,11-oxide catalyzed by rabbit microsomal EH, discussed above.

Moving to toxic compounds, we encounter the infamous *aflatoxin B1*, a mycotoxin considered to be a major cause of human liver cancer in some parts of the world. Thus, there are some estimates that, in China, as many as 1 in 10 adults die from liver cancer caused by aflatoxin [205]. Oxidation of

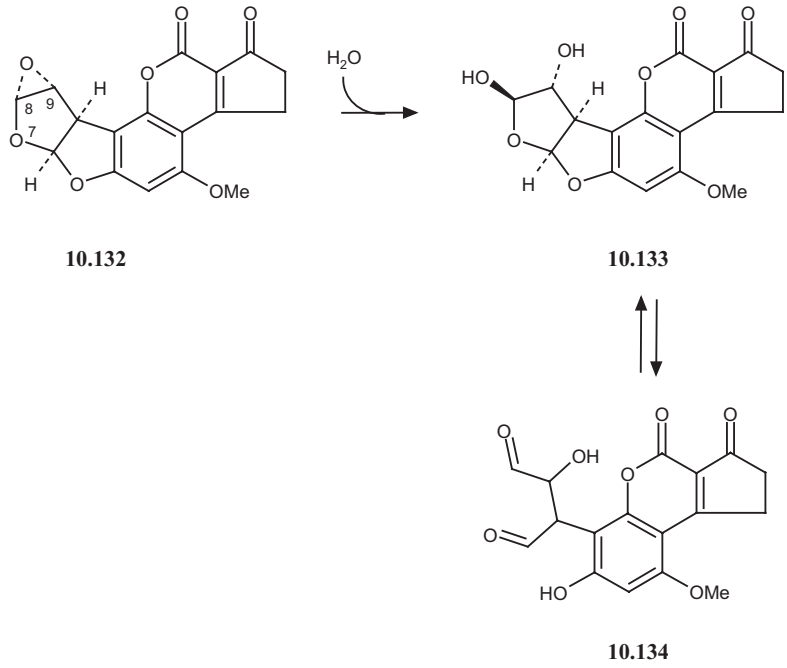


Fig. 10.30. *Structure of aflatoxin B1* exo-*8,9-epoxide* (**10.132**)*, the dihydrodiol resulting from hydrolysis* (**10.133**)*, and the reactive α-hydroxy dialdehyde* (**10.134**) *that exists in equilibrium with the diol under alkaline conditions* [204]

aflatoxin B1 at the C(8)=C(9) bond, mainly by CYP3A4, produces the *endo*-8,9-epoxide as a minor and the *exo*-8,9-epoxide (**10.132**, *Fig. 10.30*) as a major metabolite.

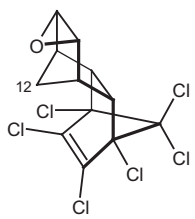
The two isomeric 8,9-epoxides of aflatoxin B1 differ remarkably in reactivity. Whereas the *exo*-8,9-epoxide is highly reactive (as discussed below) and genotoxic, its *endo*-stereoisomer is believed to be *ca.* 40-fold more stable in water, unreactive toward DNA, and essentially non-genotoxic [204]. The observed rate of hydration of aflatoxin B1 *exo*-8,9-epoxide is constant and extraordinarily high ($t_{1/2}$ *ca.* 1 s) in the pH range of 5 – 10, and increases with decreasing pH by more than one order of magnitude between pH 5 and 2. Two mechanisms account for this pH profile, namely H^+- and H_2O-catalyzed hydrolysis. The former mechanism implies protonation of the oxirane O-atom followed by heterolytic cleavage of the C(8)–O bond and addition of H_2O (see *Fig. 10.11*, *Pathway a*). In this mechanism, the (*R*)-configuration at C(9) is preserved, whereas C(8) undergoes partial inversion of configuration. In other words, the resulting product of proton-catalyzed hydrolysis is a mixture of aflatoxin (8*R*,9*R*)-dihydrodiol (**10.133**, *Fig. 10.30*) and its (8*S*,9*R*)-epimer.

In the pH range of 5 – 10, H_2O-catalyzed hydrolysis is the predominant mechanism (see *Fig. 10.11*, *Pathway b*), resulting in the formation of the *(8*R*,9*R*)-dihydrodiol* (**10.133**, *Fig. 10.30*). Thus, aflatoxin B1 *exo*-8,9-epoxide is possibly the most reactive oxirane of biological relevance. Such an extreme reactivity is mostly due to the electronic influence of O(7), as also influenced by stereolectronic factors, *i.e.*, the difference between the *exo*- and *endo*-epoxides. The structural and mechanistic analogies with the dihydrodiol epoxides of polycyclic aromatic hydrocarbons (*Sect. 10.4.4*) are worth noting.

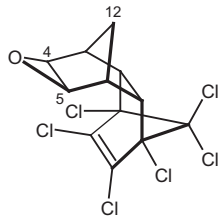
A further remarkable finding in the hydrolysis of aflatoxin B1 *exo*-8,9-epoxide is the relative instability of the dihydrodiol, which under basic conditions exists in equilibrium with an aflatoxin dialdehyde, more precisely a furofuran-ring-opened oxy anionic α-hydroxy dialdehyde (**10.134**, *Fig. 10.30*). The dihydrodiol is the predominant or exclusive species at pH < 7, whereas this is true for the dialdehyde at pH >9, the p*K* value of the equilibrium being 8.2 [204]. The dialdehyde is known to form *Schiff* bases with primary amino groups leading to protein adducts. However, the slow rate of dialdehyde formation at physiological pH and its reduction by rat and human aldo-keto reductases cast doubts on the toxicological relevance of this pathway [206].

The exceptional reactivity of aflatoxin B1 *exo*-8,9-epoxide raises the question of its potential detoxification by EHs. Despite the short half-life, the epoxide does react with DNA (toxification) and glutathione *S*-transferases (detoxification), but a role for EH appeared dubious [207]. Rat liver or recombinant rat EH has since been shown to provide a modest enhancement of up to 22% in the hydrolysis rate of aflatoxin B1 *exo*-8,9-epoxide, and to decrease somewhat the genotoxicity of aflatoxin B1 when the ratio of EH to cytochrome P450 is high (*ca.* 50-fold). Purified human EH provided no such enhancement in hydrolysis, nor did it have a clear effect on genotoxicity. Thus, little evidence exists to support a role for EH in the detoxification of aflatoxin B1 [208].

Our final (and contrasting) examples are dieldrin (**10.135**) and endrin (**10.136**), two isomeric polychlorinated insecticides that contain *exo*-epoxide



10.135



10.136

groups, which are fully stable in water and poor substrates for EH. Indeed, *dieldrin* (**10.135**) incubated with purified EH from rat and rabbit liver microsomes was hydrolyzed extremely slowly, about six orders of magnitude slower than the less hindered homologue, which lacks the CH_2(12) bridge [209]. This appears to contradict the observation that *endrin* (**10.136**) given orally to rats and rabbits did yield small amounts of the *trans*-4,5-diol [210][211]. However, there was evidence for an *oxidative mechanism* in the opening of the 4,5-epoxy group, such that the first step was cytochrome P450 catalyzed hydroxylation of C(4) to produce an unstable geminal diol epoxide. Such a product would immediately rearrange to a 5-hydroxy-4-oxo derivative, which would, in turn, be stereoselectively reduced by an oxidoreductase to the *trans*-4,5-diol.

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Chapter 11

Miscellaneous Reactions of Hydration and Dehydration

Contents

Hydrolysis in Drug and Prodrug Metabolism. Bernard Testa, Joachim M. Mayer

11.1. Introduction

In earlier chapters, we examined metabolic reactions of hydrolysis, which mainly involve ester and amide groups, and hydration of epoxides was presented in *Chapt. 10*. Yet, despite the large variety of reactions and moieties examined in the previous chapters, many reactions remain that do not fit the categories already discussed.

This chapter, therefore, ends the monograph with a *potpourri* of reactions all of which occur without a change in oxidation state. In many cases, the reaction is one of *nucleophilic attack* at an electrophilic C-atom. The result is often *hydrolytic bond cleavage* (*e.g.*, in carbohydrate conjugates, disubstituted methylene and methine groups, imines, oximes, isocyanates, and nitriles, and various ring systems) or a *nucleophilic substitution* (*e.g.*, hydrolytic dehalogenation of halocarbons and chloroplatin derivatives, and cyclization reactions). The formation of multiple bonds by *dehydration* is a special case to be discussed separately.

While some of these reactions are mediated by enzymes, *e.g.*, glucuronidase, which hydrolyze glucuronide conjugates, many others are nonenzymatic. These are driven by the electrophilicity of the substrates, which renders them susceptible to attack by the HO^- anion (specific base catalyzed hydrolysis) or by other bases (general base catalyzed hydrolysis). A semblance of order in such a heterogeneous group of reactions is, thus, far from obvious.

11.2. Hydrolysis of Carbohydrate Conjugates

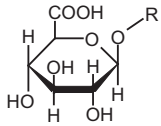
The hydrolysis of carbohydrate conjugates (*i.e.*, glycosyl conjugates) is catalyzed by enzymes classified as glycosidases (EC 3.2), the majority of which are glycosidases that hydrolyze *O*-glycosyl compounds (EC 3.2.1). As far as xenobiotic metabolism is concerned, the most important enzyme is β-glucuronidase (*Sect. 11.2.1* and *11.2.2*), but other enzymes may also be of interest. Enzymes that cleave *N*-glycosyl compounds (*Sect. 11.2.3*) and *S*-glycosyl compounds (EC 3.2.3) may also play a role in xenobiotic metabolism.

Monomeric carbohydrates in their cyclic form (furanoses and pyranoses) are hemiacetals, which, to become acetals, form *O*-glycosyl conjugates. The C-atom C(1) that bears two O-atoms is the reactive, electrophilic center targeted by glycosidases. Nonenzymatic hydrolysis is also possible, although, as a rule, under physiological conditions of pH and temperature, the reaction is of limited significance.

11.2.1. Ether *O*-Glucuronides

β-Glucuronidase (β-D-glucuronoside glucuronosohydrolase, EC 3.2.1.31) is a ubiquitous enzyme localized in the endoplasmic reticulum, in lysozymes, and in blood serum [1 – 4]. The glucuronidase of microflora in the gut plays a particular role in the hydrolysis of glucuronides, which enter the gut *via* biliary secretion. The biology of glucuronidase has been comprehensively reviewed [5].

β-D-*O*-Glucuronides of the ether type (general structure **11.1**, R = alkyl or aryl) are formed in the body as conjugates of endogenous and exogenous alcohols and phenols. *O*-Glucuronides of hydroxylamines (N–OH) and *N*-hydroxylamides (RCON–OH) are also formed and have toxicological significance. Glucuronides are particularly significant in xenobiotic metabolism as so-called phase-II metabolites, *i.e.*, glucuronides are formed subsequent to the oxidation or reduction reaction that generates the OH group to be glucuronidated. Ether glucuronides, which must be distinguished from the ester glucuronides (acyl glucuronides, **11.1**, R = acyl) discussed in *Sect. 11.2.2*, are relatively stable in the pH range of 2 – 8 [6].



11.1

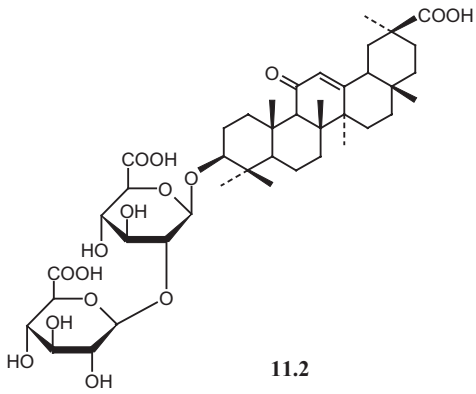
In the paragraphs below, we discuss the occurrence and significance of glucuronidation–deglucuronidation cycling, and go on to examine the hydrolysis of glucuronides of endobiotics, of a natural glucuronide, of xenobiotics prepared and used as metabolic probes, and, lastly, of glucuronide prodrugs.

Glucuronidation, followed by enzymatic hydrolysis of the glucuronide metabolic product to restore the original substrate, is called a glucuronidation–deglucuronidation cycle. Such a cycle is sometimes referred to as *futile cycle*, although the cycle can have clinical or physiological consequences by increasing the apparent half-life of a hormone, drug, or metabolite [7]. This is particularly true in the case of *enterohepatic cycling*, which involves the sequence of events hepatic glucuronidation, biliary excretion, intestinal hydrolysis, and reabsorption. The clinical importance of such an enterohepatic cycle has been convincingly demonstrated: in healthy post-menopausal women dosed orally with *estradiol*, the first absorption phase of estradiol was followed after 1 – 2 h by a second absorption phase that maintained practically constant plasma levels of estradiol for 24 h [8]. Stated differently, exogenous

estradiol and its metabolite estrone followed the circulation route of endogenous estrogen hormones, and, since constant serum levels of the hormone could be maintained, enterohepatic cycling of estrogens is hardly futile.

The formation of glucuronides is a common and frequently investigated pathway of inactivation, detoxification, and elimination of numerous other *endogenous compounds* beside estrogens. In contrast, less is known about the hydrolysis of these conjugates. That such reactions can occur is also documented, for example, by the effective hydrolysis of 3- and 25-monoglucuronides of *25-hydroxyvitamin D_3* and *25-hydroxyvitamin D_2* [9]. But the physiological significance of the glucuronidation–deglucuronidation cycling of endogenous compounds has seldom been elucidated.

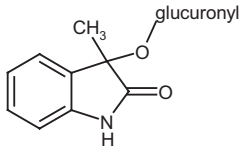
An interesting glucuronide of *plant origin*, in fact a diglucuronide, is *glycyrrhizin* (**11.2**), the well-known sweetener found as a main constituent of liquorice extract. Hydrolysis to the monoglucuronide was observed in all examined subcellular fractions of rat liver [10]. The highest activity was found in lysosomes, but the nuclear, mitochondrial, microsomal, and cytosolic fractions were also active. There was also marked species dependence in the rate of monoglucuronide formation in liver lysosomes, which was fastest in human, and decreased in the sequence pig, rat, mouse, and cattle. Another species difference is in the second hydrolysis reaction, *i.e.*, from the monoglucuronide to glycyrrhetic acid, which is always markedly slower than the first reaction of hydrolysis in rat liver lysosomes, slow in mouse and cattle, and undetectable in humans and other species. The hydrolysis of 4-nitrophenyl β-D-glucuronide was monitored in parallel as a control, and was, in general, severalfold faster than the first hydrolytic step of glycyrrhizin. Globally, two metabolic pathways of glycyrrhizin were seen, one a β-D-glucuronidase-dependent direct hydrolysis to glycyrrhetic acid, and the other consisting of two different β-D-glucuronidases hydrolyzing glycyrrhizin first to its monoglucuronide and the latter to glycyrrhetic acid [11].



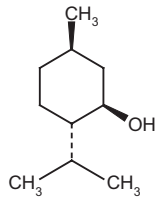
11.2

The hydrolysis of O-*glucuronides of xenobiotics* is a common metabolic pathway with marked substrate selectivity, which is well-documented in the literature. For example, *morphine 3-β-*D-*glucuronide* (the major human metabolite of morphine) is efficiently hydrolyzed in rat intestinal homogenates (rat intestinal fragments plus their content) [12]. This is indicative of the possibility of enterohepatic recycling by the gastrointestinal flora. An active metabolite of morphine, namely *morphine 6-β-*D-*glucuronide* (M6G), underwent little hydrolysis in various physiological compartments of the rat, with the exception of the colon [13], indicating good oral bioavailability.

Very few ether glucuronides are resistant to glucuronidase, as shown for the *diastereoisomeric* O-*glucuronides of 3-hydroxy-3-methyloxindole* (**11.3**) [14]. These conjugates arise from metabolic oxidation and conjugation of 3-methyl-1*H*-indole, a pneumotoxin. For unknown reasons, the two glucuronides were resistant to bovine liver β-glucuronidase, but steric and electronic shielding by the Me and C=O groups, respectively, may be evoked as possible explanations.



11.3



11.4

An aspect of glucuronide hydrolysis heavily investigated in connection with *prodrug design* [15] is the search for glucuronide prodrugs that would undergo tissue-selective hydrolysis and delivery. Given that certain tissues, *e.g.*, tumors and the intestine, contain relatively high levels of glucuronidase activity, development of tissue-targeted glucuronide prodrugs is a rational strategy. Thus, the alkylating agent *aniline mustard* was coupled to glucuronic acid, and some correlation between glucuronidase activity and tumor regression was found [16]. However, clinical results were disappointing, seemingly due to loss of glucuronidase as a mechanism of resistance. Better *in vivo* results may be obtained with glucuronide prodrugs of *anthracycline*, which proved highly cytotoxic in human melanoma cells [17]. In another project, the metabolic deglucuronidation of *estradiol* and *estrone 3β-*D-*glucuronide* was carefully examined (see also above). These prodrugs were shown to be important sources of primary estrogens and catecholestrogen metabolites in hamster kidney, a target of estrogen-induced tumorigenesis [18].

Perhaps the most promising application of glucuronide prodrugs is targeting the intestine in the treatment of ulcerative colitis and irritable-bowel syn-

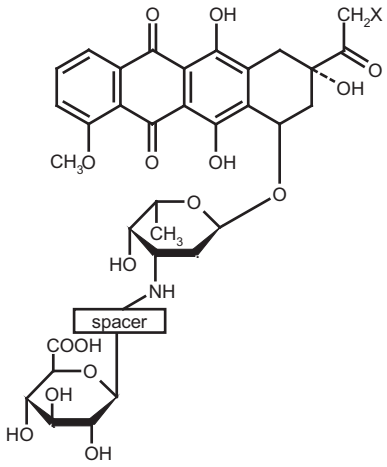
drome with menthol β-D-glucuronide. Peppermint oil, of which *(–)-menthol* (**11.4**) is the main constituent, has the ability to control spasms and motility in the large intestine, but its activity in that tissue is limited by rapid absorption upstream in the small intestine. Menthol β-D-glucuronide was, therefore, examined as a prodrug for the colon-specific delivery of menthol [19]. The glucuronide was stable at pH 4.5 – 7.4, but underwent *ca.* 20% hydrolysis in 4 h at pH 1.5 and 37°, calling for enteric coating to prevent breakdown in the stomach. The conjugate was also stable in the presence of luminal contents of rat stomach and small intestine, but was readily hydrolyzed by the glucuronidase-rich microflora of the luminal contents of rat colon and caecum. Hydrolysis was lower but evident in human stool samples. These studies suggest that menthol glucuronide may, indeed, be a useful prodrug that is able to bypass the rapid intestinal absorption of menthol for release in the colon.

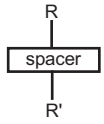
The same strategy was applied in colon-specific delivery of two *corticosteroids* used to treat inflammatory bowel disease [20][21]. Indeed, budesonide β-D-glucuronide and dexamethasone β-D-glucuronide underwent ready hydrolysis in the luminal contents of rat colon and caecum. Rat mucosal homogenates were less active, and hydrolysis in human fecal samples was quite low. Based on these and other studies, the prodrugs were found to be suitable candidates for delivery of corticosteroids to the large intestine.

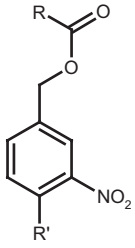
In *Chapt. 8*, we presented the therapy approach known as *ADEPT* (antibody-directed enzyme prodrug therapy), in which a tumor-specific antibody–enzyme conjugate is administered, followed, after a given time interval, by a relatively nontoxic prodrug. The latter is a specific substrate for the antibody-bound enzyme, and activation in the immediate vicinity of the tumor releases the cytotoxic agent. The administration of human lysosomal β-glucuronidase antibody conjugate appears promising. In *in vitro* studies, a number of glucuronides of antitumor antibiotics were prepared and examined for activation by human glucuronidase [22][23]. At pH 6.8 and 37°, the prodrug **11.5** (X = OH, *doxorubicin-nitrophenyl-glucuronide*, an ether glucuronide) was an excellent substrate as judged by the k_{cat}/K_m ratio. This compound, like ester glucuronides of daunorubicin to be discussed in the next subsection, were hydrolyzed by glucuronidase to release glucuronic acid, followed by hydrolysis of the carbamate ester bond to cleave the spacer and the drug.

11.2.2. Acyl Glucuronides and Other *O*-Glycosides

Acyl glucuronides (*i.e.*, ester glucuronides of general structure **11.6**) are formed as conjugates of carboxylic acids. These compounds are rather uniquely reactive: *a*) they are far more sensitive to chemical hydrolysis than ether







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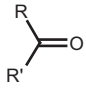

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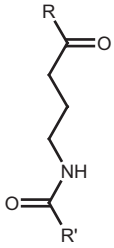
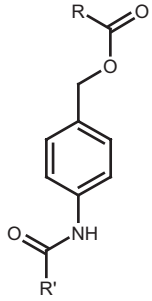


11.10



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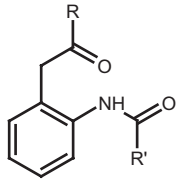

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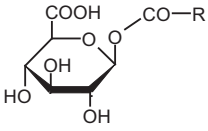

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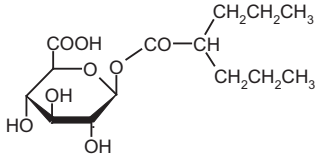


11.7

glucuronides at neutral and alkaline pH, *b*) they are sensitive to enzyme hydrolysis, as are ether glucuronides, *c*) they undergo intramolecular migration of the 1-acyl moiety from C(1) to C(2), C(3), and C(4) (*Fig. 11.1*), and *d*) they binding covalently to macromolecules *in vitro* and *in vivo* [24 – 26].

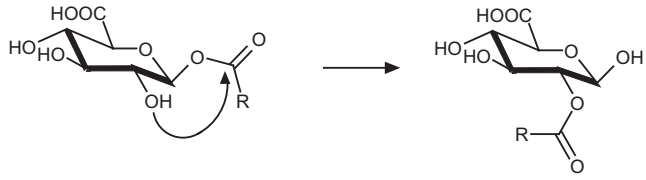
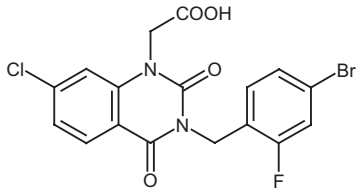


Fig. 11.1. *Regio-isomerization of acyl glucuronides.* Migration of the β-1-acyl group from C(1) to C(2) by intramolecular nucleophilic attack. Migration can continue to C(3) and then C(4). Such reactions are usually competitive with chemical hydrolysis [25][26].

Valproate glucuronide (**11.7**) is an important metabolite of valproic acid. Like for morphine 3-glucuronide (see above), convincing evidence exists that it is readily hydrolyzed by intestinal flora and in other organs to liberate the parent drug [12][27]. A number of more-general studies have also been published on the *nature of the enzymes* that act on acyl glucuronides. For example, human serum albumin (HSA) decreased the rate of hydrolysis of tolmetin glucuronide, but increased the rate of hydrolysis of oxaprozin glucuronide [26]. Studies with ketoprofen and carprofen glucuronides that support the existence of distinct sites on HSA for hydrolysis (*i.e.*, the esterase-like activity of HSA) and reversible binding (which protects the conjugate from degradation in the serum) have increased our understanding of this phenomenon, [28][29].

Involvement of *tissue esterases* is documented for zomepirac glucuronide, which undergoes rapid hydrolysis *in vivo* in the guinea pig and rabbit [30]. Co-administration of (phenylmethyl)sulfonyl fluoride (an inhibitor of esterases) dramatically decreased the apparent plasma clearance of zomepirac glucuronide, and, in other experiments, strongly increased the apparent plasma clearance of zomepirac administered as such.

A quite comprehensive study was reported on the enzymatic hydrolysis of *zenarestat acyl glucuronide* [31]. The major metabolite of the aldose reductase inhibitor zenarestat (**11.8**) in humans and rats is the acyl glucuronide. This compound was unstable at physiological pH, being converted to its positional isomers and to the aglycone with a global $t_{1/2}$ value of 25 min at pH 7.4 and 37°. In both rat and human blood, hydrolysis was accelerated by the



11.8

presence of serum albumin. In the rat, carboxylesterases from red blood cell cytosol also catalyzed the reaction, as did hepatic and renal β-glucuronidase and arylesterases.

The anti-inflammatory drug *diflunisal* provides an interesting comparison of the hydrolysis of ether and ester glucuronides. This compound contains both phenolic and carboxylic acid moieties, both of which are glucuronidated *in vivo*. A study in the rat in which D-glucaro-1,4-lactone was used to inhibit glucuronidase showed that the enzyme catalyzed the hydrolysis of the acyl glucuronide, but not detectably that of the ether glucuronide [32]. Given the bioreactivity of some acyl glucuronides [33], this effective glucuronidase-catalyzed hydrolysis was considered to have protective value.

Four ester glucuronides of the type *daunorubicin–spacer–glucuronide* (**11.9** – **11.12**, X = H) were investigated as part of the same work presented in the previous subsection on an ether glucuronide derivative of doxorubicin [22]. At pH 6.8 and 37°, the prodrugs **11.11** and **11.12**, which have an aromatic spacer, were as good as **11.5** as substrates, whereas prodrugs **11.9** and **11.10** underwent only very slow activation [22]. These results are interesting in that the potential of the drug–spacer–glucuronide motif is demonstrated along with the great sensitivity of its glucuronidase-catalyzed activation to structural variations.

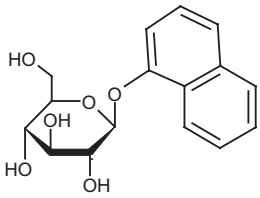
Besides *O*-glucuronides, a number of other O-*glycosides* are potential substrates for hydrolysis mediated by glycosidases (EC 3.2.1). These xenobiotics, and, in a few cases, metabolites thereof, include conjugates of glucose (glucosides), rhamnose (rhamnosides), ribose (ribosides), and many others.

O-*Glycosyl compounds*, in particular glycosides of anthocyanins, anthraquinones, benzoic acids, chalcones, chromones, cinnamic acids, coumarins, flavones, isoflavones, lignans, xanthones, *etc.*, are important natural products. Many of these conjugates are present in foods. Other naturally occurring *O*-glycosyl compounds are used as drugs in purified form (*e.g.*, cardiac glycosides) or are the active agents in herbal extracts (*e.g.*, *Ginseng* preparations) [34]. Whereas, in a number of cases, metabolic hydrolysis to the aglycone is documented, few studies have addressed basic enzymological aspects such as the nature and distribution of enzymes, the kinetics of the reactions, and structure–metabolism relationships.

One particularly telling example of the enzymes involved in *O*-glycosyl cleavage is seen in the metabolism of *digoxin* (digoxigenin tris-digitoxoside). This well-known cardiac glycoside is excreted mostly unchanged in humans, but undergoes stepwise cleavage in rats to yield digoxigenin bis-digitoxoside, digoxigenin mono-digitoxoside, and digoxigenin. Contrary to what one would expect, these cleavage reactions are not hydrolytic but occur by an oxidative mechanism catalyzed by cytochrome P450 3A [35]. Ginsenosides, on

the other hand, have been shown to undergo enzymatic hydrolysis, in particular by intestinal bacteria [36].

An interesting model compound, *1-naphthyl glucoside* (**11.13**), is excreted after oral administration to mice as hydrolysis products (*i.e.*, *ca.* 3% free 1-naphthol, and *ca.* 20 – 25% sulfate and glucuronide conjugates), with *ca.* 50% of the dose unchanged [37]. Another example is the hydrolysis of *arbutin* (**11.14**), the *O*-glucoside conjugate of hydroquinone contained in some herbal medicines used as urinary antiseptics. Under the relatively acidic conditions prevailing in urine, the active hydroquinone is liberated. In a sense, arbutin can be considered a natural prodrug that undergoes site-selective activation.



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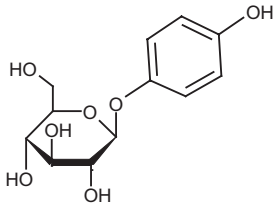
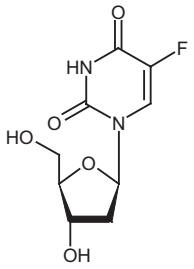


11.14

11.2.3. *N*-Glycosides

Synthetic N-*glycosyl compounds* are currently of great medicinal interest given their potential value as antiviral and antitumor agents. These compounds are analogues of endogenous nucleosides that might act as substrates for hydrolases of endogenous nucleosides. Two groups of enzymes that hydrolyze *N*-glycosyl compounds have been described, namely the *glycosidases* (EC 3.2.2) that yield a nucleobase and a pentose, and *pentosyltransferases* (EC 2.4.2) that yield a nucleobase and a phosphorylated pentose. Relevant examples in the first group are purine nucleosidase (EC 3.2.2.1), uridine nucleosidase (EC 3.2.2.3), and adenosine nucleosidase (EC 3.2.2.7). The second group includes purine phosphorylase (EC 2.4.2.1), pyrimidine phosphorylase (EC 2.4.2.2), uridine phosphorylase (EC 2.4.2.3), and thymidine phosphorylase (EC 2.4.2.4).

A few examples of C–N bond cleavage of chemotherapeutic *N*-glycosides agents are presented here. Isolated perfused rat lung and rat lung slices exhibited high capacity to cleave the cytostatic agent *5-fluoro-2′-deoxyuridine* (**11.15**), with 5-fluorouracil (**11.16**) being by far the major metabolite [38]. This reaction is catalyzed by thymidine phosphorylase. 5-Fluorouracil is also used as an antitumor agent, but, like 5-fluoro-2′-deoxyuridine, it must be activated to the nucleotide before it can block DNA synthesis. The phosphorol-



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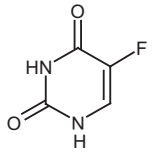

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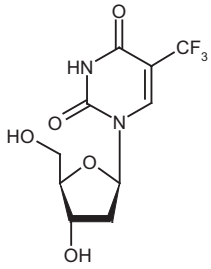
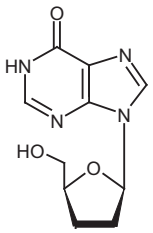


11.17

ysis of 5-fluoro-2′-deoxyuridine to 5-fluorouracil may, thus, be seen, therapeutically speaking, as a catabolic step in the wrong direction, but issues such as transport processes and site-specific activation afford counter-arguments.

The metabolic fate of *trifluridine* (**11.17**), an antiviral agent closely related to 5-fluoro-2′-deoxyuridine, was comparable. The main metabolite in the urine of mice administered the drug was the free 5-trifluorothymidine, *i.e.*, the product of C–N bond cleavage [39]. All other metabolites were products of further biotransformation of trifluorothymidine.

In analogy with the two analogues of pyrimidine nucleosides presented above, some purine nucleoside analogues are also known to undergo metabolic cleavage under various conditions and to various extents. *Didanosine* (**11.18**), *e.g.*, is an HIV drug of which *ca.* 50 or 60% of a dose is metabolized in dogs after *i.v.* or oral administration, respectively [40]. In each of the four metabolites identified (hypoxanthine, xanthine, uric acid, and allantoin; one metabolite was not identified), the dideoxyribosyl moiety is lost. Given that the total dose was fully accounted for, it appears that the primary metabolic step of didanosine is (almost) exclusively cleavage of the dideoxyribosyl moiety. The small difference in the extent of biotransformation after *i.v.* or oral administration suggests that metabolism in the intestine is marginal.



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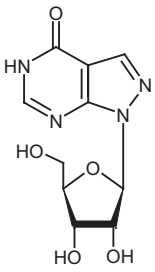

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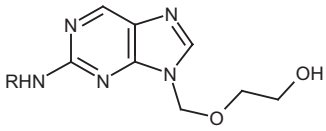
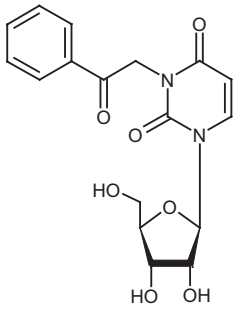


11.20

By contrast, when *allopurinol riboside* (**11.19**), a metabolite of allopurinol and an antiparasitic agent, is administered orally to humans, the drug is incompletely absorbed. The residual fraction is then extensively metabolized by the enteric flora to produce metabolites that are absorbed [41]. Bacterial metabolism proceeds by cleavage to allopurinol, a reaction that apparently occurs only in the intestine. Although the examples described pertain to different animal species, it is clear that small structural changes can elicit major differences in metabolism.

Synthetic conjugates that are quite different in molecular structure from the nucleoside analogues presented above include potential prodrugs of *desciclovir* (**11.20**, R = H), which is, itself, a prodrug of the antiherpes drug acyclovir [42]. A number of synthetic *N*-glycoside conjugates of desciclovir (**11.20**, R = *N*-D-glucosyl, *N*-D-galactosyl, *N*-D-mannosyl, *N*-L-rhamnosyl, *etc*.) were evaluated as double prodrugs of the antiherpes agent acyclovir. However, only limited amounts (usually < 10% of dose) of acyclovir were recovered in the urine of rats given an oral dose of any of these *N*-glycoside conjugates. Given the fast activation of desciclovir to acyclovir, these results demonstrate the metabolic stability of the N–C bond in these *N*-glycoside conjugates.

Cleavage of the N–C bond is also relevant in the metabolism of a few *CNS agents*. Thus, metabolism of the potential hypnotic N^3*-phenacyluridine* (**11.21**) occurred by two major routes in mice; carbonyl reduction was the predominant reaction, but *N*-deribosylation to yield N^3-phenacyluracil was also important [43]. Given the intraperitoneal (*i.p.*) mode of administration, it is doubtful that intestinal bacteria were involved.



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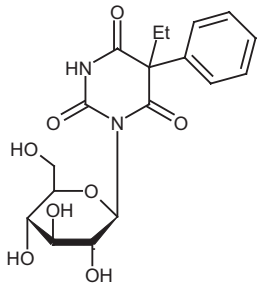

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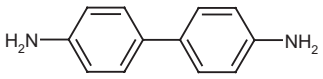

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Phenobarbital, the next example to be presented here, is biologically very different from the above synthetic *N*-glycosides. Phenobarbital is known to undergo a rather rare reaction of conjugation in humans and mice, namely *N*-glucosylation. The stereoselectivity of formation of the product *phenobarbital* N-*glucoside* (**11.22**) is species specific, with different epimers being formed in the two species. Of interest here is that, following *i.p.* administration of phenobarbital *N*-glucoside to mice, phenobarbital and *para*-hydroxyphenobarbital were excreted in the urine [44]. The glucoside was unstable under neutral and basic conditions, but stable in the slightly acidic urine. Thus, hydrolysis must have occurred in blood and/or tissues, but whether by an enzymatic or nonenzymatic route is not yet firmly established.

Earlier in this chapter, we discussed formation of ether *O*-glucuronide (*Sect. 11.2.1*) and ester *O*-glucuronide (*Sect. 11.2.2*) metabolites. The fate of *N*-glucuronide metabolites has not yet been explored in a systematic manner. A recent study of bacterial (*E. coli*) and recombinant human β-glucuronidases is, therefore, of particular interest [45]. The substrates investigated were *N*-glucuronides of benzidine (**11.23**) and closely related analogues, and the *O*-glucuronides of 3-hydroxybenzidine, *N*′-hydroxy-*N*-acetylbenzidine, and closely related analogues. The results clearly showed that both enzymes preferentially hydrolyze *O*-glucuronides over *N*-glucuronides; the bacterial enzyme is, in fact, unable to hydrolyze the latter.

11.3. Hydrolytic Cleavage of Benzhydryl Ethers

Ethers are stable toward hydrolysis, yet are hydrolyzed under severe conditions of acidity and temperature. Furthermore, the presence of certain moieties can decrease the high stability of ethers. In the case of aminoalkyl benzhydryl ethers of the general structure $(aryl)_2CH–O–(CH_2)_n–NRR'$, the decrease in stability becomes marked enough to be of pharmaceutical and even pharmacological relevance. This structural motif is a component of various drugs, including some well-known antihistamines and anticholinergics, *e.g.*, diphenhydramine, orphenadrine, and chlorphenoxamine.

The hydrolysis of *diphenhydramine* and analogues (**11.24**, *Fig. 11.2*) has been studied extensively [46 – 48]. These compounds are essentially inert toward base-catalyzed hydrolysis, but do undergo *proton-catalyzed hydrolysis*, the mechanism of which is shown in *Fig. 11.2*. The reaction begins with protonation of the ether O-atom and continues with the irreversible heterolytic cleavage of the C–O bond to produce the benzhydryl cation. This reaction is greatly facilitated by the weakening effect of the benzhydryl moiety on the adjacent C–O bond. The benzhydryl cation itself is stabilized by resonance, which also explains why the reaction is facilitated. The last step is the for-

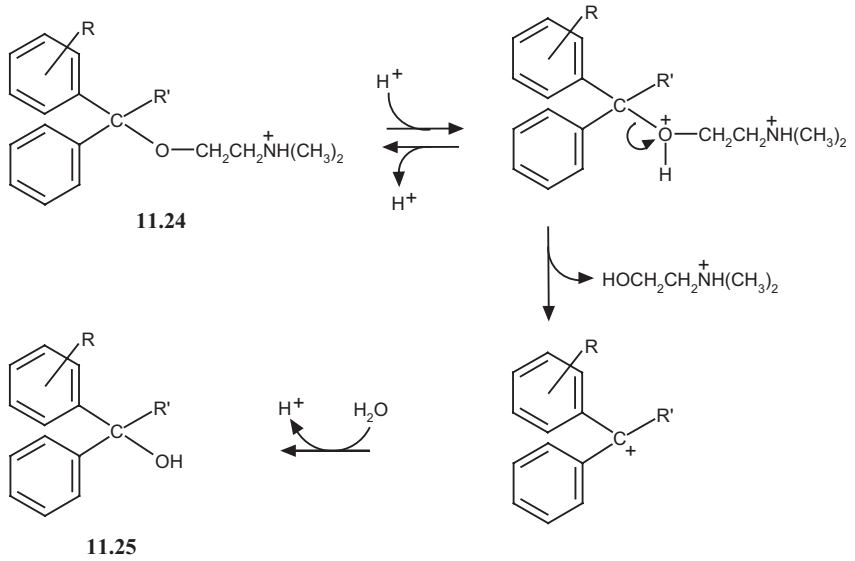


Fig. 11.2. *Mechanism of proton-catalyzed hydrolysis of aminoalkyl benzhydryl ethers* **11.24**, e.g., *diphenhydramine* (R = R′ = H), *orphenadrine* (R = 2-Me, R′ = H), *and chlorphenoxamine* (R = 4-Cl, R′ = Me)

mation of the corresponding benzhydrol (**11.25**) from the benzhydryl cation and H_2O.

Detailed kinetics investigations have shown that the reaction follows pseudo-first-order kinetics. A linear relationship exists between pH and log *k* (the log of the rate constant), such that log *k* decreases by 1.7 (*i.e.*, $t_{1/2}$ increases by a factor of *ca.* 50) with each increase of one pH unit. For example, the $t_{1/2}$ value of *diphenhydramine* (**11.24**, R = R′ = H, *Fig. 11.2*) and *orphenadrine* (**11.24**, R = 2-Me, R′ = H) at pH 0 and 25° were found to be 550 and 460 min, respectively, from which $t_{1/2}$ values of *ca.* 460 and 360 h could be calculated for pH 1. Each increase of 10° in temperature led to a decrease in the $t_{/2}$ value of 3 – 4 h. Hence the $t_{1/2}$ value of diphenhydramine and orphenadrine in the stomach at 37°, assuming pH 1 and neglecting any effect caused by ionic strength, should be *ca.* 4 d. This is clearly too slow for any significant nonenzymatic formation of benzhydrol in the body (see below).

Structure–reactivity relationships have been established for a variety of analogues [47]. For example, a 4-Cl substituent (**11.24**, R = 4-Cl, R′ = H) increased the $t_{1/2}$ value by a factor of 1.5 relative to diphenhydramine. As a rule, the influence of ring substituents was related to their *Hammett* σ constant, such that the greater the electron-withdrawing capacity, the greater the stability. In contrast, electron-donating ring substituents increased reactivity by stabilizing the benzhydryl cation. The same mechanism prob-

ably accounts for the increased reactivity of analogues that carry a Me group at the benzhydryl C-atom (**11.24**, R = H, R′ = Me; $t_{1/2}$ decreased by a factor of *ca.* 25).

From these data, it can be estimated that *chlorphenoxamine* (**11.24**, R = 4-Cl, R′ = Me) should hydrolyze *ca.* 17 times faster than diphenhydramine. This decreased stability appears sufficient to drive formation of detectable amounts of the benzhydrol metabolite (**11.25**, R = 4-Cl, R′ = Me) in the stomach of patients dosed with chlorphenoxamine. Indeed, ether bond cleavage to form this and derived metabolites was a major pathway in humans [49]. Whether the reaction was entirely nonenzymatic or resulted in part from oxidative *O*-dealkylation (Chapt. 7 in [50]) remains unknown.

Clemastine, an antihistamine closely related to chlorphenoxamine in which the dimethylamino group is replaced by 1-methylpyrrolidin-2-yl, appears to be comparable in stability to chlorphenoxamine. Indeed, cleavage of the ether was the dominant feature of metabolism in humans [51][52], and, in fact, all metabolites recovered in humans were either the benzhydrol analogue or products of its further biotransformation. Here again, the ether cleavage pathway may be entirely or only partly nonenzymatic, although the extent of the reaction suggests an enzymatic contribution.

In contrast to chlorphenoxamine and clemastine, ether cleavage is a minor metabolic reaction in orphenadrine, since urinary 2-methylbenzhydrol accounted for only 8 – 9% of the metabolism of a human dose [53]. No such metabolite seems to be formed from diphenhydramine [54]. Taken globally, these results suggest that cytochrome P450 catalyzed ether cleavage is modest in drugs having the structure **11.24** (*Fig. 11.2*), and that nonenzymatic hydrolytic cleavage is of metabolic significance for only the most labile of these drugs.

11.4. Hydrolytic Dehalogenations

11.4.1. Mechanistic Considerations

The *dehalogenases* (EC 3.8.1), a subclass of the hydrolases that act on the halide bonds in C-halide compounds, catalyze reactions of *hydrolytic dehalogenation* (*Fig. 11.3,a)*, *i.e.*, the replacement of a halide atom at a sp^3 C-atom with a OH group. Exceptions include thyroxine deiodinase (EC 3.8.1.4), which catalyzes reductive deiodination on phenyl rings, and the bacterial 4-chlorobenzoate dehalogenase (EC 3.8.1.6), which forms 4-hydroxybenzoate.

Mechanistic information on the catalytic cycle of hydrolytic dehalogenation has been obtained from crystallographic studies of bacterial enzymes,

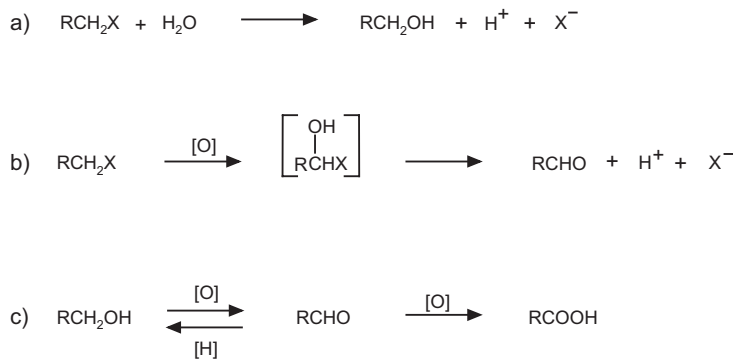


Fig. 11.3. *Comparison of* a) *hydrolytic dehalogenation and* b) *oxidative dehalogenation.* The products of *Reaction a* (an alcohol) and *Reaction b* (a carbonyl) may be interconverted by dehydrogenation/hydrogenation (*Reaction c*). When these products are a primary alcohol and an aldehyde, further oxidation to the acid is possible (*Reaction c*).

specifically the *haloalkane dehalogenase* (EC 3.8.1.5) isolated from the nitrogen-fixing bacterium *Xanthobacter autotrophicus* (*Fig. 11.4*) [55]. In the first step, the substrate 1,2-dichloroethane is bound at the active site with C(1) in the proximity of an aspartate residue (Asp^{124}) with the departing Cl-atom interacting with the NH moieties of Trp^{125} and Trp^{175} (*Fig. 11.4*, *Box a*). Nucleophilic attack of Asp^{124} at C(1) follows, resulting in the formation of an ester bond and the departure of Cl^-, which remains attached to the two Trp residues (*Box b*). Activation of a H_2O molecule (*Box c*) cleaves the ester bond (*Box d*). The product (2-chloroethanol) is liberated first (*Boxes d* → *e*), followed by Cl^-. The kinetics and specificity of the enzyme have been further elucidated [57].

The relevance of this mechanism to mammalian enzymes is an important question, but we are not aware of any detailed study that affords a definitive answer. Proof that reactions of *hydrolytic dehalogenation of haloalkyl groups* occur in animals is presented in the next subsection, but much remains to be discovered regarding the enzymes involved or the reaction mechanisms. Furthermore, *nonenzymatic reactions* remain a distinct possibility when the C-atom bearing the halogen is sufficiently electrophilic, as seen, *e.g.*, with (2-chloroethyl)amino derivatives (see *Sect. 11.4.2*).

Another reaction of dehalogenation, the *oxidative dehalogenation of haloalkyl groups*, summarized in *Fig. 11.3,b* (Chapt. 8 in [50]), has also been observed in mammals and other organisms. Here, the haloalkane is oxidized by a cytochrome P450 enzyme to form a hydroxylated intermediate that loses HX to become a carbonyl derivative. The latter is then reduced by dehydrogenases to the corresponding alcohol (*Fig. 11.3,c*), or, when the carbonyl derivative is an aldehyde, oxidation to the acid can occur (*Fig. 11.3,c*).

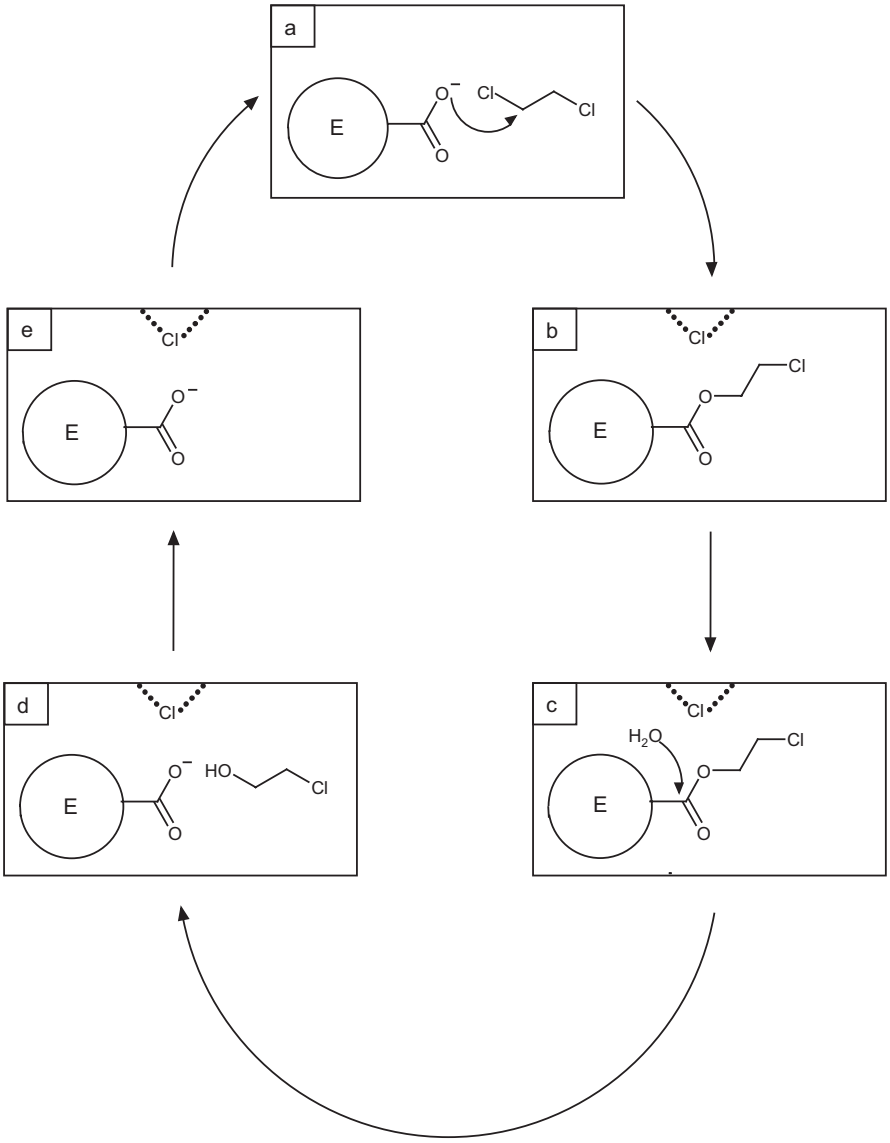


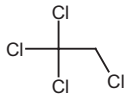
Fig. 11.4. *Catalytic cycle of the haloalkane dehalogenase of* Xanthobacter autotrophicus (modified from [56])

This reaction should not be confused with hydrolytic dehalogenation, despite apparent similarities [58]: although both hydrolytic and oxidative dehalogenation routes may produce the alcohol and carbonyl derivatives, the product that is formed as the primary or secondary metabolite is different in the two pathways. Further, it is clear that the enzymes involved cannot be identical.

Another type of dehalogenation occurs in xenobiotic metabolism, namely *halide elimination with cyclization*. These intramolecular nucleophilic substitutions are discussed in *Sect. 11.4.2* (nitrogen mustards) and *11.8*.

11.4.2. Monodehalogenation Reactions

The metabolism of *1,1,1,2-tetrachloroethane* (**11.26**), a representative aliphatic chlorohydrocarbon, provides an example of mechanistic ambiguity. When administered to mice, *ca.* 40 – 45% of the dose was excreted unchanged in the expired air, whereas *ca.* 30% and 4% of the dose was excreted in urine and feces as 2,2,2-trichloroethanol and 2,2,2-trichloroacetic acid, respectively [59]. Both *Pathways a* and *b* in *Fig. 11.3*, together with the redox reactions shown in *Fig. 11.3,c*, can explain these results.



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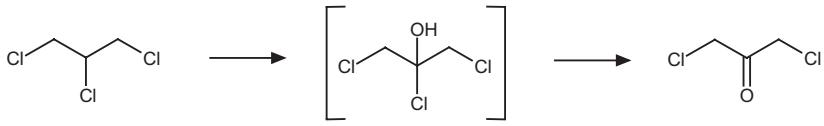
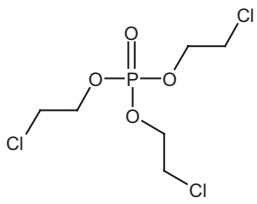


11.27 **11.28** **11.29**



11.30

The metabolism of *1,2,3-trichloropropane* (**11.27**), an industrial solvent that undergoes biotransformation *via* dechlorination at C(1) and C(2) [60], is a clearer case of oxidative dehalogenation. Following incubation with human or rat liver microsomes, the compound was converted to 1,3-dichloroacetone (**11.29**), which could *a priori* be produced by oxidative dehalogenation (*i.e.*, *via* **11.28**) or by hydrolytic dehalogenation. In this study, evidence was found

that the actual route is the oxidative one, given that pretreatment with β-naphthoflavone, a strong inducer of cytochrome P450, led to a 24-fold increase in formation of 1,3-dichloroacetone (**11.29**). Addition of alcohol dehydrogenase and NADH resulted in the reduction of **11.29** to 1,3-dichloropropan-2-ol, the corresponding alcohol. Another alcohol, 2,3-dichloropropan-2-ol, was also detected after addition of alcohol dehydrogenase and NADH, indicating minor dechlorination of the terminal C-atom, most likely also by an oxidative mechanism. It is also of interest to compare the metabolism of these saturated chloro alkanes with that of the chloro alkenes discussed in *Sect. 10.5*.

Tris(2-chloroethyl) phosphate (**11.30**), a flame retardant, also undergoes monodehalogenation in rats and mice [61]. All evidence is compatible with oxidative dehalogenation to an intermediate aldehyde, which, in turn, accounts for the CNS toxicity of the compound.

The above evidence should not be interpreted as meaning that hydrolytic dehalogenation can never be demonstrated unambiguously. Indeed, the next subsection will document reactions of multiple dehalogenation that are only consistent with a hydrolytic mechanism. But before doing so, we examine here the hydrolysis of medicinal nitrogen mustards and of their sulfur analogue, the infamous sulfur mustard.

Nitrogen mustards ((2-chloroethyl)amino derivatives, **11.31**, *Fig. 11.5*) are antitumor agents that act by DNA alkylation. This reaction is initiated by intramolecular nucleophilic substitution to form a highly electrophilic aziridinium species (see below), which reacts with nucleophiles such as nucleic acids (the target), H_2O, glutathione (see below), and phosphate, among others. Nitrogen mustards can undergo a variety of metabolic reactions depending on chemical structure and conditions, as documented, for example, with chlorambucil and cyclophosphamide [62 – 64]. In the context of this chapter, the paragraphs below focus mostly on the transformation of 2-chloroethyl into 2-hydroxyethyl derivatives (*Fig. 11.5*). The nonenzymatic and hydrolytic nature of this reaction has been demonstrated in a number of studies.

The reactivity of the agent *mechlorethamine* (**11.31**, R = Me, *Fig. 11.5*) was investigated in buffer solution by means of NMR to monitor the formation of primary, secondary, and tertiary products [66]. The reactive aziridinium derivative (**11.32**) mentioned above and resulting from intramolecular nucleophilic substitution was indeed observed and underwent hydrolysis first to the 2-hydroxyethyl derivative and then to *N*-methyl-2,2′-iminodiethanol.

The reactivity of mechlorethamine was also examined in the presence of *reduced glutathione* (L-glutamyl-L-cysteinylglycine), a major cytoprotective compound present throughout the body in physiologically high concentra-

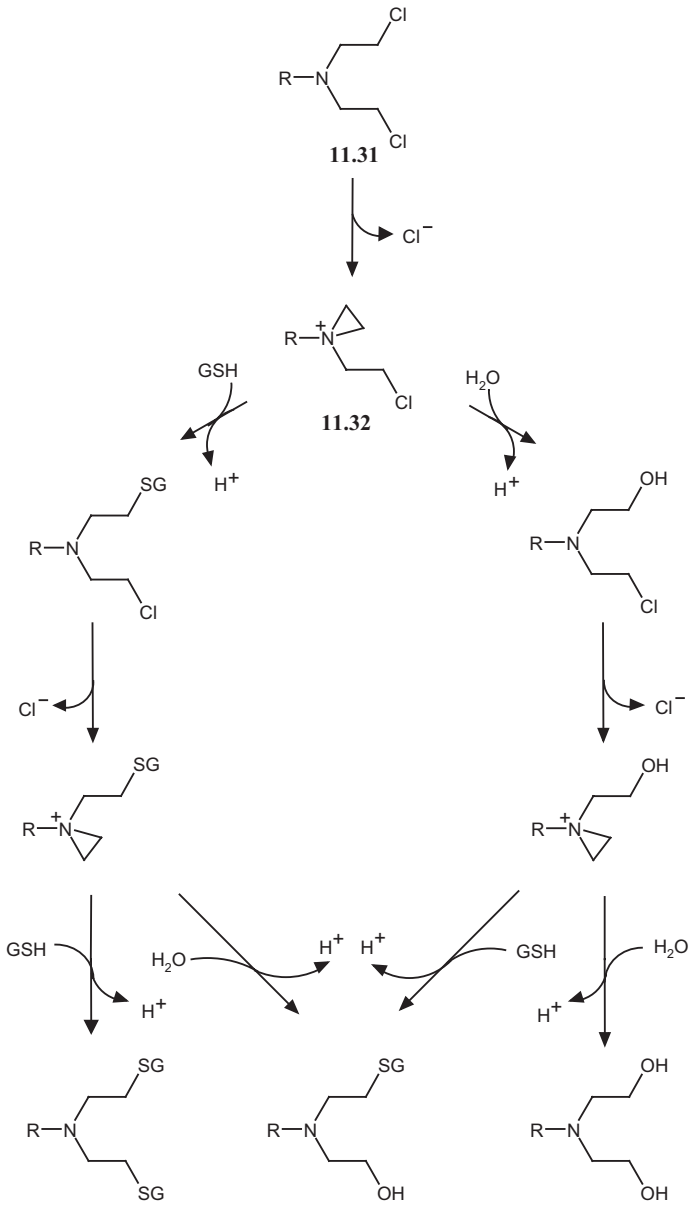
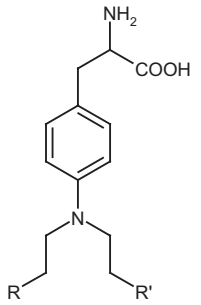


Fig. 11.5. *Mechanism of hydrolytic dechlorination of mechlorethamine* (**11.31**, R = Me) *and other antitumor nitrogen mustards* [65][66]. Also shown is the mechanism of the nonenzymatic conjugation with reduced glutathione (GSH, L-glutamyl-L-cysteinylglycine).

tions. As shown in *Fig. 11.5*, glutathione also reacts very efficiently with the aziridinium intermediate to yield mono- and diconjugates. Thus, reaction with glutathione (alone or catalyzed by glutathione *S*-transferases) offers another route for nonoxidative dechlorination, but such a reaction of conjugation is outside the scope of the present volume. The general occurrence of the reaction of glutathione with nitrogen mustards is well-known (*e.g.*, [67]).

The drug *melphalan* (phenylalanine mustard, **11.33**, R = R′ = Cl) is another good example of a nitrogen mustard that undergoes hydrolytic dechlorination. Melphalan administered to cancer patients gives rise to the monohydroxy (**11.33**, R = Cl, R′ = OH) and dihydroxy metabolites (**11.33**, R = R′ = OH), which were detected in the plasma with a combined AUC (area-under-the-curve) amounting to 29% of the AUC of the drug [68].



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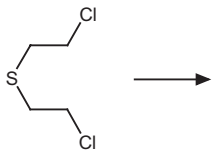

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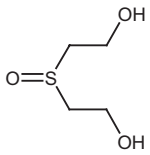


11.35

That the metabolism of melphalan occurs by the same reaction mechanism as that of mechlorethamine has been demonstrated in *in vitro* studies [65]. Under physiological conditions of temperature and pH, formation of the first and second aziridinium intermediates *en route* to the bis(hydroxyethyl) metabolite occurred with rate constants of *ca.* 0.017 and 0.041 min^{-1}, respectively. After 60 min, *ca.* 2/3 of the drug had been converted to the monohydroxy and dihydroxy products in comparable amounts. In the presence of a phosphate buffer, competition between hydrolysis and phosphatolysis was seen, such that at completion of the reaction (4 h) the two major products were the dihydroxy and the hydroxy/phosphate metabolites, with the dihydroxy derivative produced in small amounts. Similar hydrolytic dehalogenation has also been observed for ifosfamide in acidic aqueous solution [69].

That mostly electronic factors apparently control the rate of hydrolytic C–Cl bond cleavage is suggested by the reactivity of *N*,*N*-bis(2-chloroethyl)arylamines (**11.31**, R = substituted aryl, *Fig. 11.5*) [70]. A statistically meaningful negative correlation was found between the rate of hydrolysis and the electron-withdrawing capacity of the substituent on the aromatic ring, as assessed by the *Hammett* σ-substituent constant. This finding, that the

greater the electron-withdrawing effect, the slower the hydrolysis, may be interpreted to mean that the weaker the basicity of the N-atom, the smaller its capacity to act as an electrophile in the formation of the aziridinium intermediate.

Sulfur mustard (**11.34**), a chemical warfare agent first deployed at Ypres during World War I (hence the name yperite), persists in the environment for long after spreading. This mustard is a terrible vesicant and biological alkylating agent that is highly corrosive to internal mucosa. Following *i.p.* administration to rats, *ca.* 60% of the dose was excreted in the urine collected for 24 h [71]. The main route was clearly glutathione conjugation, but marked amounts were also recovered as the hydrolysis product (*i.e.*, the dihydroxy analogue) and the sulfoxide metabolite thereof (**11.35**). Comparable observations were made in two human subjects accidentally exposed to the toxin [72]. The same metabolic pathways as in the rat appear to operate in humans, and the main urinary metabolite was characterized as **11.35**.

11.4.3. Reactions of Multiple Dehalogenation

In addition to the nitrogen and sulfur mustards discussed above, a few xenobiotics that contain two or three halide atoms also undergo dehalogenation by a mechanism that is indisputably hydrolytic, based on substrate reactivity or metabolite structure. The first case discussed here is that of *dichloromethane* (**11.36**, *Fig. 11.6*). The major metabolic pathway is by enzymatic conjugation with glutathione [73], as shown in *Fig. 11.6*. The reaction yields *S*-(chloromethyl)glutathione, a metabolite that is highly sensitive to hydrolysis due to the increased electrophilicity of the C-atom adjacent to the S-atom (see also *Sect. 7.5.1*). Hydrolysis yields *S*-(hydroxymethyl)glutathione, a hemimercaptal, which hydrolyzes to glutathione and formaldehyde [58]. Here, the enzymatically catalyzed GSH conjugation reaction is followed by a nonenzymatic hydrolysis to result in the loss of two geminal halogen atoms.

Another relevant but poorly understood example is that of *trifluridine* (**11.17**), an important metabolite of which is the free 5-trifluorothymidine resulting from loss of the deoxyribosyl moiety (see *Sect. 11.2.3*). Of relevance here is the subsequent formation of 5-carboxyuracil (**11.37**) as a metabolite

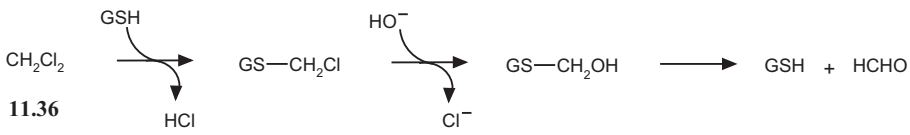
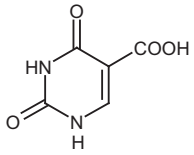


Fig. 11.6. *Mechanism of dehalogenation of dichloromethane* (**11.36**) [58]

in mice and humans [39]. CF_3 groups are generally thought to be metabolically inert, leading one to wonder about the mechanism of this reaction. Certainly, it cannot be oxidative, given the absence of a geminal H-atom. It is possible that the electronic structure of this CF_3 group promotes enzymatic or nonenzymatic hydrolysis, but we are not aware of any evidence to this point.



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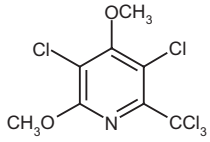


11.38

The novel antitumor agent *penclomedine* (**11.38**) provides another example of multiple dehalogenation [74]. In the perfused rat liver, rapid biotransformation occurred by two major pathways, namely oxidative *O*-demethylation at C(4), and formation of penclomic acid by dechlorination of the CCl_3 moiety. The proposed sequence of reactions:

$$–CCl_3 \rightarrow –C(OH)Cl_2 \rightarrow –C(=O)Cl \rightarrow –C(=O)OH$$

is certainly a realistic hypothesis, although no definitive proof of a hydrolytic mechanism has been reported. An alternative pathway, however, would involve a sequence of reductive, then oxidative, and, finally, hydrolytic steps, *e.g.*:

$$–CCl_3 \rightarrow –CHCl_2 \rightarrow –C(OH)Cl_2 \rightarrow –C(=O)Cl \rightarrow –C(=O)OH$$

Reductive dechlorination of penclomedine was, indeed, observed with liver microsomes under anaerobic conditions, indicating that a composite sequence such as this should not be dismissed without the benefit of further investigations. Given that the dehalogenation reactions exemplified by trifluridine and penclomedine may be less uncommon than implied here, mechanistic studies designed to clarify these processes would be most welcome.

The most important example to be discussed here is that of the drug *chloramphenicol* (**11.39**, R = 2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl, *Fig. 11.7*), the many metabolic pathways of which have yielded a wealth of information [75]. The dichloroacetyl moiety is especially of interest in that dechlorination proceeds by three proven routes: glutathione-dependent dechlorination, cytochrome P450 catalyzed oxidation, and hydrolysis. Of particular value is that the oxidative and hydrolytic routes can be unambiguously distinguished by at least one product, as shown in *Fig. 11.7*. Oxidation at the geminal H-atom produces an unstable (dichloro)hydroxyacetamido intermediate that spontaneously eliminates HCl to yield the oxamoyl

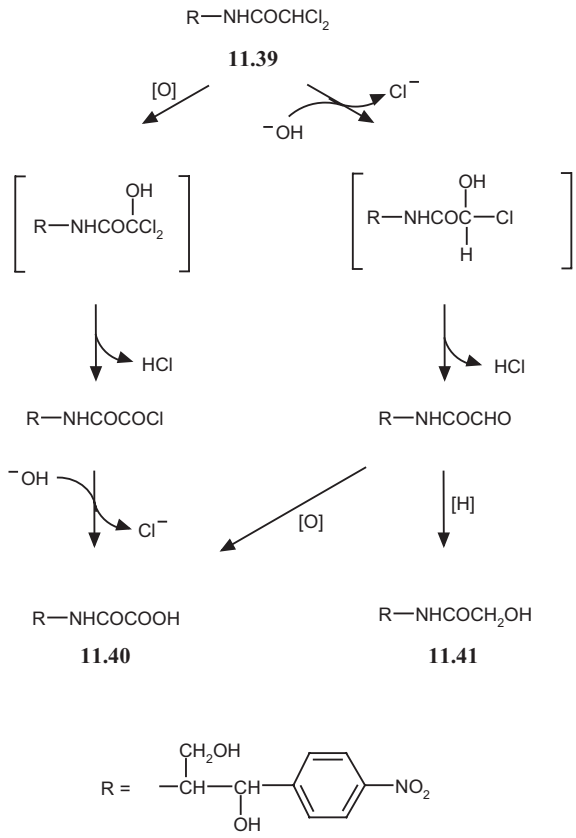


Fig. 11.7. *The two pathways of dechlorination of chloramphenicol* (**11.39**). Cytochrome P450 catalyzed oxidation to yield the oxamic acid derivative (**11.40**), and hydrolytic dechlorination to yield both oxamic acid (**11.40**) and primary alcohol (**11.41**) metabolites [75].

chloride derivative, a highly reactive acyl chloride that binds covalently to cellular macromolecules, thus accounting at least in part for the hepatotoxicity of the drug. The oxamoyl chloride function hydrolyzes to yield the oxamic acid derivative, chloramphenicol-oxamic acid (**11.40**, *Fig. 11.7*). Thus, the oxidative dechlorination of chloramphenicol is a toxification pathway.

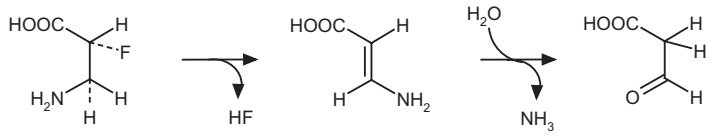
In contrast to oxidative dechlorination, the hydrolytic dechlorination of chloramphenicol replaces a Cl-atom with a OH group to yield a (monochloro)hydroxyacetamido intermediate. The latter, like the dichloro analogue, also eliminates HCl, but the product is an aldehyde that is far less reactive than the oxamoyl chloride intermediate. Chloramphenicol-aldehyde undergoes the usual biotransformation of aldehydes, namely reduction to the primary alcohol **11.41** and dehydrogenation to the oxamic acid derivative **11.40** (*Fig. 11.7*).

In conclusion, the oxamic acid derivative is produced by two distinct metabolic pathways, namely by oxidative and hydrolytic dechlorinations. In contrast, the primary alcohol metabolite **11.41** can be produced only by hydrolytic dechlorination and is, thus, an unambiguous marker of this pathway. The alcohol **11.41** is a known urinary metabolite of chloramphenicol in humans.

11.4.4. Dehydrohalogenation Reactions

The elimination of a hydrogen halide molecule (HX) has already been encountered in the previous subsections as *C(OH)X dehydrohalogenation* (*e.g.*, *Figs. 11.3,b* and *11.7*). Here, we briefly present elimination of vicinal H- and halogen-atoms, *i.e.*, *vicinal dehydrohalogenation* (α,β-elimination) in which the HC–CX bond is converted to a C=C bond.

One case where enzymatic involvement is documented is that of *(*R*)-α-fluoro-β-alanine* (**11.42**), itself the major (>80%) metabolite of 5-fluorouracil (**11.16**) in humans. With rat liver homogenates, it was demonstrated that mitochondrial L-alanine-glyoxylate aminotransferase II (AlaAT-II, EC 2.6.1.44) catalyzed the defluorination of (*R*)- and (*S*)-α-fluoro-β-alanine with catalytic efficiencies of 0.038 and 0.050 mM^{-1} s^{-1} at 37° and pH 7.0, respectively, [76]. The primary product of the reaction was β-aminoacrylate (**11.43**), but this product decomposed to ammonia and malonic semialdehyde (**11.44**).



11.42 **11.43** **11.44**

However interesting, this example is a highly specific one with little extrapolative value. Indeed, only a very few closely similar analogues, *e.g.*, 5-fluoro-5,6-dihydrouracil, were found to be substrates for AlaAT-II. Thus, β-chloro-L-alanine was metabolized to pyruvic acid, but it slowly inactivated the enzyme [76].

More general cases are encountered in the metabolism of a variety of *halogenated hydrocarbon* solvents and insecticides [58]. Examples include the dehydrochlorination of 1,1,2,2-tetrachloroethane to trichloroethylene in the mouse, and of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) to DDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethene) [58][77]. Glutathione transferases may be involved in some of these reactions.

11.5. Hydrolysis of *Mannich* Bases

In *Sect. 11.4*, we examined hydrolytic attack at sp^3 C-atoms rendered more electrophilic by the presence of one or more halogen substituents. In a logical follow-up, the present section presents the hydrolysis of *Mannich* bases [78], *i.e.*, compounds that contain the X–CH_2–N moiety, where an sp^3 C-atom is made more electrophilic by the presence of two flanking heteroatoms, one of which is an N-atom.

Mannich bases are classified according to the nature of the second heteroatom, and this section will present in turn:

- O-*Mannich* bases, *i.e.*, compounds containing an *N*-(hydroxymethyl) (HO–CH_2–N) or an *N*-(alkoxymethyl) group (RO–CH_2–N);
- S-*Mannich* bases of the type (N–CH_2–SO_3^-);
- N-*Mannich* bases (N–CH_2–N).

These moieties are found mainly in compounds that are potential prodrugs and generally undergo rapid nonenzymatic hydrolysis, as exemplified by the following overall reaction of an N-*Mannich* base:

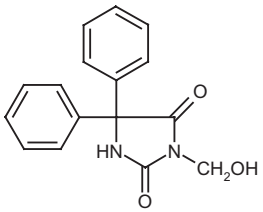
$$RR'N–CH_2–NR''R''' + H_2O \rightarrow RR'NH + HCHO + R''R'''NH$$

An unfavorable feature of this reaction is the release of formaldehyde, which, depending on the amounts produced and the location and kinetics of its release, may be a health hazard.

11.5.1. O- and S-*Mannich* Bases

In previous chapters, we discussed the hydrolysis of a number of esters of *N*-(hydroxymethyl)phenytoin, namely esters of organic acids (*N*-acyloxymethyl derivatives, *Sect. 8.7.3*) or inorganic acids (*Sect. 9.3.2*). Hydrolysis of these potential prodrugs released *3-(hydroxymethyl)phenytoin* (**11.45**), whose breakdown to phenytoin and formaldehyde was also investigated *per se* [79]. The latter reaction followed pseudo-first-order kinetics. At pH 7.4, the $t_{1/2}$ values were 4.7 and 1.6 s at 25° and 37°, respectively. The $t_{1/2}$ values decreased tenfold for each increase of pH by one unit, which, together with the absence of any buffer catalysis, indicates catalysis by the HO^- anion.

These and other interesting results allow quantitative insight into the rate of breakdown of N-*(hydroxymethyl) compounds* (*i.e.*, *carbinolamines* (hemiaminals)), the reaction mechanisms of which we examined in *Sect. 8.7.3* (see, in particular, *Fig. 8.20*) [80 – 82]. These carbinolamines, we recall, are major metabolic intermediates in *oxidative N-dealkylation* reactions resulting from cytochrome P450 catalyzed hydroxylation of the C-atom adjacent to the



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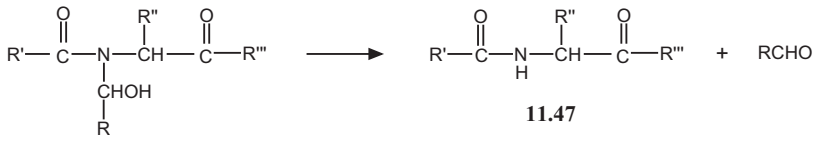


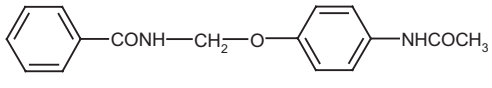
11.46 **11.47**

N-atom (Chapt. 6 in [50]). In reactions of *N*-dealkylation, the rate-limiting step is *C*-hydroxylation, whereas subsequent hydrolytic cleavage of the N–C bond is too fast under physiological conditions to be measurable, at least when the parent compound is a basic amine. Breakdown of carbinolamines of very weak amines and amides, *e.g.*, 3-(hydroxymethyl)phenytoin, is not as fast and becomes measurable.

Another factor that stabilizes carbinolamines is steric hindrance. Some interesting investigations are based on the possibility of preparing prodrugs of peptides by bioreversible derivatization of the peptide N-atom [83]. In addition to *N*-(hydroxymethyl) derivatives, N-*(α-hydroxyalkyl) derivatives* of general formula **11.46** were also prepared. As expected, hydrolysis of the N–C bond is catalyzed by HO^-, leading to formation of the aldehyde (R–CHO) and the free model compounds **11.47**. Rather unexpectedly, however, the observed range in rate of breakdown under physiological conditions was extremely large. In a broad series of derivatives, the $t_{1/2}$ values for reaction ranged from 5 min (**11.46**, R = Ph, R′ = $PhCH_2O$, R″ = H, R‴ = OH) to 64 d (**11.46**, R = H, R′ = $PhCH_2O$, R″ = H, R‴ = Ile).

Replacement of the *N*-(hydroxymethyl) group with *N*-(α-hydroxyalkyl) in compounds **11.46** decreased stability, whereas the influence of R″ seemed to be modest. In contrast, the R‴ substituent strongly affected the rate of reaction, with amides (R‴ = R⁗NH) being less stable than the free acid (R‴ = OH). Hydrophobic amino acid substituents (R = Leu, Ile, nor-Leu, or Val) markedly stabilized the derivatives. Thus, a complex interplay of electronic and steric factors appears to modulate the stability of compounds **11.46** over several orders of magnitude, allowing prodrug designers to fine-tune the rate of drug release. The possibility to release an aldehyde other than formaldehyde is a further advantage.

Not only carbinolamines, but also their ether derivatives have potential value in prodrug design. This is exemplified by O-*(benzamidomethyl) derivatives of phenols*, which undergo quantitative and rapid hydrolysis to the model or medicinal phenol, plus benzamide and formaldehyde [84]. The base-catalyzed reaction follows pseudo-first-order kinetics *via* nucleophilic attack of the HO^- ion on the electrophilic CH_2 group. *Paracetamol* (**11.49**) was, thus, derivatized to the *O*-(benzamidomethyl) prodrug (**11.48**); hydrolysis to the drug occurred with a $t_{1/2}$ value of *ca.* 5 h under physiological conditions. The rate of the reaction was strongly increased by the presence of electron-withdrawing substituents in the *para*-position of the phenol (and, hence, with increasing acidity of the phenol), and increased also with increasing acidity of the amide.



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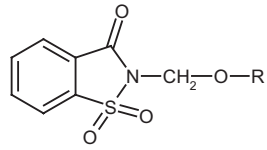

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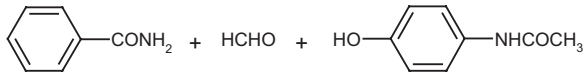
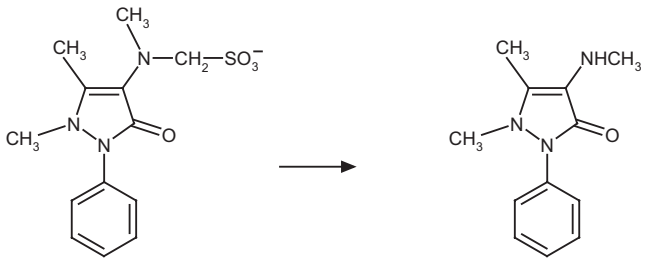


11.49

Indeed, the choice of a nitrogen compound to be used as carrier in such *O*-alkyl prodrugs is not restricted to benzamides. Other amides were examined, *e.g.*, phthalimide, succinimide, and saccharin. Thus, *the saccharin prodrug of estradiol* (**11.50**, R = 17β-estradiol) underwent rapid nonenzymatic hydrolysis in rat and human plasma [85]. Administration of the prodrug to rats led to an impressive fivefold increase in the oral bioavailability of estradiol, and it was approximately nine times more potent orally, based on the 50% effective dose.

A very rare, perhaps unique, example of a *S*-Mannich *base* of medicinal value is *dipyrone* (**11.51**), a derivative of the analgesic agent 4-(methylamino)antipyrine (**11.52**) [86][87]. Dipyrone is, in fact, a prodrug that is highly water-soluble and, hence, also suitable for parenteral administration. In an artificial gastric juice (*i.e.*, at acidic pH), breakdown to 4-(methylamino)antipyrine was complete in 1 h. The other breakdown products (HCHO and H_2SO_3 ?) do not appear to have been detected. In an artificial intestinal juice, hydrolysis reached completion in *ca.* 5 h. When dipyrone was administered to humans either orally or intramuscularly, no unchanged prodrug was detect-



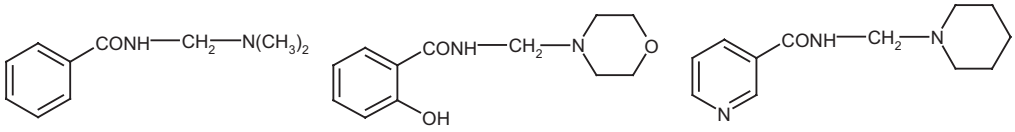
11.51 **11.52**

able in plasma, but plasma concentrations of 4-(methylamino)antipyrine peaked after 1 h. These data suggest that oral dipyrone undergoes fast breakdown in the stomach, followed by intestinal absorption of the active agent.

11.5.2. N-*Mannich* Bases

As with the O-*Mannich* bases discussed above, the rate of nonenzymatic hydrolysis of N-*Mannich* bases depends on factors such as steric hindrance and electrophilicity of the sp^3 C-atom. A rather large number of studies have been published on the value and properties of N-*Mannich* bases as potential prodrugs for amines, amides, and imides [80][82][88][89]. Here, we first review available reactivity data and then discuss selected examples of medicinal relevance.

The influence of systematic changes to structure on the *reactivity of N-*Mannich *bases* is illustrated in *Table 11.1*. The compounds shown were derived, for example, from model (benzamide (**11.53**)) and medicinal (salicylamide (**11.54**) and nicotinamide (**11.55**)) aromatic amides. The rates of breakdown to the parent amide spanned a broad range of values ($t_{1/2}$ in *Table 11.1* ranging from a few minutes to 1 d). The reactions followed strict first-order kinetics, and all went to completion. The pH–rate profiles in the acidic to neutral range usually had a sigmoidal shape, with lower rate constants for the protonated bases, and higher rate constants (by one to four orders of magnitude) for the neutral bases. Given the pK_a values of these N-*Mannich* bases, the observed rate constants at pH 7.4 were, in fact, those of the neutral species.



11.53 **11.54** **11.55**

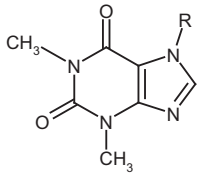
Table 11.1. *Chemical Hydrolysis of N-*Mannich *Bases of Model and Medicinal Amides at pH 7.4 and 37°* [82][88]

N-*Mannich* base	$t_{1/2}$ [min]
N-[(Methylamino)methyl]benzamide	600
N-[(Dimethylamino)methyl]benzamide (**11.53**)	58
N-[(Ethylamino)methyl]benzamide	190
N-[(Diethylamino)methyl]benzamide	4.0
N-[(Isobutylamino)methyl]benzamide	82
N-[(Benzylamino)methyl]benzamide	380
N-(Piperidinomethyl)benzamide	47
N-(Morpholinomethyl)benzamide	1400
N-[(Methylamino)methyl]salicylamide	28
N-(Piperidinomethyl)salicylamide	14
N-(Morpholinomethyl)salicylamide (**11.54**)	41
4-Nitro-*N*-(piperidinomethyl)benzamide	8.2
4-Methoxy-*N*-(piperidinomethyl)benzamide	55
4-Chloro-*N*-(piperidinomethyl)benzamide	20
N-(Piperidinomethyl)nicotinamide (**11.55**)	8.0

For some N-*Mannich* bases of benzamides and various amines, the rate constant increased with the size of the alkylamino group and with the pK_a of the free amine. The reactivity also increased strongly with increasing acidity of the parent amide. Thus, N-*Mannich* bases of toluenesulfonamide or succinimide had $t_{1/2}$ values of 1 min or less.

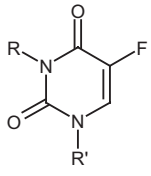
An N-*Mannich* base in therapeutic use is *rolitetracycline*, *i.e.*, *N*-(pyrrolidinomethyl)tetracycline. This highly water-soluble prodrug of tetracycline is of value for parenteral administration following *ex tempore* dissolution. The rate of breakdown of the prodrug was found to vary only slightly in the pH range of 1 – 10. At 21°, the limit of stability (10% breakdown) was 1.6 h, whereas the $t_{1/2}$ value at 37° and pH 7.4 was 43 min [82].

A few N-*Mannich* bases of the anti-inflammatory drug salicylamide are reported in *Table 11.1*. The pharmacokinetic behavior of one of these, N-*(morpholinomethyl)salicylamide* (**11.54**), was examined in the rabbit [90]. Plasma concentration curves showed that the oral bioavailability of salicylamide was increased two- to sixfold by administration of the prodrug.



11.56 R = H

11.57 R = $-CH_2-N$



11.58 R = R' = H

11.59 R = R' = $-CH_2-N$ O

N-*Mannich* derivatization has also been documented to improve skin delivery [91][92]. In the case of *theophylline* (**11.56**) and *5-fluorouracil* (**11.58**), a much improved solubility in water of the various N-*Mannich* bases examined was observed. To avoid breakdown, however, the prodrugs had to be dissolved in a polar nonaqueous solvent (isopropyl myristate) for pharmaceutical use. The delivery of theophylline and 5-fluorouracil through hairless mouse skin was, thus, accelerated approximately sixfold through use of the prodrugs **11.57** and **11.59**, respectively.

11.6. Hydrolytic Cleavage of Imines and Analogues

The present section examines the hydrolysis of C=N and C≡N moieties, as found in the following functional groups:

- imines (C=N–R, *i.e.*, *Schiff* bases) including hydrazones (C=N–NRR′) and enaminones (C(=O)–CRR′–C(R′)=N–R′′);
- imidates (RO–C(R′)=N–R′′) and amidines (N–C(R)=N–R′);
- oximes (C=N–OH) and oximines (C=N–OR);
- isocyanates (R–N=C=O);
- nitriles (–C≡N).

Such groups are relevant in our context since they occur in drugs, drug metabolites, prodrugs, and other xenobiotics. Their hydrolysis is essentially a nonenzymatic process, the rates depending not only on the biological context, but also on the structure and properties of the compounds.

11.6.1. Imines, Enaminones, and Hydrazones

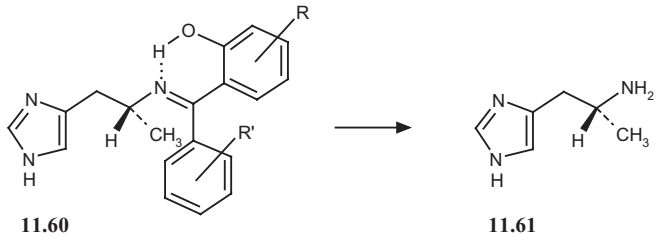
The literature contains a number of examples of *imines* of medicinal interest, including prodrugs and drug metabolites. These compounds may undergo metabolic hydrolysis as described below:

$$RR'C{=}N{-}R'' + H_2O \leftrightarrows RR'C{=}O + H_2N{-}R''$$

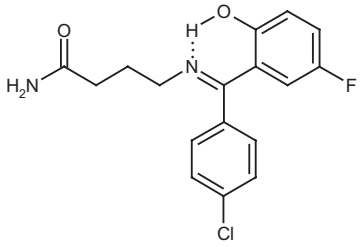
This reaction is reversible, since the condensation of a carbonyl compound and a primary amine yields the imine under dehydrating conditions. In fact, acidic conditions usually tend to favor hydrolysis, whereas neutral or slightly alkaline conditions lead to deprotonation of the amine, which then behaves as a nucleophile toward the carbonyl.

*Azomethine prodrugs of (*R*)-α-methylhistamine* (**11.60**) offer an apt illustration of this approach. (*R*)-α-Methylhistamine (**11.61**) is potent and selective as a histamine H_3 receptor agonist, but its medicinal usefulness in hu-

mans is severely limited by insufficient peroral absorption, poor brain penetration, and rapid metabolism, especially by histamine *N*-methyltransferase. To circumvent these problems, potential prodrugs were prepared by allowing the drug to react with 2-hydroxybenzophenones substituted at various positions on the ring [93]. Most compounds were readily hydrolyzed in aqueous solution, with conversion yields at room temperature ranging from *ca.* 0.2 to 80% at pH 1, and from 1 to 34% at pH 4. Monohalogenation (*i.e.*, R or R′ = halogen) favored hydrolysis, and dihalogenation (*i.e.*, R and R′ = halogen) increased it further. The majority of the compounds appeared to be hydrolyzed slightly faster in acidic than in neutral solution, whereas, for others, the rates of hydrolysis were approximately equal to or somewhat higher in neutral solution.



11.60 **11.61**



11.62

Following oral administration to mice (24 μmol kg^{-1}), the plasma AUC of (*R*)-α-methylhistamine varied from not detectable (**11.60**, R = 5-MeO, R′ = H) to 1200 ng h ml^{-1} (**11.60**, R = 4-F, R′ = H). For several of the compounds (*e.g.*, **11.60**, R = H, 4-F, or 4-Cl; R′ = H, 4-F, or 4-Cl), the plasma AUC was around 300 ng h ml^{-1}. Only compounds with the highest plasma AUC values produced measurable brain AUC values, which varied from *ca.* 1% (**11.60**, R = 4-F, R′ = H) to 22% (**11.60**, R = R′ = 4-Cl) of the plasma AUC. These pharmacokinetic studies have been complemented by *in vivo* pharmacological investigations that demonstrate anti-inflammatory and antinociceptive properties [94]. Taken together, the results clearly document the

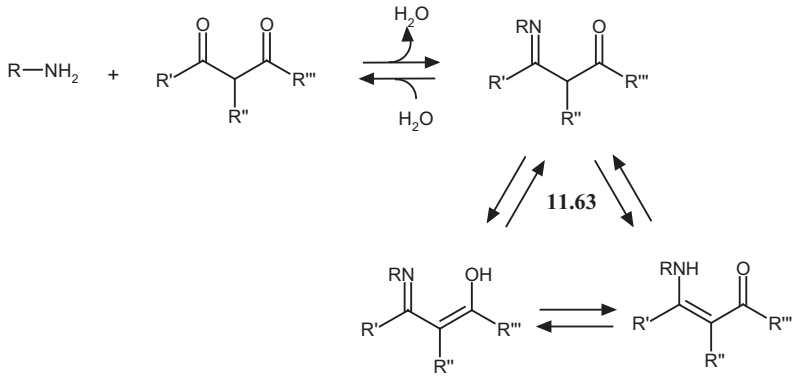


Fig. 11.8. *Formation and tautomeric equilibria of enaminones* (**11.63**) *as potential prodrugs of primary amines* [99]

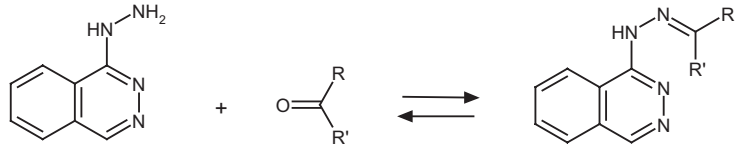
potential of this type of prodrug to improve the pharmacokinetic profile of some primary amines. Further studies have examined promising analogues of **11.60** in which the less-substituted phenyl ring is replaced by an aromatic heterocycle [95].

The above prodrug candidates show an analogy with the well-known GABA-ergic agent *progabide* (**11.62**). This compound was developed as a brain-penetrating analogue or prodrug of the neurotransmitter γ-aminobutyric acid (GABA) [96]. Metabolic studies in the rat revealed two hydrolytic reactions, the major one being hydrolysis of the terminal amide group to form the corresponding carboxylic acid and a less important one being hydrolysis of the imine link to liberate GABA and gabamide (*i.e.*, the amide of GABA) [97]. Detection of GABA and gabamide in the brain of rats dosed with progabide implies slow *in situ* generation of these active metabolites.

Rifapentin, an antituberculosis antibiotic related to rifamycin, also contains an imino group, the cleavage of which was found to be a major metabolic reaction in humans [98]. This reaction of hydrolysis was shown to be nonenzymatic, occurring primarily in the gut and in the acidic environment of the urine.

Despite chemical analogy, the reactivity of *enaminones* (**11.63**, *Fig. 11.8*) appears to be sufficiently different from that of the standard imines discussed above [82] to preclude their use as oral prodrugs. Enaminones are, however, of interest here because they teach us that thorough mechanistic investigations are necessary in prodrug design. Enaminones are formed in the reaction of a primary amine with a 1,3-dicarbonyl compound. The resulting *Schiff* base is stabilized by keto–enol and imine–enamine tautomeric equilibria (*Fig. 11.8*). A systematic study has shown that the rate of hydrolysis of such enaminones, which is strongly acid-catalyzed, is relatively insensitive to the

nature of the parent primary amine, but highly sensitive to minor differences in the structure of the 1,3-dicarbonyl parent [99]. Indeed, a linear correlation was found between the rate of hydrolysis and the pK_a of the dicarbonyl compound, suggesting that the rate-determining step in the reaction is addition of a proton to the vinyl C-atom. Unfortunately, the lability of enaminones in the low pH range is an important shortcoming, since such potential prodrugs would be broken down too fast in the acidic milieu of the stomach to reach the circulation.



11.64 **11.65**

Imines are also of medicinal interest as *metabolites*, since metabolic conjugation of some therapeutic hydrazines with endogenous carbonyl compounds yields a class of conjugates known as *hydrazones*. Typical drugs that undergo this reaction are isoniazide and *hydralazine* (**11.64**). The reaction is nonenzymatic, the reacting carbonyl compounds being, *e.g.*, acetaldehyde (CH_3–CHO), acetone (CH_3–CO–CH_3), pyruvic acid (CH_3–CO–COOH), or α-ketoglutaric acid (HOOC–CH_2–CH_2–CO–COOH). In an interesting study of lability, four hydralazine hydrazones (**11.65**) were examined in rat plasma under physiological conditions [100]. Hydralazine acetaldehyde hydrazone (**11.65**, R = H, R′ = Me) was the most-labile conjugate, exhibiting 80% hydrolysis in 1 h. The other three hydrazones were more stable by far; the acetone hydrazone yielded 10% of the parent drug in 1 h, whereas only a few percent of hydralazine were generated from the pyruvic acid and α-ketoglutaric acid hydrazones. Besides intrinsic reactivity, it seems that other factors, perhaps protection by protein binding, contributed the observed rate of hydrolysis in rat plasma.

11.6.2. Imidates and Amidines

Imidates are a different type of imine. Conceptually, imidates result from dehydration after condensation of a primary amido N-atom with the carbonyl of an ester function, although the actual synthetic route is often *via* an ortho ester ($RC(OR')_3$). A large variety of *N*-sulfonyl imidates have been prepared and examined for their value as potential prodrugs containing a sulfonamide or an ester moiety [101][102]. The hydrolysis of such compounds,

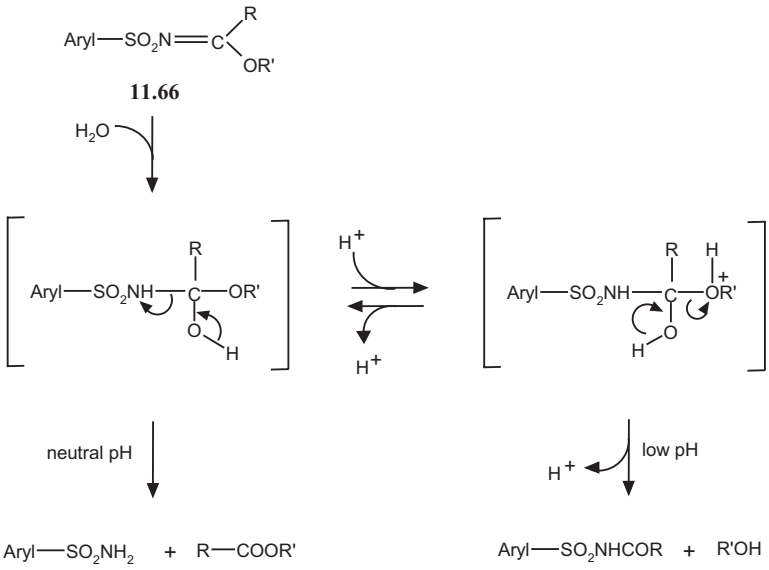


Fig. 11.9. *Mechanisms and products of hydrolysis of aromatic* N-*sulfonyl imidates* (**11.66**) *as potential prodrugs of drugs containing sulfonamide or ester moieties* [101][102]

which have the general structure **11.66** in *Fig. 11.9*, is remarkably structure-dependent. The (4-methylphenyl)sulfonamide imidate (R = Ph and R′ = Me) had a $t_{1/2}$ value of *ca.* 2 h at pH 7.4 and 37°, whereas the $t_{1/2}$ value was 17.5 h for the analogue with R = Ph and R′ = i-Pr, and 2.7 min for the analogue with R = Ph and R′ = $(CH_3)_2NCH_2CH_2$.

The stability of these compounds is maximal at pH 4 – 6, and decreases very sharply at lower and higher pH values, and the mechanism and products of the reaction differed with pH. In the neutral range, hydrolysis yielded the aromatic sulfonamide and the ester, whereas, under acid catalysis in the low pH range, the products were the *N*-acyl sulfonamide and an alcohol (R′OH, *Fig. 11.9*). Of particular interest is that the $t_{1/2}$ values for hydrolysis of the *N*-sulfonyl imidates in 80% human plasma were 3 – 150 times lower than in buffer solution at identical pH and temperature. This was taken as evidence for enzymatic hydrolysis by human plasma hydrolases. Hydrolysis under these conditions yielded the sulfonamide and the ester in quantitative amounts.

Whereas, in imidates, an O-atom is attached to the sp^2 C-atom, in another class of imines, the *amidines*, a N-atom is attached to the sp^2 C-atom. Oxidative cleavage of benzamidines has been discussed (Chapt. 5 in [50]). Here, we present studies in which *formamidines* were examined (N–CH=N–R) as potential prodrugs of anti-HIV cytidine analogues [103]. Specifically, the

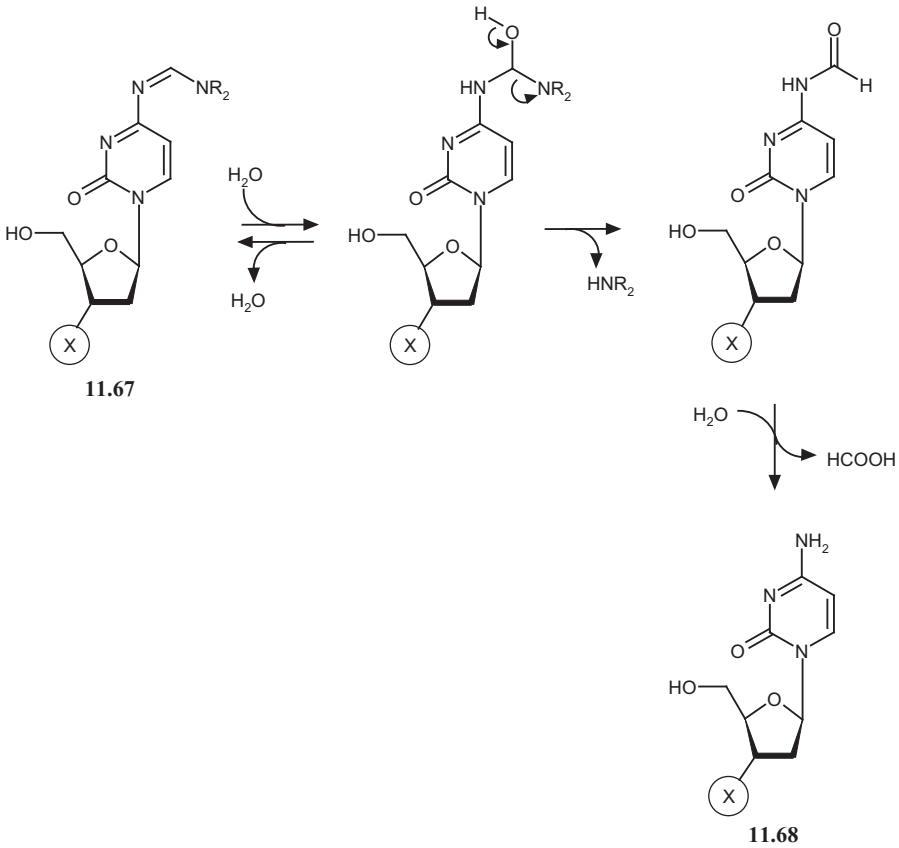


Fig. 11.10. *Mechanism postulated for activation of formamidines* (**11.67**) *as potential prodrugs of anti-HIV nucleoside analogues* [103]

NH_2 group at C(4) of the cytosine moiety was substituted with a [(dialkylamino)methylidene]amino group to yield the desired formamidine (**11.67**, X = H or F, *Fig. 11.10*).

These prodrugs underwent spontaneous hydrolysis in aqueous solution. The *mechanism of reaction* (*Fig. 11.10*) was postulated to involve nucleophilic hydration of the C=N bond to yield an intermediate carbinolamine. The latter breaks down with loss of the dialkylamine to form an N^4-formyl intermediate, which, in turn, hydrolyzes to the active agent (**11.68**, *Fig. 11.10*). The hydrolysis of these prodrugs followed pseudo-first-order kinetics with $t_{1/2}$ values at pH 7.4 and 37° that were mostly influenced by the steric properties of the dialkylamino group. Thus, $t_{1/2}$ values were *ca.* 4, 9 – 10, 14 – 15, and 48 – 52 h for R = Me, Et, Pr, and i-Pr, respectively. Interestingly, the rates of hydrolysis were decreased in human serum, indicating protective

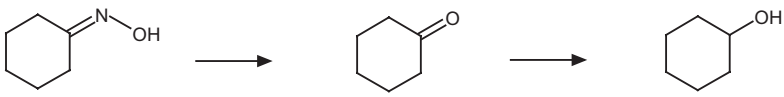
binding to serum proteins. Also, hydrolysis in 1N HCl at 37° was accelerated by one order of magnitude only, suggesting survival during gastric transit.

Amidines such as the ones presented here appear to have a number of advantages, displaying good water solubility, and not producing formaldehyde during breakdown. Varying the dialkylamino group can modulate the lipophilicity and the rate of nonenzymatic hydrolysis, and the stability of amidines under the acidic conditions that prevail in the stomach is compatible with oral administration. Future studies will certainly reveal the potential medicinal value of amidines.

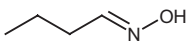
11.6.3. Oximes and Oximines

Like imines, some *oximes* are known to undergo metabolic hydrolysis by a nonenzymatic mechanism. *Cyclohexanone oxime* (**11.69**), an intermediate in the synthesis of polycaprolactam or *Nylon-6*, is a good example with which to begin our discussion. Following administration to male rats by various routes, cyclohexanone oxime undergoes rapid metabolism, and only trace amounts of the parent compound can be recovered in the urine [104]. Although cyclohexanone (**11.70**) represented a small fraction of the urinary metabolites, most of the dose was recovered as glucuronides of cyclohexanol (**11.71**) and of *cis*- and *trans*-cyclohexane-1,2-diol.

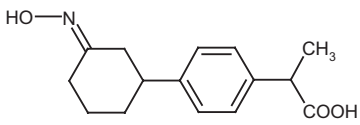
In vitro studies on rat liver preparations confirmed the postulated metabolic pathway of nonenzymatic hydrolysis of the oxime **11.69** to the ketone **11.70**, followed by enzymatic reduction to **11.71**, hydroxylation to diols, and glucuronidation. Clearly, the first step in this metabolic scheme is the nearly quantitative hydrolysis of the oxime to the ketone.



11.69 **11.70** **11.71**



11.72

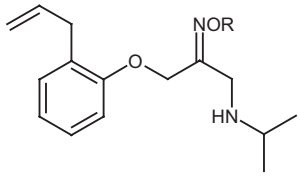


11.73

A complicating factor in the metabolism of oximes arises from the possibility that they become *reduced to the imine* ($RR'C{=}NH$) prior to hydrolysis, thus releasing NH_3 rather than hydroxylamine ($H_2N{-}OH$) as the primary metabolite. Evidence for this indirect pathway of hydrolysis comes from a study of the effect of a variety of selective enzyme inhibitors on the metabolism of butanal oxime (**11.72**) in rats [105].

But what about the *nitrogenated fragment* (N–OH or NH) liberated by hydrolysis of oximes or the corresponding imines, respectively? The primary product expected is clearly $H_2N{-}OH$ or NH_3. However, the blood of rats dosed with cyclohexanone oxime was found to contain nitrosylhemoglobin, itself a product of the reaction of hemoglobin with *nitrogen monoxide* (NO; nitric oxide) [106]. Incubation of cyclohexanone oxime with venous blood also resulted in the formation of nitrosylhemoglobin, suggesting that the blood was a possible site for the direct hydrolysis of the oxime, followed by the oxidation of hydroxylamine. These observations raise the suggestion that excessive production of NO may partly contribute to the observed hematotoxicity of cyclohexanone oxime.

The hydrolysis of *ximoprofen* (**11.73**), a potent nonsteroidal anti-inflammatory agent, has been studied in rats, baboons, and humans [107]. Again in these studies, hydrolysis of the oxime group appears to be a primary pathway, here in competition with glucuronidation of the carboxy group to form an acyl glucuronide. The ketone resulting from hydrolysis and the corresponding alcohol produced by subsequent reduction were important urinary metabolites in the three species.



11.74

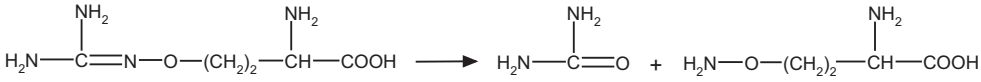
Oximes may even have some potential in prodrug design, as evidenced by studies with *ketoxime analogues of β-adrenergic blocking agents* [108]. A number of these potential prodrugs, including *alprenoxime* (**11.74**, R = H), the ketoxime analogue of alprenolol, were readily transformed into the corresponding β-blocker. The metabolic sequence involves first oxime hydrolysis to the ketone in a reaction that is probably nonenzymatic, followed by a stereospecific (and hence enzymatic) ketone reduction to the secondary alcohol of (*S*)-configuration. This is the configuration of the active enantiomer of β-blocking arylpropanolamines such as alprenolol, propranolol, and timolol.

Furthermore, by *in vitro* experiments, it has been verified that stereospecific activation of *alprenoxime* is *organ-specific*, occurring in the eye and not systemically. When administered locally to rabbits, marked decreases in intra-ocular pressure were observed, whereas oral administration elicited almost no cardiac effects. Such ketoximes represent promising chemical delivery systems in the treatment of glaucoma. However, a major limitation is their poor stability in solution, seemingly due to hydrolysis of the oxime function.

An *oximine* (*i.e.*, a substituted oxime) analogue of alprenoxime was examined in an attempt to overcome the problem of low stability in aqueous solution. To this end, the *methoxime analogue of alprenolol* (**11.74**, R = Me) was prepared and evaluated [109]. Stability in solution was greatly improved at neutral pH. Topical administration to rabbits produced a decrease in intraocular pressure that had the same onset and intensity as that produced by alprenolol, but that lasted longer. Alprenolol was, indeed, formed in eye tissues as a metabolite, with the peak concentration reached 30 min after topical administration of the methoxime.

All evidence presented here indicates that the methoxime-to-ketone reaction is enzymatic, but the nature of the reaction is unclear. Hydrolysis by an uncharacterized hydrolase, as postulated by the authors, is a valid assumption. However, one should not dismiss another possibility, namely oxidative *O*-demethylation to the oxime, followed by spontaneous oxime-to-ketone hydrolysis, and stereospecific reduction, as explained above.

Another case in point is L-*canavanine* (**11.75**), a natural oximine that exhibits antitumor activity. When administered orally or parenterally to rats, most of the dose is recovered in the urine, and practically no unchanged compound is found [110]. The predominant route of metabolism by far is C=N bond cleavage to yield urea (**11.76**) and L-canaline (**11.77**), a reaction believed to be catalyzed by arginase (arginine amidinase, EC 3.5.3.1).



11.75 **11.76** **11.77**

For oximes, which are relatively unstable in aqueous solution, nonenzymatic hydrolysis is a major pathway. In contrast, oximines appear to be more stable and not subject to nonenzymatic hydrolysis, but little is known about the enzymes that catalyze the cleavage of oximines, or whether the mechanisms by which they act are hydrolytic or oxidative.

11.6.4. Isocyanates and Nitriles

Organic isocyanates (R–N=C=O) are xenobiotics used extensively in the manufacture of paints, pesticides, and polyurethanes. The reactivity of the isocyanate group can also underlie the toxic reactions observed in patients that have been exposed to organic isocyanate monomers. Furthermore, the isocyanates formed in the body can be metabolites of various other xenobiotics, such as:

- Alkylformamides used as industrial solvents;
- Chemotherapeutic dialkylnitrosoureas;
- Organic isothiocyanates (R–N=C=S) found in dietary sources or used therapeutically. In this case, the formation of isocyanate occurs by oxidative desulfuration (Chapt. 7 in [50]).

The toxicity of organic isocyanates can be ascribed to two main mechanisms, namely:

- Massive damage to exposed areas (respiratory tract, eyes, *etc.*) in the case of heavy accidental exposure, as exemplified by the effects of the infamous methyl isocyanate [111];
- Hypersensitivity of the respiratory tract, as seen in workers exposed to polluted atmospheres.

The toxicity of isocyanates is the distant consequence of their reactivity as *electrophiles* toward alcohols, phenols, amines, and thiols [112]. These are, indeed, the mechanisms by which isocyanates react with and modify proteins and nucleic acids [113][114]. Isocyanates also react reversibly with the tripeptide glutathione (GSH), the resulting conjugates being considered to be transport forms as well as products of detoxification [115][116].

Nonenzymatic hydrolysis to yield the corresponding primary amine is, perhaps, the only genuine route of detoxification open to isocyanates. During hydrolysis, the isocyanate behaves as an electrophile; alkyl isocyanates are significantly less reactive than aryl isocyanates [112][113a]. A study of the hydrolysis of *hexane-1,6-diyl diisocyanate* (**11.78**), an industrially important compound known to be metabolized to hexane-1,6-diamine (**11.79**) and further to *N*-(6-aminohexyl)acetamide as a urinary metabolite, has led to mechanistic insights of biological relevance [117]. In phosphate or glycine buffer at pH 7.4 and 30°, only trace amounts had reacted in 10 min to hexane-

11.78 **11.79**

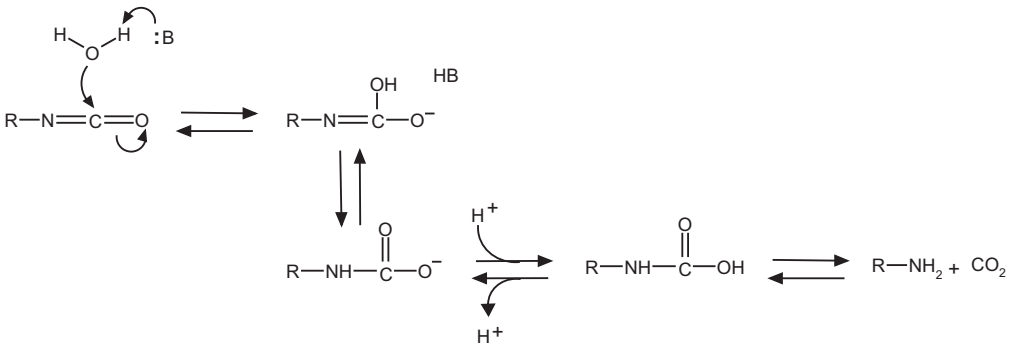


Fig. 11.11. *Mechanism postulated for hydrolysis of organic isocyanates involving electrophilic addition of H_2O with general base catalysis.* The product of hydration is a carbamic acid that spontaneously decomposes to the primary amine with loss of CO_2 [117].

1,6-diamine. In contrast, hydrolysis under the same conditions in the presence of a physiological concentration (20 mM) of bicarbonate was practically complete after 10 min. This toxicologically important finding identifies a catalyst responsible for the detoxification of hexane-1,6-diyl diisocyanate in the body.

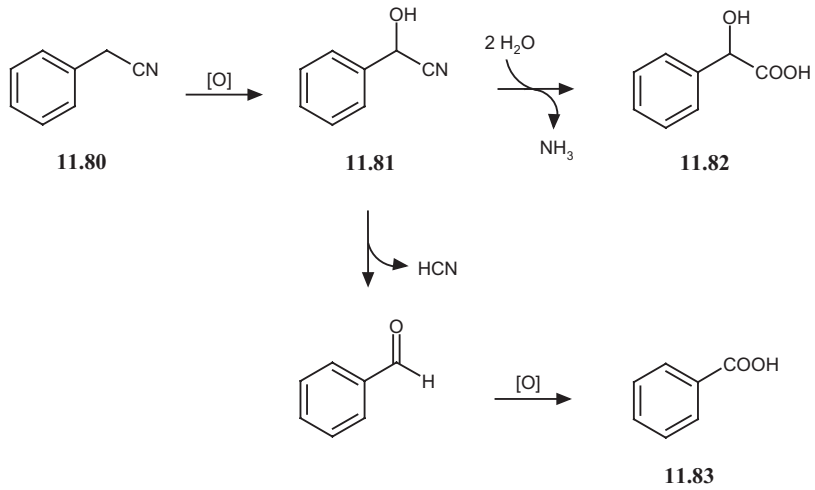
In the same study, it was shown that catalysis of hexane-1,6-diyl diisocyanate is carried out by acids having a $pK_a > 6$, whereas stronger acids ($pK_a < 5$) were less efficient catalysts. These results are compatible with the mechanism of general base catalysis with electrophilic addition of H_2O postulated in *Fig. 11.11*. Hydration of isocyanate, thus, produces a carbamic acid, which spontaneously decomposes to the primary amine with loss of CO_2.

Nitriles differ from all other classes of compounds discussed in *Sect. 11.6* in terms of both chemistry and bioreactivity. It is essential, however, to discriminate between aliphatic and aromatic nitriles.

The acute toxicity of *aliphatic nitriles*, which are important industrial compounds, is ascribed mainly to the liberation of cyanide after *oxidative denitrilation* [118 – 120]. The reaction involves cytochrome P450 mediated hydroxylation of C(α) to form an intermediate cyanohydrin, which then breaks down to inorganic cyanide and an aldehyde:

$$R–CH_2–C{\equiv}N \rightarrow R–CHOH–C{\equiv}N \rightarrow R–CHO + HCN$$

Given the acidity of the H-atoms at C(α), this metabolic reaction proceeds very rapidly and is usually the only one that involves the CN group itself. This pathway, however, falls outside of the scope of the present work and has already been reviewed (Chapt. 4 in [50]).



11.80 **11.81** **11.82** **11.83**

Fig. 11.12. *Metabolic scheme for reaction of benzyl cyanide* (**11.80**) *to mandelonitrile* (**11.81**) *as a crossroads to benzoic acid* (**11.83**) via *oxidative denitrilation, and to mandelic acid* (**11.82**) *as a minor metabolite produced by hydrolysis of the CN group* [118][122]

Of significance in the present context is that the CN group is also susceptible to hydrolysis, producing an amide according to the following mechanism:

$$R–C{\equiv}N + HO^- \rightarrow R–C(OH)=N^-$$

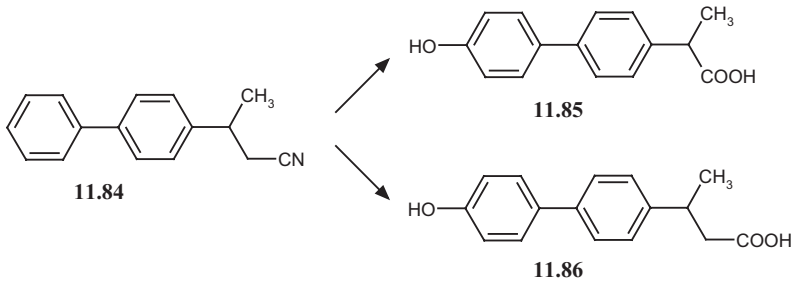
$$R–C(OH)=N^- + H^+ \rightarrow R–C(OH)=NH \rightarrow R–CONH_2$$

Bacterial enzymes have been reported to catalyze the hydrolysis of nitriles [118][121]. A *nitrilase* (EC 3.5.5.1) acts to hydrolyze aromatic nitriles directly to the carboxylic acid. A *nitrile hydratase* (a lyase, EC 4.2.1.84) acts on short-chain aliphatic nitriles to form the amide. As discussed below, the hydrolysis of nitriles to amides is also documented in mammals, but little appears known about the enzymes involved.

There are a few data in the literature to suggest that the hydrolysis of aliphatic nitriles occurs in mammals, but only as a minor or even undetectable pathway in competition with oxidative denitrilation. For example, benzyl cyanide (**11.80**, *Fig. 11.12*) undergoes cytochrome P450 catalyzed hydroxylation to mandelonitrile (**11.81**), from which cyanide and benzaldehyde are produced, the latter being oxidized to benzoic acid (**11.83**) [118]. However, a careful metabolic study of mandelonitrile has shown that, in the rat, this pathway accounts for *ca.* 90% and not 100% of the dose [122]. Only *ca.* 10% of orally administered benzyl cyanide was converted to mandelic acid (**11.82**, *Fig. 11.12*) by hydrolysis of the CN group.

A comparable metabolic pattern was observed in the rat after oral administration of *3-([1,1'-biphenyl]-4-yl)butanenitrile* (**11.84**), a nonsteroidal anti-

inflammatory agent [123], which probably acts as a prodrug. The major urinary metabolite is, indeed, the acid (**11.85**) resulting from oxidative denitrilation (leading one to suspect that cyanide is liberated), whereas another acid (**11.86**) resulting from hydrolysis was also excreted in marked quantities. In analogy with profens, one can postulate that acid **11.85** is the active metabolite.

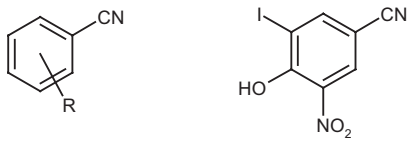


11.84 **11.85** **11.86**

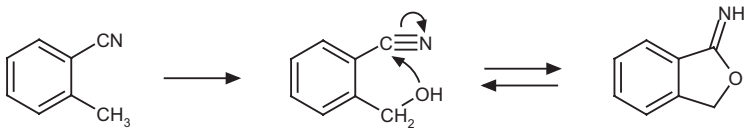
Aromatic nitriles differ from their aliphatic cousins in that they bear no H-atoms at C(α) and, hence, cannot undergo oxidative denitrilation. Here, hydrolysis of the CN group depends on the substrate properties and on the relative efficiency of competitive reactions such as ring hydroxylation. Thus, the CN group of benzonitrile (**11.87**, R = H), 2- and 4-hydroxybenzonitriles (**11.87**, R = 2-OH or 4-OH), and 4-nitrobenzonitrile (**11.87**, R = 4-NO_2) was not hydrolyzed by rat liver subcellular preparations [124].

In contrast to the above benzonitriles, 2-methylbenzonitrile (**11.88**) did undergo cyano hydrolysis, but by a very indirect route involving cytochrome P450 catalyzed hydroxylation of the 2-Me group to form 2-(hydroxymethyl)benzonitrile (**11.89**), followed by intramolecular nucleophilic addition. The cyclization reaction yielded an unstable imino ether derivative (**11.90**), which hydrolyzes to phthalide (**11.91**). The conversion of 2-(hydroxymethyl)benzonitrile to phthalide followed first-order kinetics with a $t_{1/2}$ value of 2.8 h at pH 7.4 and 37° [124].

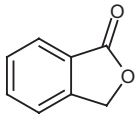
The flukicidal agent *nitroxynil* (**11.92**) upon incubation with rat liver subcellular preparations has been demonstrated to undergo hydrolysis of the aromatic CN group to yield two metabolites, both resulting from hydrolysis of the CN group, namely the corresponding benzamide (4-hydroxy-3-iodo-5-nitrobenzamide) as the major product, and the corresponding benzoic acid (4-hydroxy-3-iodo-5-nitrobenzoic acid) as the minor product [124]. The formation of these metabolites occurred preferentially in the cytosolic fraction, suggesting the involvement of one or more soluble enzymes. Why the cytosolic fraction was more effective under aerobic than anaerobic conditions has not been explained, and why nitroxynil is readily hydrolyzed when other aromatic nitriles are not is another unanswered question.



11.87 **11.92**

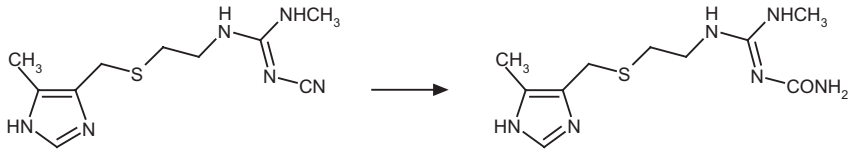


11.88 **11.89** **11.90**



11.91

The last nitrile derivative to be examined is *cimetidine* (**11.93**), in which the CN group is attached to a guanidino function. While cimetidine cannot be strictly classified as an aliphatic or aromatic nitrile, it nevertheless resembles aromatic nitriles in two ways: there are no H-atoms at C(α) to allow oxidative denitrilation, and the electrons of the CN group are delocalized over the moiety to which it is attached.



11.93 **11.94**

Interestingly, it has been shown that cimetidine undergoes hydrolysis of the CN group as a minor pathway in animals and humans [125][126]. The drug itself, being rather hydrophilic, is excreted mainly unchanged (*ca.* 2/3 of the human dose). A few metabolites have been characterized, the predominant one being cimetidine *N*-glucuronide (20 – 25% of the human dose). In our context, the relevant metabolite is cimetidine guanylurea (**11.94**), which accounts for *ca.* 1 – 2% of urinary excretion in humans.

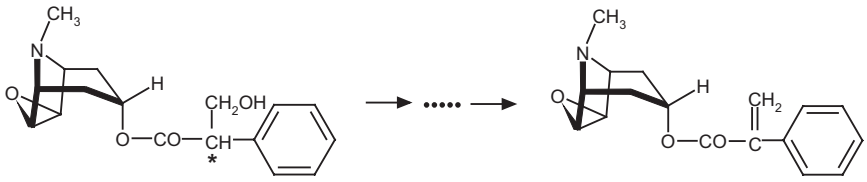
11.7. Bond-Order Increase by Elimination Reactions

In this section, we briefly examine three rather rare metabolic reactions that occur *without substrate oxidation*, *i.e.*:

- Apparent dehydration to transform C–C bonds into C=C bonds;
- Thiol elimination to transform certain single bonds into double bonds, and
- Dehydration to transform certain oximes into nitriles.

The reactions presented here must not be confused with oxidative reactions that increase bond order and are catalyzed by oxidoreductases, as discussed elsewhere. Examples of the latter reactions include the cytochrome P450 mediated oxidation of testosterone to 6,7-dehydrotestosterone, and the oxidation of 1,2,3,6-tetrahydro-1-methyl-4-phenylpyridine to 2,3-dihydro-1-methyl-4-phenylpyridinium catalyzed by monoamine oxidase (Chapt. 4 and 9 in [50]).

The first case to be considered is an increase in bond order of C–C bonds to C=C bonds by an *apparent dehydration* reaction. There are very few unambiguous examples of such a reaction, the best, in our view, being that of *scopolamine* (**11.95**). When examined in several animal species, the *in vivo* metabolism of scopolamine revealed a variety of reactions including *N*-demethylation and ester hydrolysis (see *Chapt. 7*). Of relevance here is the formation of aposcopolamine (**11.96**) and aponorscopolamine [127]. Large species differences were seen, with this pathway accounting for *ca.* 1/3 of the dose in the guinea pig, *ca.* 10% in the mouse, and a few percent in the rat and the rabbit. Such large differences cannot be attributed to an artifact and are suggestive of an enzymatic mechanism.

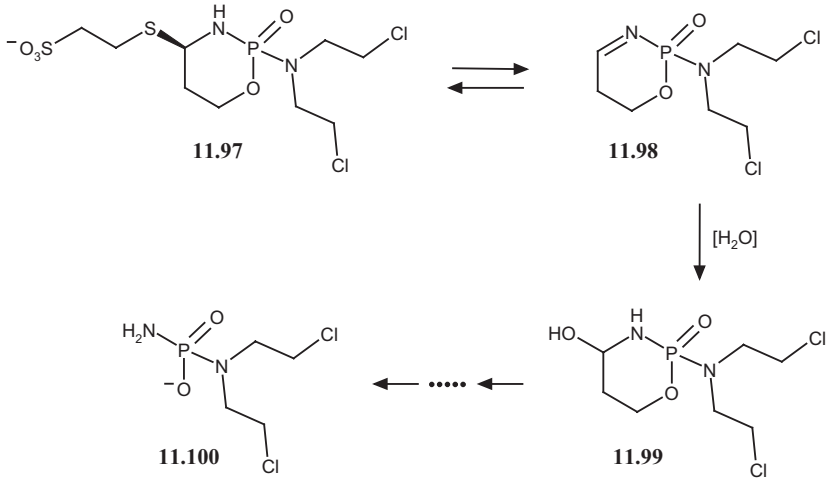


11.95 **11.96**

Indeed, it was shown that aposcopolamine was not formed by direct dehydration of scopolamine, but *via* the conjugate *scopolamine* O-*sulfate* generated by a sulfotransferase [127]. This explains the species differences observed, and indicates a mechanism of heterolytic C–O bond cleavage made possible by the electron-withdrawing capacity of the sulfate moiety. The reaction is also facilitated by the acidity of the departing proton carried by the vicinal, stereogenic C-atom. This acidity also accounts for the facile base-catalyzed racemization of scopolamine and hyoscyamine [128].

Apparent dehydration reactions, thus, appears to require certain well-defined conditions, namely conjugation of the OH group to make it a good leaving group, and acidity of the vicinal proton. Such conditions are seldom met simultaneously, offering a possible explanation for why the reaction occurs so rarely.

Thiol elimination has been characterized as the initial reaction during activation of *mafosfamide* (**11.97**), an analogue of the anticancer drug cyclophosphamide [129]. In aqueous solution under physiological conditions, this agent was converted to 4-hydroxycyclophosphamide (**11.99**), which is also an intermediate in the metabolic activation of cyclophosphamide *en route* to the ultimate cytotoxic metabolite, phosphoramide mustard (**11.100**). Kinetic and mechanistic studies showed that the mechanism of conversion of mafosfamide to 4-hydroxycyclophosphamide involves the intermediate iminocyclophosphamide (**11.98**), which is formed by specific-base catalysis. In other words, deprotonation of the NH group is the initial step in the formation of the imine **11.98**, which is supported by the failure of *N*-alkylated derivatives of mafosfamide to form iminocyclophosphamide and 4-hydroxycyclophosphamide [130].



Thiol elimination to create a C=C bond is also seen in the metabolism of *spironolactone* (**11.101**, *Fig. 11.13*) [131]. This diuretic drug undergoes a number of metabolic reactions in humans, one of which is ready hydrolysis at the thioester bond to yield deacetyl-spironolactone (see *Chapt. 7*). This reaction is in competition with other pathways such as lactone hydrolysis, *S*-oxygenation, and *dethioacetylation*. The latter reaction is the one of interest here, since the elimination of $CH_3CO–SH$ transforms the C(5)–C(6) bond into a C=C bond to produce the active metabolite canrenone (**11.102**, *Fig.*

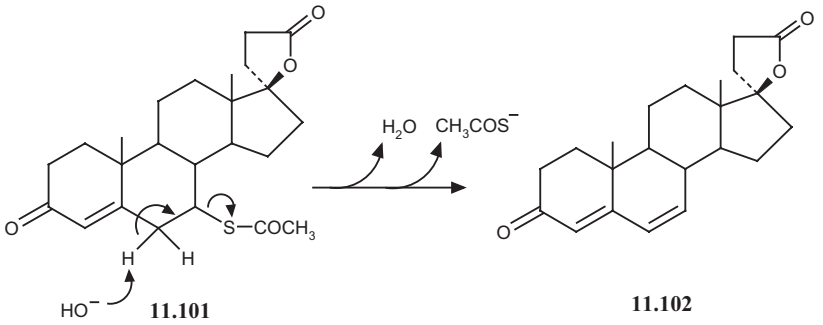


Fig. 11.13. *Mechanism postulated for base-catalyzed dethioacetylation of spironolactone* (**11.101**) *to canrenone* (**11.102**)

11.13). The mechanism discussed above for mafosfamide can also explain the formation of canrenone, as illustrated in *Fig. 11.13*.

Dehydration of oximes to form nitriles is, again, a rare but intriguing reaction. It has, indeed, been demonstrated that *cytochrome P450* (see Chapt. 3 in [50]) is able to transform butanal oxime (**11.103**, R = H, R′ = Pr, *Fig. 11.14*; also **11.72**) to butanenitrile by a *Beckmann*-type dehydration [105][133]. The reaction is inhibited by the presence of O_2, and is catalyzed by cytochrome P450 in its reduced (ferrous) state.

The mechanism of the reaction has been partly unraveled [132] and is believed to occur as depicted in *Fig. 11.14*. Ketoximes (**11.103**, R and R′ ≠ H), (*E*)-aldoximes (**11.103**, R ≠ H, R′ = H), and (*Z*)-aldoximes (**11.103**, R = H, R′ ≠ H) are all ligands of ferric cytochrome P450, but, for steric reasons, only

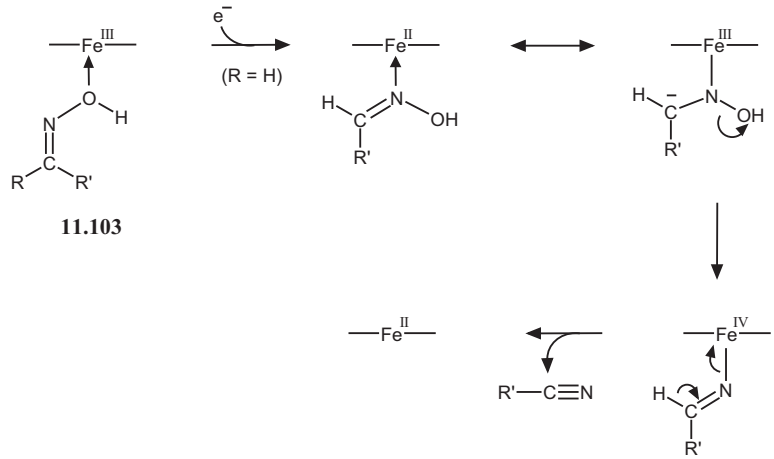
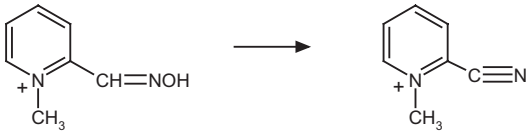


Fig. 11.14. *Mechanism postulated for* Beckmann-*type dehydration of* (Z)-*aldoximes* (**11.103**, R = H, R′ ≠ H) *catalyzed by cytochrome P450* [132][133]

(*Z*)-aldoximes remain bound after catalytic one-electron reduction of the enzyme. Charge transfer in the (*Z*)-aldoxime–P450(Fe^{II}) complex results in mesomeric forms in which elimination of the OH group is facilitated, presumably after protonation. The resulting P450(Fe^{IV})–N=CHR′ complex contains a activated H-atom at C(β) relative to the electrophilic Fe^{IV} species, and should lose this proton to yield R′–CN and regenerate P450(Fe^{II}). This is, perhaps, a unique reaction, since here cytochrome P450 acts neither as an oxidase nor as a reductase, but as a dehydratase.



11.104 **11.105**

It is of interest that oxime dehydration to the nitrile can also occur in humans. This has been shown with *pralidoxime* (**11.104**), a well-known agent used to antagonize the effects of anticholinesterase organophosphates (see *Sect. 9.3*). When administered *i.v.* to humans, pralidoxime is excreted mostly unchanged (60 – 90% of a dose in the 24 h urine) in agreement with its high polarity and facilitated urinary excretion. A single metabolite was detected in small amounts and characterized as the 2-cyano-1-methylpyridinium cation (**11.105**) [134]. Nothing is known regarding the enzyme(s) involved, but it is tempting to speculate that, here also, cytochrome P450 is the catalyst.

11.8. Hydrolytic Ring Opening

The true significance of *ring opening* and *ring formation* reactions in metabolism is not always recognized and, to the best of our knowledge, these two types of reaction have never been reviewed *per se* in a systematic manner. Here, we offer a preliminary classification of these reactions in an attempt to clarify this complex field.

A number of discriminations must be made when examining ring opening and closure. Indeed, such reactions can be:

- Enzymatic, nonenzymatic, or postenzymatic (*i.e.*, spontaneous reaction of enzymatically produced metabolic intermediates);
- Oxidative, reductive, or non-redox (*i.e.*, involving neither oxidation nor reduction of the substrate).

In the context of this work, only non-redox ring-opening reactions (mostly hydrolyses, this section) and ring closure (mostly dehydrations, *Sect. 11.9*)

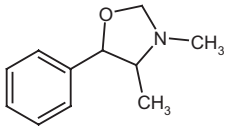
are examined, and these are often nonenzymatic or postenzymatic. The reactions of ring hydrolysis discussed here do not include *lactams* (*Chapt. 5*) and *lactones* (*Chapt. 7*).

11.8.1. Cyclic *Mannich* Bases

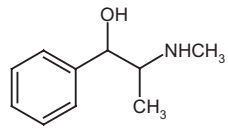
First, we examine the hydrolytic opening of cyclic *Mannich* bases, *i.e.*, rings that contain N–CH_2–O , N–CH_2–S, or N–CH_2–N fragments. The mechanism of hydrolytic fragmentation of linear *Mannich* bases was discussed in *Sect. 11.5*, and, in this section, we demonstrate that cyclic *Mannich* bases can also be of interest in prodrug design.

Oxazolidines are five-membered *cyclic O*-Mannich *bases*, some of which have, indeed, been examined as potential produgs of β-amino alcohols of medicinal relevance such as ephedrines and β-blockers. For example, 3,4-dimethyl-5-phenyloxazolidine (**11.106**), the oxazolidine of ephedrine (**11.107**) undergoes hydrolysis to ephedrine and formaldehyde slowly at pH 1 and 12, but very rapidly in the neutral pH range ($t_{1/2}$ < 1 min at 37°) [135]. Interestingly, the equilibrium reached between the reactants and products of hydrolysis was markedly pH- and concentration-dependent. However, despite its poor stability in aqueous solution, the oxazolidine was delivered through human skin significantly faster than ephedrine when applied as 1% aqueous solutions of pH 7 – 11. The lower basicity of the oxazolidine (pK_a 5.5) compared to that of ephedrine (pK_a 9.6) may explain the efficient skin permeation.

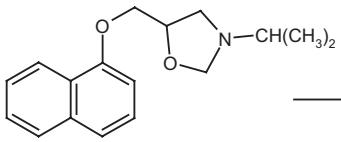
The above example clearly points to nonenzymatic hydrolysis of 3,4-dimethyl-5-phenyloxazolidine (**11.106**). However, enzymatic hydrolysis cannot entirely be ruled out. A related compound, 3-isopropyl-5-{[(naphthalen-



11.106



11.107



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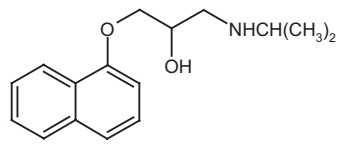
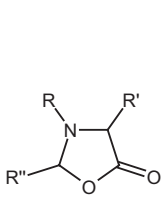


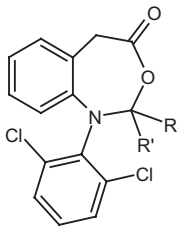
11.109

1-yl)oxy]methyl}oxazolidine (**11.108**), the oxazolidine of *propranolol* (**11.109**), was found to undergo enantioselective hydrolysis in rat liver postmitochondrial supernatant (15,000 *g* supernatant), such that the (*R*)-oxazolidine was hydrolyzed 3 – 4 times faster than the (*S*)-oxazolidine [136]. This may be taken as evidence for enzymatic catalysis, and is confirmed by the finding that hydrolysis in boiled rat liver preparations is nonstereoselective and slower by two orders of magnitude. Clearly, such results are intriguing enough to warrant further confirmation and extension.

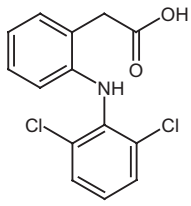
Oxazolidin-5-ones (**11.110**) are structurally related to oxazolidines, combining the motifs of a lactone and an O-*Mannich* base. These derivatives have already been discussed in *Sect. 8.7.5*. However, they serve here as a transition to *[3,1]benzoxazepin-4-ones* as an example of potential prodrugs. Thus, [3,1]benzoxazepin-4-one derivatives (**11.111**, R = H or Me, R′ = H, Me, Et, or Ph) were prepared from *diclofenac* (**11.112**) [137]. These prodrugs were stable for at least a few hours in simulated gastric juice, but, when administered to rats elicited an anti-inflammatory response comparable to that of diclofenac. One compound (**11.111**, R = Me, R′ = Et) was even more active than diclofenac without producing the gastric mucosal injury (ulcers) caused in all rats by diclofenac itself. Here again, there was no indication of whether the mechanism of hydrolysis is chemical or enzymatic.



11.110



11.111



11.112

Cyclic S-Mannich *bases* are rarely encountered in medicinal chemistry. The *(*R*)-thiazolidine-4-carboxylic acids* (**11.113**, *Fig. 11.15*), which are used as derivatives and chemical delivery systems for L-cysteine (**11.114**), provide an excellent example of S-*Mannich* bases. These compounds underwent activation by two distinct mechanisms, directly by nonenzymatic hydrolysis to cysteine and the original aldehyde (*Fig. 11.15*, *Pathway a*), and oxidatively (*Pathway b*) [138]. The latter route involved first oxidation by mitochondrial enzymes to the (*R*)-4,5-dihydrothiazole-4-carboxylic acid (**11.115**), followed by (presumably nonenzymatic) hydrolysis to *N*-acylcysteine, and, finally, cytosolic hydrolysis to cysteine (**11.114**).

A number of (*R*)-thiazolidine-4-carboxylic acids prepared from cysteine and various aldehydes have been investigated [138 – 140]. Nonenzymatic hy-

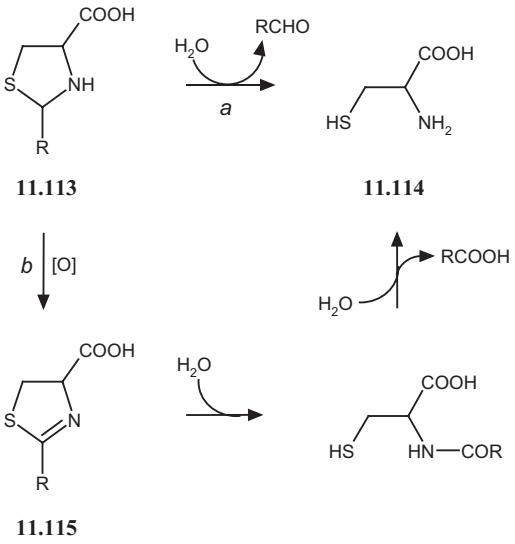
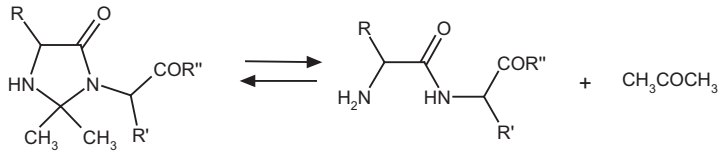


Fig. 11.15. *Mechanisms of ring opening of* (R)-*thiazolidine-4-carboxylic acids* (**11.113**) *as derivatives of and chemical delivery systems for* L-*cysteine* (**11.114**). Activation was shown to be by nonenzymatic, hydrolytic reaction (*Pathway a*), or by mitochondrial oxidation (*Pathway b*) to the (*R*)-4,5-dihydrothiazole-4-carboxylic acid (**11.115**), followed by a (presumably nonenzymatic) hydrolysis to the *N*-acylcysteine, and then by cytosolic hydrolysis to cysteine [138].

drolysis (*i.e.*, *Fig. 11.15*, *Pathway a*) was rapid when the starting aldehyde was acetaldehyde (**11.113**, R = Me) or a sugar such as glucose or xylose [138]. The same pathway was slower when the starting aldehyde was, for example, formaldehyde (**11.113**, R = H). The oxidative route (*i.e.*, *Fig. 11.15*, *Pathway b*) appears to follow the opposite selectivity, since the formaldehyde derivative was rapidly activated, and the acetaldehyde derivative was only slowly activated; the sugar derivatives were inert. Effects on the levels of low-molecular-weight thiol groups in liver and brain mitochondria were complex. A preliminary conclusion was that rapid nonenzymatic hydrolysis would overflow the cell with free SH groups, ultimately leading to a decrease in the level of these groups by overcompensating mechanisms. In contrast, enzymatic control of breakdown would prevent the initial overflow and lead to an increase in cellular SH groups.

Some *cyclic N*-Mannich *bases* have also been reported, for example, the *imidazolidin-4-ones* (**11.116**) that were investigated as potential prodrugs of peptides (**11.117**) [141]. The imidazolidin-4-ones, prepared by allowing the peptide to react with acetone under dehydrating conditions, are bases with pK_a values of 3 – 4. For most of the derivatives, hydrolysis is spontaneous; the protonated form (*i.e.*, at pH < 2) reacts *ca.* 10 – 30 times slower than does the neutral form (pH > 6). Very large differences in reactivity were noted,



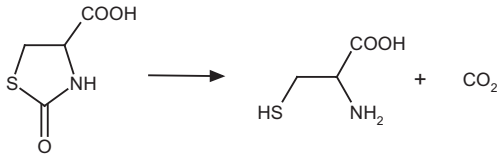
11.116 **11.117**

with $t_{1/2}$ values for hydrolysis ranging from 18 min (the Gly-Phe-Phe derivative) to 545 h (the Phe-Gly derivative) under physiological conditions of pH and temperature. Interestingly, a quantitative relation was found between the steric bulk of the R′ group in **11.116**, such that the bulkier R′, the faster the breakdown to the peptide. No enzymatic hydrolysis was observed in human plasma or in 10% rabbit intestinal homogenates.

11.8.2. Other Ring Systems

Here, we present a few other types of ring systems that also undergo metabolic hydrolytic opening.

The first cases of ring opening to be examined, involving *loss of* CO_2, is observed during activation of the cysteine prodrug, L-*2-oxo-1,3-thiazolidine-4-carboxylic acid* (**11.118**) [142]. This compound, which resembles the thiazolidines discussed above, has been shown to provide effective hepatic delivery of L-cysteine (**11.114**) [143]. L-Cysteine is then used in the liver as the limiting factor in the synthesis of the tripeptide glutathione, the major endogenous detoxifier of reactive electrophiles and radicals. All mechanistic details regarding hydrolytic ring opening of L-2-oxo-1,3-thiazolidine-4-carboxylic acid are not known, but there is evidence that the reaction is catalyzed by *5-oxo*-L-*prolinase* (EC 3.5.2.9, an enzyme that belongs to the amidohydrolases acting on lactams) [144].



11.118 **11.114**

This type of ring opening was observed in a detailed mechanistic study of the activation of the antitumor prodrug *temozolomide* (**11.119**, *Fig. 11.16*) [145]. The compound is stable in acidic media, but at pH >7 undergoes base-catalyzed activation to 5-(3-methyltriaz-2-en-1-yl)imidazole-4-carboxamide

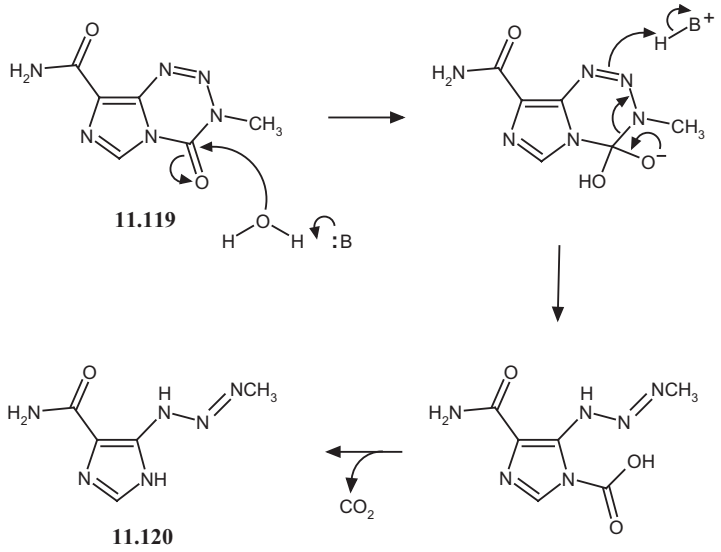
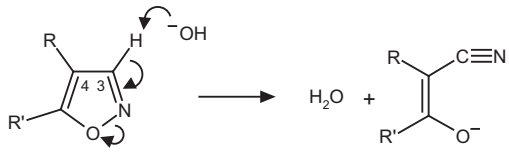


Fig. 11.16. *Base-catalyzed mechanism of activation of the antitumor prodrug temozolomide* (**11.119**) *to 5-(3-methyltriaz-2-en-1-yl)imidazole-4-carboxamide* (MTIC, **11.120**, the precursor of the reactive methyldiazonium species) [145]

(MTIC, **11.120**) according to the mechanism summarized in *Fig. 11.16*. The $t_{1/2}$ values for the reaction in 0.1M phosphate buffer at pH 7.4 and 37° was 1.8 h. The mean plasma half-life in patients after *i.v.* or oral dosing was also 1.8 h, suggesting that the prodrug is resistant to other routes of metabolism. The proximate DNA methylating agent is not MTIC, but the methyldiazonium species that is rapidly liberated upon acid-catalyzed hydrolysis of MTIC.

Additional interesting ring systems are the *1,2-oxazoles* (isoxazoles) and *1,2,4-oxadiazoles*. Reductive ring opening of the isoxazole ring has been previously reviewed (Chapt. 12 in [50]). Here, we discuss the mechanism of base-catalyzed hydrolysis reaction involving O–N bond cleavage and rearrangement of *isoxazoles unsubstituted at C(3)* (**11.121**, *Fig. 11.17*) [146]. Briefly, the reaction occurs by concerted abstraction of the proton at C(3) and ring cleavage to yield the cyanoenolate.

Of interest here is that certain drugs and prodrugs that contain an isoxazole ring are metabolized to a cyano enol, *e.g.*, *leflunomide* (**11.122**, R = CF_3, R′ = H), a prodrug of the potential anti-arthritic agent 2-cyano-3-hydroxy-*N*-[4-(trifluoromethyl)phenyl]but-2-enamide (**11.123**). After oral administration of 50 mg/kg of **11.122** to rats, the plasma concentration of the prodrug peaked at 1 μg/ml and dropped rapidly, whereas the concentration of the active metabolite **11.123** was maintained at *ca.* 100 μg/ml for 24 h [147]. This

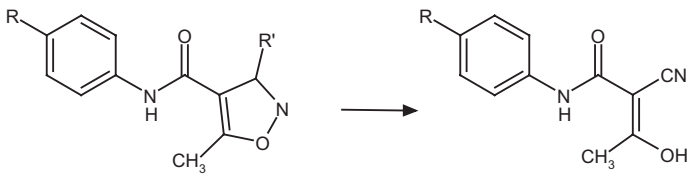


11.121

Fig. 11.17. *Concerted, base-catalyzed opening of 3-unsubstituted isoxazoles*. As demonstrated for 4-phenylisoxazoles (**11.121**, R = Ph or substituted Ph, R′ = H) and 5-phenylisoxazoles (**11.121**, R = H, R′ = Ph or substituted Ph), the first product of the reaction is a cyano enolate [146].

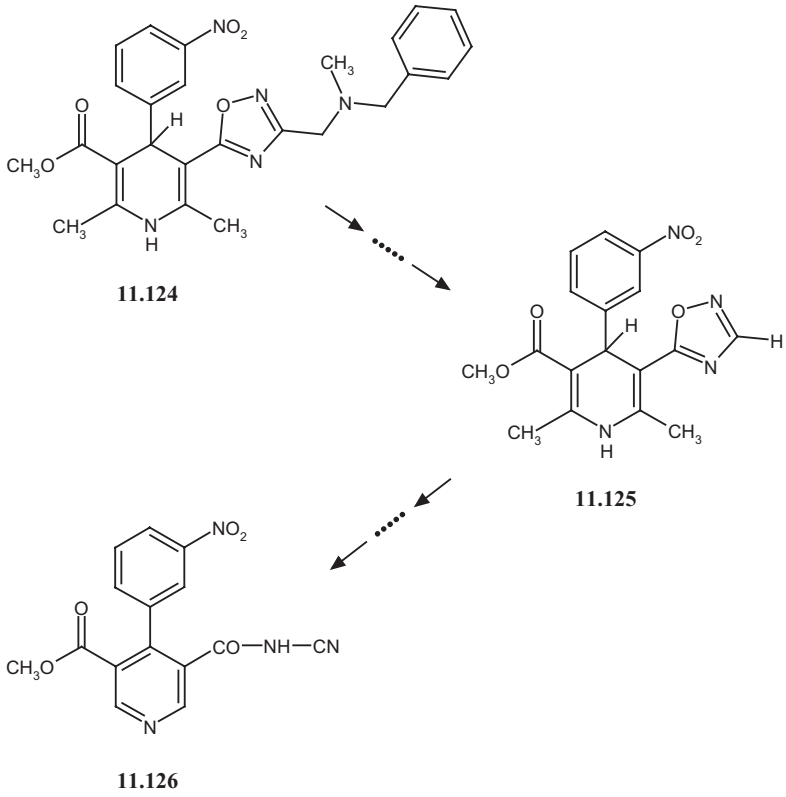
finding clearly indicates that ring cleavage, most likely by the base-catalyzed mechanism depicted in *Fig. 11.17*, is very efficient or perhaps complete, but whether the metabolic reaction is enzymatic or nonenzymatic remains to be elucidated.

The same metabolite, **11.123**, is produced when a carboxy substituent is present at C(3) (*i.e.*, **11.122**, R′ = COOH) [147][148]. The mechanism of this activation reaction is also concerted, but, in this case, more extended, with involvement of the carboxylate group and decarboxylation simultaneous with ring cleavage. *In vivo* in rats, this reaction was clearly slower than for the unsubstituted analogue, with plasma levels of both the prodrug and the metabolite **11.123** being maintained at comparable levels for 24 h.



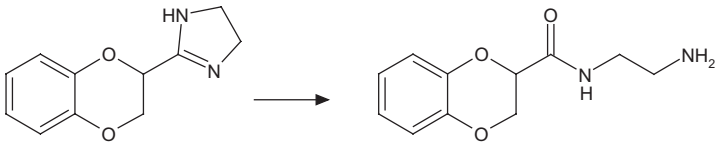
11.122 **11.123**

1,2,4-Oxadiazoles appear to exhibit comparable metabolic behavior. For example, *SM-6586* (**11.124**), a dihydropyridine calcium antagonist, yielded two major and rather unexpected biliary metabolites following oral administration to rats [149]. One metabolite (**11.125**), in which the basic side chain is lost, is probably the result of oxidative deamination to remove benzyl(methyl)amine, and oxidative decarboxylation of the remaining 3-formyl group (Chapt. 6 and 7 in [50]). The second metabolite (**11.126**) is most likely derived from the first by dehydrogenation of the dihydropyridine ring, plus cleavage of the 1,2,4-oxadiazole ring by a reaction mechanism that has not been elucidated but is probably similar to that depicted in *Fig. 11.17*.

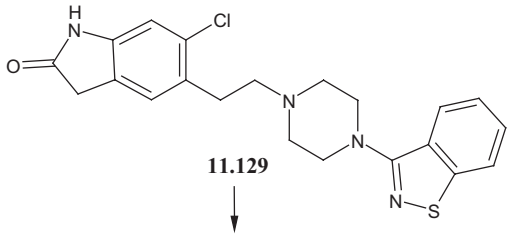


The reactivity of *cyclic imines*, the last ring system to be presented here, is difficult to compare to that of linear imines (see *Sect. 11.6*) because of the electronic and steric influence of the ring system. Only a small number of cyclic imines are drugs, and, indeed, hydrolytic cleavage of the C=N moiety plays a role in the metabolism. Foremost among cyclic imines are the *α-adrenergic imidazolines*, for example *idazoxan* (**11.127**), a selective α_2-adrenoceptor antagonist. When administered to rats, the aromatic ring of this compound is extensively oxidized, but oxidative opening of the imidazoline ring is also an important reaction [150]. It is relevant that the formation of a minor metabolite identified (**11.128**) may be easily explained by hydrolytic opening of the imidazoline ring.

To further illustrate the diversity of hydrolytic opening reactions, we turn our attention to an *isothiazole* ring as found in the antipsychotic agent *ziprasidone* (**11.129**). This drug is subject to various reactions of oxidation and reduction, but also undergoes hydrolytic cleavage of the C=N bond of the isothiazole ring. Evidence for this reaction was afforded by detection of radioactive metabolite **11.130**, a sulfonamide, in the urine of patients dosed



11.127 **11.128**



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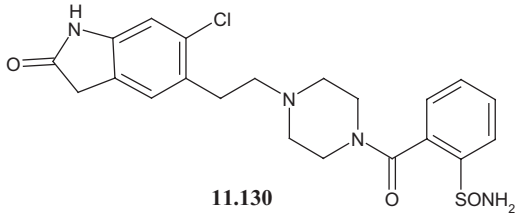


11.130

with labeled ziprasidone [151]. Two reactions contribute to the formation of this metabolite, namely sulfoxidation and C=N hydrolysis, in unknown order.

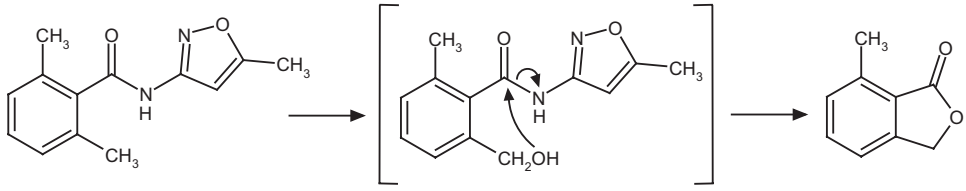
11.9. Nucleophilic Cyclizations

A large variety of metabolic cyclization reactions, counterparts to the reactions of hydrolytic ring opening discussed above, occur without any change in the degree of oxidation, and often nonenzymatically. Such reactions proceed by various mechanisms of *intramolecular nucleophilic substitution*, with *elimination* of amine, phenol, halide, or H_2O.

From a pharmacological viewpoint, the compounds of interest that undergo metabolic cyclization include ring-opened prodrugs of cyclic drugs [82], or linear drugs that yield a cyclic (and often inactive) metabolite. In the subsections below, we first examine cyclizations, which eliminate a halide or a relatively large molecule, and then proceed to discuss reactions of dehydration.

11.9.1. Cyclizations That Eliminate an Amine, a Phenol, or a Halide Anion

Cyclization–elimination reactions play an ever increasing role in prodrug design [152][153]. In most cases, the molecule eliminated is an active amine, phenol, or alcohol. This topic has been extensively reviewed in connection with *prodrug design* (see *Chapt. 8*, particularly *Sect. 8.5.7*), and, in this context, will not be given further attention here. However, cyclization–elimination reactions are not restricted to prodrugs, as we illustrate with a few selected examples. Thus, the anticonvulsant agent *D2916* (**11.131**) was found to undergo two major gender-selective oxidative reactions in rats [154]. In female rats, the Me group on the isoxazolyl ring was predominantly hydroxylated to form an active metabolite, while the Me group preferentially hydroxylated in male rats was one on the phenyl ring. The latter metabolite could not be isolated, since it underwent rapid intramolecular cyclization to the form the inactive metabolite **11.132** with elimination of 3-amino-5-methylisoxazole. There is an obvious analogy with cases discussed in *Sect. 8.5.7*.



11.131 **11.132**

Another interesting reaction is *cyclization with halide elimination*. The case of formation of aziridinium rings from *N*-(2-chloroethyl) derivatives (nitrogen mustards) has been reviewed in *Sect. 11.4.2*, as was sulfur mustard. The remainder of this subsection is devoted to comparable reactions in which the product is not a strongly electrophilic aziridinium ion, but another ring type.

2-Bromoethylamine (**11.133**, R = Br, *Fig. 11.18*) is a potent nephrotoxin used to create an experimental model of nephropathy. Its mechanism of toxicity is postulated to involve perturbation of mitochondrial function, and its metabolism was investigated in a search for toxic metabolites. In rat plasma, 2-bromoethylamine was converted to aziridine (**11.134**), formed by intramolecular nucleophilic substitution and bromide elimination [155]. Another major metabolite was oxazolidin-2-one (**11.136**). This peculiar metabolite resulted from the reaction of 2-bromoethylamine with endogenous carbonate to form carbamic acid **11.135**, followed by cyclization–elimination to oxazolidin-2-one. In aqueous media containing excess carbonate, the formation of

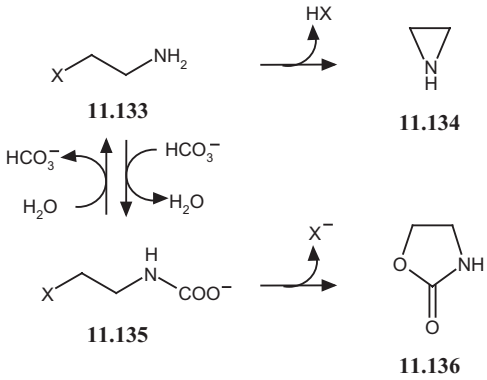
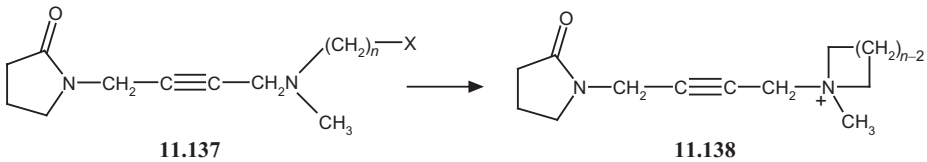


Fig. 11.18. *Cyclization–elimination reactions in the* in vitro *and* in vivo *metabolism of nephrotoxic 2-haloethylamines* (**11.133**). Aziridine (**11.134**) formation is probably a reaction of toxification, whereas oxazolidin-2-one (**11.136**) is clearly a reaction of detoxification [155][156].

oxazolidin-2-one was 200 times faster for 2-bromoethylamine than for 2-chloroethylamine (**11.133**, R = Cl), and formation of oxazolidin-2-one was even slower with 2-fluoroethylamine (**11.133**, R = F).

In rats administered 2-bromoethylamine, urinary aziridine accounted for 15 – 45% of the dose. The carbamate **11.135** was not detected in urine, whereas oxazolidin-2-one and a tertiary metabolite, 5-hydroxyoxazolidin-2-one, accounted for 0 – 20% and 2 – 12% of the dose, respectively [156]. The innocuity of oxazolidin-2-one led to the suggestion that either aziridine or 2-bromoethylamine itself is responsible for mitochondrial toxicity. These studies show that the nephrotoxic 2-haloethylamines undergo two competitive cyclizations with halide elimination, one probably a reaction of toxification, the other clearly a reaction of detoxification.

Analogues of oxotremorine with the general formula **11.137** (n = 3 or 4; X = Cl, Br, or I) have been examined as prodrugs of potent muscarinic agonists [157]. These compounds, themselves practically inactive, cyclized to form the corresponding azetidinium (**11.138**, n = 3) and pyrrolidinium (**11.138**, n = 4) ions, which are active. The cyclization reaction followed first-order kinetics at pH 7.4 and 37°, with **11.137** (n = 3, X = Cl) cyclizing slowly ($t_{1/2}$ 436 min); cyclization of the bromo and iodo analogues was more rapid ($t_{1/2}$ 11 and 14 min, respectively). Cyclization of lower homologues



($n = 2$) was one order of magnitude faster, and that of the higher homologue **11.137** ($n = 4$, X = Cl) was almost instantaneous ($t_{1/2} < 0.4$ min). Thus, the data available in this series indicate that the rate of cyclization increased in the series $n = 3$, $n = 2$, $n = 4$.

The azetidinium (**11.138**, $n = 3$) and pyrrolidinium (**11.138**, $n = 4$) analogues of oxotremorine were more stable than aziridium ions under physiological conditions, and, therefore, less likely to react with nucleophiles, *i.e.*, to alkylate biomacromolecules (see *Sect. 11.4.2*). Indeed, aziridinium analogues of oxotremorine (**11.138**, $n = 2$) exhibited brief muscarinic agonism followed by long-lasting antagonism caused by receptor alkylation. This was not observed with the azetidinium and pyrrolidinium analogues, which behaved as genuine muscarinic agonists. An interesting feature of the bioprecursor type **11.137** is its ability to permeate the blood–brain barrier, unlike the corresponding active metabolite, whose permeation is impeded by the presence of the quaternary ammonium ion.

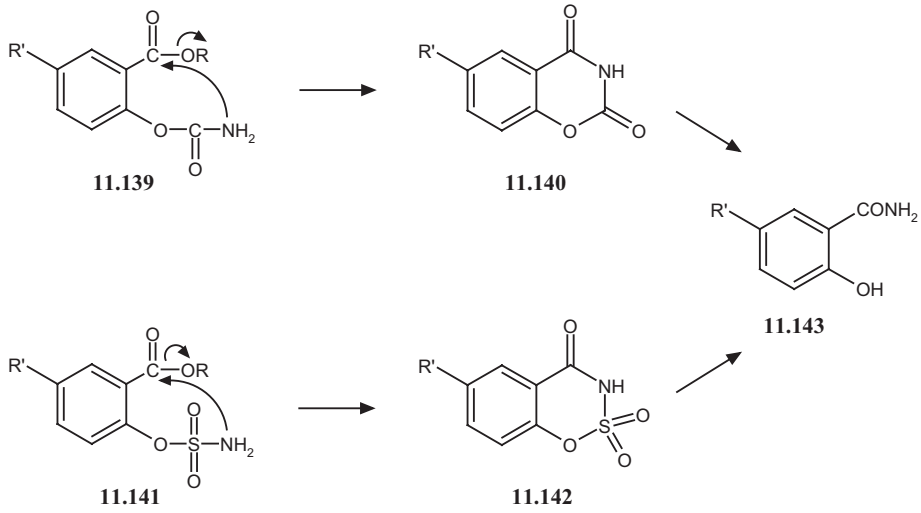
11.9.2. Formation of Lactams

The most common cyclization reactions in drug metabolism proceed with elimination of a H_2O molecule. These reactions produce lactams, lactones, cyclic *Schiff* bases, and even more-complex metabolites.

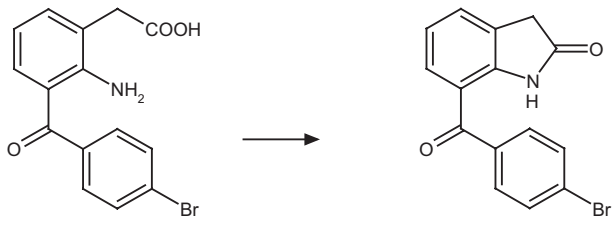
Chapt. 5 is entirely devoted to the hydrolytic ring opening of lactams. The reverse reaction, namely *lactam formation* by cyclization–dehydration, has been seldom reported in the literature, suggesting that the reaction is, indeed, uncommon. However, it is also conceivable that lactam formation has sometimes been overlooked, and that much remains to be discovered regarding structural conditions for lactam formation, the factors that influence the lactam/amino acid equilibrium, and the role of enzymes.

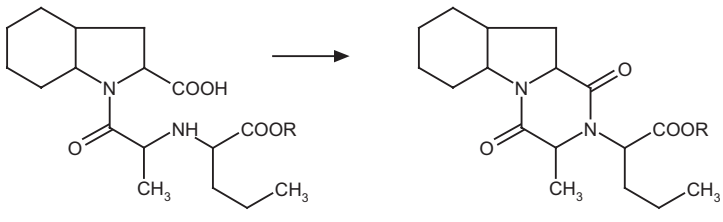
Very few *enzymes* have been characterized as lactam-forming dehydrases. One exception is carbamoylaspartic dehydrase (EC 3.5.2.3), which catalyzes the cyclization of *N*-carbamoylaspartic acid to dihydroorotic acid *en route* to pyrimidines (this enzyme is also known as dihydroorotate amidohydrolase when it catalyzes the reverse reaction).

Lactam-forming activity in rat liver microsomes has been demonstrated with the cyclization of *2-(carbamoyloxy)benzoates* (**11.139**) and *2-(sulfamoyloxy)benzoates* (**11.141**) (R = alkyl or aryl; R′ = H, Cl, Br, Me) [158]. Good yields (60 – 80%) of the products **11.140** and **11.142**, respectively, were obtained following incubation for 20 h at 20 – 25°. Incubation at 35 – 37° yielded mostly salicylamides **11.143**, *i.e.*, the common products of ring hydrolysis. No reaction was seen with heat-inactivated microsomes, seemingly ruling out nonenzymatic cyclization. Again, similarity with *Sect. 8.5.7* is clear.



Lactam formation has been documented for a few drugs or drug metabolites. For example, the *in vivo* formation of a primary lactam metabolite was reported for bromfenac and perindopril. *Bromfenac* (**11.144**) is an anti-inflammatory agent, which, in humans and monkeys, is cyclized to the lactam **11.145**, the first metabolite to appear in urine [159]. At later times post-dose, hydroxylated and conjugated metabolites of **11.145** are excreted. Thus, cyclization by dehydration appears to be the major if not the sole primary re-



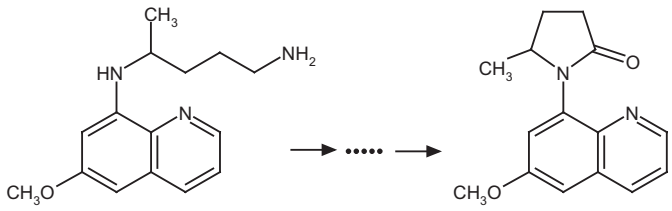


action in the metabolism of bromfenac; that this reaction is not an artifact is evidenced by its further biotransformation.

In the metabolism of *perindopril* (**11.146**, R = Et), *in vivo* cyclization to the lactam **11.147** (R = Et) was also observed [160]. In monkeys, dogs, and rats, hydrolysis of the ethyl ester group predominated, yielding perindoprilate (**11.146**, R = H). In humans, perindopril lactam (**11.147**, R = Et) and perindoprilate lactam (**11.147**, R = H) were the major metabolites. Such large interspecies differences seem to exclude artifactual formation of the lactams during sample workup.

The examples of bromfenac and perindopril clearly demonstrate that, given properly positioned amino and carboxylate groups, lactam formation is, indeed, a distinct metabolic possibility.

Formation of a lactam as a late metabolite is also possible, as documented by the identification of an important and novel lactam metabolite of the antimalarial drug *primaquine* (**11.148**) [161]. When **11.148** was incubated with hamster liver fractions for periods of up to 1 d in the absence of added cofactors, oxidative deamination at both amino groups was the primary metabolic reaction. The metabolite resulting from loss of the primary amino group was further oxidized to the carboxylic acid, which was recovered partly as such and partly as the lactam **11.149**.



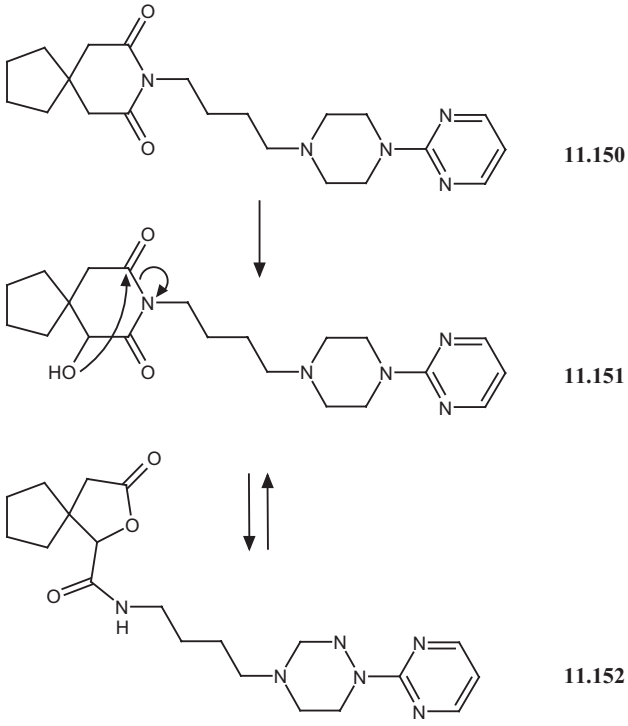
11.148 **11.149**

11.9.3. Lactam–Lactone Tautomeric Equilibrium

Although ring–ring and ring–chain tautomeric equilibria are infrequently observed in drugs or metabolites, they offer intriguing and informative examples of intramolecular reactions. The examples examined here involve lactam–lactone equilibria important for several lactamic drugs. The possibility of distinct yet comparable tautomeric equilibria is illustrated by the *ring–chain keto–hemiketal tautomeric equilibrium* shown by the immunosuppressive macrolide *tacrolimus* (FK-506) and its C(15)-*O*-demethylated metabolite [162]:

$$RR'C{=}O + HO{-}R'' \leftrightarrows RR'C(OH){-}O{-}R''$$

The tautomeric equilibrium existing in the case of tacrolimus is *metabolically relevant*, meaning that it occurs under physiological conditions and is not a chemical artifact that arises during analysis. This is, indeed, a relevant point, and great care must be taken when characterizing such equilibria to ascertain whether they are metabolically relevant or artifactual. The anti-anxiety drug *buspirone* (**11.150**) provides an example of artifactual equilibrium: the 6′-hydroxyglutarimide metabolite (**11.151**) was converted to the 5-carbamoyl-γ-lactone (**11.152**) under mildly basic conditions during purification, but seemingly not under physiological conditions [163].



The two antimigraine drugs *proxibarbal* and *valofan* (**11.153** and **11.154**, respectively, *Fig. 11.19,a*) attain lactam–lactone equilibrium, the mechanism of which is summarized in *Fig. 11.19,b*. In aqueous media, the proxibarbal/valofan ratio increased linearly with pH, from 78:22 at pH 6.75 to 84:16 at pH 7.4. As for the two diastereoisomers of valofan, their *cis/trans* ratio remained constant and close to 63:37 in the pH range of 6 – 9 [164].

To understand the pharmacokinetic relevance of the proxibarbal–valofan equilibrium, the kinetics and thermodynamics of the reaction were carefully examined in aqueous and biphasic media. The various pseudo-first-order rate constants shown in *Fig. 11.19* were determined in the pH range of 6.7 – 8.0

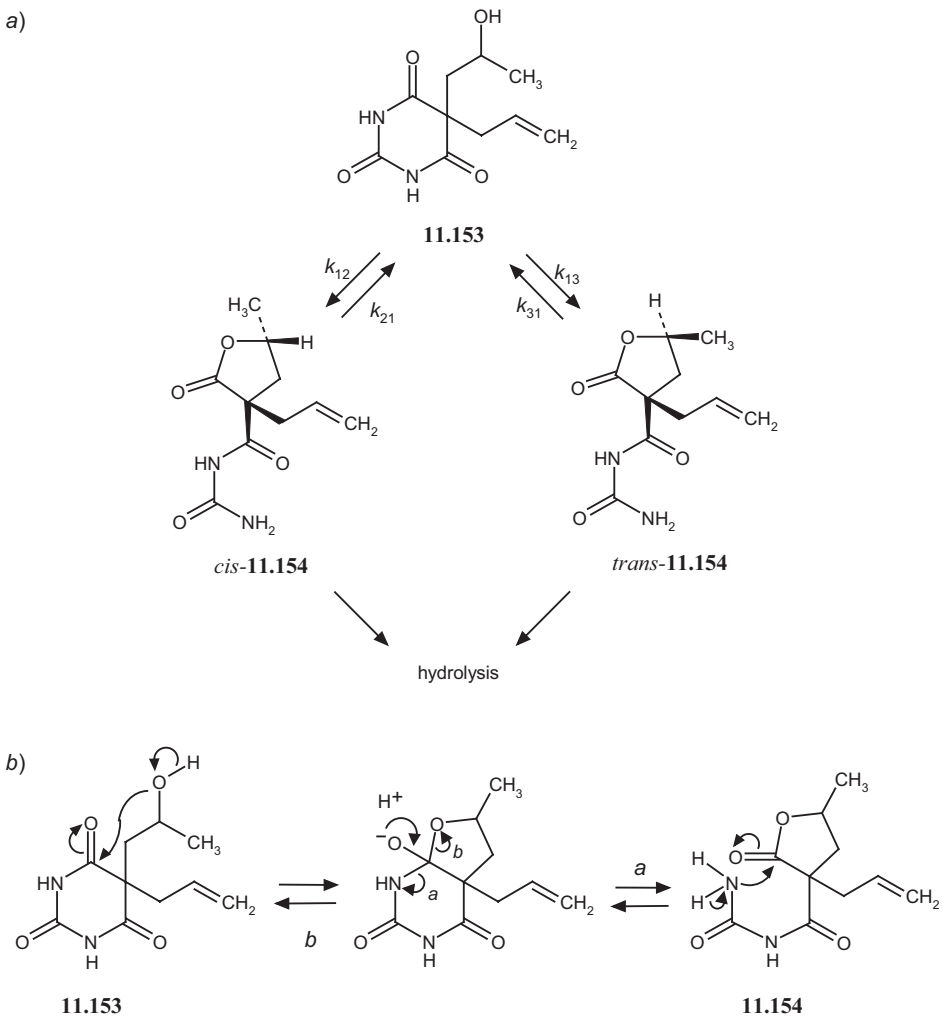


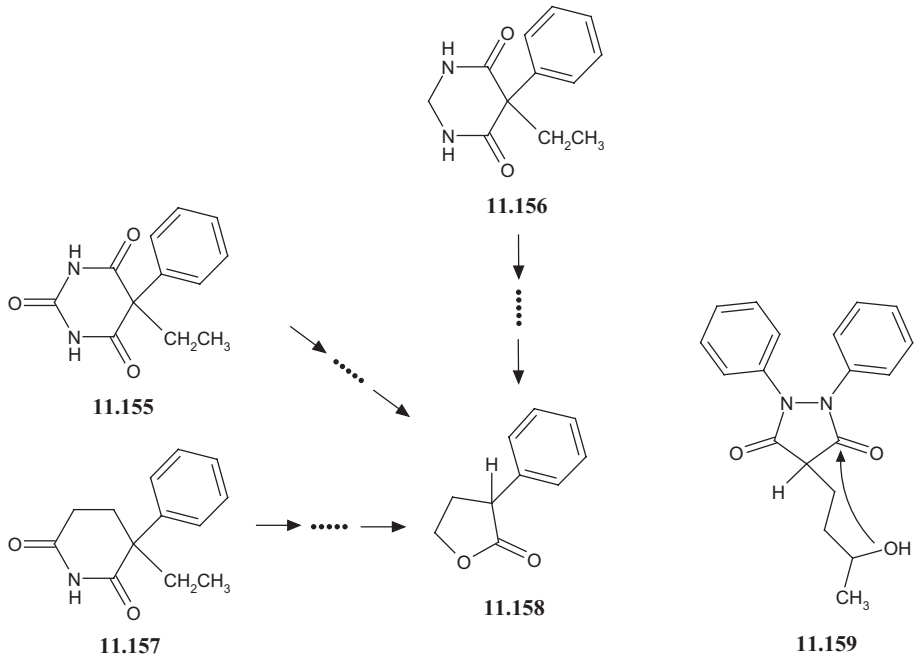
Fig. 11.19. a) *Lactam–lactone tautomeric equilibrium between proxibarbal* (**11.153**) *and valofan* (**11.154**)*, and* b) *the mechanism of interconversion* [164]

and in the temperature range of 20 – 45°. Thus, at pH 7.4 and 37°, the times for the reaction to proceed halfway to equilibrium ($t_{1/2} = 0.693/(k_{\text{forward}} + k_{\text{backward}})$) were 21 and 19 min for the conversion of proxibarbal to the *cis*- and *trans*-valofan, respectively (41 and 34 min for the conversion of *cis*- and *trans*-valofan, respectively, to proxibarbal).

These values obtained under physiological conditions prove that the proxibarbal–valofan equilibrium is metabolically relevant. However, little if any valofan was recovered in humans dosed with either proxibarbal or valo-

fan, indicating extensive biodegradation of the latter [165]. This is consistent with findings from stability studies of valofan, which, at pH 8.0 or higher, undergoes irreversible hydrolysis of the allophanyl side chain (*Fig. 11.19,a*). Presumably the same reaction occurs enzymatically *in vivo*.

Beyond proxibarbal and valofan, these results also have relevance for a number of drugs that are known or suspected to form lactone metabolites. Thus, α-phenyl-γ-butyrolactone (**11.158**) was found in human urine following ingestion of intoxicating amounts of *phenobarbital* (**11.155**), *primidone* (**11.156**), or *glutethimide* (**11.157**) [166]. This metabolite is generated by β-hydroxylation of the Et side chain, followed by lactone formation (according to the mechanism shown in *Fig. 11.19,b*) and loss of the amide side chain. A lactone metabolite analogous to **11.158** but retaining the amide side chain was also found in humans undergoing chronical treatment with *aminoglutethimide* (**11.157** with a *para*-amino group) [167].



A similar metabolic pathway has been characterized in the rat for *phenylbutazone*, whose γ-hydroxylated metabolite (**11.159**) slowly attained equilibrium with its tautomeric δ-lactone [168]. The two tautomers together represented one-third of the dose in 48 h urine.

In a number of *barbiturates*, the 5-alkyl side chain is known to undergo metabolic hydroxylation at C(2′) and/or C(3′). Examples include allobarbi-

tal, amobarbital, barbital, butalbital, nealbarbital, pentobarbital, and secobarbital. Such hydroxylated metabolites are candidate precursors of lactones and could help explain the fraction of the dose that remains unaccounted for in studies of the *in vivo* metabolism of barbiturates.

11.9.4. Cyclodehydrations by Nucleophilic Attack at a Keto Group

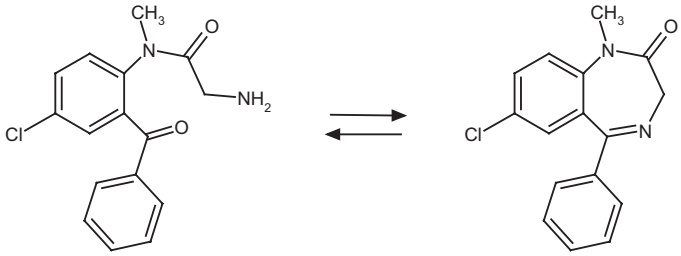
In this subsection, two types of cyclodehydrations will be discussed, namely the formation of cyclic *Schiff* bases, and the formation of cyclic enol ethers and cyclic enamines.

Both reactions involve nucleophilic attack at a keto group, in the former case by an amino group, in the latter case by an alcohol or amino group. Our objective here and throughout is not to be encyclopedic, but to reveal analogies in apparently unrelated cases.

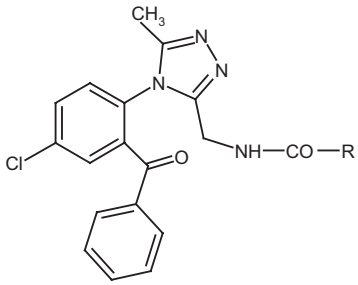
Intramolecular formation of an imino group is possible in compounds that contain properly positioned carbonyl and amino groups (primary or secondary). The equilibrium between the open-chain parent and the *cyclic* Schiff *base* product will be pH-dependent, as explained in *Sect. 11.6.1*.

Benzodiazepines, triazolobenzodiazepines, and their analogues are known to undergo reversible ring-opening reactions in acidic media at body temperature [169]. These finding have been put to good use in a prodrug strategy based on *open-chain derivatives of benzodiazepines*, which have received some attention in attempts to prepare water-soluble forms of these drugs. Thus, the *ring-opened prodrug of diazepam* (**11.160**), when administered orally to a human volunteer, yielded plasma concentration profiles for diazepam (**11.161**) that were identical to those seen after oral administration of an equivalent dose of the drug [170]. It was postulated that ring closure occurs in the gastrointestinal tract.

Another example is that of the triazolobenzodiazepine *alprazolam* [171]. Under acidic conditions, this drug undergoes ring opening to form a triazolyl-benzophenone; at neutral pH, the latter rapidly and quantitatively cyclizes back to alprazolam. Water-soluble *N*-acylated derivatives of the triazolylbenzophenone (**11.162**) were examined for their ability to liberate alprazolam by sequential hydrolysis and cyclization. In human serum, only the glycyl amide (**11.162**, R = H_2NCH_2) and the leucyl amide (**11.162**, R = $(CH_3)_2CH_2CH_2CH$ (NH_2)) were able to regenerate alprazolam. Other derivatives (**11.162**, R = H, Me, $HOCOCH_2CH_2$, or $H_2NCH_2CH_2CH_2$) were poorly hydrolyzed. *Ex vivo* receptor binding experiments in mice confirmed these results, which indicate that the rate-limiting step in alprazolam regeneration is enzymatic hydrolysis of the amide group, and not the subsequent, nonenzymatic reaction of cyclization.



11.160 **11.161**



11.162

Intramolecular imine formation was also observed during the metabolism of *cis*-3,4-dichloro-*N*-methyl-*N*-[2-(pyrrolidin-1-yl)cyclohexyl]benzamide (*U-54494A*, **11.163**, *Fig. 11.20*), an anticonvulsant/antiseizure agent [172]. In the dog, this compound was extensively oxidized at the pyrrolidine ring, in particular during first-pass metabolism. Two of the potential metabolites of pyrrolidine oxidation were the secondary amine **11.164** and the primary amine **11.165**. Interestingly, these metabolites were not observed as such, but as their cyclized products **11.166** and **11.167**, respectively. Artifactual formation appears unlikely in view of the analytical conditions used. The plasma AUC of the minor metabolite **11.167** represented *ca.* 20% that of U-54494A after oral dosing. In contrast, the imidazolinium cation **11.166** was by far the major metabolite; its AUC after oral administration was seven times greater than that of U-54494A.

The minor metabolite **11.167** in *Fig. 11.20* can be viewed as a cyclic *Schiff* base formed by reaction of the NH_2 and C=O groups. In contrast, the major and unexpected metabolite **11.166** is also a *Schiff* base, but, more precisely, it is a *permanent iminium cation* formed between a secondary amino and a keto group. Presumably, formation of **11.166** is facilitated sterically by the spatial proximity of the two reacting groups. Another factor might well be the stability of the iminium cation, which is expected to be high in acidic media (proton repulsion by the positive charge) and in alkaline media (ab-

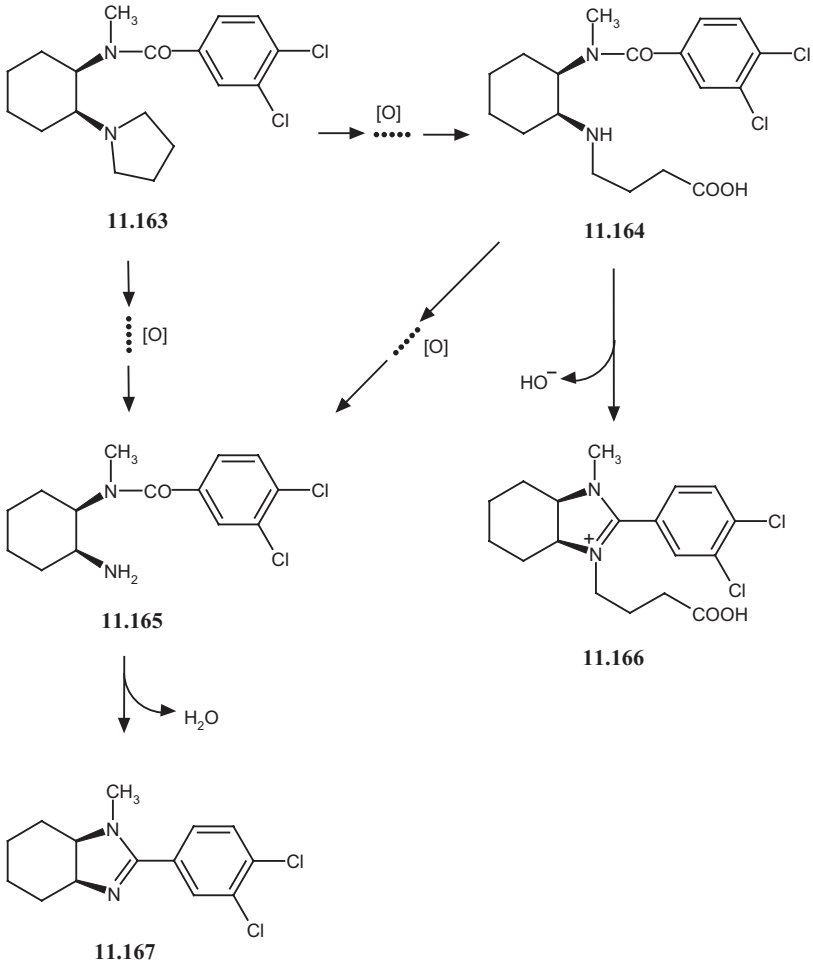


Fig. 11.20. *Examples of intramolecular imine formation in the metabolism of U-54494A* (**11.163**) [172]

sence of an abstractable proton at the C-atom). These points warrent further investigation.

A comparable reaction was seen decades ago in the metabolism of *methadone* [173 – 175]. This well-known synthetic opiate undergoes *N*-demethylation as a major metabolic reaction in humans and laboratory animals. The resulting secondary amine (**11.168**, *Fig. 11.21*) has never been isolated, as it undergoes practically instantaneous cyclization. The reaction is believed to proceed *via* the carbinolamine with formation of metabolite **11.169** as the major urinary metabolite in humans. This structurally intriguing basic compound is, in its neutral form, a pyrrolidine with an exocyclic C=C bond,

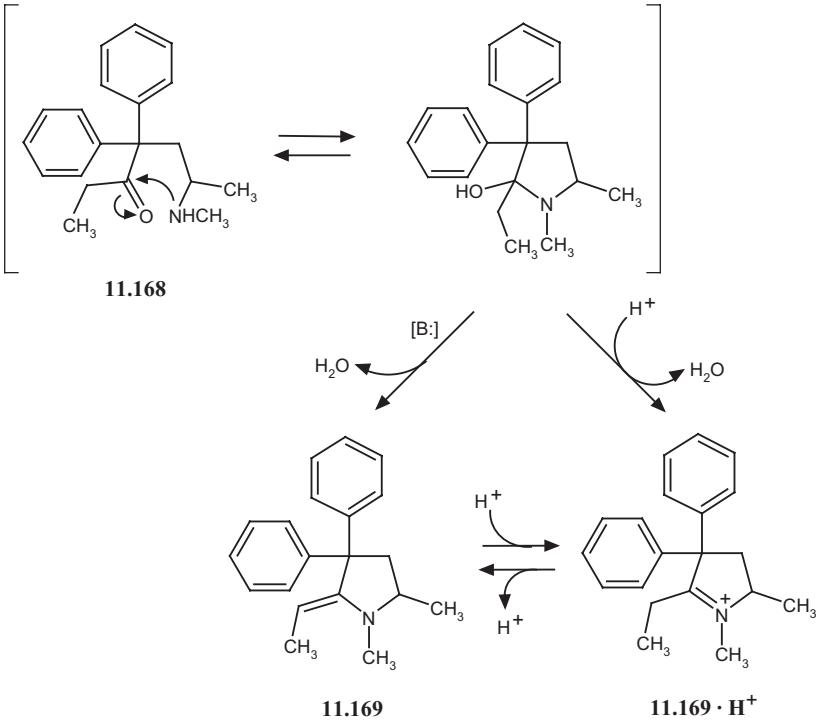


Fig. 11.21. *Following oxidative* N-*demethylation, methadone yields a secondary amine metabolite* (**11.168**) *that undergoes practically instantaneous cyclization to* **11.169** (the major urinary metabolite in humans)

whereas the protonated form (**11.169·H$^+$**) has an endocyclic iminium structure; the pK_a does not appear to have been reported. The product isolated from biological samples may be either protonated or unprotonated depending on isolation conditions.

The cyclic metabolite **11.169** was also a substrate in further biotransformations, being *N*-demethylated to the corresponding endocyclic imine, and oxidized to phenolic metabolites. Very little if any of the secondary amine metabolite (**11.168**) appeared to undergo direct *N*-demethylation to the primary amine, in contrast to many other tertiary amines, presumably due to very rapid cyclization of the secondary amine facilitated by steric and electronic factors. The possibility for the iminium cation (**11.169·H$^+$**) to become deprotonated (a reaction impossible for the iminium **11.166** in *Fig. 11.20*) should also drive the cyclization reaction.

The second type of cyclodehydration to be presented is the *formation of cyclic enol ethers and cyclic enamines*. Here, an alcohol or amino group reacts with an enol, resulting in ring closure with loss of H_2O. Such reactions

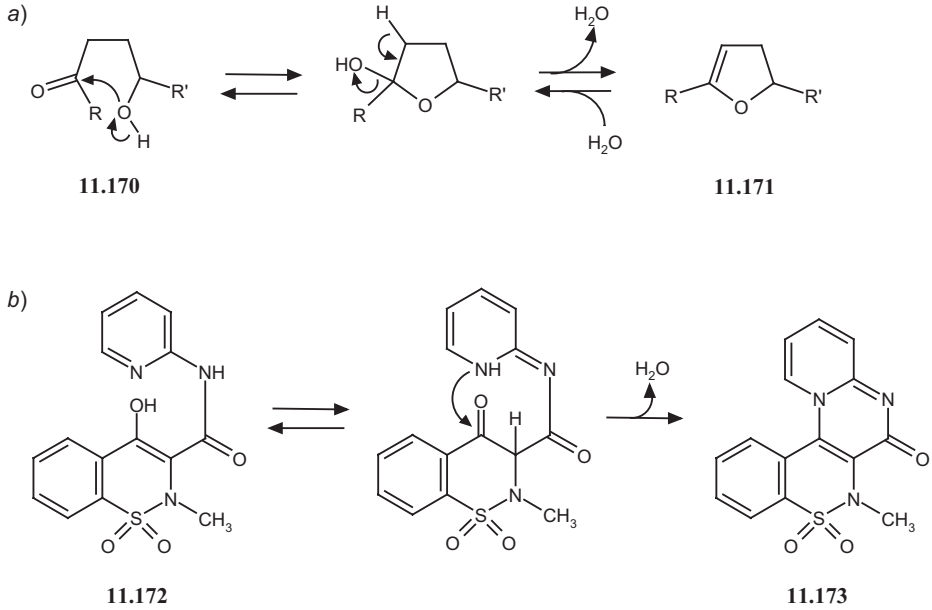


Fig. 11.22. *Mechanisms postulated for cyclodehydration of* a) *linear γ-hydroxy ketones* (**11.170**) *as metabolites of alkanes and alkanones to form 2,5-dialkyl-2,3-dihydrofurans* (**11.171**)*, and* b) *piroxicam* (**11.172**) *to form its cyclodehydrated metabolite* **11.173**

have been rarely reported and have received limited attention. In particular, little appears to be known regarding their mechanism or catalyst. The hypothetical parallels between cyclodehydration in the metabolism of alkanes and oxicams outlined here reveal clear analogies.

Rats exposed to a *heptane*-containing atmosphere excreted a variety of metabolites resulting from oxidative pathways [176]. The major metabolites were isomeric mono-alcohols and ketones, but small amounts of 2-ethyl-5-methyl-2,3-dihydrofuran (**11.171**, R = Et, R′ = Me, *Fig. 11.22,a*) and 5-ethyl-2-methyl-2,3-dihydrofuran (**11.171**, R = Me, R′ = Et) were also detected. These metabolites are believed to arise from 6-hydroxyheptan-3-one (**11.170**, R = Et, R′ = Me) and 5-hydroxyheptan-2-one (**11.170**, R = Me, R′ = Et). The postulated mechanism of formation of 2,3-dihydrofurans involves their equilibrium with the corresponding linear γ-hydroxy ketones, as shown in *Fig. 11.22,a*. Such a reaction has been documented for linear γ-hydroxy aldehydes [177].

The analogous 2,3-dihydro-2,5-dimethylfuran (**11.171**, R = R′ = Me) was formed in rats from 5-hydroxyhexan-2-one (**11.170**, R = R′ = Me), itself a primary metabolite of *hexan-2-one* [178]. These studies are relevant in the search for the toxic metabolites of alkanes and ketones responsible for

producing peripheral neuropathy in humans and animals. Much remains to be understood regarding the formation of dihydropyrans in biological systems.

A seemingly quite different reaction of cyclodehydration is found in the metabolism of the anti-inflammatory drug *piroxicam* (**11.172**, *Fig. 11.22,b*) [179][180]. Indeed, rats, dogs, monkeys, and humans receiving the drug consistently excrete the cyclodehydrated metabolite **11.173**, a major product in the dog and a minor one in the other species. Given the rigorous conditions needed to form this derivative chemically, the likelihood that it is formed artifactually is considered remote. The origin of this metabolite has not been elucidated, but, in analogy to the mechanism in *Fig. 11.22,a*, we propose the hypothetical mechanism shown in *Fig. 11.22,b*. In this mechanism, it is postulated that piroxicam reacts as the keto–imino tautomeric form, with the endocyclic NH group initiating nucleophilic substitution at the keto group. How the reaction is catalyzed in biological systems remains an intriguing question.

11.10. Hydration of Cisplatin and Analogues

The serendipitous discovery of the antitumor activity of cisplatin opened a huge field of research, leading to significant advances and successes in cancer chemotherapy [181]. Cisplatin and its analogues are reactive complexes that exhibit pharmacokinetic, pharmacodynamic, and toxicological behaviors so closely interdependent as to be all but impossible to untangle. Our focus here is and remains metabolism, but some considerations regarding activity and toxicity are also addressed.

11.10.1. Cisplatin

The discovery by *Rosenberg* of the antitumor activity of platinum compounds, most notably *cisplatin* (*cis*-diamminodichloroplatinum(II), **11.174**, *Fig. 11.23*), opened a new era in cancer chemotherapy [182][183]. These agents act as electrophiles to bind covalently to nucleophilic sites on DNA, and particularly N(7) of adenine and guanine. However, their strong electrophilicity is also responsible for reactions with other endogenous nucleophiles of low or high molecular weight. Simply stated, reactions with cellular proteins lead to cytotoxicity, whereas the reaction with H_2O results in activation. In contrast, the reaction with glutathione or serum albumin results in inactivation. The following discussion focuses on the reaction of cisplatin and analogues with H_2O, without ignoring the involvement of other reactions.

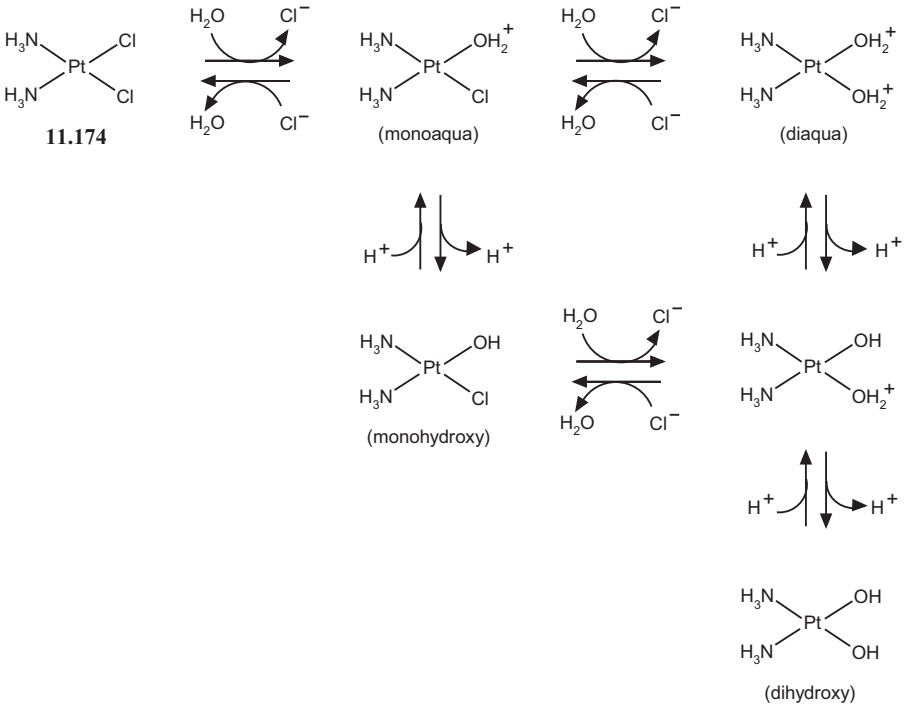


Fig. 11.23. *Aquation of cisplatin* (**11.174**) *to the cationic monoaqua and dicationic diaqua species, and ionization equilibria of these species*

Diamminodichloroplatinum(II) is a square-planar molecule that exists as the *cis*- and *trans*-diastereoisomers. Only the *cis*-diastereoisomer (cisplatin) is active. In aqueous solution, cisplatin *exchanges* Cl^- *for* H_2O, a reaction of hydration sometimes called *aquation* (*Fig. 11.23*) [184]. In phosphate buffer at pH 7 and 37°, the reaction follows first-order kinetics and proceeds to completion, cisplatin disappearing with a $t_{1/2}$ value of 2 h [185]. The first product formed, the *monoaqua species*, is a positively charged molecule with a pK_a value of *ca.* 6.6, meaning that the monohydroxy species predominates at physiological pH [186]. The second chloride can also be exchanged, but the equilibrium constant for this displacement is *ca.* 30-fold smaller than for the first. This reaction yields the *diaqua species*, a doubly charged molecule whose pK_a values have been reported at *ca.* 5.6 – 5.9 for the first deprotonation, and at *ca.* 7.3 – 7.8 for the second deprotonation to yield the dihydroxy species.

Aquation is a significant activation reaction, since the monoaqua and diaqua species are 10 – 70 times more reactive toward nucleophiles than cisplatin itself [187]. In fact, the monoaqua species is considered to be the most

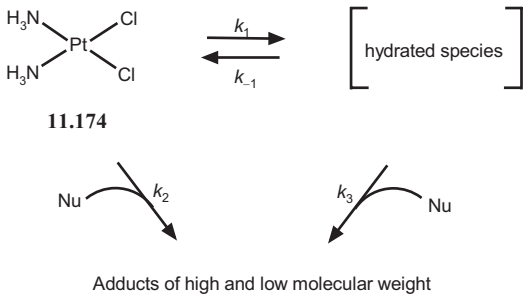


Fig. 11.24. *Nucleophilic substitution reactions of cisplatin to form low- and high-molecular-weight adducts*

pharmacodynamically significant, in agreement with the finding that lowering the intracellular pH in a tumor cell line increases the cytotoxicity and, hence, efficacy of cisplatin.

As indicated in *Fig. 11.23*, the hydration of cisplatin is reversible in the presence of Cl^- ions, and proceeds to an equilibrium that is dependent on pH and Cl^- concentration. Thus, the equilibrium was reached in *ca.* 24 h at 37° in the presence of 7 mM Cl^-, yielding the following levels of remaining cisplatin: *ca.* 84% at pH 3, *ca.* 72% at pH 5, *ca.* 30.6% at pH 6, *ca.* 4.3% at pH 7, and *ca.* 1.6% at pH 9 [185]. The rate constant of the forward reaction (k_1 in *Fig. 11.24*) was nearly constant over the pH range of 3 – 9 and was 0.2 – 0.3 h^{-1} at 37° ($t_{1/2}$ *ca.* 2.3 – 3.5 h); the second-order rate constant for the backward reaction (k_{-1} in *Fig. 11.24*) varied from 99 M^{-1} h^{-1} at pH 3 to 0.82 M^{-1} h^{-1} at pH 7 to 0.39 M^{-1} h^{-1} at pH 9.

These results are of great pharmacodynamic significance given the various levels of Cl^- concentration in the body. Indeed, the extracellular Cl^- concentration is *ca.* 0.1M, whereas intracellular concentrations are *ca.* 5 – 25 times lower [187]. As a result, the concentration ratios of cisplatin over the aqua/hydroxy species are markedly higher outside than inside cells. In other words, the less reactive, more lipophilic parent drug tends to predominate extracellularly, from where it may penetrate into cells. In contrast, the more polar, more reactive species is formed intracellularly, where it remains trapped and can act.

Many addition factors can influence the hydration of cisplatin. Indeed, cisplatin reacts with many nucleophilic compounds of high or low molecular weight, be they endogenous compounds or even inadequate pharmaceutical additives [184][188 – 192]. The reaction with high- and low-molecular-weight endobiotics leads to *mobile* and *fixed metabolites*, respectively. A scheme representing this global reaction is shown in *Fig. 11.24*. Kinetics studies with various nucleophiles indicate that the rate constant k_3 is gener-

ally much higher than k_{-1}, and that the rate constant for disappearance of cisplatin can be approximated by the sum of k_1 and k_3 (*Fig. 11.24*) [185].

The binding of cisplatin to the two major proteins in human serum, namely *serum albumin* (HSA) and *globulin*, is of particular clinical relevance. In the absence of NaCl, cisplatin binds covalently to the lone SH group (Cys^{34}) of HSA. The reaction was slower in the presence of NaCl (in agreement with the greater reactivity of the aqua species), and involved two sites per HSA molecule, but the nature of the additional site(s) was not determined [193]. Binding of cisplatin to HSA is irreversible, accounting for the very long (perhaps life-long) persistence of platinum adducts in the blood of cured patients. The binding of cisplatin to HSA and globulin is *ca.* 9 and 16 times faster, respectively, than the reaction with *glutathione*, a thiol-containing tripeptide [185]. The reaction with glutathione leads to mobile metabolites and is considered a detoxification pathway.

In human plasma at 37°, the disappearance of cisplatin occurred with a $t_{1/2}$ value of 1.3 h. Kinetics analysis demonstrated that the major components of reaction were formation of hydrated species ($t_{1/2}$ 3.4 h), irreversible binding to proteins ($t_{1/2}$ 2.1 h), and formation of mobile conjugates (*e.g.*, with glutathione; $t_{1/2}$ 24 h) [185]. A very large quantity of relevant data are compiled in [181].

11.10.2. Other Platinum Complexes

Thousands of platinum complexes have been synthesized and tested in recent years, and the pace of research remains very fast. The effort has yielded a number of compounds that exhibit higher activity, better selectivity, and/or increased therapeutic index compared to cisplatin. Some of these drugs are discussed herein.

The analogue *carboplatin* (**11.175**, *Fig. 11.25*) is a drug that exhibits activity equivalent to that of cisplatin, but it is markedly less nephrotoxic, the limiting toxicity being shifted to myelosuppression. Among other nucleophiles, carboplatin reacts with Cl^- and H_2O as shown in *Fig. 11.25*. Stepwise nucleophilic attack replaces one and then a second carboxylate ligand [194]. The end products thus formed are cisplatin (**11.174**) and its diaqua form (*Fig. 11.25*). The monoaqua and diaqua species are thought to be the active, DNA-binding forms of carboplatin. In buffered aqueous solution, the disappearance of carboplatin can be described by the equation:

$$k_{obs} = k_o + k_1[Cl^-]$$

where k_{obs} is the apparent (observed) rate constant of disappearance, k_o the pseudo-first-order rate constant of hydration, and k_1 the second-order rate

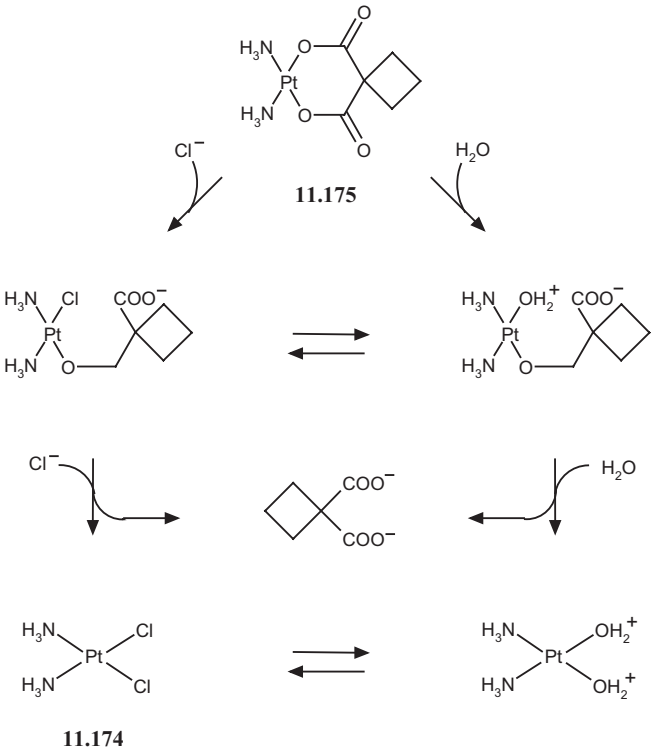
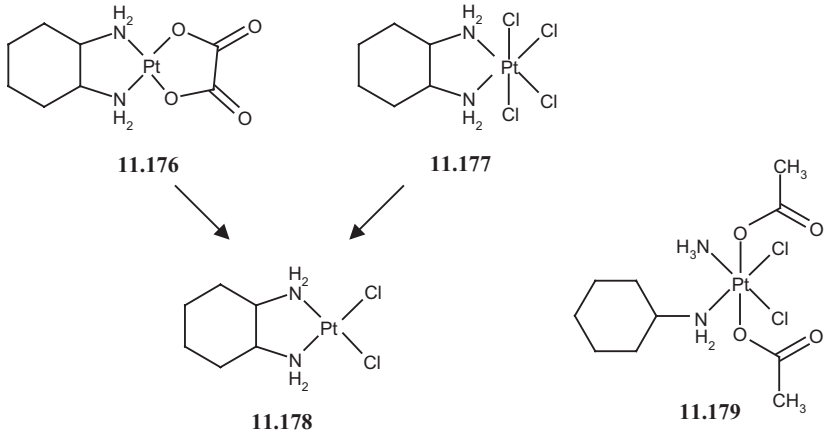


Fig. 11.25. *Nucleophilic degradation of carboplatin* (**11.175**) *by* Cl^- *and* H_2O *to yield cisplatin* (**11.174**) *and diaqua-cisplatin* [194]

constant for reaction with Cl^-. In 0.1M NaCl solution at pH 7.0 and 37°, the $t_{1/2}$ value corresponding to k_{obs} is 245 h (*ca.* 10 d!), with $k_o = 0.00207\ h^{-1}$ ($t_{1/2}$ *ca.* 14 d) and $k_1 = 0.0076\ M^{-1}\ h^{-1}$ [194]. The irreversible binding to plasma proteins is also a slow process, with a $t_{1/2}$ value of *ca.* 2 d.

A few more drugs deserve mention here. *Oxaliplatin* (**11.176**) has a chemical structure closely related to that of carboplatin, the major difference being the presence of the *trans*-diaminocyclohexane moiety (abbreviated as dach). In human plasma at 37°, oxaliplatin rapidly exchanges its carboxylate ligands with chloride to yield dichloro(*trans*-diaminocyclohexane)platinum(II) (**11.178**, abbreviated as $[PtCl_2(dach)]$). After incubation for 1 h, 30% of the drug had been transformed into $[PtCl_2(dach)]$, which itself underwent further transformation. The formation of $[PtCl_2(dach)]$ is considered to be a pathway of activation, since the metabolite is taken up by tumor cells faster than the parent drug. Another relevant reaction is covalent binding to plasma proteins, which exhibits an average $t_{1/2}$ value of 1.7 h



[195]. Rapid binding to albumin, globulins, and hemoglobin has been demonstrated in patients [196].

Tetraplatin (**11.177**) is another dach derivative, one that belongs to the subclass of Pt^{IV} complexes. In water, Cl^- is displaced by H_2O, with equilibrium at *ca.* 70% of the initial concentration being reached in 1 h at 25° [189]. In 0.9% NaCl solution, no Cl^- displacement was observed. In plasma, tetraplatin undergoes rapid reduction to [$PtCl_2$(dach)] (**11.178**), and reactivity toward plasma proteins is comparable to that of oxaliplatin [195]. Thus, both oxaliplatin and tetraplatin yield the same major metabolite in plasma, which might account for their similar cytotoxicity [197].

A Pt^{IV} complex with two axial acetoxy ligands is *JM216* (**11.179**). Upon incubation in human plasma for 1 h at 37°, it undergoes exchange of Cl for OH as the major pathway [198]. The two major metabolites were one of the isomeric monochloro monohydroxy derivative (JM518) and the dihydroxy analogue (JM383). The other isomeric monochloro monohydroxy complex (JM559) was a minor metabolite. Other metabolic routes were reduction of JM216 to amminedichloro(cyclohexylamine)platinum(II) (JM118), *i.e.*, loss of the acetoxy ligands, and conjugation with glutathione and other low-molecular-weight thiols. Irreversible plasma protein binding accounted 95% of incubated JM216 [199]. In the plasma ultrafiltrate of patients treated orally, the major metabolite was the reduction product JM118, followed by the hydroxy metabolites JM383 and JM559 [200]. These metabolites exhibited cytotoxicities similar to that of JM216.

In conclusion, nucleophilic substitution by H_2O, Cl^-, low- and high-molecular-weight thiols, and other nucleophiles plays a major role in the metabolism of platinum complexes. These reactions direct the activation, deactivation, toxification, detoxification, distribution, and excretion of platinum anticancer drugs. Given the large differences in reactivity, and the multiplicity

and interdependence of the factors involved, our understanding of the pharmacokinetic–pharmacodynamic relationships of platinum complexes remains incomplete.

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Chapter 12

Conclusion: The Biochemistry of Water

Molecular oxygen and water are the two compounds most essential for the support of life. In fact, deprivation of these two most important compounds leads rapidly to the death of aerobic organisms. But while the role of molecular oxygen is purely that of a reagent, water is seen primarily as a solvent.

Indeed, water is the medium in which life is believed to have emerged and evolved. This was made possible by the evolution of hydrophobic membranes that allow formation of compartments in which optimal concentrations and gradients of physiological compounds can be maintained and biochemical hypercycles could emerge. Imagine life appearing on a planet of methane seas – it would most likely be supported by hydrophilic membranes.

As multicellular life forms reached a sufficient degree of complexity, they improved their physical separation from the water medium, allowing migration to land and continued evolution in novel environments. In terrestrial plants and animals, water is no longer both external and internal, but solely the internal milieu, the *milieu intérieur*, in constant flux with the environment yet carefully separated from it. Such, in a nutshell, is the *biophysical role* of water as a medium.

But there is more to water than its role as solvent, since, like oxygen, it is also a reagent (as well as a product, but this aspect is irrelevant here) in innumerable biochemical reactions. This is the *biochemical role* of water, a double one, in fact. First and foremost, water is a reagent in innumerable life-supporting reactions. Secondly, and like for oxygen, evolution has recruited water as a major player in the detoxification and elimination of xenobiotics. This biological function of water, we believe, is presented here for the first time in its full depth and breadth.

Biological systems have 'learned' to generate and 'tame' highly reactive chemical species such as $NO^{\bullet}$, H^{-}, $O_2^{\bullet-}$, $HO^{\bullet}$, and oxene, in other words to produce and trap them in nano-environments where they can react productively rather than deleteriously. But biological systems have also learned a contrasting strategy, which is to enhance the chemical reactivity of a mild molecule such as water. As illustrated in this book, a diversity of enzyme mechanisms have been discovered by nature to *render water more nucle-*

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ophilic and, hence, more reactive. These more nucleophilic forms of water lie in a continuum between the isolated water molecule at one extreme, and at (or near) the other extreme water having lost one proton (*i.e.*, having become the HO^- ion). One can only wonder at the contrast between the chemical simplicity of this principle, and the complexity of the underlying mechanisms.

To summarize what was discussed mainly in *Chapt. 3* and *10*, enzyme mechanisms of hydrolysis/hydration fall into two large subdivisions:

- In one subdivision, the enzymatic cycle begins with an activated functional group in the catalytic site attacking the bound and polarized substrate to form an acylated intermediate. This group is a OH function in serine hydrolases (*Fig. 3.3*), a SH group in cysteine hydrolases, and a carboxylate group in epoxide hydrolases (*Fig. 10.6*). *Water enters the catalytic cycle in a second step* to hydrolyze the acylated intermediate, its reactivity being enhanced by a basic residue at the active site. In the hydrolases of this subdivision, the base is generally a histidine imidazole, the reactivity of which is fine-tuned by neighboring amino acid side chains.
- In the other subdivision, *water activation occurs in the first step* of the enzymatic cycle. This activation is achieved by a carboxylate group in aspartic hydrolases (*Fig. 3.10*), Zn^{2+} and a carboxy group in metallopeptidases (*Fig. 3.12*), a histidine side chain in calcium-dependent hydrolases (*Fig. 3.14*), or a Zn^{2+} in carbonic anhydrase (*Fig. 3.15*). The substrate, on the other hand, is polarized (activated) by a carboxy group in aspartic hydrolases or by a cation in metallopeptidases and calcium-dependent hydrolases. In this manner, the reactivity of both the water molecule and the substrate is enhanced and fine-tuned to drive formation of a tetrahedral intermediate that will break down to form the hydrolysis products.

Also noteworthy here is the diversity as well as the underlying similarity of the *substrates of hydrolases*. The vast majority of such substrates are either amides or carboxylic acid esters, in other words, molecules in which a carbonyl group is polarized by an adjacent heteroatom. Other substrates are epoxides, the target motif of which is, again, a highly polarized C–O bond. In esters of inorganic acids, the carbonyl C-atom is replaced by an electron-deficient S- (*e.g.*, sulfates), P- (*e.g.*, phosphates), or N-atom (*e.g.*, nitrates). Other types of substrates include the polarized C–halogen and C=N motifs. Thus, the common target of all hydrolases is a $Z^{\delta+}$–$X^{\delta-}$ motif with Z = C, S, P, or N, and X = O, S, N, or halogen, *i.e.*, an electron-deficient atom (often a C-atom) linked to an electron-rich atom (often an O-atom).

This book has had as its first objective a comprehensive and systematic presentation of hydrolytic reactions in the metabolism of drugs, prodrugs, and other xenobiotics. As such, it aims to fulfill a utilitarian and an educa-

tional role by providing information and knowledge. But those who take the global view of the work presented here cannot fail to be impressed by the simultaneous unity and diversity of hydrolytic reactions. To repeat and to summarize, there are, indeed, intriguing contrasts in the impressive variety of enzymes and the underlying correspondence of their catalytic sites, the complexity of the individual catalytic mechanisms and the chemical simplicity of water activation, and the immense chemical diversity of substrates and the simplicity of their common motifs.

Science progresses by accumulating disparate evidence and uncovering the underlying unifying principles. The unity inherent in hydrolytic reactions is but one aspect of the simplicity of the principles by which Nature operates.

Index*)

*) For compound names, inverted indexing (*CA*) has been used; *e.g.,* '4-(4-Chlorophenyl)-2-methylpyrrolidin-3-ol' is indexed under 'Pyrrolidin-3-ol, 4-(4-chlorophenyl)-2-methyl-'.

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