CHAPTER SEVENTEEN

Approaches to the Rational Design of Enzyme Inhibitors

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Burger's Medicinal Chemistry and Drug Discovery
Sixth Edition, Volume 1: Drug Discovery
Edited by Donald J. Abraham
ISBN 0-471-27090-3 © 2003 John Wiley & Sons, Inc.

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1 INTRODUCTION

Many of the top 100 drugs sold worldwide are enzyme inhibitors. In recent years, enzyme inhibitors not only have provided an increasing number of potent therapeutic agents for the treatment of diseases, but also have significantly advanced the understanding of enzymatic transformations. The aim of this chapter is to present current approaches to so-called rational inhibitor design, which uses knowledge of enzymic mechanisms and structures in the design process. Rational inhibitor design is intended to complement laborious and resource-consuming screening processes, which consist of testing large numbers of synthetic chemicals or natural products for inhibitory activity against a chosen target enzyme.

1.1 Enzyme Inhibitors in Medicine

A human cell contains thousands of enzymes each of which can, theoretically, be selectively inhibited. These enzymes constitute the various metabolic pathways that, in concert, provide the requirements for the viability of the cell. A selective inhibitor may block either a single enzyme or a group of enzymes, leading to the disruption of a metabolic pathway(s). This will result in either a decrease in the concentration of enzymatic products or an increase in the concentration of enzymatic substrates. The effectiveness of an enzyme inhibitor as a therapeutic agent will depend on (1) the potency of the inhibitor, (2) its specificity toward its target enzyme, (3) the choice of metabolic pathway targeted for disruption, and (4) the inhibitor or a derivative possessing appropriate pharmacokinetic characteristics. Higher potency will mean less drug is required to obtain a physiological response, whereas high specificity means that the inhibitor will react only with its target enzyme and not with other sites in the body. Taken together, low dosage and high specificity combine to reduce both the toxicity caused by inhibition of other vital enzymes and the problems arising from the formation of toxic decomposition products. Further, high specificity will generally avoid depletion of the inhibitor concentrations in the host by nonspecific pathways. The areas of potency and specificity will both be addressed in this chapter. Clearly, the choice of target enzyme is also of prime importance for chemotherapy, although that is beyond the scope of this review. However, there are a number of texts available that provide a good introduction to this subject (1–4). Good bioavailability of the drug is also crucial for the drug to reach its site of action in the body in effective therapeutic concentrations. For example, highly polar or charged compounds, such as phosphorylated compounds, frequently cannot readily cross cell membranes and are therefore generally less useful as drugs. Physical approaches to facilitate the transport of this class of compounds into the cell include the use of liposomes or nanoparticles (5–7). Chemical approaches may also be employed. These include the use of prodrugs, in which functional groups on the inhibitor are modified in such a manner that they are able to be taken up by the cell and, later, metabolically converted to the active drug. Prodrugs are discussed in more detail in Volume **2,** Chapter 14.

As indicated earlier, a wide variety of enzyme inhibitors have found use in the clinic. Tables 17.1–17.3 show a number of these compounds and, although they provide by no means an exhaustive list, they do give an indication of the range of human disease states that can be ameliorated with the use of enzyme inhibitors.

The human body, even though its defenses are constantly on guard, is still susceptible to invasion by foreign pathogens. Since the de-

Table 17.1 Examples of Enzyme Inhibitors Used in the Treatment of Bacterial, Fungal, Viral, and Parasitic Diseases

Clinical Use	Enzyme Inhibited	Inhibitor
Antibacterial	Dihydropteroate synthetase	Sulphonamides
Antibacterial	Dihydrofolate reductase	Trimethoprim, methotrexate
Antibacterial	Alanine racemase	D-Cycloserine
Antibacterial	Transpeptidase	Penicillins, cephalosporins
Antifungal	Fungal sterol 14α-demethylase	Clotrimazole, ketoconazole
Antifungal	Fungal squalene epoxidase	Terbinafine, naftifine
Antiviral	Thymidine kinase and thymidylate kinase	Idoxuridine
Antiviral	DNA, RNA polymerases	Cytosine arabinoside (Ara-C)
Antiviral	Viral DNA polymerase	Acyclovir, vidarabine
Antiviral	HIV reverse transcriptase	Dideoxyinosine, zidovudine
Antiviral	HIV protease	Saquinavir
Antiviral	Influenza virus neuraminidase	Zanamavir, oseltamivir
Antiprotozoal	Pyruvate dehydrogenase	Organoarsenical agents
Antiprotozoal	Ornithine decarboxylase	a-Difluoromethylornithine

velopment of the sulfa drugs (sulfonamides), enzyme inhibitors have played a vital role in controlling these infectious agents. Table 17.1 provides a list of enzyme inhibitors that have been used in the treatment of the various diseases caused by these agents. All these compounds needed to satisfy the usual requirements for specificity and low toxicity.

This can be achieved in a variety of ways. For instance, it is possible to inhibit an essential pathway in the pathogen that does not exist in the host. D-Cycloserine **(1)** (Fig. 17.1), for example, inhibits alanine racemase, an enzyme involved in bacterial cell wall biosynthesis and not found in humans (8, 9). D-Cycloserine is active against a broad spectrum of both gram-positive and gram-negative bacteria (10), but plays its major role in the treatment of tuberculosis (11). Conversely, even if both host and pathogen contain the same enzymes, it may be possible to exploit subtle structural differences between the isozymes to obtain a highly specific inhibitor that preferentially binds to the invader's version. Trimethoprim (2) shows this selective toxicity. An inhibitor of dihydrofolate reductase, trimethoprim is a potent antibacterial agent because the bacterial enzyme is inhibited at a concentration several thousand times lower than that required for inhibition of the mammalian isozyme (12). Acyclovir (**3a**), an antiviral drug used for the treatment of herpes infections **(13,** 14), also fits into this category. It binds very tightly to the ***Herpes simplex*** DNA polymerase with an estimated half-life of about 40 days. Acyclovir is a prodrug because it requires transformation by a viral thymine kinase and cellular phosphotransferases to the corresponding triphosphate (**3b**) to serve ***in vivo*** as an inhibitor of the viral DNA polymerase **(15).**

Table 17.2 Examples of Enzyme Inhibitors Used in the Treatment of Cancer

Type of Cancer	Enzyme Inhibited	Inhibitor
Benign prostatic hyperplasia	Steroid 5α-reductase	Finasteride
Estrogen-mediated breast cancer	Aromatase	Arninoglutethimide, fadrozole
Leukemia, osteosarcoma, head, neck, and breast cancer	Dihydrofolate reductase	Methotrexate
Colorectal cancer	Thymidylate synthase	5-Fluorouracil
Leukemia	Glutamine-PRPP amidotransferase	6-Mercaptopurine, azathioprine
Small-cell lung cancer, non-Hodgkin's lymphoma	Topoisomerase II	Etoposide
Hairy-cell leukemia	Adenosine deaminase	Pentostatin

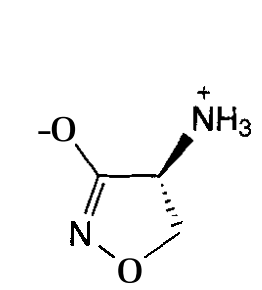
Table 17.3 Examples of Enzyme Inhibitors Used in Various Human Disease States

Clinical Use	Enzyme Inhibited	Inhibitor
Epilepsy	GABA transaminase	γ-Vinyl GABA
Epilepsy	Carbonic anhydrase	Sulthiame
Epilepsy	Succinic semialdehyde dehydrogenase	Sodium valproate
Antidepressant	Monoamine oxidase (MAO)	Tranylcypromine, phenelzine
Antihypertensive	Angiotensin converting enzyme	Captopril, enalaprilat
Cardiac disorders	Na^+,K^+-ATPase	Cardiac glycosides
Gout	Xanthine oxidase	Allopurinol
Ulcer	H^+,K^+-ATPase	Omeprazole
Hyperlipidemia	HMG-CoA reductase	Atorvastatin, simvastatin
Anti-inflammatory	Prostaglandin synthase, Cyclooxygenase (COX) I and II	Aspirin, naproxen, ibuprofen
Arthritis	Cyclooxygenase (COX) II	Celecoxib
Glaucoma	Acetylcholinesterase	Neostigmine
Glaucoma	Carbonic anhydrase II	Acetazolamide, dichlorphenamide

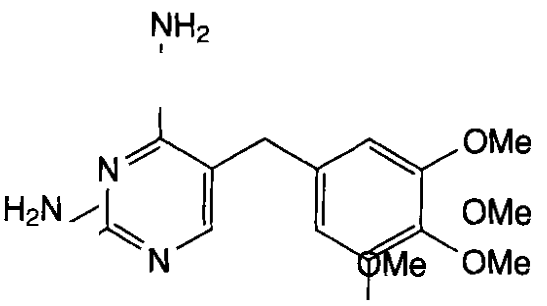
Although their inhibitors are not specifically therapeutic agents in themselves, the p-lactamases are another important target for drug design. These are bacterial enzymes and, as with the alanine racemases, are not found in humans. Inhibitors of β-lactamases include clavulanic acid (4) (16–20) and sulbactam (penicillanic acid sulfone) (5) (18, 21–24). These two compounds act to prevent the bacterial degradation of penicillins and cephalosporins by p-lactamases, thereby extending their lifetime and effectiveness. Accordingly, both clavulanic acid (4) and sulbactam (5) have reached the market as drugs that act synergistically with these commonly prescribed antibacterial agents.

Even though it has proved possible to selectively inhibit the enzymes of a number of pathogens, the enzymes of cancer cells have proved to be a far more elusive target. Indeed, the majority of the currently employed antitumor agents can be described as antiproliferative agents. These take advantage of the fact that many, but not all, tumor cells grow and divide more rapidly than normal cells. Lymphomas, for example, proliferate more rapidly than solid tumors, whereas, conversely, acute leukemia cells divide more slowly than the surrounding bone marrow cells. Most of the enzyme inhibitors used as these antiproliferative agents (Table 17.2) can also be described as antimetabolites (i.e., they inhibit a metabolic pathway), often those involved in DNA biosynthesis, which are important for cell survival or replication. 5-Fluorouracil (6), the prodrug form of an inactivator of thymidylate synthase (25), and methotrexate (7), an inhibitor of dihydrofolate reductase (26, 27), both fit into this category. Unfortunately, rapidly dividing normal cells, such as hair follicles, the cells lining the gastrointestinal tract, and the bone marrow cells involved in the immune system are also significantly affected. The resultant hair loss, nausea, and susceptibility to infection means that this type of chemotherapy is seldom employed as a first-line defense against cancer.

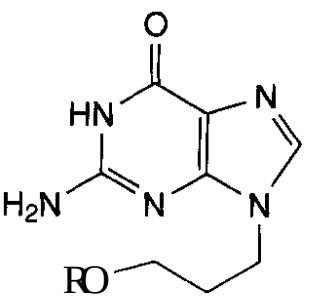
The inhibition of enzymes involved in metabolic pathways is not restricted to anticancer agents. A variety of diseases have been correlated with either the dysfunction of an enzyme or an imbalance of metabolites. A cross section of the disease states treated with enzyme inhibitors is shown in Table 17.3. Practically, these may be treated by the inhibition of an individual enzyme or by using enzyme inhibitors to regulate the metabolite concentration in the body. For example, an imbalance of the two neurotransmitters, glutamate and γ-aminobutyric acid, is responsible for the convulsions observed in epileptic seizure. The latter is metabolized by y-aminobutyric acid aminotransferase (GABA-T) and, consequently, inhibitors of this enzyme offered themselves as potential antiepileptic candidates. This led to the development of the GABA-T inhibitor, vigabatrin (8)(28), which clinically results in an increase of the brain concentration of γ-aminobutyric acid and cessation of epileptic convulsions. As with the anticancer agents, block-



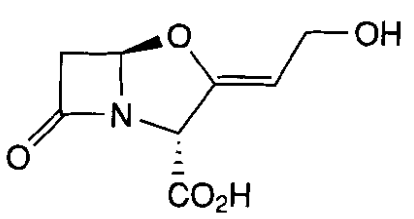
(1)



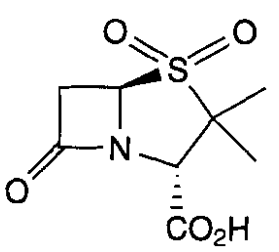
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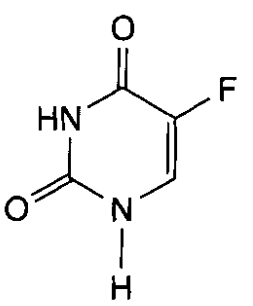
(3a) R = H
(3b) R = PPP



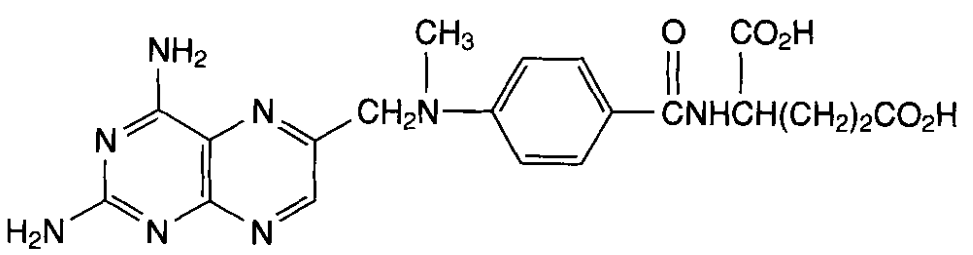
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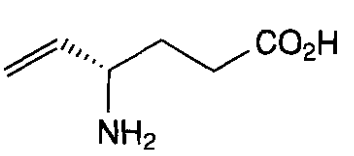
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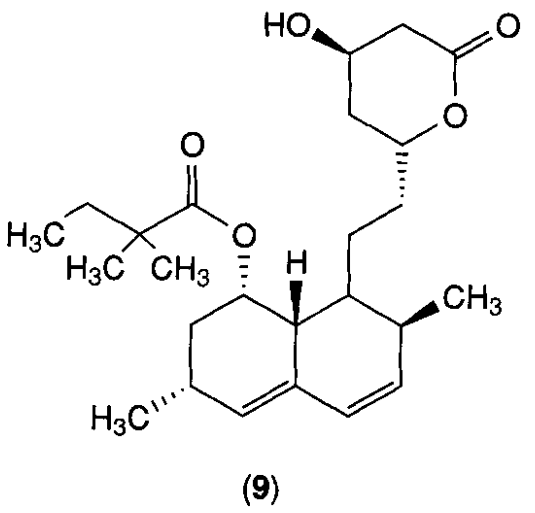
(6)



(7)



(8)



(9)

Figure 17.1. Examples of enzyme inhibitors used clinically.

ade of a metabolic pathway may also have therapeutic benefits. The **statins**, a group of serum cholesterol-lowering drugs, are inhibitors of hydroxymethylglutaryl-CoA (**HMG-CoA**) reductase (29). HMG-CoA reductase catalyzes the irreversible conversion of HMG-CoA to mevalonic acid, the rate-determining step in cholesterol biosynthesis (30–32). Inhibitors such as simvastatin (9) have been found to be effective in the treatment of **hyperlipidemia** and familial hypercholesteremia (33, 34) and have become some of the world's best-selling drugs.

Finally, enzyme inhibitors can also be used to induce an animal model of a genetic disease. Inactivation of **γ-cystathionase** by **propargylglycine**, for example, produces an experimental model of the disease state known as **cysta-**

Table 17.4 Classification of Enzyme Inhibitors Employed in This Chapter

Noncovalent Inhibitors	Covalent Inhibitors
Rapid reversible inhibitors (ground-state analogs)	Chemical modifiers
Tight, slow, slow-tight binding inhibitors	Affinity labels
Multisubstrate analogs	Mechanism-based inhibitors
Transition-state analogs	Pseudoirreversible inhibitors

thioninuria (35). Deficiency of this enzyme leads to the accumulation of cystathionine in the urine and has sometimes been associated with mental retardation **(36).**

1.2 Enzyme Inhibitors in Basic Research

In basic research enzyme inhibitors have found a multitude of uses. They serve as useful tools for the elucidation of structure and function of enzymes, as probes for chemical and kinetic processes, and in the detection of short-lived reaction intermediates **(37).** Product inhibition patterns provide information about an enzyme's kinetic mechanism and the order of substrate binding **(38).** Covalently binding enzyme inhibitors have been used to identify active-site amino acid residues that could potentially be involved in substrate binding and catalysis of the enzyme **(39, 40).** Reversible enzyme inhibitors are routinely used to facilitate enzyme purification by using the inhibitor as a ligand for affinity chromatography (41, 42) or as eluants in affinity-elution chromatography **(43).** Immobilized enzyme inhibitors can also be used to identify their intracellular targets (44), whereas irreversible inhibitors can be used to localize and quantify enzymes in *vivo* **(45).**

In Table **17.4** we have provided the classification of the various types of enzyme inhibitors that we employ in this chapter. The classification may appear somewhat arbitrary, in that some inhibitors may fit into more than one category. This can arise because these categories are attempting to bring together some nonrelated properties such as structure, mechanism of action, and kinetic behavior. Thus, what we have classed as a reversible inhibitor may, simply because it has a slow dissociation rate, be described elsewhere in the literature as being irreversible. In each instance we will discuss approaches to the design of that type of inhibitor, as well as indicating how it may be evaluated. The discussion will be accompanied by references to recent, representative examples from the literature. Where appropriate, these examples will be of inhibitors of therapeutic interest.

It should be noted that we will concentrate on inhibitors directed at the active site of the enzyme. While recognizing that there are inhibitors that bind to regions other than the active site, such as allosteric effectors, these are not the focus of this chapter and will not be included. There are many reviews of enzyme inhibitors available in the literature **(37,** 46–48) and the reader is referred to them for more detailed analysis.

2 RATIONAL DESIGN OF NONCOVALENTLY BINDING ENZYME INHIBITORS

As their name indicates, this class of inhibitors binds to the enzyme's active site without forming a covalent bond. Therefore the affinity and specificity of the inhibitor for the active site will depend on a combination of the electrostatic and dispersive forces, and hydrophobic and hydrogen-bonding interactions. Traditionally, noncovalently binding enzyme inhibitors were analogs of substrates, products, or reaction intermediates. More recently, an explosion in the use of combinatorial chemistry and rapid screening techniques has seen the development of large numbers of enzyme inhibitors that bear little or no resemblance to the substrate or products, yet still bind selectively to their target enzyme. Computer-aided drug design, in the broadest sense, encompasses both structure-based drug design and quantitative structure-activity relationship (QSAR) methods. A complement to the rapid screening techniques, computer-aided methods provide a more focused approach to the

design and discovery of both substrate and nonsubstrate analog inhibitors.

In structure-based design, the structure of a drug target interacting with small molecules is used to guide drug discovery. Consequently, either the three-dimensional enzyme structure or, at a minimum, the pharmacophore structure must be known. A pharmacophore represents the nature of the chemical groups of a given ligand and their relative orientation important for inhibitor binding. Today, structure-based design, used in conjunction with docking techniques, combinatorial chemistry, and rapid screening not only leads more quickly to novel enzyme inhibitors but also greatly reduces the number of compounds that must be synthesized. More information on these approaches may be found in Chapter 10 and some recent monographs (49–52).

Traditionally, an increase in inhibitory or biological activity was achieved by synthesizing an analog of the substrate and then making gradual empirical changes in the structure by adding or removing functional groups. QSAR methods provide a means of making this empirical testing more focused. In this technique there is no need to know the structure of the active site. Instead, computer algorithms are employed to correlate the biological activity of a series of inhibitors with their chemical structure, thereby allowing better predictions as to how to change the structure to obtain a more potent inhibitor. This topic is discussed further in Chapter 1, and detailed reviews are also available (53–56).

Table 17.4 shows the classification of noncovalent inhibitors we use in this chapter. Based on their kinetics it is possible to distinguish among rapid reversible, tight-binding, slow-binding, slow-tight-binding, irreversible, and pseudoirreversible inhibitors. Conversely, inhibitors classified on the basis of structure, such as ground-state analogs, multisubstrate inhibitors, and transition-state analogs, which mimic the structures of substrates and products, reaction intermediates, and transition states, may fall into any of the kinetic categories. However, before introducing these categories, it is important to have an understanding of the forces involved in the binding of substrates and inhibitors to an enzyme's active site.

2.1 Forces Involved in Forming the Enzyme-Inhibitor Complex

To understand the design concepts of the various types of noncovalently binding enzyme inhibitors, a basic knowledge of the binding forces between an enzyme's active site and its inhibitors is required. The forces involved in a substrate or an inhibitor binding to an enzyme's active site are, as with a drug binding to a receptor, the same forces that are experienced by all interacting organic molecules. These include ionic (electrostatic) interactions, ion-dipole and dipole-dipole interactions, hydrogen bonding, hydrophobic interactions, and van der Waals interactions. A brief overview of the forces involved follows. More comprehensive treatments can be found in Chapter 4 and elsewhere (57–60).

The binding of an inhibitor is dependent on a variety of interactions, and it is the sum of these interactions that will determine the degree of affinity of an inhibitor for the particular enzyme. The reversible binding of an inhibitor to an enzyme's active site can be described as shown in Equation 17.1.

$$\mathrm{E} + \mathrm{I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E} \cdot \mathrm{I} \qquad (17.1)$$

There is an equilibrium between the free enzyme (E), inhibitor (I), and the enzyme-inhibitor complex (E . I). The affinity of an inhibitor for the enzyme is measured by the inhibition constant K_i, which is the dissociation constant of the enzyme-inhibitor complex, at equilibrium (Equation 17.2).

$$K_i = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{E} \cdot \mathrm{I}]} \qquad (17.2)$$

The lower the K_i value, the better the inhibitor, given that the equilibrium lies more in favor of enzyme-inhibitor complex formation. The affinity of an inhibitor for an enzyme may be related to the standard free energy (ΔG°) of a system by Equation 17.3.

$$\Delta G^\circ = RT \ln K_i \qquad (17.3)$$

where R is the universal gas constant and T the temperature in degrees Kelvin. The more negative the value of ΔG°, the more favorable the interaction at equilibrium, and the smaller the K_i value. It should be noted that, from Equation 17.3, at physiological temperature relatively small changes in free energy, only 2-3 kcal/mol, will have a significant effect on K_i.

The standard free energy (ΔG°) can also be expressed in terms of enthalpic (ΔH°) and entropic (AS") components (Equation 17.4).

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \qquad (17.4)$$

Equation 17.4 states that the free energy of a system is lowered (i.e., the reaction is made more favorable) by either a decrease in enthalpy or an increase in entropy. This is also an important concept because there are both enthalpic and entropic components to the forces that contribute to the strength of the enzyme-inhibitor interaction.

When discussing the forces involved in the noncovalent binding of a substrate/inhibitor to an enzyme, or drug to a receptor, it must be recognized that these interactions will be carried out in an aqueous medium. The physical properties of water mean that noncovalent interactions in aqueous solution will be significantly different from those interactions observed in either an organic medium or in the gas phase. A water molecule has electronic asymmetry; the strongly electronegative oxygen atom withdraws electron density from the hydrogen atoms. This creates partial positive charges on the hydrogens and a partial negative charge on the oxygen. As a result a water molecule possesses a permanent dipole moment, facilitating strong interactions with other water molecules as well as with any charged or polar species.

Water is both a donor and acceptor of hydrogen bonds. Consequently, in bulk solvent, water molecules are extensively hydrogen bonded to each other. These are relatively weak bonds (~5 kcal/mol) and, at physiological temperature, are rapidly broken and reformed. However, the hydrogen-bonding network affects many of the properties of water. For example, water has a higher melting point, boiling point, and heat of vaporization than those of comparable hydrides such as H_2S and NH_3. The heat capacity of water indicates that it is highly structured and its surface tension (73 dyne cm^{-1} at 20°C) is considerably higher than that of most liquids (20–40 dyne cm^{-1}). The dielectric constant of water (80) is also considerably higher than that of most liquids, which are generally less than 30. Ethanol, for example, has a dielectric constant of 24, whereas those of benzene and hexane are 2.3 and 1.9, respectively. All told, water is a unique solvent, and one that has a major influence on binding interactions between an enzyme and an inhibitor.

Hydrogen bonds are readily formed between water and biologically important atoms such as the hydrogen bond acceptors N and O and, to a lesser extent, S. The conjugate acids NH and OH may act as hydrogen bond donors. Molecules containing these atoms have the capacity for many hydrogen-bonding interactions with water and, as a result, are usually soluble in water. However, solute-solute hydrogen bonding interactions are less favorable because their formation will require the disruption of favorable solute-water hydrogen bonds. Thus, what may be strong hydrogen bonds in the gas phase, or in organic media, are often considerably weaker in aqueous media.

Water's high dielectric constant makes it extremely effective in solvating, dissociating, and dissolving most salts. Because of its permanent dipole, water is readily able to interact with ionic species, with the result that ionic solute-solute interactions are less favored. The situation is analogous to that observed for hydrogen bonding and again results in a weakening of the normally strong interactions between ions that occur in the gas phase or nonpolar media. This is sometimes described as a "leveling effect."

Small amounts of many nonpolar substances can also dissolve in water. However, these substances do not interact very well with water and prefer to interact with each other. The force driving this interaction, known as the hydrophobic force, is not so much an attraction between hydrophobic molecules as an entropic effect arising from the

displacement of water. Indeed, there are no hydrophobic forces in the gas phase or in nonpolar solvents. However, collectively, hydrophobic forces are thought to transcend other types of forces, particularly in the folding of proteins, in all biological systems.

2.1.1 Electrostatic Forces. Although we recognize that, in essence, all forces between atoms and molecules are electrostatic, here we use the term to describe ion-ion, ion-dipole, and dipole-dipole interactions. At physiological pH, the side-chains of basic residues such as lysine and arginine and, to a lesser extent, the imidazole ring of histidine will be protonated, whereas the acidic groups on the side chains of aspartic and glutamic acid residues will be deprotonated. In addition, the *N*-terminal amino groups and C-terminal carboxylates will be ionized. Therefore, in addition to atoms with permanent and induced dipoles, an enzyme potentially will have several charged groups available for binding to charged or polarized groups on a substrate or inhibitor. As described by Equation 17.5, the electrostatic force (F) between the charged atoms (q_1 and q_2) will depend on the distance between the charged groups (r) and the dielectric constant of the surrounding medium (D).

$$F = \frac{q_1 q_2}{r^2 D} \qquad (17.5)$$

The strength of an ion-ion interaction is inversely related to the square of distance between the ions, whereas ion-dipole and dipole-dipole interactions have $1/r^4$ and $1/r^6$ relationships, respectively. Because the strength of the interaction decreases more slowly with distance, ion-pair interactions can be thought of as long-range interactions. Conversely, interactions involving dipoles are effective over only a short range, although, because they are much more prevalent, dipole interactions may be more significant to the overall binding process. Clearly, the dependency of the strength of interaction on the distance between atoms is an important consideration when designing potential enzyme inhibitors.

Equation 17.5 also leads to the fact that electrostatic interactions are less favorable in polar solvents. As discussed above, because of its high net permanent dipole moment, water is very polar and has a large dielectric constant. The high polarity of water greatly diminishes the attraction or repulsion forces between any two charged groups giving rise to the leveling effect of water. It is somewhat difficult to predict the exact strength of a charge-charge interaction between an enzyme and an inhibitor. For example, the formation of a salt bridge (charge-charge) interaction between an enzyme (Enz) and an inhibitor (I) may be described by Equation 17.6.

$$\text{Enz—}\overset{\oplus}{\text{N}}\text{H}_3 \cdot (\text{H}_2\text{O})_x + \text{I—COP} \cdot (\text{H}_2\text{O})_y \rightleftharpoons \text{Enz—}\overset{\oplus}{\text{N}}\text{H}_3 \cdot {}^{\ominus}\text{O}_2\text{C—I} + (\text{H}_2\text{O})_{x+y} \qquad (17.6)$$

Both the charged species are initially solvated by water, and to form the salt bridge both ions must be desolvated. This comes at some enthalpic cost, but the freeing of water molecules leads to a concomitant, favorable increase in entropy. The strength of the ion pair will depend on the stability of the salt bridge vs. that of the individual solvated ions. If the salt bridge is buried in a relatively hydrophobic active site, it is less solvated and will be more favored than the same interaction in a solvent-exposed active site.

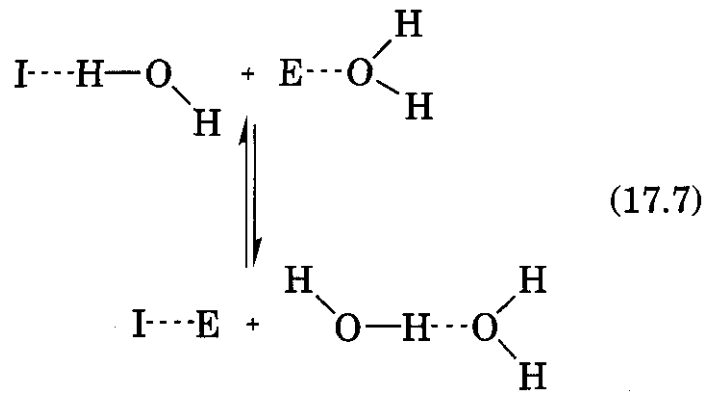
2.1.2 van der Waals Forces. Also called nonpolar interactions or London dispersion forces, these are the universal attractive interactions that occur between atoms. As two molecules closely approach each other there is an interpenetration of their electron clouds. As a consequence, temporary local fluctuations in the electron density occur, giving rise to a temporary dipole in each molecule, even though the molecules may, in themselves, have no net dipole moment. Thus there will be an attractive force between the two molecules, with the magnitude of the force depending on the polarizability of the particular atoms involved and the distance between each other. Electronegative oxygen has, for example, a much lower polarizability than that of a nonpolar methylene group. Accordingly, dispersion forces are considerably stronger between nonpolar compounds than between nonpolar com-

pounds and water. The optimal distance between the atoms is the sum of each of their van der Waals radii, so these forces come into play only when there is good complementarity between enzyme and inhibitor. Although van der Waals forces are quite weak, usually around 0.5-1.0 kcal/mol for an individual atom-atom interaction, they are additive and can make an important contribution to inhibitor binding.

2.1.3 Hydrophobic Interactions. Hydrophobic interactions may be described as entropy-based forces. When a nonpolar compound is dissolved in water, the strong water-water interactions around the solute lead to an effective "ordering" of the structure of the solvent. This is entropically unfavorable; that is, there is negative entropy of dissolution. When a nonpolar inhibitor binds to a nonpolar region of an enzyme, all the ordered water molecules become less ordered as they associate with bulk solvent, leading to an increase in entropy. According to Equation 17.4 any increase in entropy will lead to a decrease in free energy and, through Equation 17.3, stabilization of the enzyme-inhibitor complex. It has been calculated that a single methylene-methylene interaction releases about 0.7 kcdmol of free energy. Even though this figure is not high, given that enzymes and inhibitors usually have large regions of hydrophobic surface, this type of bonding may also play a significant role in inhibitor binding.

2.1.4 Hydrogen Bonds. A hydrogen bond occurs when a proton is shared between two electronegative atoms (i.e., —X—H· · ·Y). Electron density is pulled from the hydrogen by X, giving the hydrogen a partial positive charge that is strongly attracted to the nonbonded electrons of Y. The bond is usually asymmetric, with one of the heteroatoms, the hydrogen bond donor, having a normal covalent bond distance to the proton. The other heteroatom, the hydrogen bond acceptor, is usually at a distance somewhat shorter than the van der Waals contact distance and, for optimal hydrogen bonding, the atoms should be arranged linearly. A hydrogen bond is a special type of dipole-dipole interaction and, as we have seen, although these forces can be quite significant in nonpolar solvents, water greatly diminishes their magnitude. The energy of the amide-amide N H . ·O hydrogen bond is about 5 kcdmol, and is typical for hydrogen bonds (60).

It should be remembered that, for a hydrogen bond to form between an enzyme and an inhibitor, any hydrogen bonds between the inhibitor and water, as well as those between the enzyme and water, must be broken (Equation 17.7).



(17.7)

Overall, the total number of hydrogen bonds remains constant and, provided that the hydrogen bonds between the inhibitor and enzyme are not significantly more favorable than those between water and the inhibitor or those between water and the enzyme, the net change in enthalpy is usually insignificant. On the other hand, formation of the enzyme-inhibitor complex usually leads to an overall increase of entropy because the inhibitor remains bound to the enzyme and the formerly bound water molecules are released.

2.1.5 Cation-π Bonding. Recently it has become apparent that there is another important noncovalent binding force that may be exploited when designing enzyme inhibitors. Cations, from simple ions such as Li^+ to more complex organic molecules such as acetylcholine, are strongly attracted to the electron-rich (π) face of benzene and other aromatic compounds (61, 62). Cation-T bonds, as well as other amino-aromatic interactions, are common in structures in the protein data bank (63), and it has been estimated that more than 25% of tryptophan residues are involved in interactions of this type (64). The finding that the cationic group of acetylcholine was bound primarily by aromatic residues, most especially by a tryptophan residue, not by the ex-

pected carboxylate anion, provided evidence that cation-π interactions may play an important role in ligand binding (65, 66). Model systems suggest that, energetically, the cation-π interaction can compete with full aqueous solvation in binding cations (61), and there is now significant effort being expended in studying the contribution of these interactions to molecular recognition (62, 66).

In summary, the K_i provides an indication of the relative stability of the enzyme-inhibitor complex compared to stability of the enzyme and inhibitor free in solution. Moreover, it is clear that entropy, enthalpy, and water will all have a major impact on the binding of an inhibitor to an enzyme.

2.2 Steady-State Enzyme Kinetics

Just as an appreciation of the forces involved is essential to comprehending the binding of an inhibitor to an enzyme, so is an understanding of the kinetic analysis of an enzyrne-catalyzed reaction essential to any kinetic evaluation of an inhibitor. In this section we provide a brief introduction to the study of enzyme kinetics, particularly steady-state kinetics. Regardless, the reader is advised to refer to other sources for more in-depth reviews of the kinetic equations and mathematical derivations involved (38, 60, 67–71).

2.2.1 The Michaelis-Menten Equation. In the simplest case, an enzyme-catalyzed reaction involves the conversion of a single substrate to a single product, as shown in Equation 17.8.

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{E}\cdot\mathrm{S} \rightleftharpoons \mathrm{E}\cdot\mathrm{P} \rightleftharpoons \mathrm{E} + \mathrm{P} \qquad (17.8)$$

The free enzyme (E) binds the substrate (S) to form a noncovalent enzyme-inhibitor complex (E . S). This is assumed to be a rapid, reversible process, not involving any chemical changes, and with the affinity of the substrate for the enzyme's active site being determined by the binding forces discussed above. A chemical transformation of substrate to product (P), initially in complex with enzyme (E . P), then takes place. Finally, the product (P) is released into the medium with concomitant regeneration of free enzyme (E).

As can be seen from the following discussion, it is not difficult to carry out a kinetic analysis of a single-substrate reaction such as that described in Equation 17.8. However, as more substrates are added the task becomes more complex. Fortunately, kinetic analysis of enzymatic reactions involving two or more substrates can be made easier by varying the concentration of only one substrate at a time. By keeping all but one of the substrates at fixed, saturating concentrations, the reaction rate will depend only on the concentration of the varied substrate. This permits the use of the kinetic analysis employed for enzyme-catalyzed, single-substrate reactions even for complex multisubstrate reactions. In a further simplification, the dissociation of the E . P complex is assumed not to be rate limiting, and the reversion of product to substrate is assumed to be negligible. The latter assumption is valid under what are known as initial velocity conditions, that is, when less than about 5% of substrate has been consumed. Under these conditions, the concentration of P is low, and Equation 17.8 simplifies to Equation 17.9.

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{S} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \qquad (17.9)$$

Generally, kinetic analyses are carried out by studying the reaction under steady-state conditions, that is, when the concentration of the enzyme is well below that of the substrate. Under those circumstances, following a brief preequilibrium period, the concentrations of the various enzyme-bound species, E . S and E . P in Equation 17.8, become effectively constant and the rate of conversion of substrate to product will greatly exceed the change in concentration of any enzyme species. This is an approximation but, provided the substrate concentration does not greatly change (e.g., under initial velocity conditions), it is a very useful approximation. Given steady-state conditions, the Michaelis-Menten equation (Equation 17.10) is a quantitative description of the reaction described by Equation 17.9.

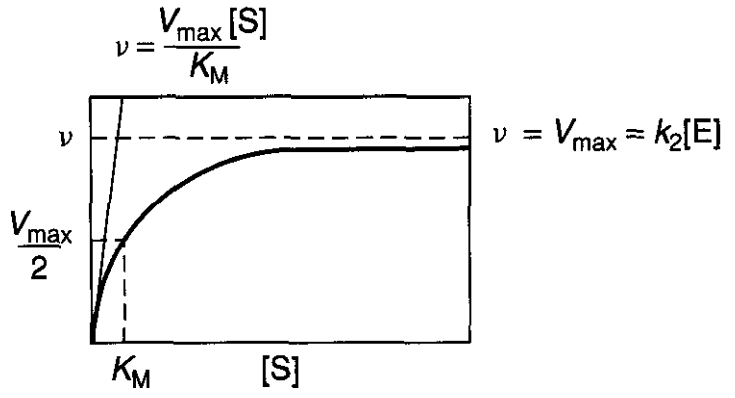


Figure 17.2. Plot showing dependency of the initial velocity (v) on substrate concentration [S] for an enzyme-catalyzed reaction obeying Michaelis-Menten (saturation) kinetics.

$$v = \frac{[S]V_{max}}{K_M + [S]} \qquad (17.10)$$
$$V_{max} = k_2[E]$$

This implies that the initial velocity (v) is directly proportional to the enzyme concentration [E], and that v follows saturation kinetics with respect to the substrate concentration [S]. This is shown graphically in Figure 17.2 and explained as follows: at very low substrate concentrations v increases in a linear fashion, so that $v = V_{max}[S]/K_M$. As the substrate concentration increases, the observed increase in v is less than the increase in [S]. This trend continues until, at high (saturating) substrate concentrations, v becomes effectively independent of [S] and tends toward the limiting value V_{max}.

V_{max} is the maximal velocity that can be achieved at a specific enzyme concentration. In the simple Michaelis-Menten mechanism described by Equation 17.9, there is only one E . S complex and all binding steps are rapid. In this instance, V_{max} is the product of the enzyme concentration [E] and k, (also known as k_{cat}), which is the first-order rate constant for the chemical conversion of the E . S complex to free enzyme and product. The catalytic constant k_{cat} is often referred to as the turnover number because it represents the maximum number of substrate molecules converted to products per active site per unit time. In a more complicated reaction, k_{cat} is a function of all the first-order rate constants and, effectively, sets a lower limit on all the chemical rate constants.

The Michaelis-Menten constant K_M is a combination of rate constants and is independent of enzyme concentration under steady-state conditions. It is equal to the substrate concentration at which half the maximum velocity of the enzyme-catalyzed reaction is reached; that is, when [S] = K_M, then v = ½ V_{max}. For the reaction illustrated in Equation 17.9, K_M is described by Equation 17.11.

$$K_M = \frac{k_2 + k_{-1}}{k_1} \qquad (17.11)$$

If, for a given reaction, $k_{-1} \gg$ k,, then Equation 17.11 simplifies to $K_M = K_S$, where K_S is the dissociation constant for the enzyme substrate complex. It is important to remember that the Michaelis-Menten equation holds true not only for the mechanism as stated above, but for many different mechanisms that are not included in this treatment. In summary, K_M can be described as an apparent dissociation constant for all enzyme-bound species and, in all cases, it is the substrate concentration at which the enzyme operates at half-maximal velocity.

Another parameter often referred to when discussing Michaelis-Menten kinetics is k_{cat}/K_M. This is an apparent second-order rate constant that relates the reaction rate to the free (not total) enzyme concentration. As described above, at very low substrate concentrations when the enzyme is predominantly unbound, the velocity (v) is equal to [El[S]k_{cat}/K_M. The value of k, $_t/K_M$ sets a lower limit on the rate constant for the association of enzyme and substrate. It is sometimes referred to as the specificity constant because it determines the specificity of the enzyme for competing substrates.

Again, for more detailed treatment of this subject the reader should refer to more specialized texts **(38,** 60, 67–69).

2.2.2 Treatment of Kinetic Data. Analysis of Michaelis-Menten kinetics is greatly facilitated by a linear representation of the data. Converting the Michaelis-Menten Equation 17.10 into Equation 17.12 leads to the popular Lineweaver-Burk plot.

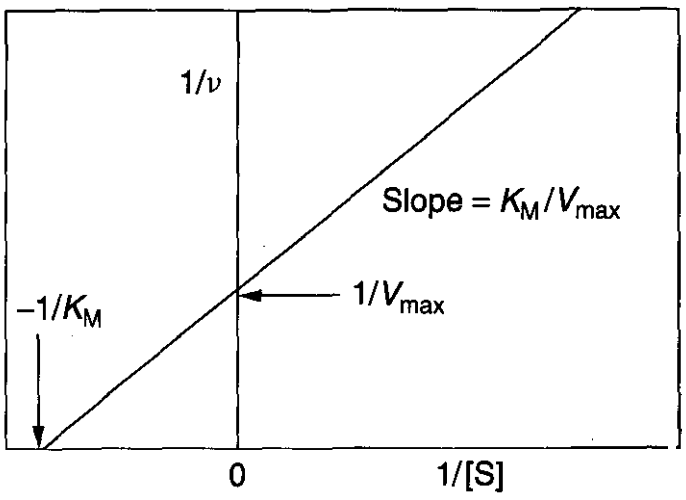


Figure 17.3. The Lineweaver-Burk plot.

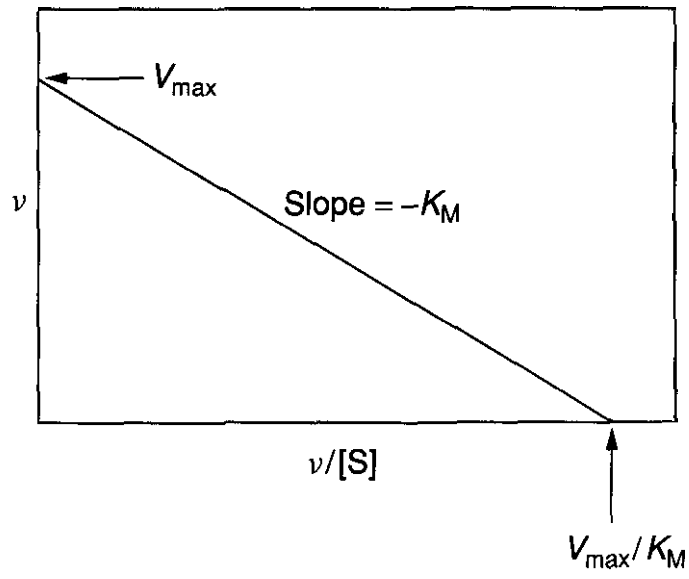


Figure 17.4. The Eadie-Hofstee plot.

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}[S]} \qquad (17.12)$$

If you plot $1/v$ against $1/[S]$ (Fig. 17.3), the y-intercept gives a value of $1/V_{max}$ and the x-intercept gives a value of $-1/K_M$. The slope of the line is equal to K_M/V_{max}. Although very popular, the Lineweaver-Burk plot suffers from the disadvantage that it emphasizes points at lower concentrations and compresses data points obtained at high concentrations (67). *As* a result it is not recommended for obtaining accurate kinetic constants.

A preferable, alternative form of the Michaelis-Menten equation is that of the Eadie-Hofstee plot (Equation 17.13)

$$v = V_{max} - \frac{K_M v}{[S]} \qquad (17.13)$$

As shown in Fig. 17.4, plotting v against $v/[S]$ results in the y-intercept providing a value of V_{max}, whereas the x-intercept provides V_{max}/K_M, and the slope of the line is equal to $-K_M$.

Another linear representation of the Michaelis-Menten equation is the Hanes-Woolf plot (Equation 17.14).

$$\frac{[S]}{v} = \frac{1}{V_{max}}[S] + \frac{K_M}{V_{max}} \qquad (17.14)$$

Thus a plot of $[S]/v$ vs. $[S]$ is linear, with a slope of $1/V_{max}$ (Fig. 17.5). The y-intercept gives K_M/V_{max} and the x-intercept gives $-K_M$.

Finally, it is possible to directly plot pairs of v, [S] data in such a way as to directly determine K_M and V_{max} values. Of the linear graphical methods, the direct linear plot of Eisenthal and Cornish-Bowden (72), shown in Fig. 17.6, is often considered to provide the best estimates of K_M and V_{max} values. In this method pairs of v and [S] values are obtained in the usual manner. A v value is plotted on the y-axis and a corresponding negative value of [S] is plotted on the x-axis. A straight line is then drawn, passing through the points on the two axes and extending beyond the "point of intersection." This is repeated for each set of v and [S] values. Thus, there are n sets of lines for n pairs of values. A horizontal line drawn from the point of intersection to the y-axis provides the V_{max} value, whereas a vertical line from the point of intersection to the x-axis provides the K_M value.

Each of these linear plots has its own merits, particularly for plotting inhibition data

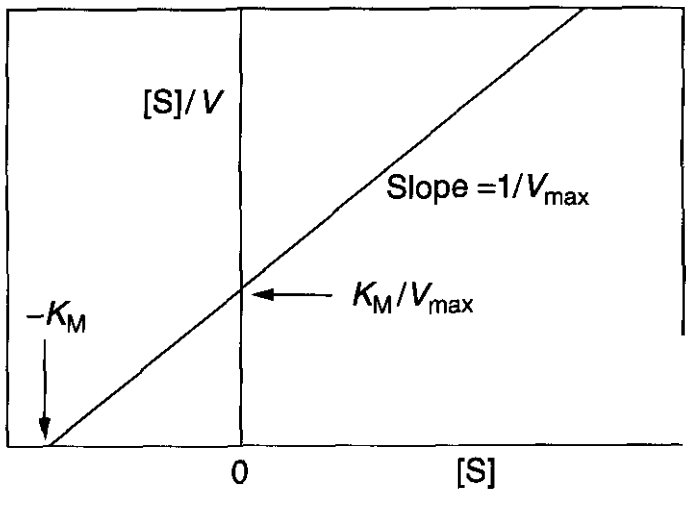


Figure 17.6. The Hanes-Woolf plot.

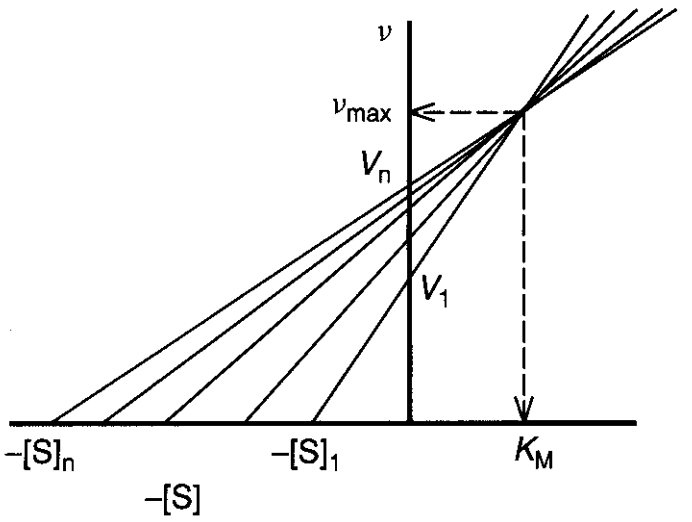


Figure 17.6. Eisenthal-Cornish-Bowdendirect linear plot of enzyme kinetic data fitting the Michaelis-Menten equation.

(38), and its own drawbacks (67). However, the rapid advances in personal computing make it relatively easy to fit kinetic data to the Michaelis-Menten equation (or other appropriate hyperbolic functions) by use of a variety of commercial graphical or spreadsheet packages. One simple package, HYPER, which is readily available on the Internet (http://www.ibiblio.org/pub/academic/biology/molbio/ibmpc/hyper102.zip), simultaneously furnishes Michaelis-Menten parameters obtained using hyperbolic regression analysis, as well as those obtained using three of the plots described here. As such, it provides a rapid contrast of these graphical methods but, unfortunately, is not suitable for the study of inhibition kinetics. In addition, the recent monograph by Copeland (71) provides a list of useful computer software and Internet sites for the study of enzymes.

2.3 Rapid, Reversible Inhibitors

This class of inhibitors acts by binding to the target enzyme's active site in a rapid, reversible, and noncovalent fashion. The net result is that the active site is blocked and the substrate is prevented from binding. Accordingly, in designing inhibitors of this type, optimization of the noncovalent binding forces between the inhibitor and the active site of the enzyme is of paramount importance.

2.3.1 Types of Rapid, Reversible Inhibitors. Binding of these inhibitors follows simple Michaelis-Menten kinetics and, depending on their preference of binding to the free enzyme and/or the enzyme-substrate complex, competitive, uncompetitive, and noncompetitive inhibition patterns can be distinguished. For the purposes of this discussion it will be assumed that the initial equilibrium of free and bound substrate is established significantly faster than the rate of the chemical transformation of substrate to product, that is, $k_1, k_{-1} \gg k_2$ (Equation 17.9). As discussed in section 2.2.1, this reduces K_M to the dissociation constant K_S of the E . S complex.

2.3.1.1 Competitive Inhibitors. A competitive inhibitor often has structural features similar to those of the substrates whose reactions they inhibit. This means that a competitive inhibitor and enzyme's substrate are in direct competition for the same binding site on the enzyme. Consequently, binding of the substrate and the inhibitor are mutually exclusive. A kinetic scheme for competitive inhibition is shown in Equation 17.15.

$$\begin{array}{ccccc} & & \overset{S}{\rightleftharpoons} & & \\ E & & E \cdot S & \rightarrow & E + P \\ & \rlap{\Big\uparrow\downarrow I} & & & \\ & E \cdot I & & & \end{array} \qquad (17.15)$$

The enzyme-bound inhibitor may either lack an appropriate functional group for further reaction, or may be bound in the wrong position with respect to the catalytic residues or to other substrates. In any event, the enzyme-inhibitor complex E . I is unreactive (it is sometimes referred to as a dead-end complex) and the inhibitor must dissociate and substrate bind before reaction can take place.

Solving this kinetic scheme for simple Michaelis-Menten kinetics leads to Equation 17.16.

$$v = \frac{[S]V_{max}}{[S] + K_M\left(1 + \dfrac{[I]}{K_i}\right)} \qquad (17.16)$$

Here, K_i, sometimes called the inhibition constant, is the equilibrium constant for the

dissociation of the enzyme-inhibitor complex, and is described by Equation 17.17.

$$K_i = \frac{[E][I]}{[E \cdot I]} \qquad (17.17)$$

Competitive inhibitors do not change the value of V_{max}, which is reached when sufficiently high concentrations of the substrate are present so as to completely displace the inhibitor. However, the affinity of the substrate for the enzyme *appears* to be decreased in the presence of a competitive inhibitor. This happens because the free enzyme E is not only in equilibrium with the enzyme-substrate complex E . S, but also with the enzyme-inhibitor complex E . I. Competitive inhibitors increase the *apparent* K_M of the substrate by a factor of $(1 + [I]/K_i)$. The evaluation of the kinetics is again greatly facilitated by the conversion of Equation 17.15 into a linear form using Lineweaver-Burk, Eadie-Hofstee, or Hanes-Woolf plots, as shown in Fig. 17.7.

2.3.1.2 Uncompetitive Inhibitors. Uncompetitive inhibitors do not bind to the free enzyme. They bind only to the enzyme-substrate complex to yield an inactive E · S • I complex (Equation 17.18).

$$\begin{array}{ccccc} & \overset{S}{} & & & \\ E & \rightleftharpoons & E \cdot S & \rightarrow & E + P \\ & & \downarrow\uparrow I & & \\ & & E \cdot S \cdot I & & \end{array} \qquad (17.18)$$

Uncompetitive inhibition is rarely observed in single-substrate reactions but is frequently observed in multisubstrate reactions. An uncompetitive inhibitor can provide information about the order of binding of the different substrates. In a bisubstrate-catalyzed reaction, for example, a given inhibitor may be competitive with respect to one of the two substrates and uncompetitive with respect to the other. The linear plots for classical uncompetitive inhibition patterns are described by Equation 17.19 and are illustrated in Fig. 17.8.

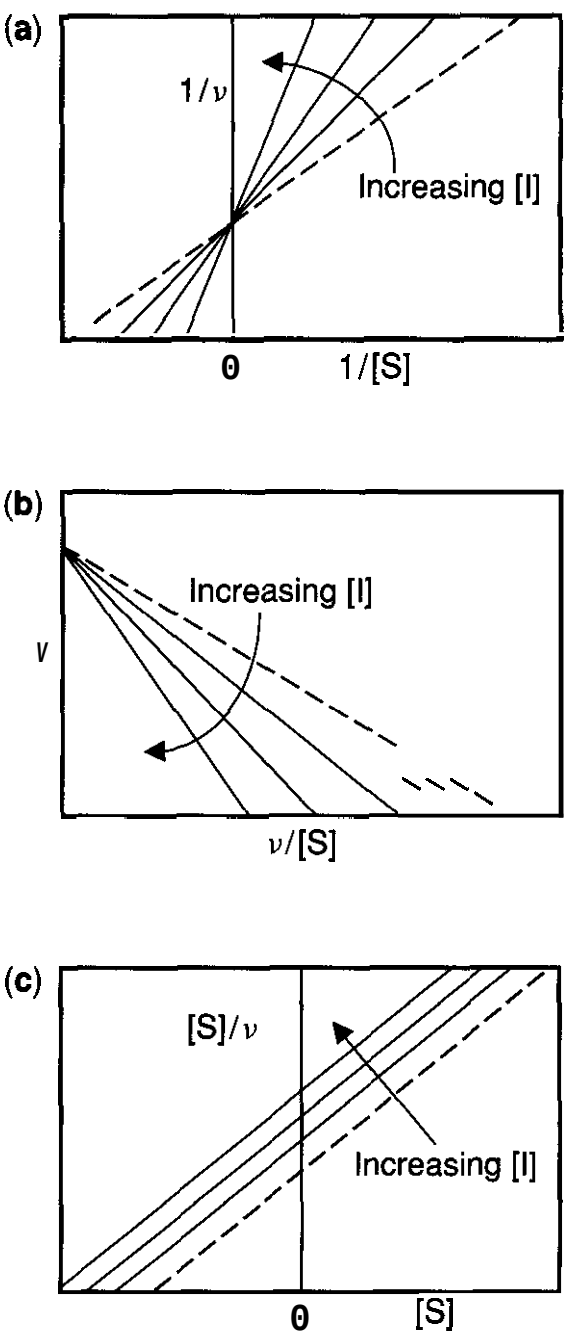


Figure 17.7. **(a)** Lineweaver-Burk, (b) Eadie-Hofstee, and (c) Hanes-Woolf plots exhibiting competitive inhibition patterns. The dashed line indicates the reaction in the absence of inhibitor, whereas the solid lines represent enzymatic reactions in the presence of increasing concentrations of inhibitor.

$$v = \frac{\dfrac{[S]V_{max}}{1 + [I]/K_i}}{[S] + \dfrac{K_M}{1 + [I]/K_i}} \qquad (17.19)$$

As with a competitive inhibitor, the apparent K_M for the substrate decreases by a factor of $(1 + [I]/K_i)$ because the formation of E · S · I will use up some of the E . S, thereby shifting the equilibrium further in favor of E . S formation. However, uncompetitive inhibitors also decrease V_{max} by the same factor because

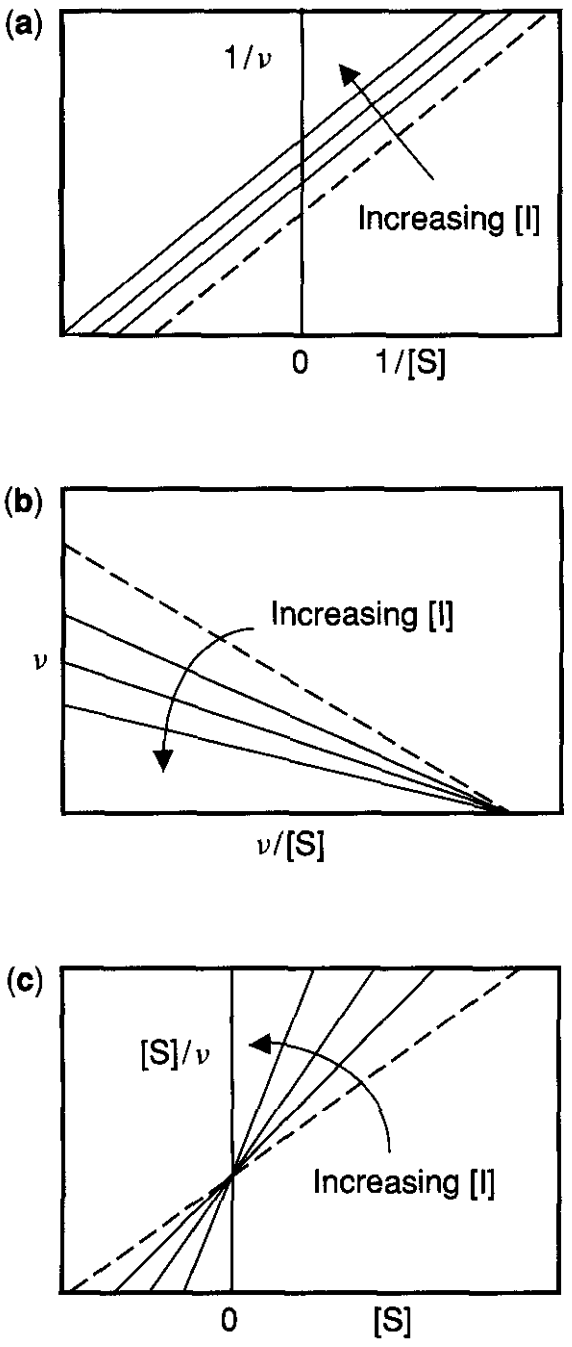
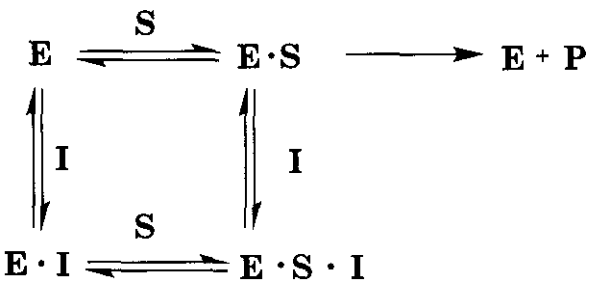


Figure 17.8. (a) Lineweaver-Burk, (b) Eadie-Hofstee, and (c) Hanes-Woolf plots exhibiting uncompetitive inhibition patterns. The dashed line indicates the reaction in the absence of inhibitor, whereas the solid lines represent enzymatic reactions in the presence of increasing concentrations of inhibitor.

some of the enzyme remains in the E.S.I form, even at infinite substrate concentration.

2.3.1.3 Noncompetitive Inhibitors. Classical noncompetitive inhibitors have no effect on substrate binding and vice versa, given that they bind randomly and reversibly to different sites on the enzyme. They also bind with the same affinity to the free enzyme and to the enzyme-substrate complex. Both the enzyme- inhibitor complex E.I and the enzyme-substrate-inhibitor complex E.S.I are catalytically inactive. The equilibria are outlined in Equation 17.20.



(17.20)

Simple Michaelis-Menten kinetics of noncompetitive inhibitors are described in Equation 17.21.

$$v = \frac{\dfrac{[S]V_{max}}{1 + [I]/K_i}}{[S] + K_M} \qquad (17.21)$$

From Equation 17.21 it is clear that noncompetitive inhibitors have an effect only on V_{max}, decreasing it by a factor of $(1 + [I]/K_i)$, consequently giving the impression of reducing the total amount of enzyme present. As with an uncompetitive inhibitor, a portion of the enzyme will always be bound in the nonproductive enzyme-substrate-inhibitor complex E·S·I, causing a decrease in maximum velocity, even at infinite substrate concentrations. However, because noncompetitive inhibitors do not affect substrate binding, the K_M value of the substrate remains unchanged. Linear plots for noncompetitive inhibition are shown in Fig. 17.9.

Again, this type of inhibition is rarely seen in single-substrate reactions. It should also be noted that, frequently, the affinity of the noncompetitive inhibitor for the free enzyme, and the enzyme-substrate complex, are different. These nonideally behaving noncompetitive inhibitors are called mixed-type inhibitors, and they alter not only V_{max} but also K_M for the substrate. Further discussion of inhibitors of this type may be found in Segel (38).

Sometimes steady-state kinetics are insufficient to analyze the mechanism of inactivation for a given inhibitor. For example, irreversible enzyme inhibitors that bind so tightly to the enzyme that their dissociation rate (k_{off}) is effectively zero also exhibit noncompetitive inhibition patterns. They act by destroying a portion of the enzyme through irreversible binding, thereby lowering the overall enzyme concentration and decreasing V_{max}. The apparent K_M remains unaffected because irre-

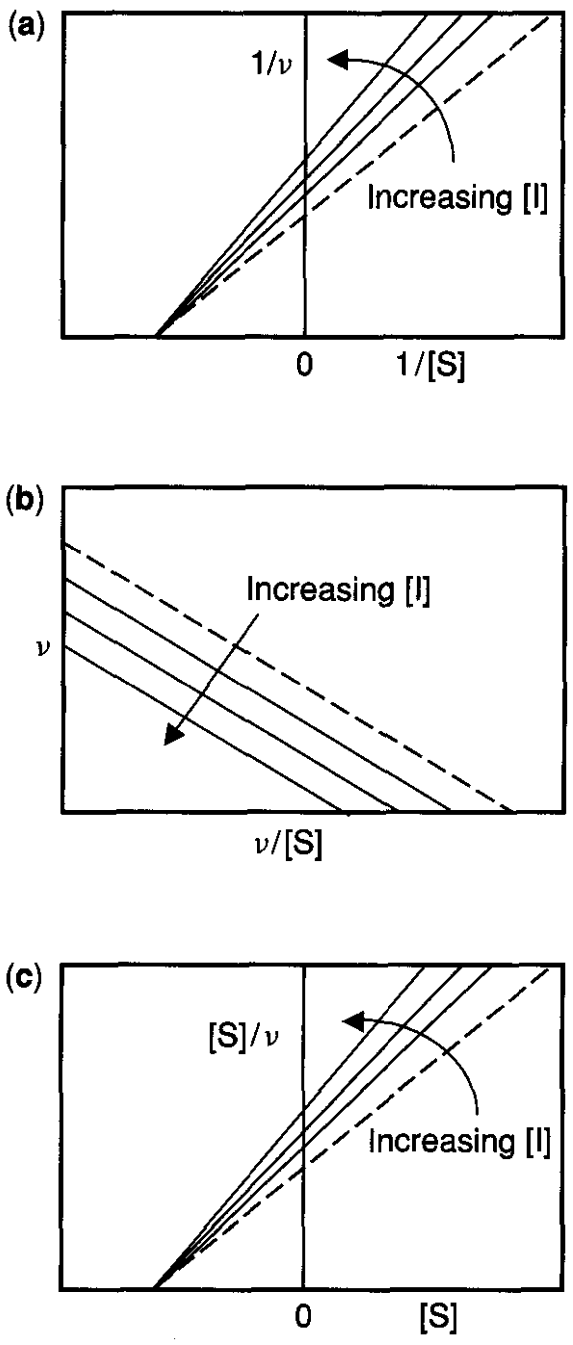


Figure 17.9. (a) Lineweaver-Burk, (b) Eadie-Hofstee, and (c) Hanes-Woolf plots exhibiting noncompetitive inhibition patterns. The dashed line indicates the reaction in the absence of inhibitor, whereas the solid lines represent enzymatic reactions in the presence of increasing concentrations of inhibitor.

versible inhibitors do not influence the dissociation constant of the enzyme-substrate complex. A simple experiment to distinguish between a reversible noncompetitive inhibitor and irreversible inhibitor is shown in Fig. 17.10, and a comprehensive review describing the kinetic evaluation of irreversibly binding enzyme inhibitors is available (73). Allosteric effectors may also show noncompetitive kinetic patterns by rendering the enzyme in the E · S . I complex less active than that in the E . S complex. Again, additional analyses are often required in these less well defined

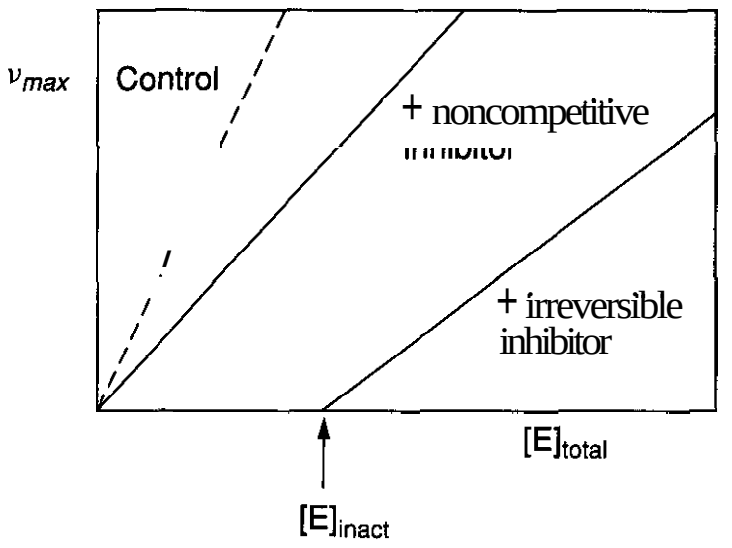


Figure 17.10. Plot showing dependency of V_{max} on the total enzyme concentration, $[E]_{total}$. An irreversible inhibitor will titrate a fraction of the enzyme $[E]_{inact}$.

situations. Such analyses may include more in-depth steady-state kinetics, as well as pre-steady-state kinetics, and testing for irreversible inhibition. Irreversible covalently binding enzyme inhibitors are discussed extensively later in this chapter.

2.3.2 Dixon Plots. Another linear method for plotting inhibition data, the Dixon plot, is shown in Fig. 17.11 (74). In this method the initial velocity is measured as a function of inhibitor concentration at two or more fixed substrate concentrations. By plotting $1/v$ against [I] for each substrate concentration, the different types of inhibition can easily be distinguished. Further, in cases of competitive or noncompetitive inhibition, the value of K_i may be determined from the x-axis value at which the lines intercept. Overall, the Dixon plot is probably the simplest and most rapid graphical method for obtaining a K_i value.

2.3.3 IC_{50} Values. The potencies of enzyme inhibitors evaluated using rapid screening techniques are often reported in terms of IC_{50} values rather than K_i values. An IC_{50} value is the inhibitor concentration that is required to halve the activity of the enzyme, that is, that concentration that leads to 50% enzyme inactivation. It is important to recognize that an IC_{50} value is not a constant, except in the case of noncompetitive inhibition, and is dependent on the substrate concentration used in the experiment. IC_{50} values are com-

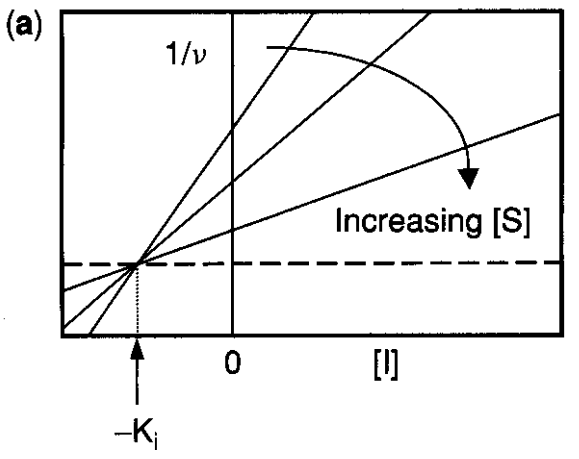


Figure 17.11. Dixon plots for (a) competitive, (b) uncompetitive, and (c) noncompetitive inhibitors. The solid lines represent enzymatic reactions in the presence of increasing concentrations of substrate. The dashed line represents the reaction at infinite substrate concentration.

monly determined by keeping the concentration of the substrate and the enzyme constant and incrementally varying the concentration of the inhibitor. This simple experimental approach makes it relatively easy to screen large numbers of potential inhibitors. Industrial high-throughput screens often employ half-log increments, and the value of IC_{50} provides a ready means of comparing the extent of inhibition. It should also be noted that the IC_{50} value can be no less than half the concentration of the enzyme, a factor that becomes important if the inhibitor is very potent or if high concentrations of enzyme are employed.

For a competitive inhibitor, a K_i value may be obtained using the relationship described by Equation *17.22.*

$$IC_{50} = K_i\left(1 + \frac{[S]}{K_M}\right) \qquad (17.22)$$

Provided that a reasonable substrate concentration ($\leq 0.1\ K_M$) is employed for the experiment, the IC_{50} value may be a reasonable approximation of the true K_i. Equation *17.22* indicates that substrate concentrations greater than about 0.1-fold of the K_M value will lead to an underestimation of the K_i value, an underestimation that becomes quite significant at high substrate concentrations.

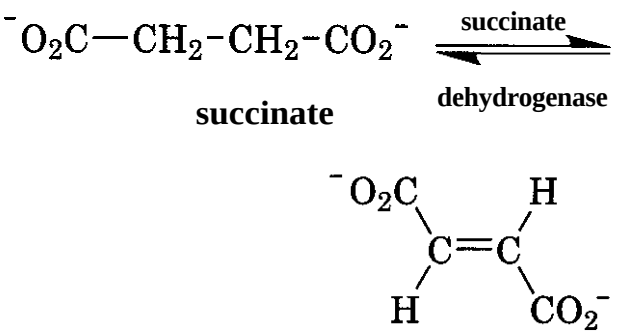
The dependency of the IC_{50} value on the substrate concentration for uncompetitive inhibitors is given in Equation *17.23.*

$$IC_{50} = K_i\left(1 + \frac{K_M}{[S]}\right) \qquad (17.23)$$

In this instance it is at high concentrations of the substrate that the K_i value is comparable to the IC_{50} value, and a significant underestimation will occur at lower substrate concentrations.

From these two equations it is clear that, for preliminary screening when the type of inhibition is unknown, substrate concentrations close to the K_M value should be used. This minimizes the deviation of the IC_{50} value from the K_i value to, in the cases of competitive and uncompetitive inhibitors, a factor of *2.* If necessary, a Dixon plot can be used to provide a quick indication of the K_i and the type of inhibition *(38,74).* It should be noted that the relationship between IC_{50} and K_i requires the initial velocity to be linearly dependent on the concentration of inhibitor. In the cases of mixed-competitive and irreversible inhibitors, the dependency of the inhibitor concentration and the initial velocity is nonlinear. Therefore, in those cases, the use of the IC_{50} value is limited.

2.3.4 Examples of Rapid Reversible Inhibitors. Competitive inhibitors are often similar in structure to one of the substrates of the reaction they are inhibiting. Inhibitors of this type are sometimes called substrate analogs and their binding affinity (K_i) usually approximates that of the substrate. One of the first reactions inhibited by a substrate analog was that catalyzed by succinate dehydrogenase (Equation 17.24).

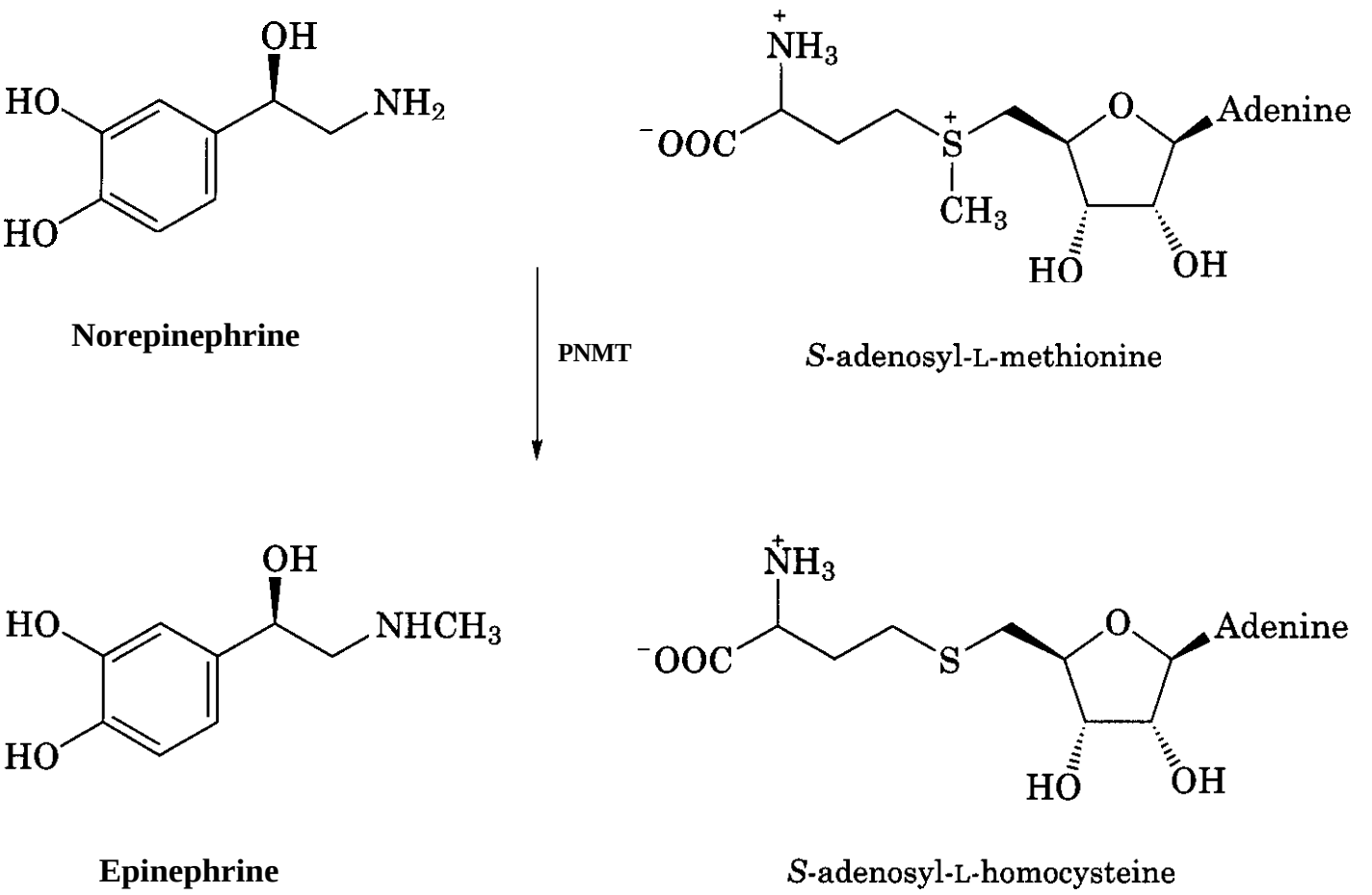


(17.24)

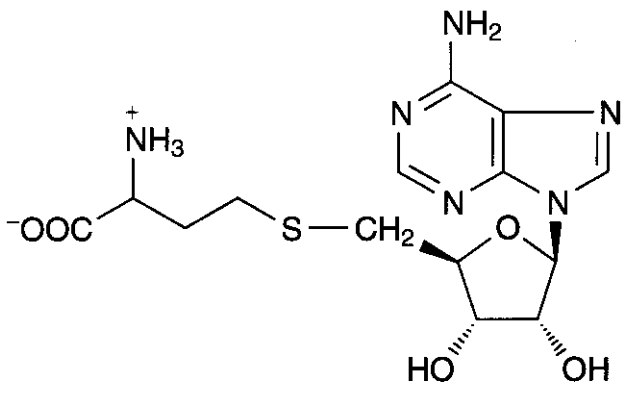
This reaction is competitively inhibited by malonate ($^-OOCCH_2COO^-$) that has, like succinate, two carboxylate groups. It is therefore able to bind to the enzyme's active site but, with only one carbon atom between the carboxylates, further reaction is impossible.

Substrate analogs are rarely useful as enzyme inhibitors, given that large concentrations are required for inhibition, and their inhibition is readily overcome by any buildup of substrate. However, they are often useful probes for determining enzyme specificity and even mechanism. Phenylethanolamine *N*-methyltransferase (PNMT) catalyzes the terminal step in epinephrine (adrenaline) biosynthesis, the conversion of norepinephrine to epinephrine (Equation 17.25), with concomitant conversion of S-adenosyl-L-methionine (SAM, AdoMet) to S-adenosyl-L-homocysteine (SAH, AdoHcy).

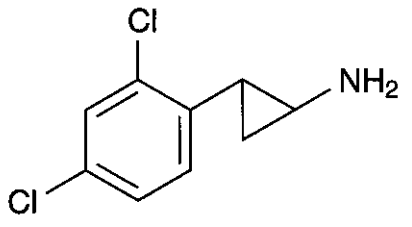
S-Adenosyl-L-homocysteine (10) (Fig. 17.12), the product of the reaction, and 2-(2,5-dichlorophenyl)cyclopropylamine (11) are analogs of S-adenosyl-L-methionine and norepinephrine, respectively. Using these inhibitors it was possible to ascertain the binding order of the two substrates (75). Kinetic analyses showed that SAH was a competitive inhibitor of SAM and a noncompetitive inhibitor of norepinephrine, whereas (11) was a competitive inhibitor of norepinephrine and an uncompetitive inhibitor of SAM. This indicates that the binding of substrates is ordered, with SAM binding first. If norepinephrine bound first, it would be expected that SAH would be an uncompetitive inhibitor and (11) would be noncompetitive with respect to SAM. If a random



(17.25)



(10)



(11)

Figure 17.12. Inhibitors of phenylethanolamine N-methyltransferase.

binding mechanism were in operation, it would be expected that both inhibitors would be competitive with either substrate. More detail on similar uses of reversible inhibitors may be found elsewhere (76).

2.4 Slow-, Tight-, and Slow-Tight–Binding Inhibitors

Not all reversible inhibitors have an instantaneous effect on the rate of an enzymatic reaction. Some inhibitors, known as slow-binding enzyme inhibitors, can take a considerable time to establish the equilibrium between the free enzyme and inhibitor, and the enzyme-inhibitor complex. This time period may be on the scale of seconds, minutes, or even longer. The enzyme-inhibitor complexes have slow off (dissociation) rates, but the on (association) rates may be either slow or fast. Hence, the term slow binding does not necessarily indicate a slow binding of inhibitor to enzyme but rather the fact that reaching equilibrium is a slow process. Other inhibitors, known as tight-binding inhibitors, bind their target enzyme with such high affinity that the population of free inhibitor molecules is significantly depleted when the enzyme-inhibitor complex is formed. Often, tight-binding inhibitors also have a slow onset of action, and are termed slow-tight-binding inhibitors. What these three types of inhibitors have in common is that, generally, the major assumptions of Michaelis-Menten kinetics do not hold true.

As with rapid reversible inhibition, for slow-binding inhibition to take place a significantly larger concentration of inhibitor than enzyme is required. However, reaching equilibrium slowly is incompatible with the assumption of Michaelis-Menten kinetics that inhibitors bind much more quickly than the enzyme turns over. Unlike rapid reversible and slow-binding inhibitors, both tight-binding and slow-tight-binding inhibitors are effective at concentrations comparable to that of the enzyme. At that point, the inhibitor concentration is no longer independent of the enzyme concentration, as assumed for Michaelis-Menten kinetics. A summary of the properties of reversible enzyme inhibitors is shown in Table 17.5. Although we give a brief overview of these types of inhibitors, excellent and more in-depth descriptions of slow-, tight-, and slow-tight-binding inhibitors have appeared elsewhere (71, 77–80).

2.4.1 Slow-Binding Inhibitors. Two different mechanisms have been suggested to rationalize the slow-binding behavior of competitive inhibitors (71, 78, 80). In the one-step mechanism A, the direct binding process of the inhibitor to the enzyme is slow (Equation 17.26); that is, the magnitude of k_3[I] is small relative to k_1[S] and k,, the rate constants for the conversion of substrate to product.

Table 17.5 Classes of Reversible Inhibitors

Inhibitor Class	Ratio of Inhibitor to Enzyme Necessary for Inhibition	Rate at Which Equilibrium is Attained E + I ⇌ E . I
Rapid, reversible	I ⩾ E	Fast
Tight binding	I ≈ E	Fast
Slow binding	I ⩾ E	Slow
Slow-tight binding	I – E	Slow

$$\begin{array}{l} \mathrm{E} \underset{k_{-1}}{\overset{\mathrm{S},k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{S} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \\ k_{-3} \Big\uparrow\downarrow \mathrm{I},k_3 \\ \mathrm{E}\cdot\mathrm{I} \end{array} \qquad (17.26)$$

The slow on rate (k_3) has been attributed to the inhibitor encountering some barrier to binding at the active site. The inhibitor has to overcome this barrier by correct alignment. Once aligned properly, it binds so tightly that it is released very slowly from the enzyme, making the overall equilibrium process extremely slow. The equilibrium dissociation constant for the E . I complex K_i, derived from Equation 17.26, is given by Equation 17.27.

$$K_i = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{E}\cdot\mathrm{I}]} = \frac{k_{-3}}{k_3} \qquad (17.27)$$

This is the same equilibrium as that for a rapid reversible inhibitor (Equation 17.17). From Equation 17.27, it should be noted that, if K_i is very small (as with a tight-binding inhibitor) and [I] is varied in the region of K_i, even if the on rate (k_3) is diffusion controlled, both k_3[I] and k_{-3} will be very small. Thus, the onset of inhibition for a tight-binding inhibitor can appear to be slow, even though k_3 is in the range expected for rapid reversible inhibitors (78). It is possible to carry out kinetic analyses of tight-binding inhibitors. This can be done either by including a preincubation step, to allow sufficient time for the enzyme and inhibitor to reach equilibrium, or by carrying out the reaction at very high concentrations of both substrate and inhibitor. More detailed discussion of these methods, with appropriate references, can be found in a recent volume by Copeland (71).

If the slow-binding inhibitor described by Equation 17.26 also binds very tightly, it is referred to as a slow-tight-binding inhibitor. For inhibitors of this type, K_i is given by Equation 17.28, where [E_T] represents the total enzyme concentration (in all forms) present in solution.

$$K_i = \frac{([\mathrm{E_T}] - [\mathrm{E}\cdot\mathrm{I}])([\mathrm{I}] - [\mathrm{E}\cdot\mathrm{I}])}{[\mathrm{E}\cdot\mathrm{I}]} \qquad (17.28)$$

For slow-tight-binding inhibitors, k_{-3} is very small and formation of the E . I complex is essentially irreversible. Use of Equation 17.28 ensures that depletion of free enzyme and free inhibitor by formation of the E . I complex is taken into account.

In mechanism B, the more common mechanism for slow-binding inhibition (80), the initial equilibrium between the enzyme, inhibitor, and the E · I complex is fast. However, there is a subsequent slow rearrangement to form the final, more stable enzyme-inhibitor complex (E . I*) (Equation 17.29).

$$\begin{array}{l} \mathrm{E} \underset{k_{-1}}{\overset{\mathrm{S},k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{S} \xrightarrow{k_2} \mathrm{E} + \mathrm{P} \\ k_{-3} \Big\uparrow\downarrow \mathrm{I},k_3 \\ \mathrm{E}\cdot\mathrm{I} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{I}^* \end{array} \qquad (17.29)$$

Here the dissociation constant for the initial E · I complex is still k_{-3}/k_3, but there is also a dissociation constant for the formation of the E . I* complex. The second dissociation constant is given by Equation 17.30.

$$K_i^* = \frac{K_i k_{-4}}{k_4 + k_{-4}} = \frac{[\mathrm{E}][\mathrm{I}]}{[\mathrm{E}\cdot\mathrm{I}] + [\mathrm{E}\cdot\mathrm{I}^*]} \qquad (17.30)$$

To observe the slow onset of inhibition and the E . I complex, K_i^* must be smaller than K_i and k_{-4} smaller than k_4. However, if k_{-4} is considerably smaller than k_4, then the formation of the E . I* complex will be effectively irreversible (i.e., the inhibitor is of the slow-tight-binding variety). Under those circumstances it will again be necessary to take depletion of free enzyme and free inhibitor into account when determining K_i and K_i^* (78).

The slow rearrangement step has been correlated with conformational changes of the enzyme following initial binding of the inhibitor. It is possible that the enzyme in its transition state conformation may be better equipped to

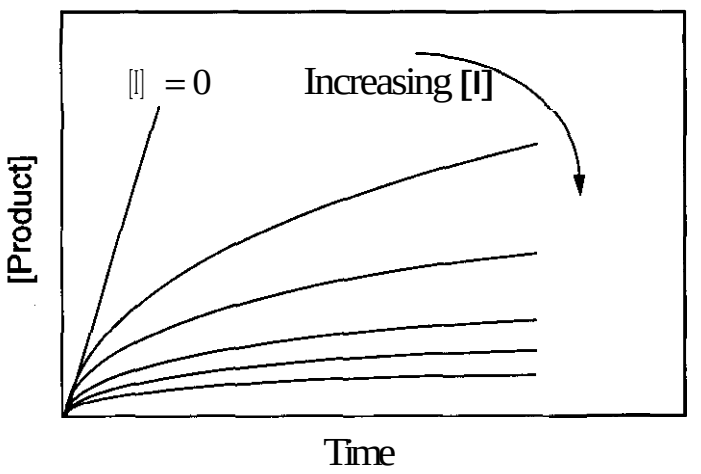
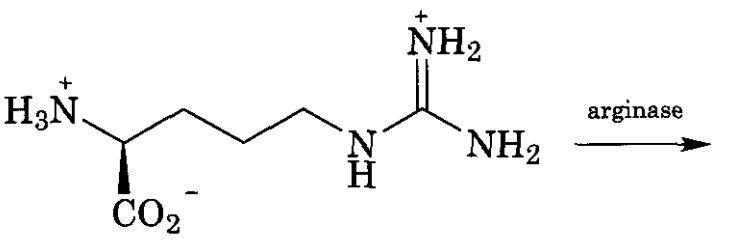


Figure 17.13. Reaction progress curves in the presence of increasing concentrations of a slow-binding inhibitor.

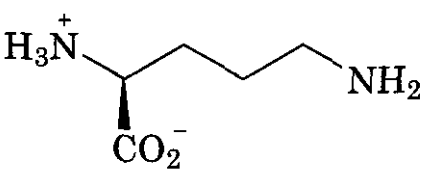
accommodate the inhibitor. **A** slow change to reach this optimal conformation will lead to tighter binding of the inhibitor and even slower release from the enzyme. **An** alternative suggestion is that the slow-binding process is linked to a requisite displacement of water molecules from the active site (81). Initially the inhibitor binds loosely to the enzyme, but upon release of water molecules the gain in entropy leads to a more stable E . I* complex.

One way of quickly identifying a potential slow-binding inhibitor is to examine the progress of the reaction at increasing concentrations of inhibitor. Under initial velocity conditions (Section 2.2.1), an enzyme-catalyzed reaction will exhibit a linear increase in the amount of product formed over time. **A** reaction progress plot for a reaction carried out in the presence of a rapid reversible inhibitor will also be linear. However, a slow-binding inhibitor will initially show a linear relationship, although this will change as the inhibitor binds, resulting in a biphasic plot. Typical biphasic progress curves for a reaction in the presence of increasing concentrations of a slow-binding inhibitor are shown in Fig. 17.13. The initial burst of the reaction, the linear section of the graphs, can be described by competitive Michaelis-Menten kinetics. The higher the concentration of the inhibitor, the shorter the initial linear section of each curve and the slower the subsequent final steady-state rate, as observed in the asymptotes in Fig. 17.13. If the inhibitor concentration is small, the substrate might be too depleted to permit observation of steady-state rates.

A good comparison of rapid reversible and slow-binding inhibition can be found in a recent study on the inhibition of arginase, an enzyme that catalyzes the hydrolysis of L-arginine to yield L-ornithine and urea (Equation 17.31).

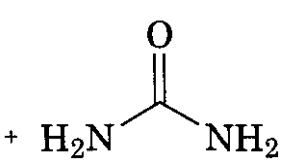


L-arginine



L-ornithine

(17.31)



Urea

Arginase competes with nitric oxide synthase (NOS) for arginine and, in doing so, helps regulate NOS. **As** a consequence, inhibitors of arginase may have therapeutic use in treating NO-dependent smooth muscle disorders, including erectile dysfunction (82). **A** series of arginine analogs were prepared and tested as inhibitors of arginase (83). Three examples are shown in Fig. 17.14. One of these, N^{ω}-hydroxy-L-arginine (**12**), is a competitive inhibitor of arginase at both pH 7.5 and pH 9.5 with K_i values of 2 and 1.6 μM, respectively. The two boronic acid derivatives, 2(*S*)-amino-6-boronohexanoic acid (13) and S-(2-boronoethyl)-L-cysteine (**14**), were also competitive inhibitors at pH 7.5 with K_i values of 0.25 and 0.31 μM, respectively. However, at pH 9.5, the boronic acid derivatives both became slow-binding inhibitors, apparently binding by mechanism B and with lowered K_i values of 8.5 and 30 nM, respectively. It was suggested that, at low pH, the trigonal form of the boronic acid derivative predominates, and that

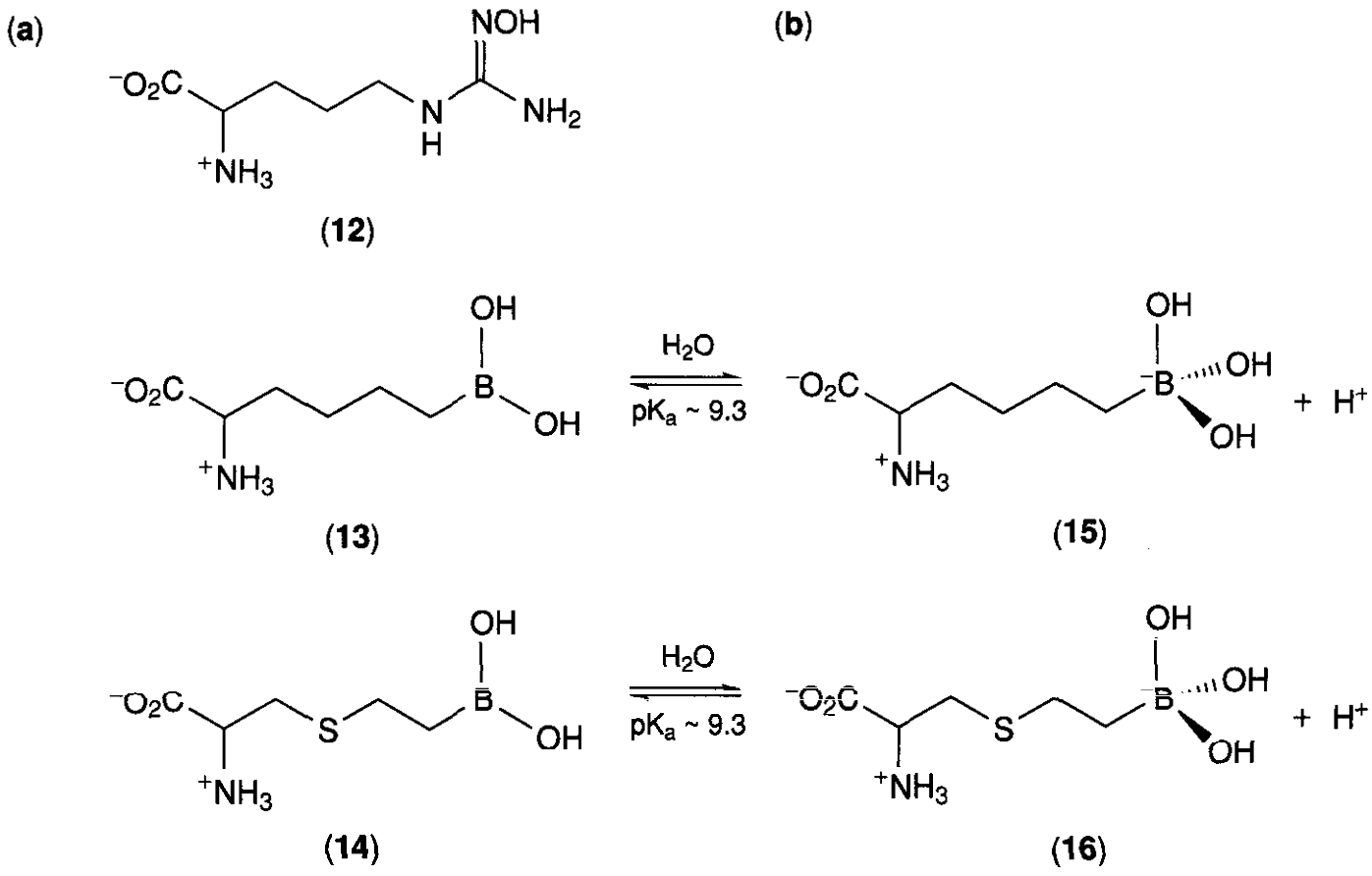
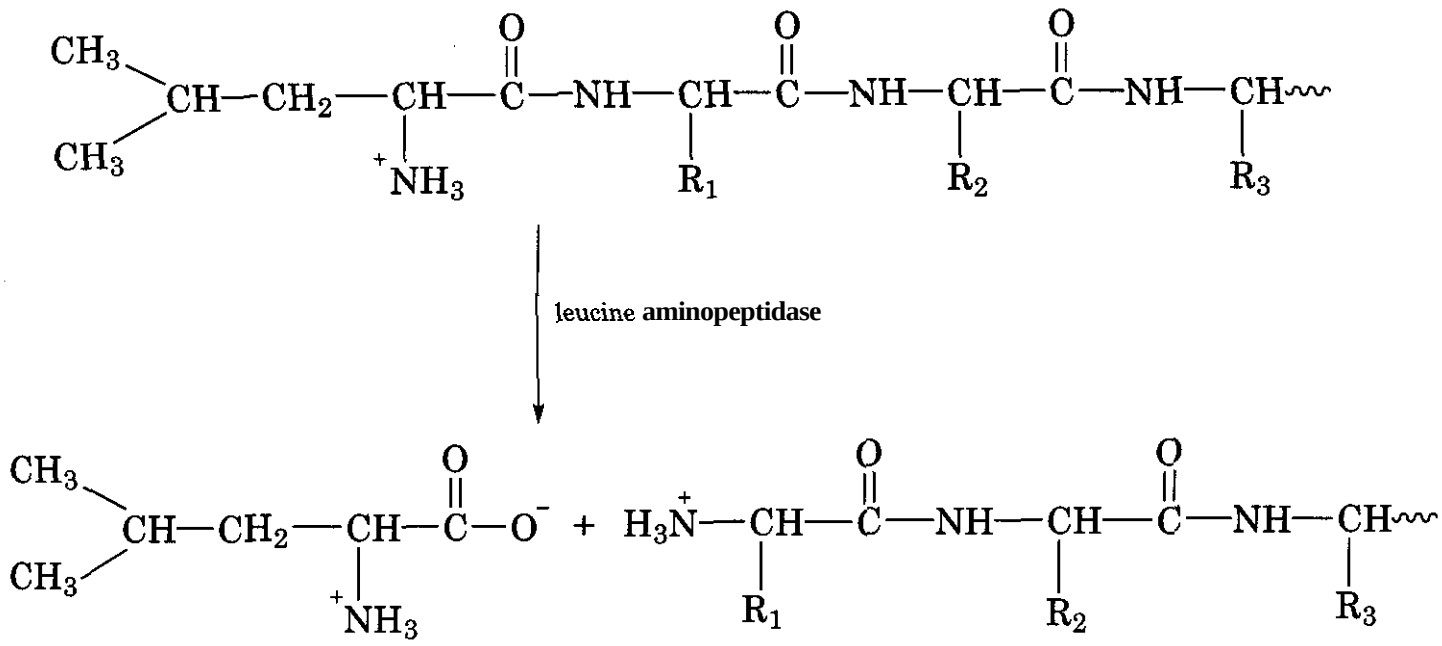


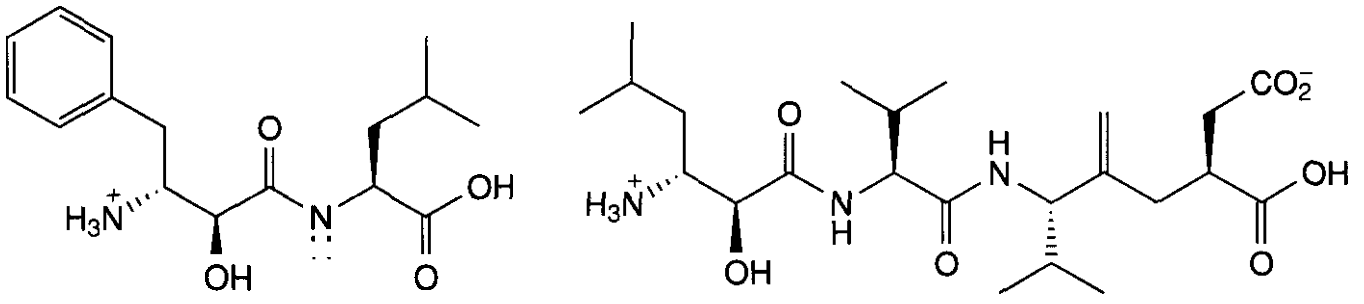
Figure 17.14. (a) Competitive and (b) slow-binding inhibitors of arginase.

this species binds with one hydroxyl, coordinating to one of the two requisite manganese ions. At pH 9.5 the tetrahedral species is the major form and this initially binds also with one hydroxyl coordinated to **a** manganese ion. Then, in a second, slower step, a water molecule that bridges the two active-site manganese ions is displaced by a second hydroxyl group on the boronic acid **(83).** Support for this mechanism is provided by crystal structures, showing both **(15)** and **(16)** are bound in the active site of **arginase** as tetrahedral species at alkaline pH **(82).** Of course, compound (12) is unable to form the tetrahedral species and is a competitive inhibitor at all times.

Leucine arninopeptidase (LAP) is a **metalloenzyme** that has been inhibited in a **slow-binding** manner. This exopeptidase catalyzes the hydrolysis of N-terminal amino acids, particularly those with a **leucine** at the *N*-terminus, although it does have a broad specificity (Equation **17.32).**



(17.32)



(17) (18)

Figure 17.15. Slow-tight-binding inhibitors of leucine aminopeptidase.

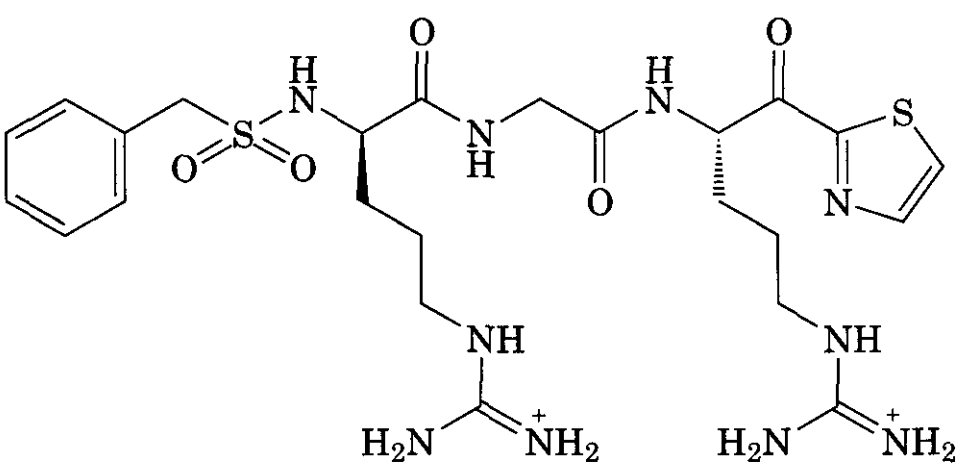
Bestatin (17) (Fig. 17.15) and amastatin (18) have been identified as slow-tight-binding inhibitors of LAP from porcine kidney, with K_i values in the low nanomolar range (84). Later, bestatin was shown to be a slow-binding inhibitor of LAP employing mechanism B, with a K_i value of 0.11 μM and a K_i^* value of 1.3 nM. Values of 1.5×10^{-2} s^{-1} and 2×10^{-4} s^{-1} were obtained for k, and k_{-4} (Equation 17.29), respectively (85). It was assumed that the inhibition of bovine lens leucine aminopeptidase (blLAP) by amastatin would also proceed by mechanism B. This prediction was supported by an X-ray crystallography study of the amastatin-blLAP complex (86), which suggested that (18) (and, by analogy, 17) initially binds to a Zn^{2+} atom in a groove in the active site. The slow step in binding was seen as a subsequent coordination to a second Zn^{2+} atom located deeper in the active site (86).

It is difficult to find clear-cut examples of slow-binding inhibition occurring by mechanism A. However, the inhibition of Factor Xa by a peptidyl-α-ketothiazole was found to be unusual because it appeared that the formation of E . I was partially rate limiting. Factor Xa is a trypsinlike protease found in the blood coagulation pathway, which cleaves prothrombin forming thrombin that, in turn, promotes blood clotting (Equation 17.33).

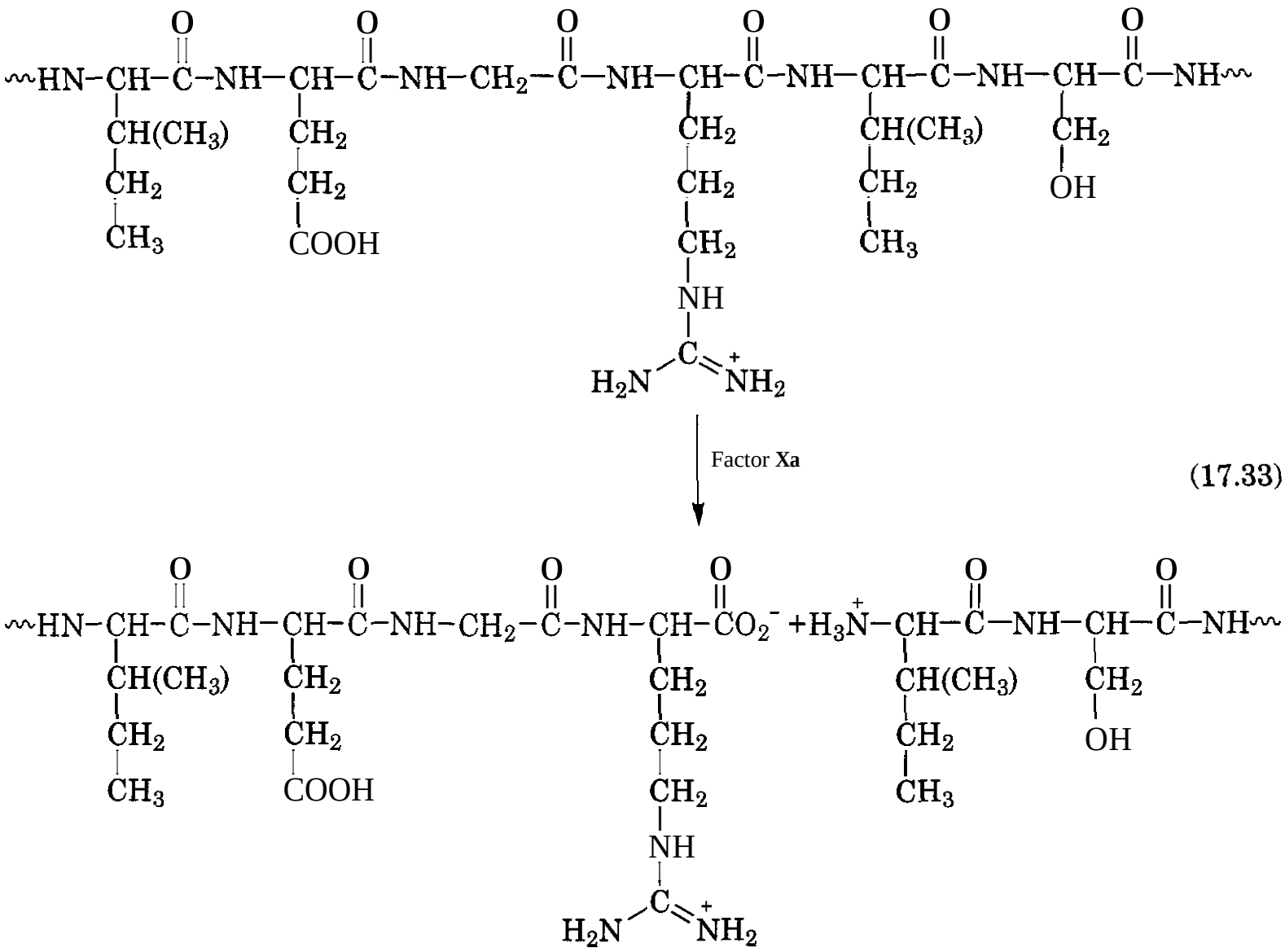
Inhibitors of Factor Xa activity offer potential as anticoagulants and several irreversible inhibitors of Factor Xa have been developed. One of the few tight-binding reversible inhibitors of Factor Xa is $BnSO_2$-D-Arg-Gly-Arg-ketothiazole (19).

The inhibitor could be displaced from Factor Xa by substrates and, based on steady-state assumptions, the dissociation constant for (19) was found to be 14 pM (87). However, the reaction progress curves indicated a slow-binding process, probably by mechanism B. Stopped-flow fluorescence studies, combined with kinetic analysis, showed that the isomerization step (E . I + E . I*) is unusually fast and that the formation of E · I is, at least, partially rate limiting.

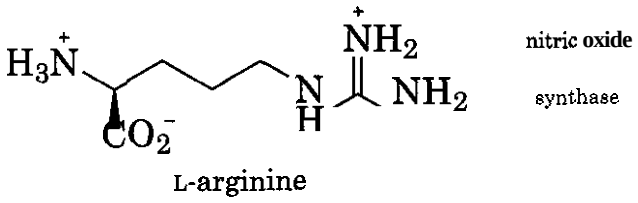
In some instances the type of inhibition has been found to be isozyme specific. For example, inducibly expressed isozymes (iNOS) and constitutively expressed isozymes (cNOS) of nitric oxide synthase (NOS) all catalyze the conversion of L-arginine to L-citrulline and nitric oxide (Equation 17.34).



(19)

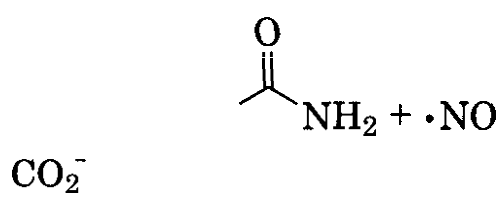


(17.33)



L-arginine

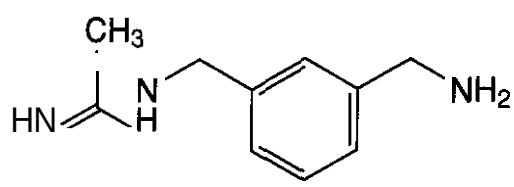
(17.34)



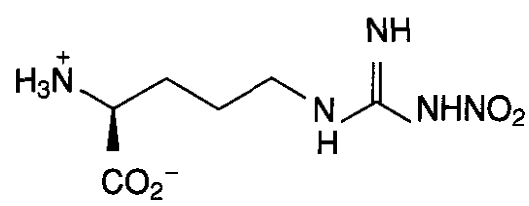
L-citrulline Nitric oxide

The inhibition of human iNOS by *N*-(3-(aminomethyl)benzyl)acetamidine (20) (Fig. 17.16) was found to proceed by mechanism B, with an overall K_d of <7 n*M*. Conversely, inhibition of constitutive isoforms of the human enzyme was found to be rapidly reversible, with K_i values in the micromolar range (88). This is in contrast to results obtained for the arginine analog, L-N^G-nitroarginine (21), which was found to be a rapid reversible inhibitor of mouse macrophage iNOS, with a K_i of 4.4 μM, and a slow-binding inhibitor of brain cNOS with a K_d (assuming mechanism A) of 15 n*M* (89).

Many more examples of these types of inhibitors can be found in the review by Morrison and Walsh (78).

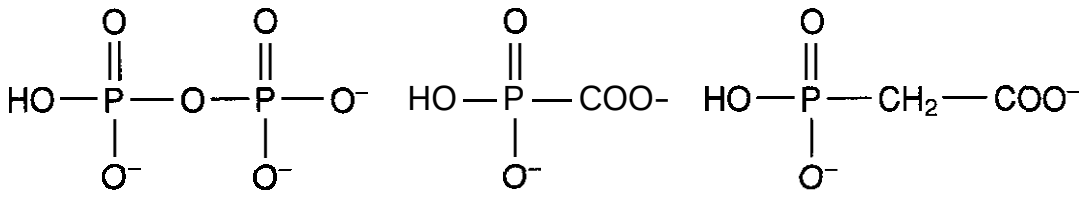


(20)



(21)

Figure 17.16. Inhibitors of nitric oxide synthase.



Pyrophosphate (PP_i) (22) (23)

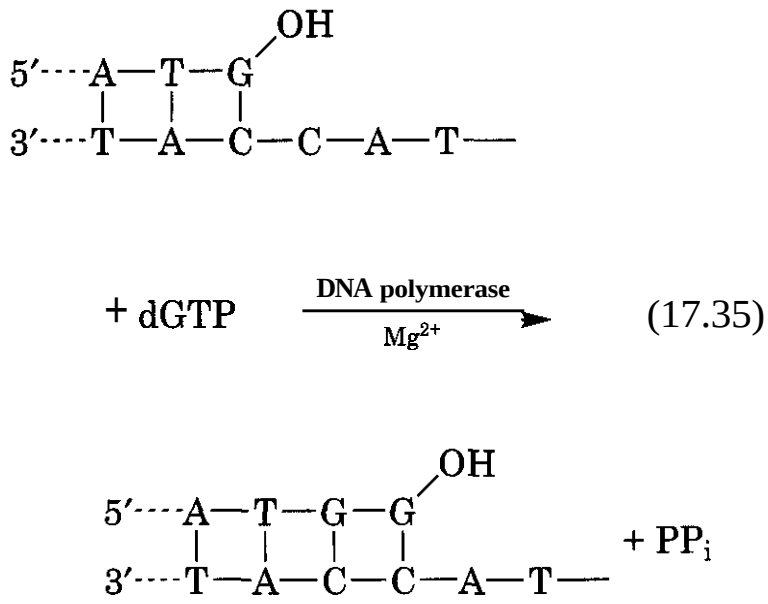
Figure 17.17. *Pyrophosphate* analogs used to inhibit DNA **polymerase.**

2.5 Inhibitors Classified on the Basis of Structure/Mechanism

As with any reaction, an enzyme-catalyzedreaction must proceed from the ground state through a transition state before products are formed. In addition, there are often some high-energy intermediates along the pathway. Knowledge and understanding of an enzyme's mechanism permits the identification of the high-energy intermediates and the prediction of the structures of the transition states. Armed with that knowledge, it is possible to design enzyme inhibitors based on the structures of the various intermediates along the reaction pathway. Inhibitors designed in this manner are occasionally referred to as mechanism-based inhibitors. However, for purposes of this chapter, we will reserve that term for the covalently binding inhibitors described in Section 3.

2.5.1 Ground-State Analogs. The ground state of an enzymatic reaction consists of the substrates and the products. Compounds that mimic the substrate of an enzymatic reaction have been examined earlier (Section 2.3) and are not discussed again here. There are many examples of enzymatic reactions that are inhibited by some or all of the reaction products. Both epinephrine and S-adenosyl-L-homocysteine, for example, are inhibitors of phenylethanolamine N-methyltransferase (Equation 17.35). In much the same way as described earlier for substrate analogs, product analogs can also be used to obtain information about the binding mechanism of enzymes (90).

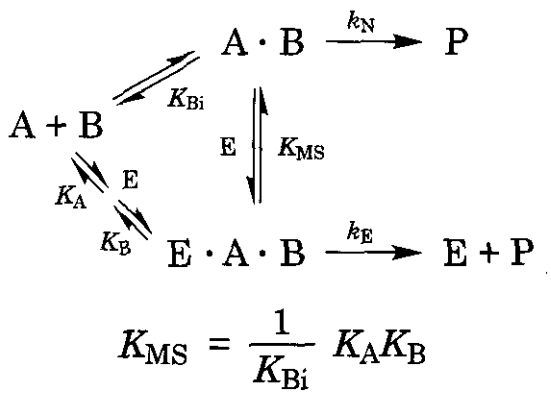
Phosphonoformate (22) (Fig. 17.17) is an antiviral agent that is used clinically in the treatment of herpes simplex virus (HSV) and human cytomegalovirus (HCMV) (91). It acts as a product analog, blocking the pyrophosphate-binding site, in the reaction catalyzed by DNA polymerase (Equation 17.35). It is also effective, using the same mechanism, against HIV reverse transcriptase (91).



(17.35)

DNA polymerase catalyzes the transfer of a complementary deoxynucleoside monophosphate moiety from its triphosphate (dNTP) to the 3' hydroxyl of the primer terminus, with subsequent release of pyrophosphate (PP_i, eq. 17.35). Initially, phosphonoformate (22) and phosphonoacetate (23) were identified as inhibitors of HSV DNA synthesis (92). Detailed kinetic studies (93), using DNA polymerase induced by avian herpes viruses, showed that phosphonoacetate (23) was a noncompetitive inhibitor of the four dNTPs. At low levels of dNTPs it was a noncompetitive inhibitor of the substrate DNA, becoming uncompetitive at saturating dNTP levels. It was also found that (23) was a competitive inhibitor of pyrophosphate, with a K_i value in the low micromolar range, in the dNTP-PP_i exchange reaction catalyzed by a turkey virus DNA polymerase (93). The inhibition patterns were identical to those observed using pyrophosphate as an inhibitor. Therefore it was concluded that (23) acted as an analog of pyrophosphate and competed for the same binding site (93). Later, both (22) and (23) were confirmed as acting as pyrophosphate (i.e., product) analog inhibitors of isolated HSV DNA polymerase (94).

2.5.2 Multisubstrate Analogs. A large number of enzymatic reactions involve the simultaneous binding of two or more substrates at the active site. The bound substrates must be in close proximity to each other and positioned in such a way as to facilitate covalent bond formation or the transfer of a functional group from one substrate to another. Multisubstrate analog inhibitors mimic the simultaneous binding of two or more substrates at the active site of the enzyme. The advantage of this, for a bireactant system, is shown in Equation *17.36.*



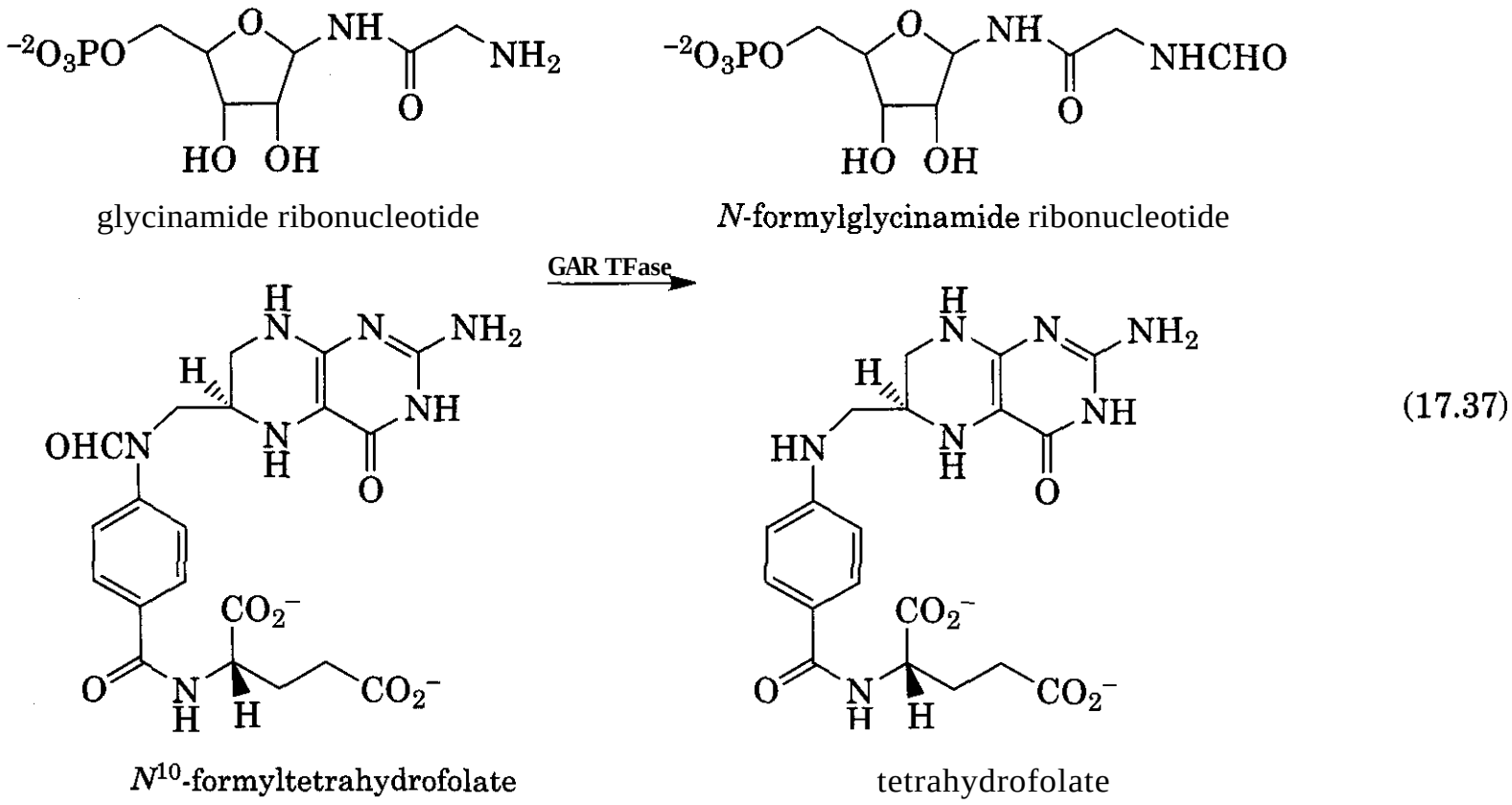
(17.36)

$$K_{MS} = \frac{1}{K_{Bi}} K_A K_B$$

There are two ways the two substrates, A and B, may bind to the enzyme to form an E · A · B complex. First, and most likely, they bind individually (in either a random or an ordered fashion) with dissociation constants of K_A and K_B. Second, the substrates may come together, positioned in such a way as to facilitate their subsequent reaction with a dissociation constant of K_{Bi}. This reactive complex A · B then binds to the enzyme with a dissociation constant of K_{MS}. In general, the formation of A · B is entropically unfavorable. However, a bisubstrate analog, designed to mimic A · B, can often be prepared by covalently connecting the corresponding substrates or substrate analogs with a suitable linker group. Linking the two groups effectively overcomes the unfavorable entropic barrier. It has been calculated that an ideal bisubstrate analog inhibitor can bind up to 10^8 times more tightly than the product of the substrate-binding constants (i.e., $1/K_{Bi}$ may be as high as 10^{-8} *M*). This figure is based on entropic considerations and also assumes a perfect fit of the bisubstrate analog inhibitor to the two binding sites on the enzyme (57).

Where does this high affinity come from? A multisubstrate analog inhibitor will bind more tightly than substrate analog inhibitor because it has (*1*) the entropic advantage of reduced molecularity and (2) an additive binding contribution from each of the substrates it mimics. For example, when two single-substrate analog inhibitors bind separately, but next to each other, two sets of translational and rotational entropies are lost. However, when a bisubstrate analog inhibitor binds it loses only a single set of translational and rotational entropies (57, ***60***). Further, let us assume that the bisubstrate analog binds to the same two sites as two single-substrate analog inhibitors. In that case there will be a gain in entropy from the release of water molecules from each substrate-binding site, as well as the favorable enthalpic contributions from the formation of hydrogen bonds, buried salt bridges, and so forth in each site. These favorable free-energy contributions will be the same for a bisubstrate analog as for the two individual inhibitors binding simultaneously. On the other hand, compared to the binding of a single-substrate analog, the multisubstrate analog inhibitor gains favorable binding enthalpies and entropies from the additional binding site(s), while still losing only one set of translational and rotational entropies. Thus the binding of a multisubstrate analog should be very tight, without needing any assistance from transition-state complementarity.

Inhibitors that combine two substrates are termed bisubstrate analogs, whereas those combining three substrates are termed trisubstrate analogs and so on, with the former being the most common. The design of a bisubstrate analog inhibitor ordinarily requires the development of two single-substrate analog inhibitors of reasonable affinity. The two single-substrate inhibitors are then connected by an appropriate linker, and the optimal length of the linker is determined experimentally. Under normal circumstances, the K_i value for a bisubstrate analog inhibitor can be expected to approximate the product of the K_i values of the two substrate analogs. A guide to a reasonably achievable K_i for a bisubstrate analog also may be obtained from the product of the K_M values of the individual substrates. For example, if two substrates of an enzymatic reaction have binding constants in the millimolar range, a bisubstrate analog would be expected



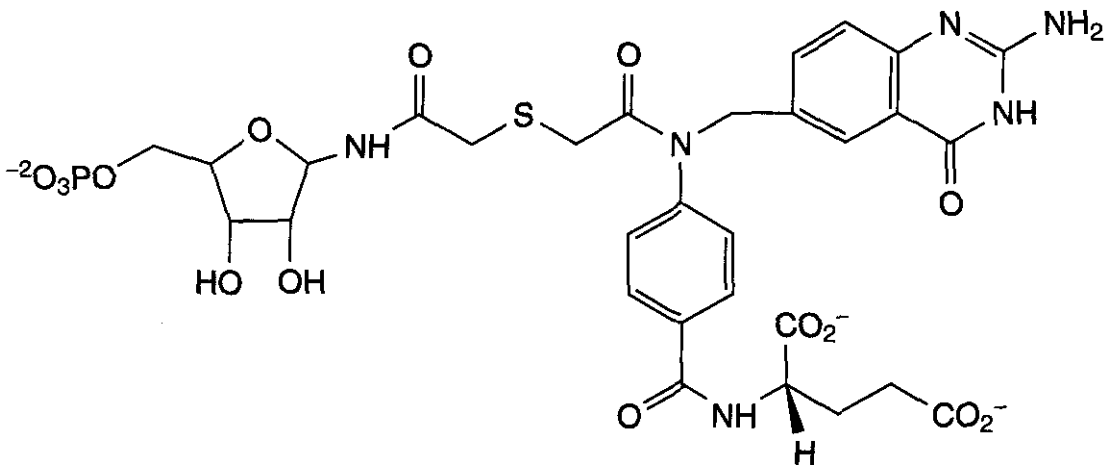
(17.37)

to have a K_i value in the micromolar range. Note also that, if the enzyme binds substrates in a random manner, then a multisubstrate inhibitor should exhibit competitive inhibition patterns with each substrate it mimics because the binding of the inhibitor should be mutually exclusive with that substrate. If the enzyme employs an ordered mechanism, then the inhibitor should be competitive with the first substrate to bind and uncompetitive with other substrates.

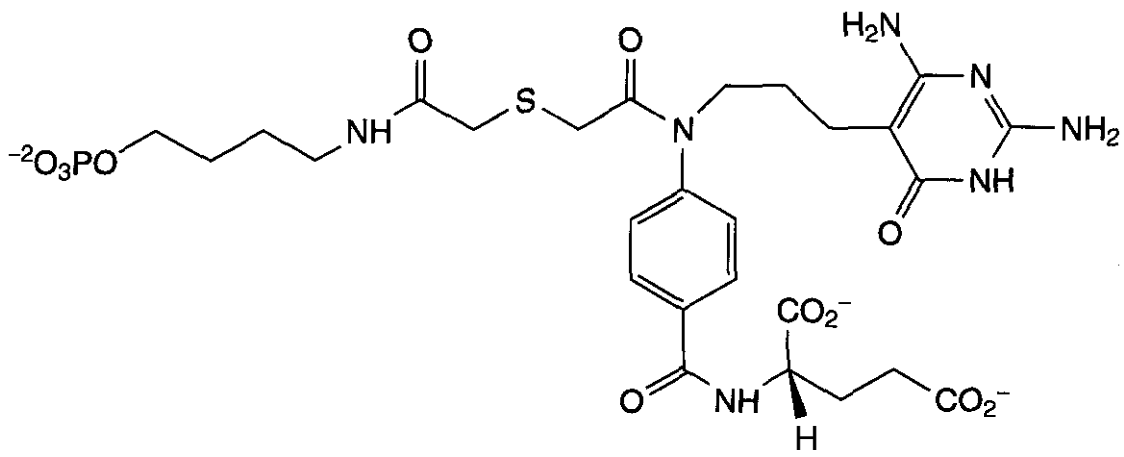
The multisubstrate analog approach to enzyme inhibition has the additional advantage in that it provides a high degree of specificity. The combination of two or more substrates will usually produce a unique structure, unlikely to bind to other enzymes that may utilize any one of the substrates. This approach has even been used to design isozyme-specific inhibitors (95). It should also be noted that the distinction between a transition-state analog (Section 2.5.3) and a multisubstrate analog inhibitor is often quite arbitrary. In fact many inhibitors described as transition-state analogs are often actually analogs of high-energy reaction intermediates that, in turn, may have structures somewhat akin to those of multisubstrate analog inhibitors. However, multisubstrate analog inhibitors are intended to mimic the combined substrates in their ground-state forms and do not require any contribution from transition-state stabilization. Several general reviews on multisubstrate analog inhibitors have appeared (96–98), and multisubstrate analogs also receive some discussion in reviews on transition-state analogs (99–101).

Glycinamide ribonucleotide transformylase (GAR TFase) catalyzes the transfer of a formyl group from N^{10}-formyltetrahydrofolate to glycinamide ribonucleotide (Equation 17.37). This is a crucial step in *de novo* purine biosynthesis, which is essential for cell division, and GAR TFase has become a target enzyme for the development of antineoplastic agents.

Inglese et al. (102) were able to synthesize the bisubstrate inhibitor β-thioGARdideazafolate (β-TGDDF) (24) (Fig. 17.18). This compound combines nearly all the features of both substrates, linked by a stable thioether bridge, and was found to inhibit GAR TFase with a K_i value of 250 pM (102). β-TGDDF acted as a slow, tight-binding inhibitor (Section 2.4) and the K_i value was about three times lower than the product of the K_M values of the substrates. More recently, the crystal structure of the complex between BW1476U89 (25) and GAR TFase was obtained (103). BW1476U89 is another multisubstrate analog and has a K_i value of about 100 pM (104). The structure confirms that the inhibitor binds in those sites identified previously as substrate-



(24)



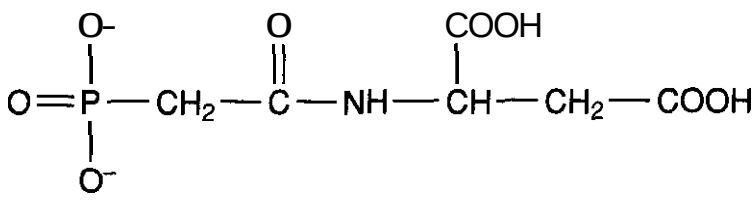
(25)

Figure 17.18. Bisubstrate analog inhibitors of GAR-TFase.

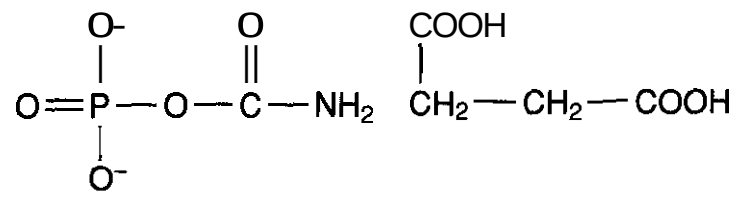
binding sites, and provides a starting point for development of even more potent transition-state analogs.

The condensation of carbamyl phosphate and L-aspartate, catalyzed by aspartate transcarbamoylase (ATCase), produces *N*-carbamyl-L-aspartate (Equation 17.38). This is one of the early steps in ***de novo*** pyrimidine biosynthesis, also a requirement for cell division, making ATCase also a target for potential anticancer agents.

N-Phosphonoacetyl-L-aspartate (PALA) (**26**) (Fig. 17.19) was initially designed as a transition-state analog inhibitor of ATCase (105). It was found to have a K_i value of 27 nM, a value that is considerably lower than the K_M values of 27 μM and 17 mM for carbamyl phosphate and L-aspartate, respectively (105). PALA was

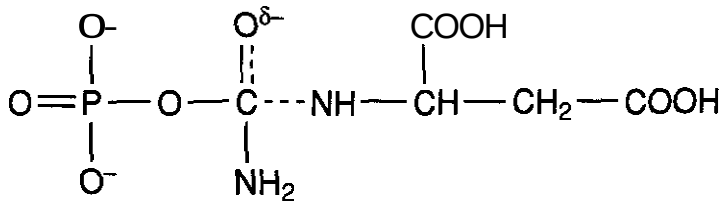


(26)

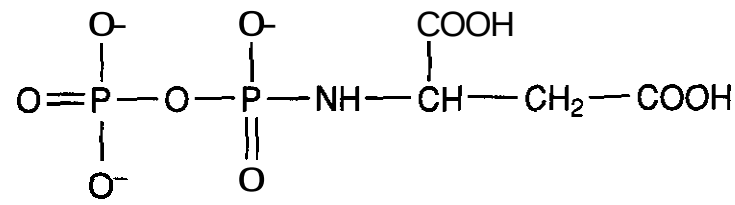


(27)

(28)

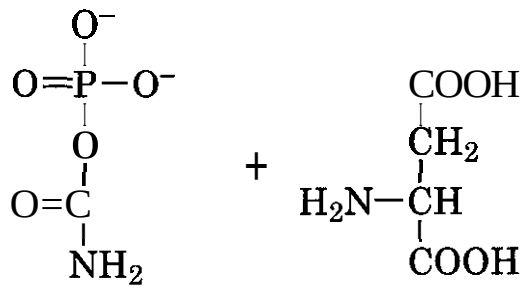


(29)

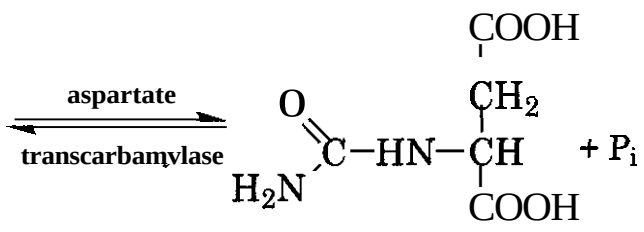


(30)

Figure 17.19. Putative transition state, substrate, and inhibitors of aspartate transcarbamylase.



carbamyl phosphate L-aspartate (17.38)

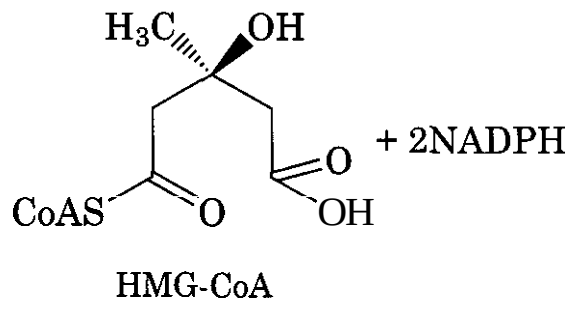


N-carbamyl-L-aspartate

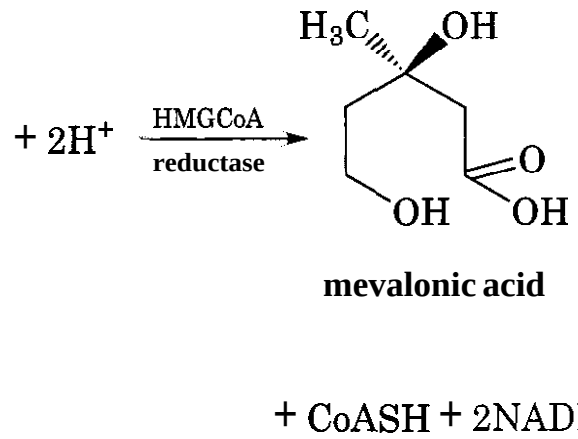
found to inhibit cell growth in *vivo* (106) and, eventually, underwent clinical trials as an anticancer agent (107).

PALA provides an example of the difficulties in distinguishing between a multisubstrate analog and a transition-state analog. As shown in Fig. 17.19, in effect PALA (26) combines two fragments, an analog of carbamyl phosphate (27) and succinate (28). The tight binding of PALA also suggested it was a potential transition-state analog. However, succinate has a K_i value of 90 μM, and the product of the K_i values of succinate and carbamyl phosphate is 24 nM, which is almost identical to the K_i value of PALA (105). As shown in Fig. 17.19, the transition-state structure (29) for the ATCase-catalyzed reaction is tetrahedral. The pyrophosphate analog (30) was expected to provide a much better mimic of the transition state, yet its K_i value of 0.24 μM was tenfold higher than that of PALA (108). It is not clear why there is this discrepancy, but a recent X-ray structure of the ATCase-PALA complex identified several groups that are positioned to bind to a tetrahedral transition state (109). Two of these, the side chain of Gln137 and the backbone carbonyl of Pro266, were positioned to interact with the amino group of the putative transition state (29). However, these groups would not be expected to interact so well to the analogous oxygen atom of the pyrophosphate transition-state analog (30), perhaps leading to its weaker-than-expected binding.

The statins are a group of cholesterol-lowering agents that have become some of the largest selling drugs in the world. They lower serum cholesterol levels by competitively inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in cholesterol biosynthesis (Equation 17.39).



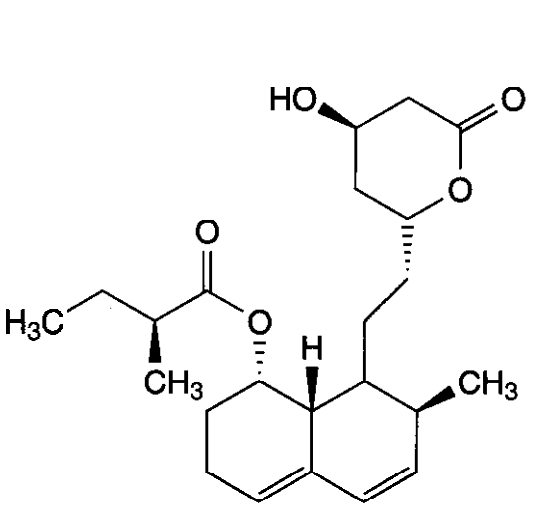
HMG-CoA



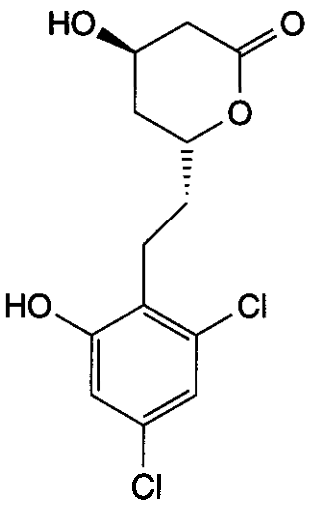
mevalonic acid (17.39)

Several statin inhibitors of HMG-CoA reductase are shown in Fig. 17.20. They consist of rigid, hydrophobic groups connected to an HMG-like group that, in inhibitors such as mevastatin (compactin) (31), simvastatin (9) (Fig. 17.1), and the dichlorophenol derivative (32) is present in the form of a lactone. In *vivo*, the lactone is converted to the free acid, as shown in Fig. 17.20 for mevastatin (33). More recently developed statins, such as fluvastatin (34) and atorvastatin (35), are prepared as the free acids. These inhibitors have K_i values in the low nanomolar range (110), significantly lower than the K_M value of the substrate HMG-CoA, which is in the micromolar range (110,111). Given that these inhibitors did not appear to be transition-state analogs, Nakamura and Abeles (112,113) conducted a number of experiments to determine the basis of the enhanced affinity of, in particular, (31) and (32).

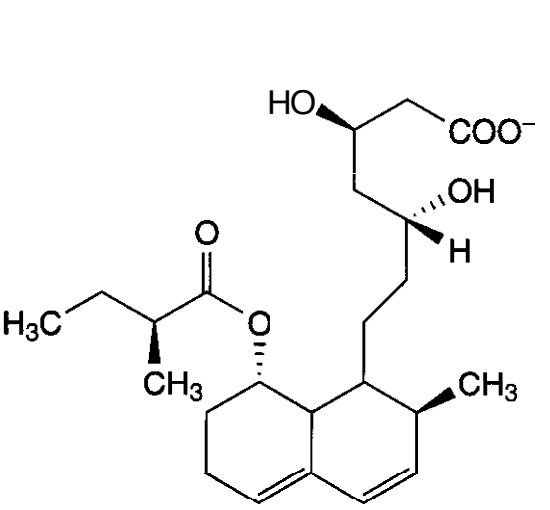
Both mevastatin (31) and (32) were found to bind to the hydroxymethylglutarate portion



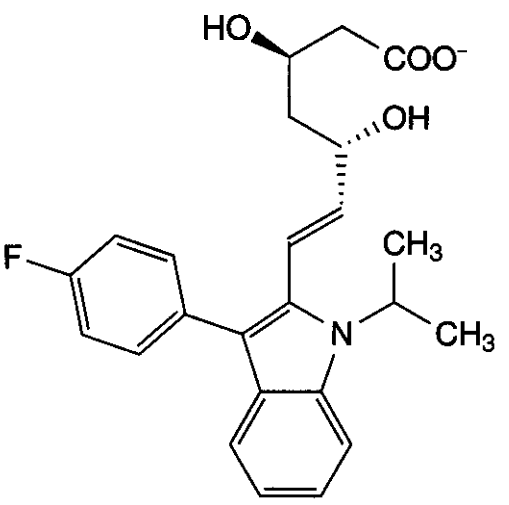
(31)



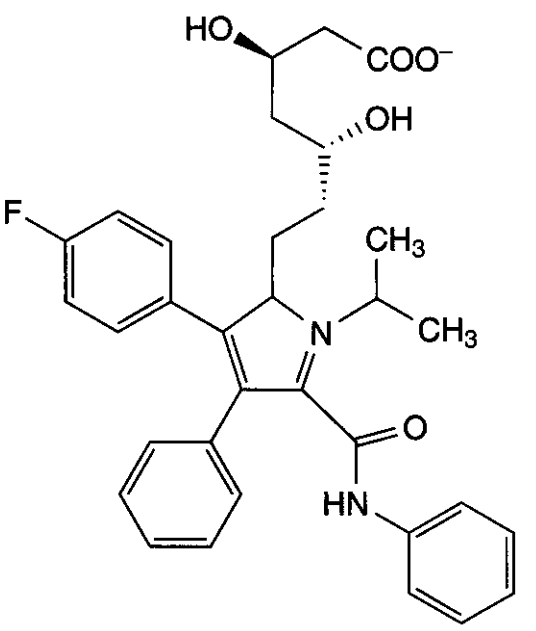
(32)



(33)



(34)

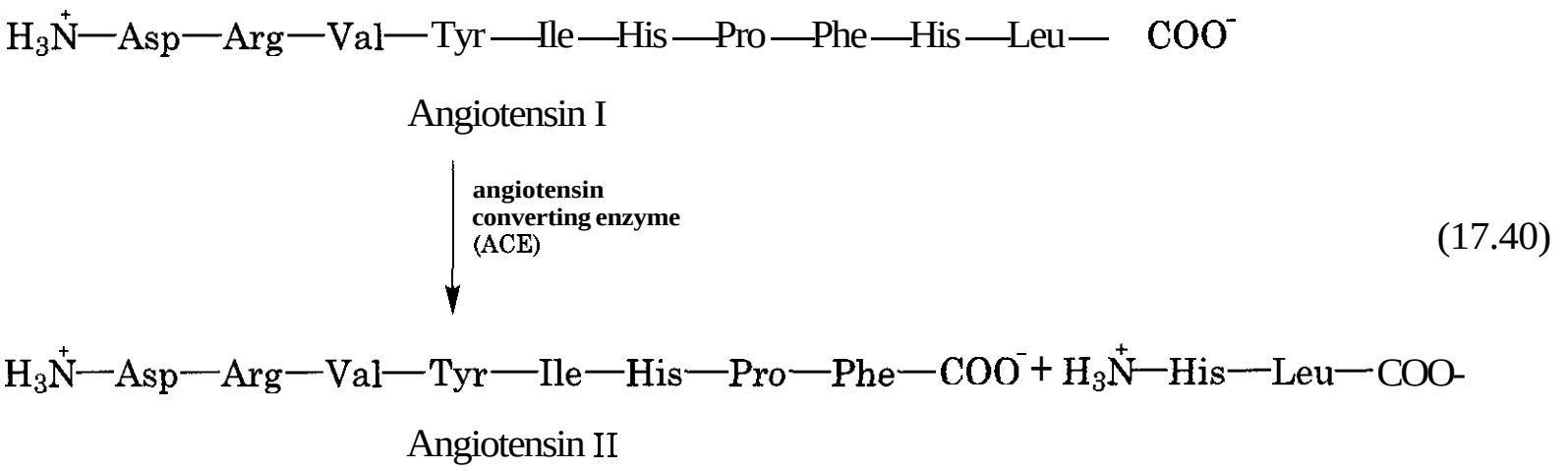


(35)

Figure 17.20. Statin inhibitors of HMGCoA-reductase.

of the active site, but not the NADPH region, whereas only (31) bound to the coenzyme A portion. D,L-Mevalonate and D,L-3,5-hydroxyvalerate, used as analogs of the upper portion of the statins, were both poor inhibitors, with K_i values in the millimolar range; however, analogs of the hydrophobic decalin region of mevastatin showed no inhibitory effect (112). Given that the K_i value for mevastatin is almost eight orders of magnitude lower than that of D,L-3,5-hydroxyvalerate, it is clear that the hydrophobic lower portion (and its covalent link) must play a significant role in the binding of (31)and, by implication, the binding of all the statins. Presumably, the upper portion of the inhibitor is necessary for specificity and the hydrophobic region for binding affinity. The hydrophobic region must be relatively nonspecific because a variety of hydrophobic groups (Fig. 17.20) are accepted. In some cases (e.g., mevastatin), the hydrophobic group overlaps the CoASH site and in others, such as the dichlorophenol group of (32), it does not (112). The structure of the statin is analogous to that of a bisubstrate inhibitor, in that there is linked binding to two distinct binding sites on the enzyme, leading to greatly enhanced inhibition of the enzyme. For mevastatin, the entropic advantage provided by linking the mevalonate and decalin portions together is estimated to be approximately 5×10^4 M (113). This is quite a reasonable enhancement, given that the theoretical maximum is 10^8 M (57), and it has been suggested that such a "hydrophobic anchor" is responsible for the enhanced binding of some inhibitors of alcohol dehydrogenase and adenosine deaminase (113).

Although this explanation appeared quite reasonable, it was thrown into doubt when X-



(17.40)

ray structures of HMG-CoA reductase complexed with both substrates and products were obtained (114, 115). These structures showed that, if the statins bound so the HMG-like groups bound the HMG-binding pocket of the active site, the bulky hydrophobic groups of the statins would clash with the residues lining the narrow pocket into which part of the coenzyme A bound (115). However, recently, Istvan and Deisenhofer have obtained X-ray structures of HMG-CoA reductase bound to six individual statins, including (**9**), (**31**), (**34**), and (35) (116). This study showed the substrate-binding pocket rearranges to accommodate the statins, that the statins do bind to the *HMG-binding* region, that a shallow hydrophobic groove now accommodates the hydrophobic groups, and that none of the NADP(H)-binding pocket is occupied (116). In *toto*, the structural studies supported all interpretations made some 15 years earlier based on kinetic studies, and provided definitive evidence for a hydrophobic anchor enhancing the binding of the mevalonate portion of the statins.

The evolution of the angiotensin converting enzyme (ACE) inhibitors is an illuminating story in the development of enzyme inhibitors as therapeutic agents. As shown in Equation 17.40, ACE catalyzes the conversion of angiotensin I to angiotensin II.

Angiotensin II, itself a potent hypertensive agent, also stimulates the release of a second hypertensive agent, aldosterone. In addition, ACE catalyzes the cleavage of the nonapeptide vasodilating agent, bradykinin (not shown). Therefore an ACE inhibitor was seen to have the potential to limit three hypertensive actions. This premise was validated by in *vivo* results with teprotide, a peptide inhibitor of ACE, which had been isolated from a South American pit viper (117).

At that time the structure of ACE was unknown, although it had been identified as a zinc metalloprotease. It was surmised that its mechanism and active site may resemble that of another metalloprotease, carboxypeptidase A, whose X-ray structure was known. (*R*)-2-Benzylsuccinic acid (36) (Fig. 17.21) had been identified as a potent inhibitor of carboxypeptidase A, and it was suggested that (36) resembled the collected products of the hydrolysis reaction (Fig. 17.21). In other words, (36) was a biproduct analog and, not unexpectedly, it was found to bind with an affinity resembling the combined affinities of the two products (118). Carboxypeptidase A appeared to have three main interactions with (36). Two substrate-binding sites bound the phenyl group and one carboxylate, and the Zn^{2+} ion, usually coordinated to the carbonyl of the amide bond being cleaved, was now bound to the second carboxylate. Combining those suggestions with studies with viper venom peptides, indicating that a C-terminal proline was effective in inhibiting ACE, a number of carboxyalkanoylproline derivatives were tested as ACE inhibitors (119). Of these, the succinyl-L-proline derivative (37) was found to be the most effective, with an IC_{50} value of **330** μM. Given that one carboxylate bound to the Zn^{2+} ion, a better zinc ligand, a thiol group, was substituted for this carboxylate, resulting in (38) with the IC_{50} value now reduced to 0.2 μM. Finally, after taking into account the differences between the active sites of ACE and carboxypeptidase A, captopril (**39**) was prepared. Captopril was found to be a competitive inhibitor of ACE, with a K_i value of 1.7 nM, and was

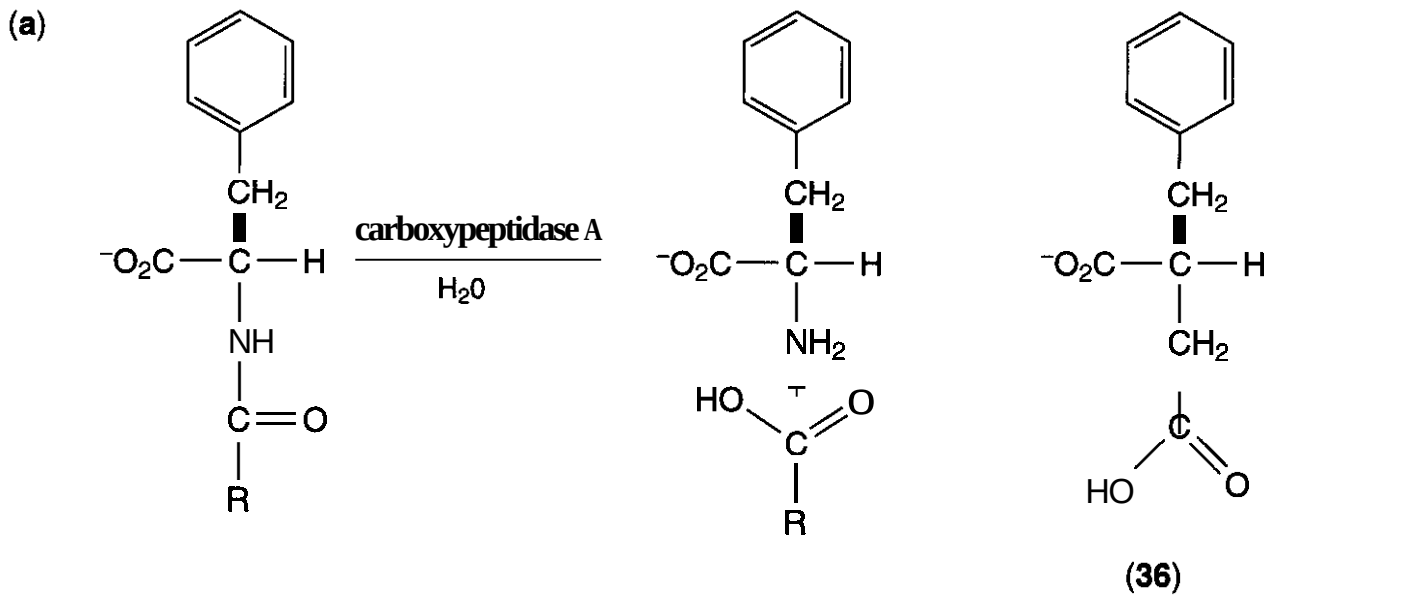


Figure 17.21. (a) Biproduct analog inhibitor of carboxypeptidase A and (b) several ACE inhibitors.

the first ACE inhibitor to be marketed. It was not long before attempts were made to make captopril more productlike, with the resultant development of **enalaprilat** (i.e. enalaprilat) (**40**). Enalaprilat was found to be a slow-tight-binding inhibitor (Section 2.4) of ACE with a K_i value below 1 nM, (**120**), but it was poorly absorbed orally. However, the ethyl ester (enalapril), (41) acted as a **prodrug**, had good oral activity, and was marketed. Note that it is also possible that enalaprilat acts as a **transition-state analog** (Section 2.5.3), thereby accounting for its performing as a slow-tight-binding inhibitor (121). Following enalapril, many more ACE inhibitors have been developed mainly aimed at increasing oral **bioavailability**, removing side effects, or improving **metabolism.These** include **ramipril** (**42**), the ester **prodrug** of ramiprilat (**43**), with 10 times better bioavailability than that of enalapril.

Ramaprilat was also shown to be a slow-tight-binding inhibitor of ACE, operating by mechanism B, with K_i^* (Equation 17.30) of 7 pM (122). A more detailed discussion of the development of the ACE inhibitors is available (121).

2.5.3 Transition-State Analogs. As a chemical reaction proceeds from substrates to products, it will pass through one or more transition states. The energy barrier imposed by the highest energy transition state controls the overall rate of the reaction. Enzymes bring about rate enhancements of 10^{10}–10^{15} (123) by lowering this energy barrier. They do this by having a greater affinity to the structure of the transition state than to the structures of either substrates or products. Although an enzyme may have good *affinity* for its substrate, as evidenced by a low dissociation constant (K_S, Equation 17.411, for the Michaelis ($E \cdot S$) complex, the enzyme can further stabilize the inherently unstable transition state, for example, by forming extra electrostatic or hydrogen bonds, by providing more effective hydrophobic interactions, or by using structural rearrangements to exclude solvent, thereby strengthening existing electrostatic contacts.

$$\begin{array}{ccccc} E + S & \overset{K_N^{\ddagger}}{\rightleftharpoons} & E + S^{\ddagger} & \xrightarrow{k_N} & E + P \\ \Big\uparrow\downarrow K_S & & \Big\uparrow\downarrow K_T & & \Big\uparrow\downarrow \\ E \cdot S & \overset{K_E^{\ddagger}}{\rightleftharpoons} & E \cdot S^{\ddagger} & \overset{k_E}{\rightleftharpoons} & E \cdot P \end{array} \qquad (17.41)$$

$$\frac{K_T}{K_S} = \frac{K_E^{\ddagger}}{K_N^{\ddagger}} = \frac{k_E}{k_N}$$

Simple transition-state theory states that the rate of an enzyme-catalyzed reaction is correlated with the rate of a noncatalyzed reaction by the same factor as the affinity of an enzyme for the transition state to the affinity of an enzyme for a substrate (Equation 17.41) (99).

Therefore, the magnitude of enzymatic catalysis (k_E/k_N) is related to the enhanced binding of the transition state to the enzyme (K_T/K_S). Compounds that can take advantage of this enhanced binding to the transition state can prove to be potent and selective enzyme inhibitors. Such compounds, referred to as transition-state analogs, can theoretically have ratios of the binding constants of inhibitor to substrate (K_i/K_S) on the order of 10^{-8} to 10^{-14}. In addition, transition-state analogs may have the further advantage of reduced molecularity, as outlined earlier (Section 2.5.2) for multisubstrate analog inhibitors. Several reviews on the theory and general aspects of transition-state analog inhibitors are available and are recommended for a more complete understanding of this topic (37, 96, 99, 100, 124, 125).

The design of a good transition-state mimic is quite challenging. It requires, at the least, sufficient knowledge of the mechanism of the target enzyme to predict transition-state structure(s). This is why transition-state analogs are sometimes (but not in this review) referred to as mechanism-based inhibitors. A detailed knowledge of the true energy profile, including details such as the existence of distinct chemical steps, high-energy intermediates, and their associated transition states, is also useful (126). Further, by definition, the transition state is unstable, often highly charged, and possesses partially broken/formed covalent bonds. Designing a ***stable compound*** that will closely mimic a transition state is impossible. However, the Hammond postulate states that the transition state between a reactant and a high-energy reaction intermediate will resemble the intermediate rather than the reactant. It is possible to design/synthesize an analog of a high-energy intermediate. Indeed, the majority of so-called transition-state analogs are actually analogs of high-energy reaction intermediates. Although a clear distinction exists, the design process is, for all practical purposes, the same.

It should also be noted that an enzyme is designed to initially recognize the features of its substrates. Often substrate binding brings about a conformational change in the enzyme that will then maximize the'attractive forces between the enzyme and transition state. The transition-state analog may not possess those features of the substrate that facilitate rapid binding, even though its affinity for the enzyme is extremely high. Although some transition-state analogs bind rapidly to enzymes,

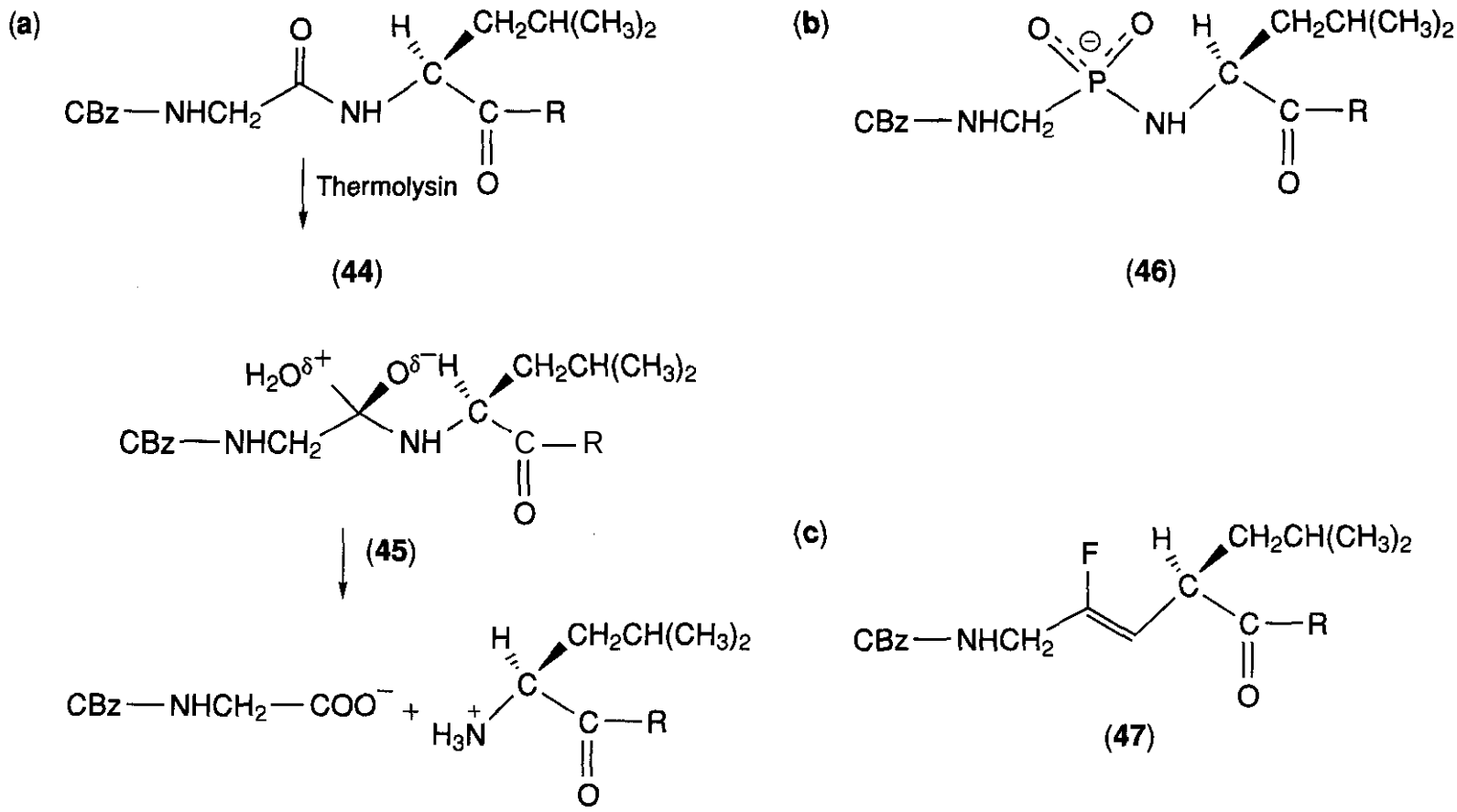


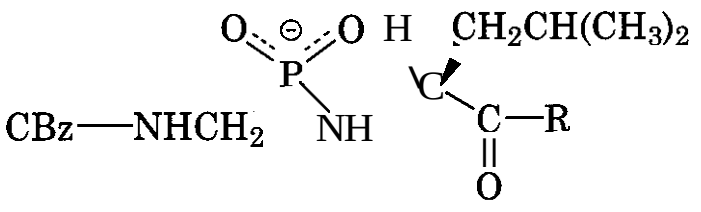
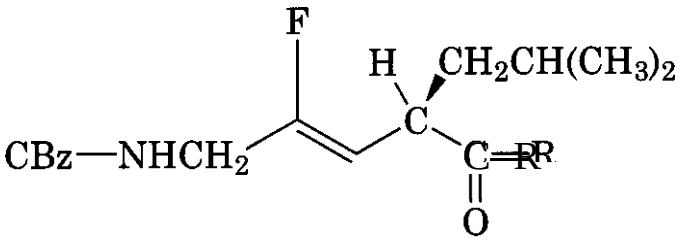
Figure 17.22. (a) Thermolysin-catalyzed hydrolysis of peptide analogs showing putative transition state, (b) phosphonamidate peptide analog, and (c) fluoroalkane peptide analog.

others bind slowly and show the properties of the slow-binding inhibitors described earlier in Section 2.4.

Slow binding, tight binding, or structural similarity to the assumed transition-state structure are not, in themselves, sufficient criteria to establish that an inhibitor is a true transition-state analog (127). Methotrexate (7), for example, is an extremely high-affinity ($K_i = 58$ pM), slow-binding inhibitor of dihydrofolate reductase (128). On the surface, it would appear that methotrexate could be classified as a transition-state analog. However, crystallographic studies have shown that methotrexate binds with its pterin ring in the opposite orientation to that of the substrate, dihydrofolate (129, 130). To distinguish between a high-affinity, ground-state analog and a putative transition-state analog requires a careful appraisal. There is a fundamental difference between the entropy change of a unimolecular enzymatic reaction and that of a multimolecular solution reaction (131). In addition, the appropriate rate constant for the nonenzymatic reaction is often either not available or hard to obtain (132, 133). These factors combine to make it difficult to evaluate quantitatively the correlation between the enhanced rates of enzymatic reactions and the tight binding of transition-state analogs.

In an attempt to develop stringent criteria for the distinction between transition- state and ground-state analogs, Bartlett and Marlowe (127) overcame some of these inherent difficulties by comparing the binding affinities of a series of substrate analogs with those of the corresponding transition-state analogs. One consequence of Equation 17.41 is that, if there is a change in structure of a substrate that alters k_{cat}/K_M without altering the nonenzymatic rate of reaction, then an analogous structural change in the transition-state mimic should bring about a similar change in K_i. Put simply, there should be a linear relationship between the values of K_i for the transition-state analog and k_{cat}/K_M for the corresponding substrate. Bartlett and Marlow (127) designed a series of dipeptide analog substrates (44) (Fig. 17.22) for thermolysin in which the structural variation was remote from the reactive center and therefore unlikely to affect the nonenzymatic reaction rate. The reaction catalyzed by thermolysin is proposed to proceed by the tetrahedral transi-

Table 17.6 Correlation of K_i Values for Inhibitors of Thermolysin with K_M and K_M/k_{cat} Values for the Corresponding Substrates[a]

	Inhibitor Data	Corresponding Substrate Data	
	K_i (nM)	K_M (mM)	K_M/k_2 (μM s^{-1})
R = D-Ala	1700	16.6	3200
R = NH_2	760	20.6	196
R = Gly	70	10.8	165
R = L-Phe	78	2.4	20
R = L-Ala	16.5	10.6	13.6
R = L-Leu	9.1	2.6	7.0
	K_i (mM)		
R = Gly	1.80	10.8	165
R = L-Ala	1.48	10.6	13.6
R = L-Leu	0.32	2.6	7.0
R = L-Phe	0.19	2.4	20

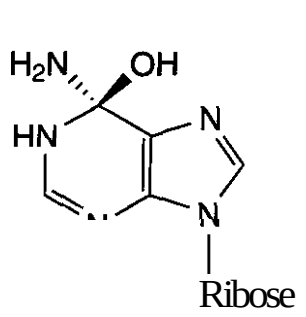
[a] Data are from Ref. 127.

tion state (45), and with their long P—O bonds, the phosphonamidate compounds (46, Table 17.6) were expected to a d as transition-state analog inhibitors. It was found that the K_i values for the putative transition-state analog inhibitors correlated linearly with the K_M/k_{cat} values of the corresponding substrates, although no correlation was found between K_i and K_M (Table 17.6). The fact that substrate binding (K_M) was relatively unaffected by a change at a remote site was not unexpected, but the observation that the binding of the phosphonamide inhibitors was greatly affected suggests that these inhibitors were, indeed, transition-state rather than ground-state analogs. Conversely, the K_i values for a series of fluoroalkene isosteres of the same substrates (47) (Fig. 17.22) correlated strongly with K_M but not K_M/k_{cat} (Table 17.6), indicating that the latter inhibitors were ground-state analogs (134). This approach has also been used to confirm that phosphonic acid peptides were transition-state analog inhibitors of pepsin (135).

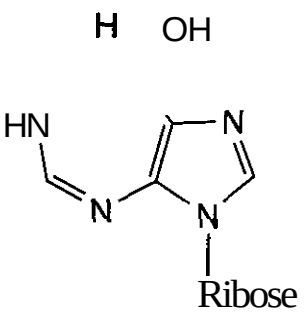
One of the most popular targets for design of transition-state analogs is adenosine deaminase. Inhibitors of this enzyme have been used as immunosuppressants and are also potential antitumor agents, whereas lack of adenosine deaminase results in severe combined immunodeficiency disease (SCID).

Adenosine deaminase (ADA), which catalyzes the conversion of adenosine to inosine (Equation 17.42), is an extremely proficient enzyme, providing a rate enhancement of more than 12 orders of magnitude (123). The enzyme-catalyzed reaction is thought to pass through an unstable hydrated intermediate (48) (Fig. 17.23), with a K_T (Equation 17.41) in the region of 10^{-17} M (123). Clearly, even a crude analog of (48) would have the potential to be an extremely powerful inhibitor of ADA.

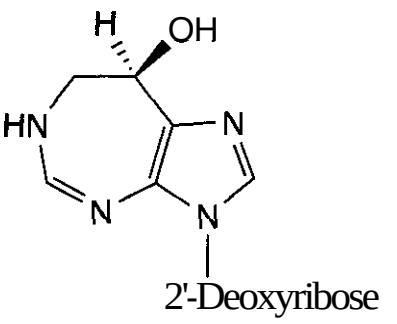
The structures of several inhibitors of ADA are shown in Fig. 17.23. Of these, the antibiotics coformycin (49) and (*R*)-deoxycoformycin (pentostatin, 50) were found to be potent ADA inhibitors, with K_i values of 1×10^{-11} M (136) and 2.5×10^{-12} M (137), respectively. The K_M for adenosine is around 30 μM (138, 139), whereas the K_i of the product, inosine, is 10^{-4} M. Thus, the two antibiotics show at least 10^6-fold greater affinity for ADA than for



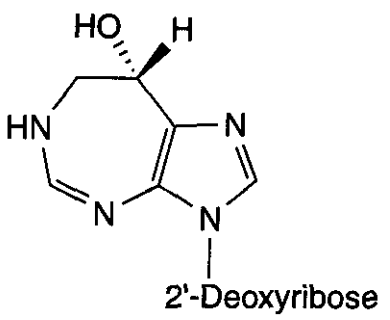
(48)



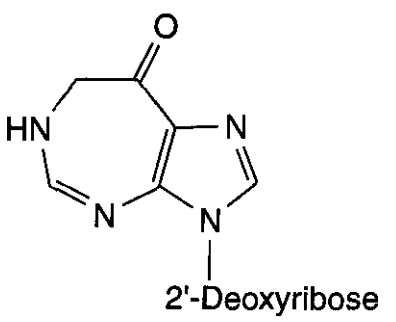
(49)



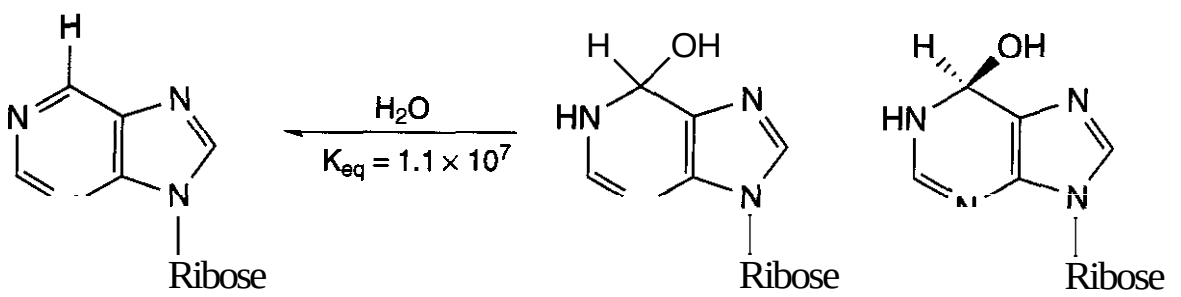
(50)



(51)

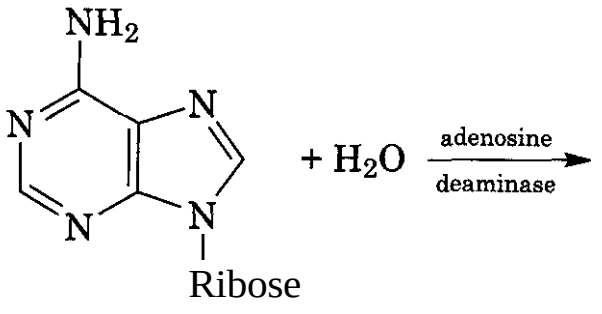


(52)



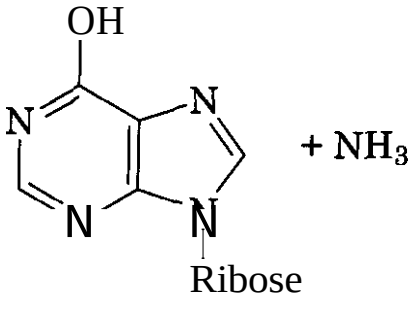
(53) (54) (55)

Figure 17.23. Reaction intermediate and inhibitors of the reaction catalyzed by adenosine deaminase.



adenosine

(17.42)



inosine

the substrate, suggesting that they are acting as transition-state analogs. By contrast, (*S*)-deoxycoformycin **(51)** and 8-ketodeoxycoformycin **(52)**, with K_i values of **33** and **40** μM, respectively, are clearly ground-state analogs. The differences in binding affinities of (*R*)- and (*S*)-deoxycoformycin translate to a difference in binding energy of almost **10** kcal/mol and provide an estimate of the energy that can be applied to substrate distortion in formation of the transition-state complex **(139).**

Purine ribonucleoside **(53)** was initially thought to bind to **ADA** as a ground-state analog, with an apparent K_i of **3** μM **(138, 140).** However, it was observed that the structure of the ligand and the enzyme were perturbed when purine riboside bound to ADA. ^{13}C-NMR spectroscopy showed that the ADA-bound purine riboside was sp^3 hybridized at **C-6** (141).The NMR and UV spectra suggested that it was the hydrated form of purine riboside (**54**) that was binding to **ADA** and, using the unfavorable equilibrium constant for hydration in solution **(1.1** $\times 10^{-7}$ M, Fig. **17.231,** a true K_i value of 3×10^{-13} M could be calculated for **(54)** (142). Given the low concentration of the free hydrate in solution, and the

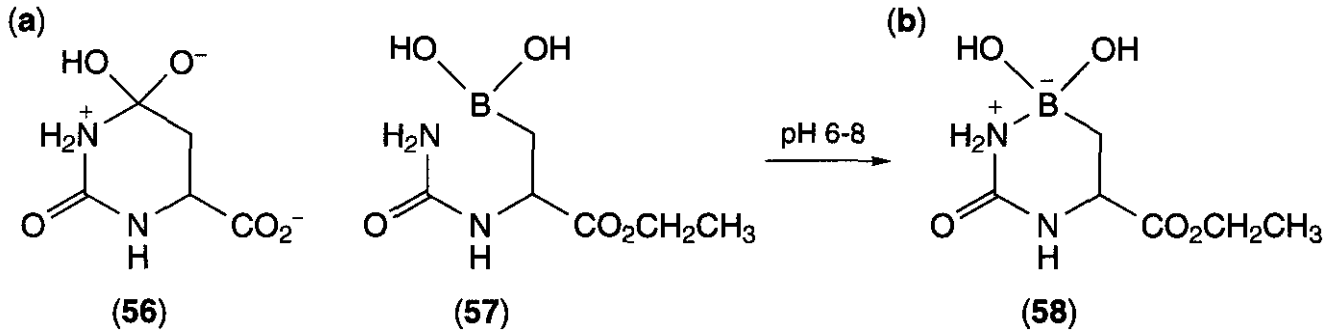
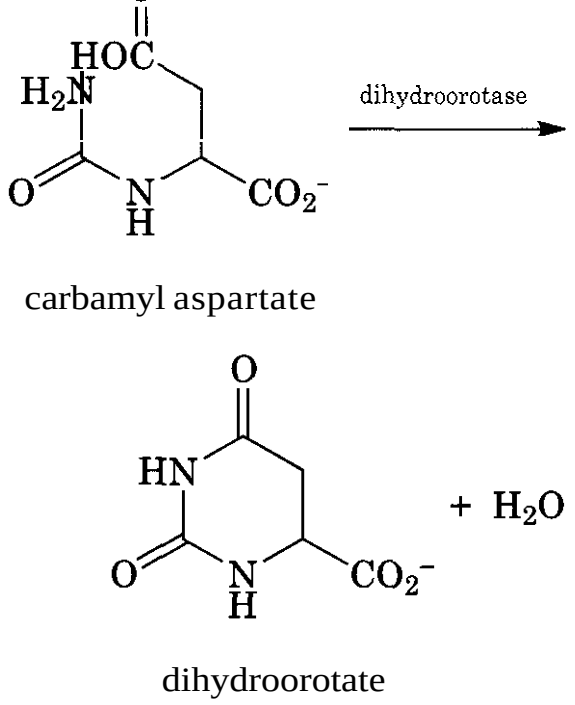


Figure 17.24. (a) Putative transition state for the dihydroorotase reaction, and (b) boronic acid transition-state analogs.

rapid onset of inhibition, it appears that purine riboside (53) itself initially binds and is then rapidly converted to (54) in the active site (141). This result, along with the high affinities of (*R*)-coformycin and (*R*)-deoxycoformycin, argues that the reaction proceeds by a stereospecific, direct attack of water rather than the double-displacement mechanism that also had been proposed (141). More recently, an X-ray study on adenosine deaminase, which had been crystallized in the presence of purine ribonucleoside, confirmed that it was the hydrated species of purine ribonucleoside that was present in the active site (143). Further, a triad of a zinc atom, a histidine residue, and an aspartic acid residue ensured that the binding was stereospecific, with the 6R isomer (55) being favored.

The adenosine deaminase story, in many ways, provides a perfect example of the general principles of enzymatic catalysis and the utility of enzyme inhibitors. ADA is an extremely efficient catalyst, producing a rate enhancement of 12 orders of magnitude. 6*R*-Hydroxy-1,6-dihydropurine riboside (55) has an affinity for ADA about 8 orders of magnitude greater than that for substrates or products; that is, it expresses a substantial fraction of the free energy of binding that separates the transition state from the ground state in an enzymatic reaction. Evidence of the extraordinary ability of an enzyme to discriminate between stereoisomers is provided by the 10^7-fold difference in binding affinities of the 8*R*-OH (50) and 8*S*-OH (51) stereoisomers of 2'-deoxycoformycin. Inhibitors were used to differentiate among several potential reaction mechanisms for ADA and, finally, an ADA inhibitor (pentostatin) has proved to be of therapeutic benefit.

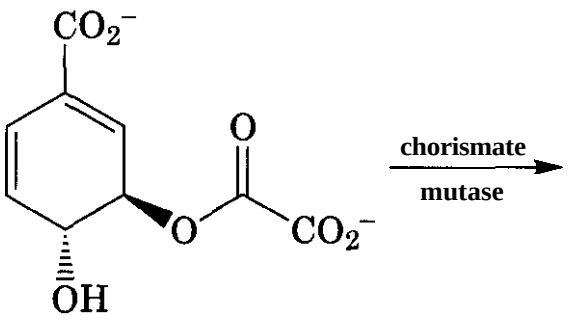
Inhibitors of pyrimidine and purine biosynthesis are used as antineoplastic agents. As a consequence, dihydroorotase, which catalyzes the third step of *de novo* pyrimidine biosynthesis, the conversion of carbamyl aspartate to dihydroorotate (Equation 17.43), is a target for therapeutic intervention.



The reaction is thought to proceed through the tetrahedral-activated complex (56) (Fig. 17.24), which is a highly charged, unstable sp^3 carbon species (144, 145). At around neutral pH, compound (57), a boron-containing analog of carbamyl aspartate, rearranges to the stable, tetrahedral boronic acid derivative (58). The affinity of (58) for dihydroorotase (K_i = 5 μM) was found to be 10-fold greater than that of the carbamyl aspartate

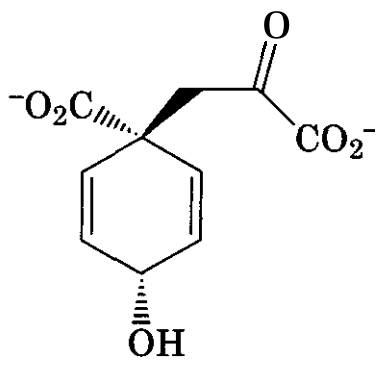
$K_M = 50\ \mu M$, indicating that (**58**) was probably acting as a transition-state analog (145). Tetrahedral boronic acid structures are stable, unlike the analogous sp^3 carbon species, and boronic acid derivatives of substrate peptides have proved to be quite potent inhibitors of a variety of proteases, particularly serine proteases (146).

Chorismate mutase catalyzes the conversion of chorismate to prephenate (Equation 17.44). This reaction is unusual, in that it is the only pericyclic [3,3] sigmatropic rearrangement (Claisen rearrangement) that is catalyzed by an enzyme.



chorismate

(17.44)



prephenate

Although chorismate mutase does provide a rate enhancement of 2×10^6 (147), this unimolecular reaction readily occurs without enzyme, under mild conditions. The reaction was expected to pass through a chairlike transition state (59)(Fig. 17.25) but early molecular orbital calculations indicated that the boatlike transition state (60) was not out of the question (147). In an attempt to define the transition-state structure, several compounds, each designed to mimic a putative transition state, were synthesized and tested as chorismate mutase inhibitors (147). The enzyme was found to be inhibited by the *exo*-carboxy nonane (**61**), with an apparent K_i value of 3.9×10^{-4} M. Conversely, the endo-carboxy nonane (62) did not inhibit the enzyme. The apparent K_i value of the adamantane derivative (**63**), in which the chair-chair conformation is fixed, was about the same as that of (61). Taken together, this implied that the reaction proceeded through a chairlike transition state (147).

This approach was later refined by Bartlett and Johnson, who suggested that IC_{50}/K_M ratios of 7 for compound (61) and 12 for compound (63) indicated that these inhibitors were not particularly good transition-state analogs (148). In an attempt to improve potency, and to further define the stereochemistry of the transition state, they synthesized several compounds including the exo- and *endo*-carboxy unsaturated oxabicylic ethers, (64) and (**65**), respectively (148). The exo-compound (64) was not significantly better than its saturated carbocyclic analog (**61**), but the endo-derivative (65) bound chorismate mutase some 100-fold more tightly than did chorismic acid under the same conditions, with a K_i value of 120 nM (148,149). Later, monoclonal antibodies elicited against (65) were found to be effective catalysts for the conversion of chorismate to prephenate, with rate enhancements of 200-fold in one case (150) and 10,000-fold in another (151). In both instances it was suggested that the rate enhancement was attributable to increased binding of the transition state by the antibody (150, 151).

X-ray structures are now available of the complexes of (65) with two chorismate mutase enzymes (152, 153), as well as with the less-efficient catalytic antibody (154). Although each active site was found to employ a different constellation of interactions with (**65**), the dissociation constants for the binding of (65) to the three proteins were strikingly similar, ranging from 0.6 to 3 μM (153, 154). However, the micromolar affinity of (65) for both enzyme and antibody is considerably weaker than might be expected for a good mimic of the transition state, and the antibody is not a particularly efficient catalyst. Wiest and Houk (155) have calculated that the bond lengths for the breaking and forming bonds in the transition state are considerably longer than those for (**65**), and the neutral inhibitor does not mimic the charge separation that builds up in the transition state. Although the two enzyme-active sites have evolved to complement the larger, polarized transition state, the anti-

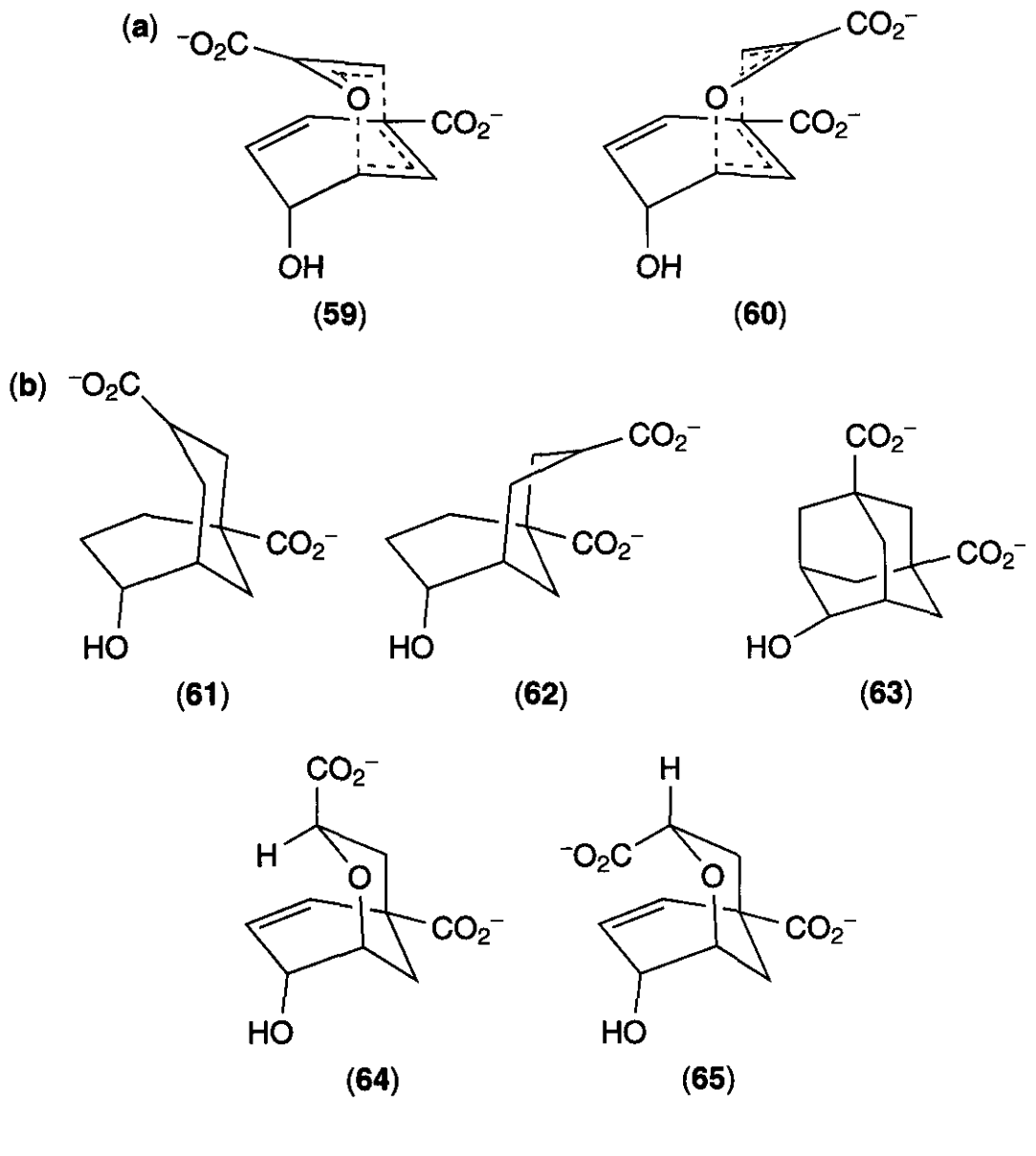


Figure 17.25. (a) Putative transition states for the chorismate-prephenate rearrangement and (b) structures of potential transition-state analogs.

body has no residues positioned to stabilize the polar-transition state. Further, the active site is smaller and makes more van der Waals contacts with the inhibitor, again features likely to impede catalysis. These features provide evidence for the innate difficulties associated with designing both good transition-state analogs and efficient catalytic antibodies.

3 RATIONAL DESIGN OF COVALENTLY BINDING ENZYME INHIBITORS

For the purposes of this chapter we have divided covalently binding enzyme inhibitors into categories according to Table 17.4. Pseudoirreversible inhibitors are discussed separately and the others are, in order of increasing specificity, chemical modifiers, affinity labels, and mechanism-based inhibitors. The targets for these inhibitors are the chemically reactive groups found within the enzyme's active site. These groups, in the majority of cases, are nucleophiles such as the —OH groups of serine, threonine, and tyrosine, the —SH group of cysteine, and the —COOH groups of aspartic and glutamic acid residues. Other nucleophilic groups include the ϵ-amino group of lysine and the imidazole ring of histidine. In some cases the $-NH_2$ and —COOH groups of the enzyme's N- and C-termini, respectively, are also active-site nucleophiles, whereas enzymic cofactors may also provide targets for covalently binding inhibitors. Arginine is the only common amino acid that has an electrophilic side chain and it also can be modified with suitable nucleophilic agents. Kyte has recently provided an excellent overview of the general area of active-site modification and labeling (156).

The first group of covalently binding enzyme inhibitors, the chemical modifiers, are small organic molecules, generally electrophiles, that are used to modify the enzyme's side chains in such a way as to produce a stable covalent bond. These are often used to study enzyme inactivation and to identify residues potentially involved in binding and catalysis. Some of the commonly used reagents are

Table 17.7 Commonly Used Reagents for Chemical Modification

Residue Targeted	Reagent	Other Residues Labeled
Lysine	Acetic anhydride Isothiocyanates Trinitrobenzenesulfonate (TNBS) Cyanate	These reagents can also react with the N-terminal amino group
Histidine	Diethylpyrocarbonate (DEPC)	DEPC should be used at neutral pH to minimize reaction with lysines, cysteines, and tyrosines
Cysteine	Iodoacetamide, iodoacetate p-Hydroxymercuribenzoate Methyl methanethiosulfonate Ellman's reagent (DTNB) *N*-Ethylmaleimide	Iodoacetamide has the potential to modify histidines and lysines
Arginine	Phenylglyoxal Butanedione	Phenylglyoxal can react with lysine Butanedione should be used in the dark to prevent reaction with tryptophans, histidines, and tyrosines
Tyrosine	Tetranitromethane Chloramine T	Chloramine T also modifies histidines and methionines
Tryptophan	*N*-Bromosuccinimide 2-Hydroxy-5-nitrobenzyl bromide	
Serine	Diisopropylfluorophosphate Halomethyl ketones	
Aspartic Acid Glutamic Acid	Carbodiimides Trimethyl oxonium fluoroborate Isoxazolium salts	

listed in Table 17.7. These compounds are chemically reactive and may lead to the modification of both catalytic and nonessential residues. As a consequence, experimental design (such as choice of reagent and reaction conditions, use of substrate protection, etc.) is of utmost importance in carrying out and interpreting chemical modification studies. Although inhibitors of this type are not the prime focus of this chapter (and are not discussed further), it should be noted that most of the kinetic equations that apply to affinity labels also apply to chemical modifiers, and there are a number of texts available that cover this topic (40, 157, 158).

Although the organic modifiers are usually not specific for a given enzyme, the second group, the affinity labels, have a degree of specificity built in. Sometimes described as active-site directed, irreversible inhibitors, affinity labels are usually substrate or product analogs that contain an additional chemically reactive moiety. They first bind to the enzyme's active site in a noncovalent fashion, like rapid reversible inhibitors. However, upon formation of the enzyme-inhibitor complex ($E \cdot I$), they react by various mechanisms with one or more amino acid residues in close proximity in the enzyme's active site. This results in covalent bond formation between the enzyme and the inhibitor (E-I) (Equation 17.45).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_2} E - I \qquad (17.45)$$

Usually the inhibitor contains an electrophilic moiety that labels amino acids containing nucleophilic groups. However, in some cases, a nucleophilic species may be formed, which can react either with arginine or with any tightly bound organic or inorganic low molecular weight cofactors possessing electrophilic sites. Unlike the mechanism-based in-

hibitors described below, affinity labels do not require activation by catalysis at the enzyme's active site. Most often, the covalent bond formation occurs by an S_N2 alkylation-type mechanism, Schiff base formation, or acylation (156, 159).

Affinity labels, some of which have become successful therapeutic agents, are often used to identify catalytically important residues. In some cases, by examining the pH dependency of the rate of inactivation, it is possible to determine the pK_a of the labeled residue. Again, there are a number of excellent reviews on this topic (160–163), including a complete volume in the Methods in Enzymology series (159).

Recently, Pratt (164) and Krantz (165) have suggested that any inactivator that utilizes an enzyme's mechanism, in the broadest sense, should be described as a mechanism-based inhibitor. Although this is not unreasonable, we have, for the purposes of this chapter, adopted the more narrow view of Silverman (166). In this view, mechanism-based inhibitors (also called suicide substrates, Trojan horse inactivators, enzyme-induced inactivators, k_{cat} inhibitors, and latent inactivators) are described as unreactive compounds, the structure of which usually resembles that of a substrate or product of the target enzyme, and that undergo a catalytic transformation by the enzyme to species that, before release from the active site, inactivate the enzyme. Thus, these compounds usually contain a latent, reactive functional group that gets activated during the normal catalysis of the enzyme. Upon formation of the initial reversible enzyme-inhibitor complex E . I, the enzyme starts its normal catalytic cycle, leading in a usually rate-determining step to the formation of a highly reactive species, E . I' (Equation 17.46).

$$\mathrm{E} + \mathrm{I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{I} \xrightarrow{k_2} \mathrm{E}\cdot\mathrm{I}' \begin{cases} \xrightarrow{k_3} \mathrm{E}\text{–}\mathrm{I}'' \\ \xrightarrow{k_4} \mathrm{E} + \mathrm{P} \end{cases} \qquad (17.46)$$

The reactive species can either react with one of the enzyme active-site amino acid residues, to form a covalent bond between the enzyme and the inhibitor (E-I"), or be released into the medium to form product (P) and free active enzyme (E). In some instances the reaction may occur between the reactive species and the enzyme's cofactor, again resulting in inactivation of the enzyme.

It should also be noted that the activation of a mechanism-based inhibitor by its target enzyme is, formally, an example of metabolic activation. However, there is a clear distinction between the activation of a mechanism-based inhibitor described above and the metabolic activation of a prodrug. In the latter case, an inactive precursor is metabolized in the body (either chemically or enzymatically) to metabolites that possess the desired activity. For example, Acyclovir (3a) must be metabolically converted to the triphosphate (3b) and released into the medium before it will inhibit viral DNA polymerase. Further discussion on prodrugs may be found in volume 2, chapter 14.

3.1 Evaluation of the Mechanism of Inactivation of Covalently Binding Enzyme Inhibitors

The inherent complexity of the inactivation mechanisms of covalently binding enzyme inhibitors makes it necessary to evaluate their proposed modes of action carefully. An overview of the criteria for the study of irreversible inhibitors is provided below.

3.1.1 Criteria for the Study of Affinity Labels. The evaluation of affinity labels is based on the fulfillment of the following criteria:

1. ***Irreversible inactivation.*** Inactivation by affinity labels leads to irreversible covalent bond formation between the enzyme and the inhibitor. Unlike the complex between and enzyme and a rapid, reversible inhibitor, the covalent enzyme-inhibitor complex is no longer in equilibrium with free enzyme and inhibitor. Therefore, exhaustive dialysis or gel filtration of the covalent enzyme-inhibitor complex cannot lead to the recovery of free, active enzyme. However, such experiments do not allow distinction among tight-binding, noncovalent inhibitors, affinity labels, and mechanism-based inactivators.
2. ***Time- and concentration-dependent inactivation showing saturation kinetics.*** The

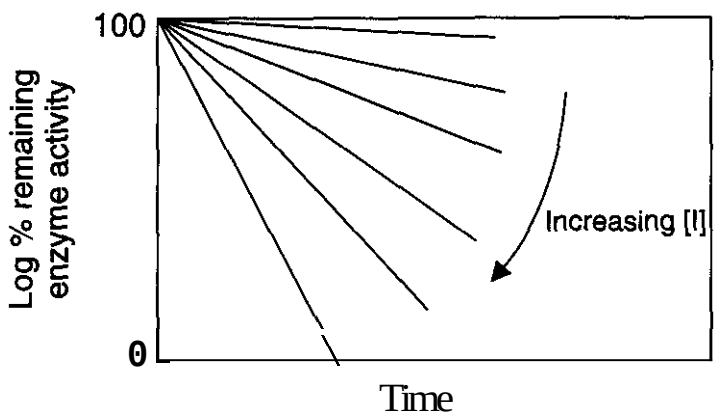


Figure 17.26. Pseudo first-order inactivation kinetics of an active-site directed irreversible inhibitor.

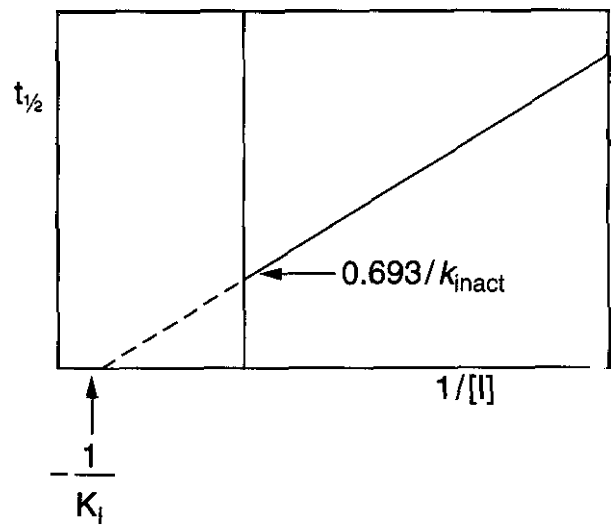


Figure 17.27. Kitz and Wilson plot.

scheme described by Equation 17.45 is analogous to that described for a simple enzyme-catalyzed reaction (Equation 17.9). This scheme can be described by Equation 17.47, which is analogous to the Michaelis-Menten equation (Equation 17.10).

$$-\frac{d[\mathrm{E}]}{dt} = \frac{k_2[\mathrm{I}]}{K_\mathrm{I} + [\mathrm{I}]} \quad \text{where} \quad K_\mathrm{I} = \frac{k_1}{k_{-1}} \tag{17.47}$$

According to Equation 17.47, an affinity label should exhibit time- and concentration-dependent inactivation. The rate of inactivation is proportional to low concentrations of inhibitor, whereas at high inhibitor concentrations saturation occurs and no further increase in the rate of inactivation is observed. A typical pseudo first-order plot of log enzyme activity vs. time is illustrated in Fig. 17.26. In some cases nonlinear plots may be obtained, particularly for mechanism-based inhibitors (166, 167).

3. Saturation kinetics and determination of K_I and k_{inact}. To distinguish the rate and binding constants of rapid reversible inhibitors (k_{cat} and K_i, respectively) from the rate and binding constants of irreversible inhibitors, the terms k_{inact} and K_I have been used. To determine k_{inact} and K_I, saturation kinetics must be obeyed. Saturation is reached when all of the free enzyme is converted to the reversible enzyme-inhibitor complex E . I. At that point, the rate of inactivation is independent of k_1/k_{-1} (Equation 17.45), assuming that the rate of formation of the initial reversible enzyme-inhibitor complex (k_1) is significantly greater than the rate of formation of the covalent enzyme-inhibitor complex (k_2). Consequently, a higher concentration of inhibitor will not lead to an increased rate of inactivation. The K_I value represents the concentration of inhibitor leading to the half-maximum rate of inactivation (in analogy to a K_M value), and k_{inact} is the maximum rate of inactivation at the point of saturation (in analogy to k_{cat}). To determine the K_I and k_{inact} values, the enzyme is incubated at various subsaturating concentrations of the inhibitor, from which the half-life of inactivation at each inhibitor concentration is deduced. Using Kitz and Wilson plots (168), the half-life of inactivation at each inhibitor concentration is plotted against 1/[I]. A typical plot is illustrated in Fig. 17.27. The y-intercept represents the half-life of inhibition at infinite inhibitor concentration, with k_{inact} equal to $0.693/t_{1/2}$. K_I can be determined from the x-intercept, which is equal to $-1/K_I$. If no saturation occurs with a tested inhibitor, the curve will intercept at the origin of the graph, implying that k_{inact} is much faster than the formation of the initial reversible enzyme-mechanism-based inhibitor complex. If this is observed, one might use a lower temperature or a different pH to lower k_{inact}. It should also be noted that, in general, if the affinity label is reacting with an ionizable group that is involved in catalysis, then the pH dependency of k_{inact}/K_I should mirror that of k_{cat}/K_M.

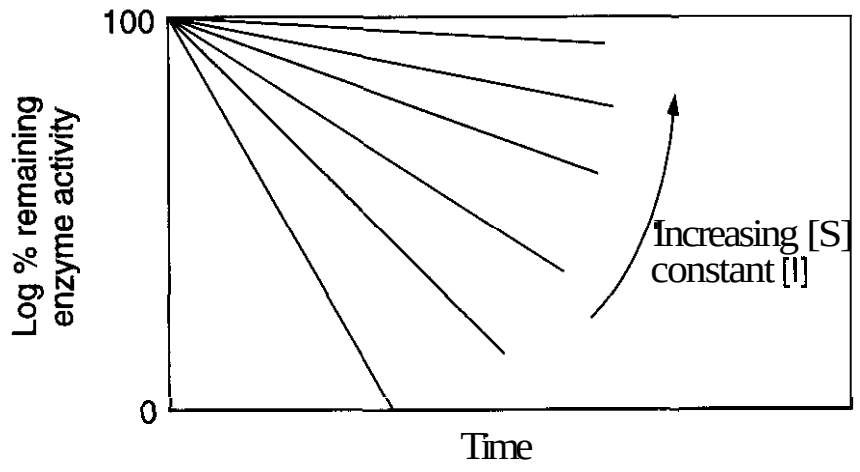


Figure 17.28. Using a substrate to protect an enzyme from inactivation by an active site-directed irreversible inhibitor.

4. **A** binding stoichiometry of *1:1* of inhibitor to the enzyme's active site. In general, complete inactivation of an enzyme requires the binding of one mole of inhibitor per mole of enzyme active site. Exceptions can be certain multimeric enzymes that are inactivated by binding of only one-half mole of inhibitor per mole of enzyme subunit, a phenomenon called half-site reactivity. The stoichiometry of binding is usually determined by incubating an excess of radiolabeled inhibitor with the enzyme to ensure complete irreversible inactivation, followed by either exhaustive dialysis or gel filtration. The binding stoichiometry of the obtained enzyme-inhibitor complex in the absence of free inhibitor is then examined for its radiolabel and protein content. More recently, developments in high-resolution mass spectrometry have allowed the determination of binding stoichiometry without the need for radiolabeled inhibitor.
5. Substrate protection. Ligands of the enzyme, either substrates or reversible inhibitors, should greatly decrease the rate of modification by the affinity label. Both affinity labels and mechanism-based inhibitors should be active-site directed, thereby competing with the substrate for the same binding site on the enzyme. This can be tested by incubating the enzyme with increasing amounts of substrate at constant inhibitor concentrations. As the substrate concentration is increased, the rate of inactivation will become slower because, under initial velocity conditions, a portion of the enzyme is protected as the E · S complex. **A** typical plot of the log of enzyme concentration vs. time at different substrate concentrations is shown in Fig. 17.28.
6. Verification of covalent bond formation. In many cases it can be difficult to differentiate between a covalently binding enzyme inhibitor and a very tight but noncovalently binding inhibitor. Although strongly denaturating conditions may not lead to the release of tight, noncovalently bound inhibitors, the covalent linkage between an enzyme and its inhibitor can sometimes be quite labile to nucleophiles and extremes of pH. **A** frequently used method to determine the covalently modified amino acid residue of an enzyme's active site is peptide mapping. Enzyme-inhibitor complexes, usually prepared from radioactive labeled inhibitor, are treated under mildly denaturing conditions with an appropriate protease. Subsequently, the peptide fragments obtained are usually resolved by high-pressure liquid chromatography and isolated. Analysis of the labeled peptides can be accomplished by Edman degradation and/or mass spectrometry. **A** good description and example of this method can be found elsewhere **(169).** Alternatively, electrospray ionization mass spectrometry has been used as a tool to determine the accurate mass of the proteins and enzyme-inhibitor complexes. In a study by Knight et al. (170), this method was successfully used to distinguish between covalent and noncovalent complexes because the latter did not survive the experimental conditions.

3.1.2 Criteria for the Study of Mechanism-Based Inactivators. In addition to the requirements described above for an affinity label, a mechanism-based inhibitor should also demonstrate the following:

1. Occurrence of a catalytic step. The major difference between the mechanism of inactivation of mechanism-based inactivators vs. that of any other type of inhibitor is the obligate involvement of a catalytic step, that is, step 2 in Equation 17.46. Initially, the mechanism-based inhibitor binds reversibly to form the E . I complex. The enzyme then starts its normal catalytic cycle, resulting in the conversion of the inhibitor into a reactive species (I^r). If the reactive species is electrophilic, it may react with an active-site nucleophile, much like an affinity label. If the reactive species is nucleophilic, it may react with an electrophilic species on the enzyme, probably an oxidized cofactor. Finally, a radical species may be generated that has the potential to react with an enzyme radical, or generate one by hydrogen atom abstraction. The experiments necessary to provide evidence for a catalytic step are obviously strongly dependent on the individual catalytic mechanism involved. The experiments may include spectrophotometric detection of oxidized or reduced cofactor, observing C–H bond cleavage by monitoring the release of tritium, or the detection of some component of cleaved inhibitor (such as fluoride ion as in some examples shown below).
2. No release of the activated species before enzyme inactivation. For a mechanism-based inactivator to retain its high specificity during inactivation, release of the reactive species from the active site must not be part of the normal mechanism of inactivation. A time-dependent increase in the rate of inactivation points to the release of an activated species before inactivation. This increase in the rate of inactivation is brought about by the accumulation of free reactive species in solution. Inhibitors generated in this manner have been termed metabolically activated affinity labels (171). In these cases, as with affinity labels, nonspecific covalent modification of residues other than those located in the active site cannot be excluded. A second test for a metabolically activated affinity label is to add an additional aliquot of fresh enzyme to the incubation buffer. The fresh enzyme should be inactivated at a higher rate than that of the first equivalent of enzyme because there is more reactive species present in solution. By contrast, the mechanism-based inhibitor should show no difference in rate until the concentration of inhibitor is depleted. It should also be noted that the observation of such rate increases necessitates that the reactive species is relatively stable and is not immediately quenched by the incubation buffer.

   Additional tests such as the addition of nucleophilic scavengers (e.g., thiols such as dithiothreitol or β-mercaptoethanol) can provide further evidence for the presence of a free, reactive electrophilic species. The scavengers should quench all of the free reactive species, thereby protecting the enzyme from inhibition. Unfortunately, this method cannot exclude the possibility that a nucleophilic thiol may even attack the bound reactive species at the active site of the enzyme (which would also give rise to protection from inactivation). However, the use of a bulky thiol, such as reduced glutathione, should limit that possibility. An alternative scenario occurs wherein the released reactive species returns and reacts faster with an active-site nucleophile than with the added thiol. Clearly this is a complex problem and, consequently, it is advisable to use several different tests to avoid misleading conclusions.
3. Partition ratio. The partition ratio is the ratio of product release to enzyme inactivation and is a measure of the efficiency of the mechanism-based inhibitor. Formally, it refers to the ratio k_4/k_3 (Equation 17.46). The most efficient inactivators will have partition ratio of zero. In those cases, theoretically, every enzymatically processed inhibitor molecule will result in the inactivation of a molecule of enzyme. Even though the partition ratio is independent of

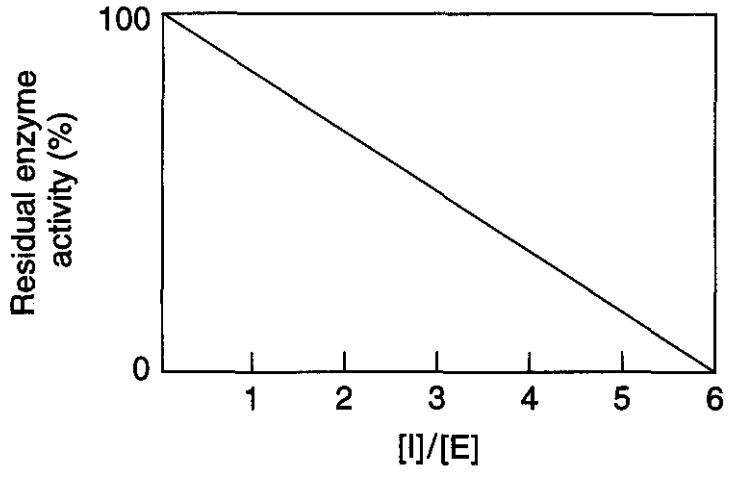


Figure 17.29. Determination of the partition ratio.

the initial concentration of inhibitor, it will depend on factors such as the rate of diffusion of the reactive species from the active site, its reactivity, and the proximity of the target for covalent bond formation. A number of different methods have been used to determine the partition ratio. For example, if, under the experimental conditions, the rate of inactivation is relatively fast compared to the chemical stability of the enzyme or the inhibitor, the partition ratio can be determined kinetically by titration of the enzyme activity. The titration measures the number of inhibitor molecules required to completely inactivate the enzyme. In an experiment of this type, increasing amounts of inhibitor are added to a known, fixed amount of enzyme, and the reaction is allowed to go to completion. After gel filtration or dialysis, a plot of the amount of inhibitor per enzyme active site and the remaining enzyme activity is drawn (Fig. 17.29). The intercept with the x-axis represents the minimum number of equivalents of inhibitor necessary to inactivate the enzyme completely (turnover number). A turnover number of 6, such as that shown in Fig. 17.29, indicates that on average 5 equivalents of inactivator are converted to product and only every sixth equivalent of inhibitor leads to irreversible covalent bond formation (i.e., the partition ratio equals the turnover ratio minus 1). Unfortunately, there are a number of factors associated with this method that may lead to misleading results (166). Another method for determining partition ratios is equilibrium dialysis of the enzyme with radiolabeled inactivator, followed by determination of the amount of radiolabeled metabolites produced per radiolabeled enzyme. Perhaps the simplest method is for cases where the rate of product formation (i.e., $k_{cat} = k_4$ in Equation 17.46) can easily be measured. In this instance, both k_{cat} and k_{inact} are measured directly, with k_{cat}/k_{inact} being the partition ratio (166,167).

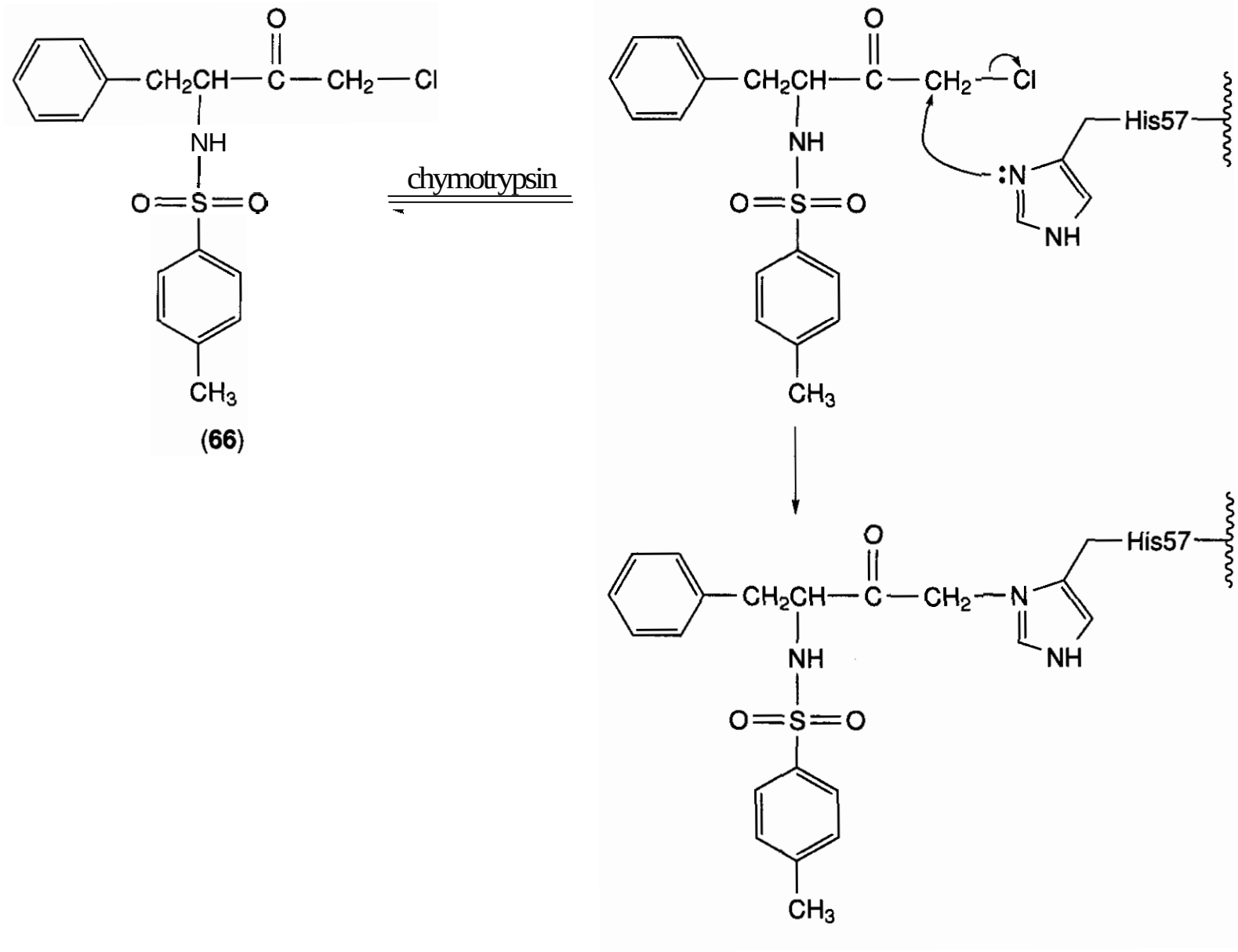
A more detailed discussion of the requirements for mechanism-based inhibition can be found in a recent review by Silverman (166).

3.2 Affinity Labels

Affinity labels are potentially good drugs, although the presence of a reactive functional group can make them somewhat nonselective and prone toward reaction with other proteins and metabolites. If the affinity label is highly selective toward its target enzyme and has a great affinity for the enzyme's active site, this drawback can be overcome kinetically. Once the inhibitor is bound, the unimolecular reaction between the inhibitor and an amino acid residue in close proximity is entropically quite favorable compared to a bimolecular reaction between two free molecules in solution. This proximity effect has resulted in rate enhancements as great as 10^8 (172) and means that a reagent that is, in itself, only weakly active, may be highly reactive when it is reformulated as an affinity label. More in-depth discussion on this topic can be found elsewhere (39, 173, 174).

The design of a potent affinity label requires the study of the initial requirements for the inhibitor to bind to the active site. Next, regions of bulk tolerance are determined that are useful for the introduction of a reactive functional group. In some cases, it might be advantageous to place the reactive group at the end of a spacer arm, particularly if no nucleophilic amino acid residue is in close proximity to the reactive group. However, not only the location and orientation, but also the size and inherent reactivity of the reactive functional group are critical for its potential as an affinity label.

Perhaps the archetypical example of an affinity label is TPCK (66) (Fig. 17.30). This

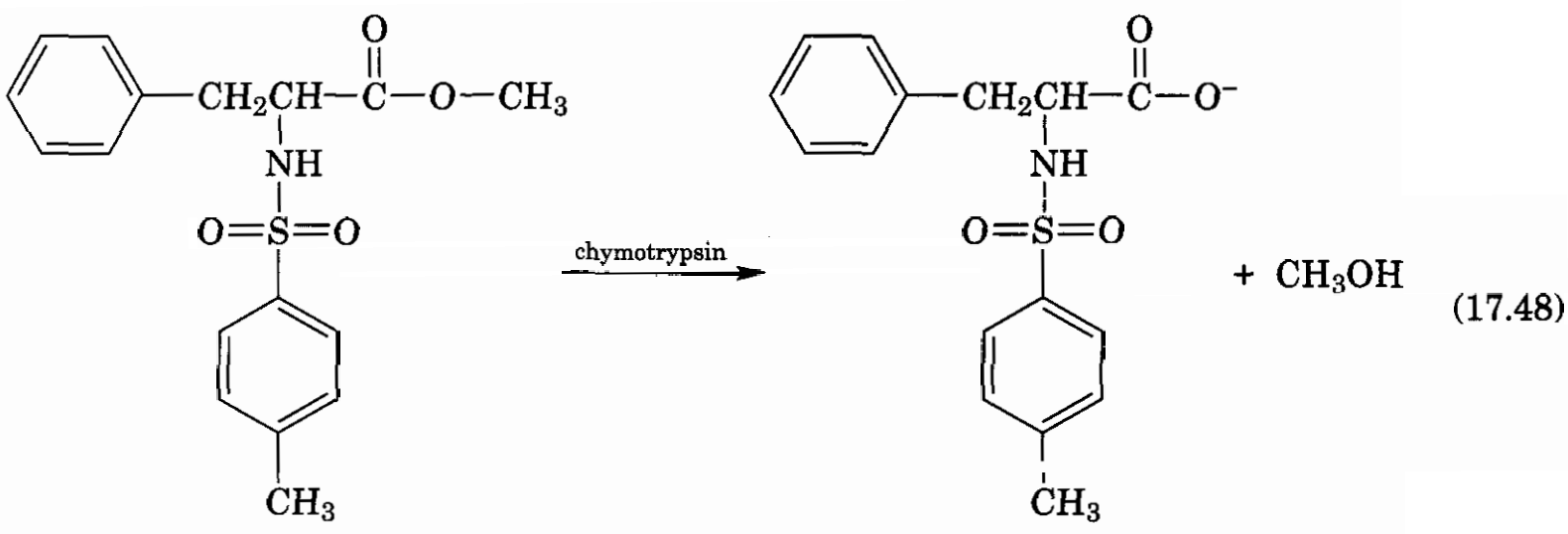


(66)

Figure 17.30. Inhibition of chymotrypsin by **TPCK.**

compound was designed to mimic substrates of chymotrypsin such as the tosyl-L-phenylalanine methyl ester (Equation **17.48),** thereby providing a basis of affinity for the chymotrypsin-active site.

In addition to mimicking a substrate, it contains the halomethyl ketone moiety, to provide a point of covalent attachment (175). TPCK **was** shown to irreversibly inhibit **chymotrypsin** (it is still employed today to remove chymotrypsin from trypsin preparations) by specifically labeling a histidine residue (175), later identified as His57 (176). After the success of TPCK, chloromethyl ketones became

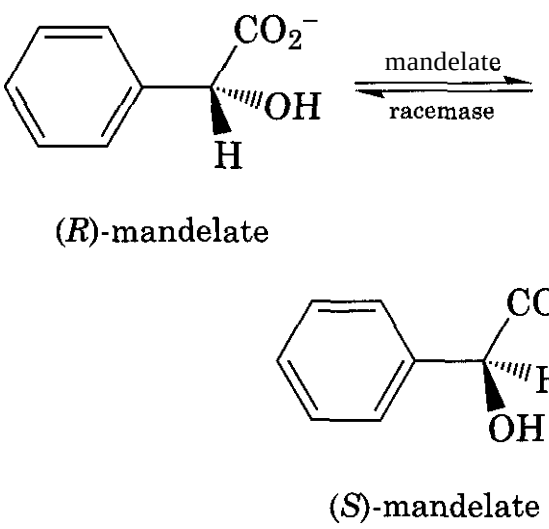


(17.48)

methyl N-tosyl-L-phenylalanine

extremely popular for the inactivation of proteases. By incorporating part of the sequence of the physiological substrate into the halomethyl ketone, it was possible to obtain selective inactivation of individual proteases (177). This selective inactivation also meant that chloromethyl ketones became widely used as probes for the binding requirements and chemically reactive residues in the active sites of serine proteases, in particular. Replacement of the chloromethyl ketone moiety by a diazomethyl group provided a specific inactivation of cysteine proteases (172). The use of TPCK has not been restricted to chymotrypsin, as elegantly demonstrated in a recent report on the inhibition of human aldehyde dehydrogenase (178). As a group, proteases remain major targets for therapeutic intervention, and peptide-based affinity labels are still playing a major role in drug design (179).

The interconversion of (*R*)-mandelate and (*S*)-mandelate is catalyzed by mandelate racemase (Equation 17.49). The reaction can be reversibly inhibited by the substrate analog atrolactate (**67**), (Fig. 17.31). Because of its structural similarity to both (67) and the substrate and, given the reactivity of the epoxide group to nucleophiles, (*R*,*S*)-α-phenylglycidate (68) was synthesized as a potential affinity label of mandelate racemase. The compound was found to be an irreversible inhibitor, fitting all the criteria described in section 3.1.1 (180). Later it was established that (*S*)-α-phenylglycidate (*S*-αPGA) did not irreversibly inactivate the enzyme, binding noncovalently and with less affinity than *R*-αPGA (69). As shown in Figure 17.31, the epoxide ring of *R*-αPGA potentially is subject to attack at either of two carbons. Attack at the distal endocyclic carbon atom of (69) (path a) will result in the formation of (**70**), whereas attack at the a-carbon (path b) will yield (71). The crystal structure of the inactivated complex revealed that nucleophilic attack of the ϵ-amino group of Lys166 resulted in adduct (**72**), which is consistent with attack on the distal carbon of the epoxide ring (181). This structure confirmed the original design premise of Fee et al. (180), wherein it was thought that the distal oxirane carbon occupied the position similar to the a-proton in mandelate. Therefore, on binding of the a-phenylglycidate to the enzyme, the electrophilic epoxide group would be subject to attack by the nucleophile responsible for a-proton abstraction in the normal catalytic cycle. Further confirmation is provided by the X-ray structure of (*S*)-atrolactate bound to the racemase (181), which reveals that Lys166 has been pushed away by the a-methyl group of (*S*)-atrolactate (which is positionally equivalent but much larger than the a-proton in (*S*)-mandelate). In both structures the positions of the remaining active-site residues are almost identical.



(17.49)

Perhaps the best-known affinity labeling reagent is aspirin (73)(Fig. 17.31), a member of the class of drugs known as the nonsteroidal anti-inflammatory drugs (NSAIDS), and whose activity was initially reported to result from its inhibition of prostaglandin biosynthesis (182, 183). Prostaglandins are involved in the inflammatory response and can cause headache and vascular pain in humans.

Prostaglandin synthase, which catalyzes the first step in the arachidonic acid cascade, is a heme protein and possesses two activities. As illustrated in Equation 17.50, a cyclooxygenase activity is used in the conversion of arachidonic acid to the bicyclic endoperoxide PGG_2, whereas a peroxidase activity catalyzes the subsequent reduction of PGG_2 to prostaglandin H_2. The latter serves as a branch point in the production of various prostaglandins as well as thromboxane A_2 and prostacyclin (PGI_2).

Aspirin (acetylsalicylicacid) was ultimately confirmed as an inhibitor of prostaglandin synthetase (184). Incubation of [*acetyl*-^{3}H] aspirin showed that one acetyl group was in-

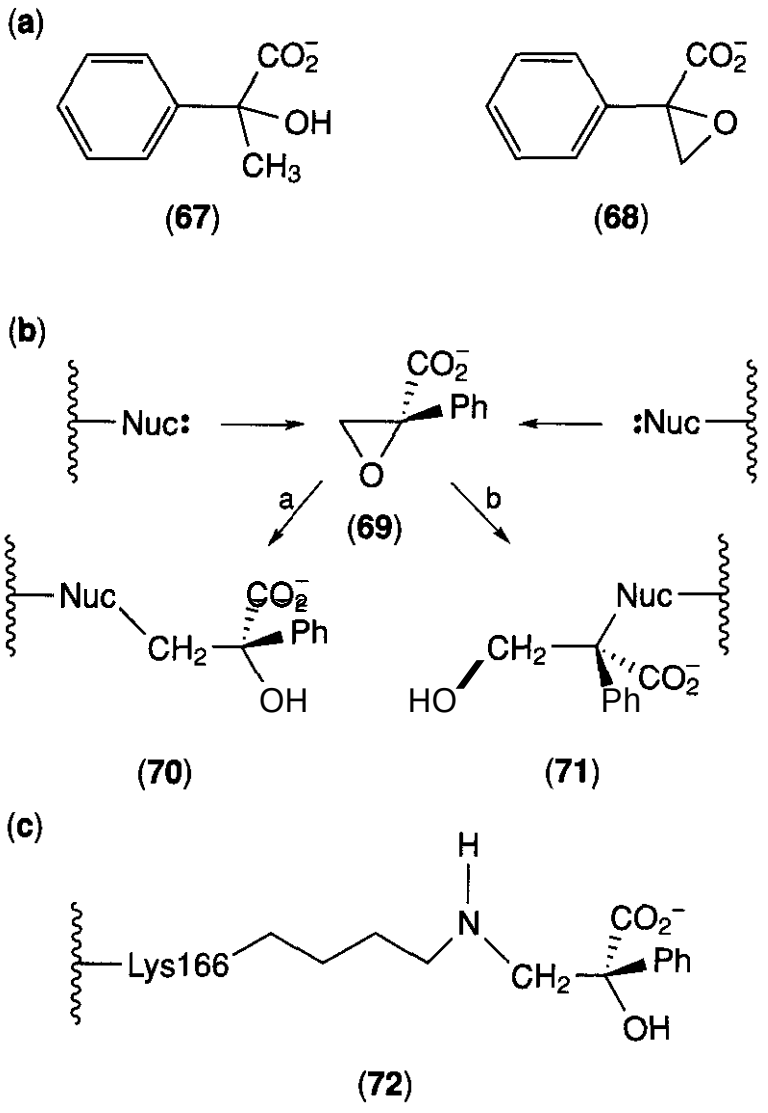
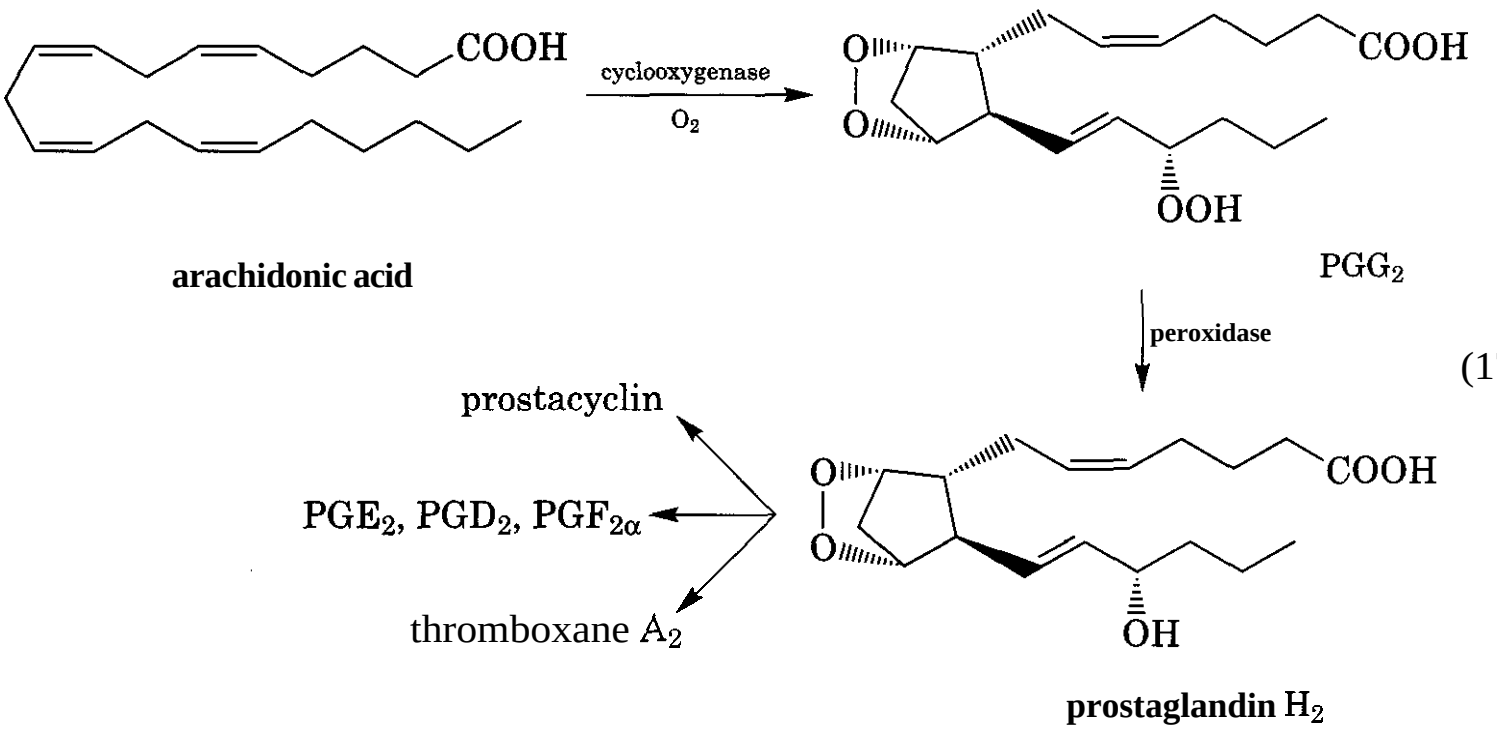


Figure 17.31. (a) Inhibitors of mandelate racemase, (b) potential products from nucleophilic attack on the epoxide of *R*-α-phenylglycidate, and (c) adduct formed by attack of Lys166.

corporated per mole of enzyme (185,186). Enzymatic digest of the labeled enzyme provided evidence that a serine residue, later identified as Ser530, was acetylated (186, 187), probably by the mechanism shown in Fig. 17.32. The X-ray structure of bromoaspirin (74) inactivated prostaglandin synthase has been solved, and the bromoacetylation of Ser530 confirmed (188). The structure was similar to that of flurbiprofen (**75**), complexed with prostaglandin synthase (189). Aspirin is the only NSAID known to inactivate prostaglandin synthase through covalent modification, and the brominated aspirin analog was also determined to be a potent irreversible inhibitor. Conversely, flurbiprofen (**75**), another NSAID, has been classified as a slow-tight-binding inhibitor (190) and was expected to induce a conformational change upon binding. However, there were no significant differences between the two X-ray structures, and it is yet to be determined whether the binding of aspirin also induces a conformational change in the enzyme.

Although affinity labels have played a major role in characterizing the active sites of a large number of proteases, they have also proved to be particularly useful in mapping nucleotide-binding sites (161, 163, 191). Numerous compounds, some of which are shown in Fig. 17.33, have been designed to be analogs of the various nucleosides and nucleotides. Perhaps the best known of these is 5'- *p*-fluorosulfonylbenzoyl adenosine (5'-FSBA) (**76**), which was designed to be an analog of ADP or ATP (77). It has both the adenosine and ribose moieties, as well as a carbonyl group adjacent to the 5' position. The latter mimics the first phosphoryl group of the purine nucleotides. If



(17.50)

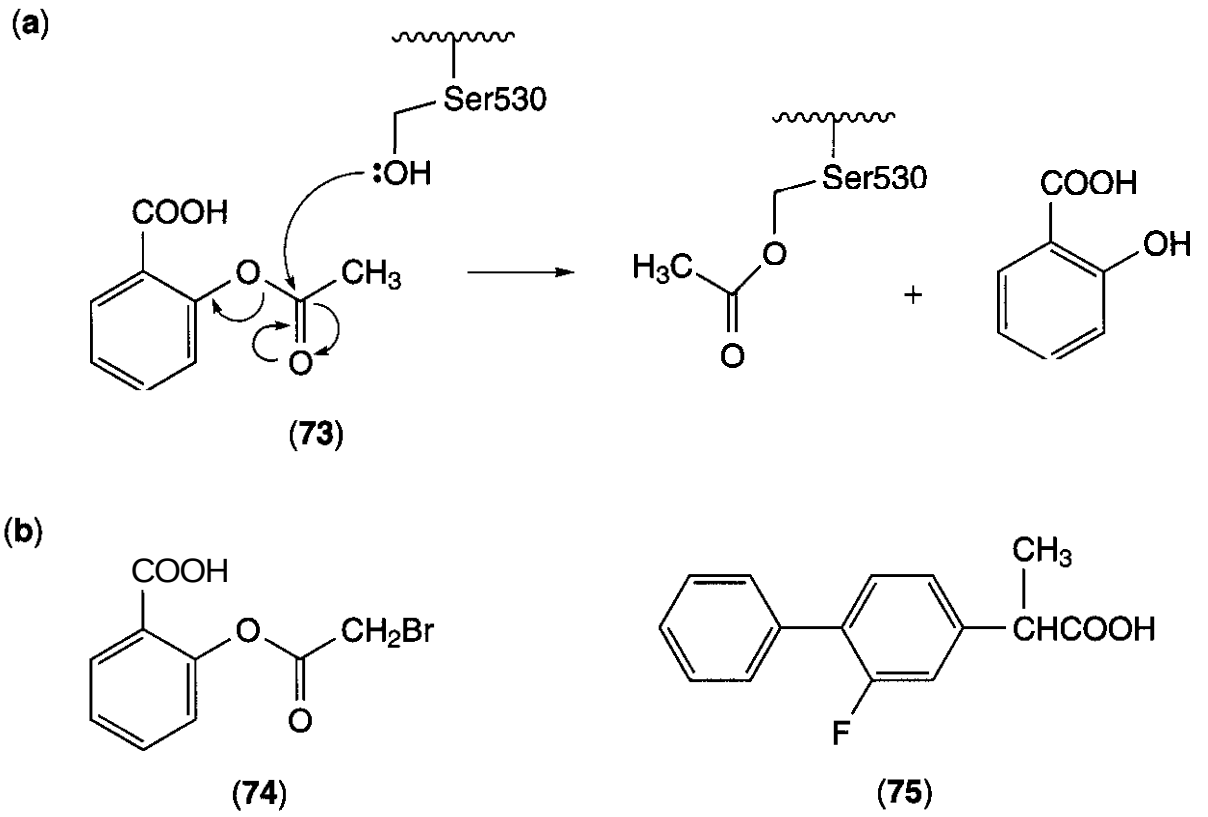


Figure 17.32. (a) Inactivation of prostaglandin H2 synthase by aspirin, and (b) inhibitors cocrystallized with prostaglandin synthase.

the molecule is arranged in an extended conformation, the reactive sulfonyl fluoride group would be found in a position analogous to that occupied by the y-phosphoryl group of ATP. This was initially used to explore the regulatory site of glutamate dehydrogenase (192) and the active site of pyruvate kinase (193). It has now been employed to label the NAD and ATP sites of more than 50 proteins (163). Modifications to 5'-FSBA have provided the fluorescent probe (78) as well as the bifunctional affinity label (79), which has a photoactivatable azido group as well as the electrophilic fluorosulfonyl moiety (163). The bromodioxybutyl compound (80)contains the adenine, ribose, and 5'-monophosphate of adenosine monophosphate (AMP). It is also water soluble and negatively charged at neutral pH. As described above, a bromomethyl ketone group will react with a number of nucleophiles, whereas the dioxo group can potentially react with arginine residues. This reagent has a structural similarity to adenylosuccinate (81) and was used to identify a critical arginine residue in the active site of adenylosuccinate lyase, an enzyme whose deficiency in humans leads to severe mental retardation and autism (163).

3.3 Mechanism-Based Inhibitors

Mechanism-based inactivators have great potential as drugs because they are designed to be specific toward their target enzyme. Furthermore, because these compounds are unreactive until activated within their target enzyme, they are expected to show little or no cellular toxicity. The design of mechanism-based inhibitors requires an understanding of the binding specificity requirements for the ligand-recognition site of the enzyme, to promote the formation of the initial noncovalent enzyme-inhibitor complex E . I (Equation 17.46). In addition, the choice of an appropriate latent functional group requires knowledge of the catalytic mechanism of the target enzyme with its normal substrate. Finally, covalent bond formation by the activated inhibitor (I′) will strongly depend on its inherent chemical reactivity, and its proximity to a susceptible amino acid residue or cofactor. A number of excellent reviews and monographs have appeared on the general design of mechanism-based inhibitors (166, 167, 194–201). The following examples have been chosen to emphasize both the potential for the use of

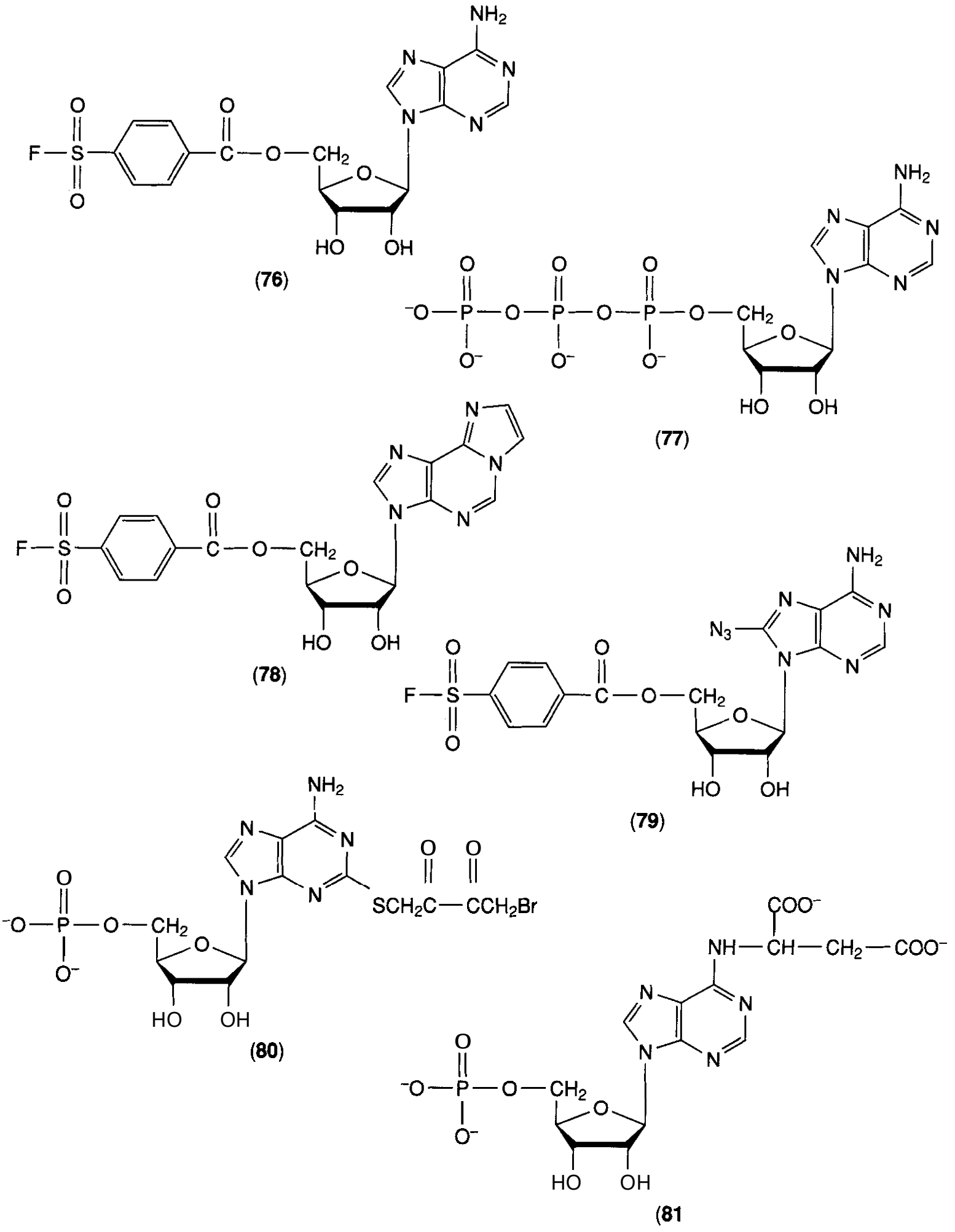
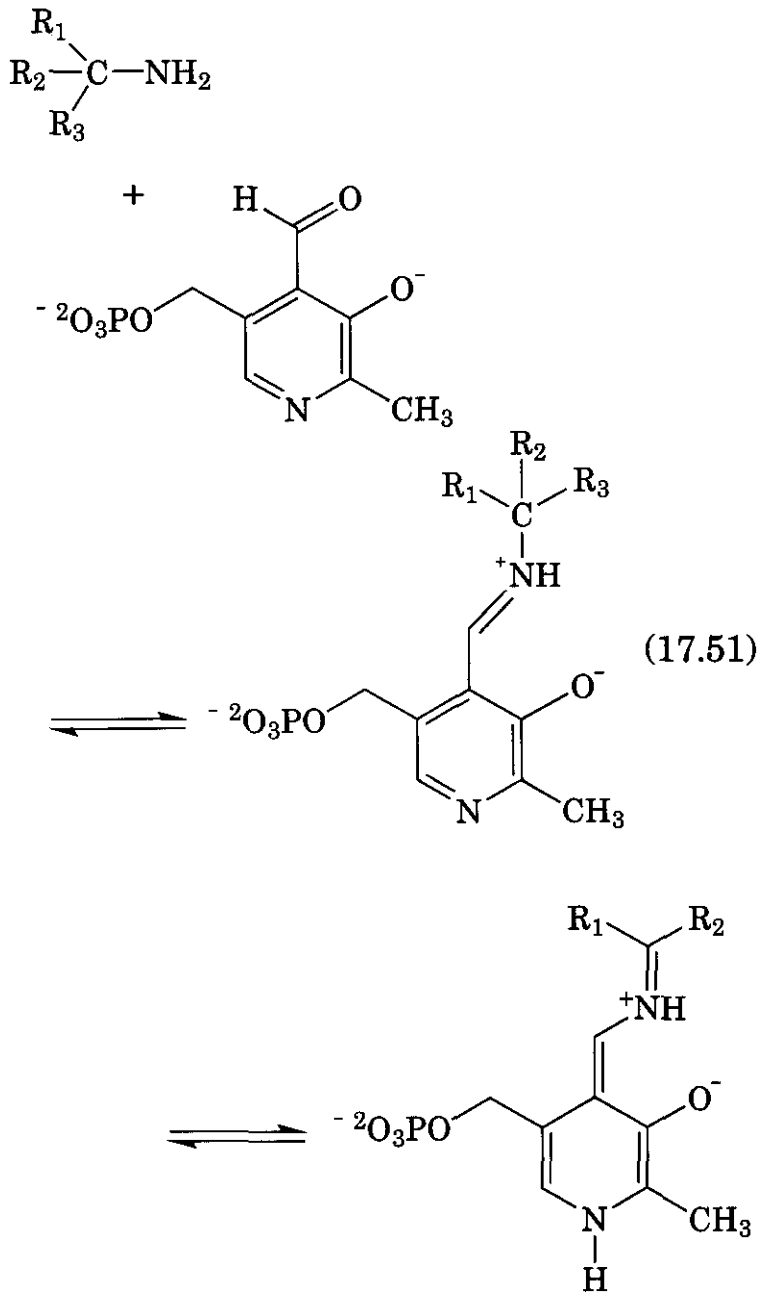


Figure 17.33. ATP (**77**), adenylosuccinate (**81**), and representative affinity label analogs.

mechanism-based inhibitors as drugs and the diversity of their mechanisms of inactivation.

Of all the classes of enzymes, the pyridoxal phosphate (PLP)-dependent enzymes have been found to be most susceptible to mechanism-based inhibitors (202). To some extent this is because the mechanism of catalysis by

the PLP-dependent enzymes is extremely well characterized, making the design process somewhat easier. The initial steps in the mechanism for a PLP-dependent enzyme are shown in Equation 17.51.

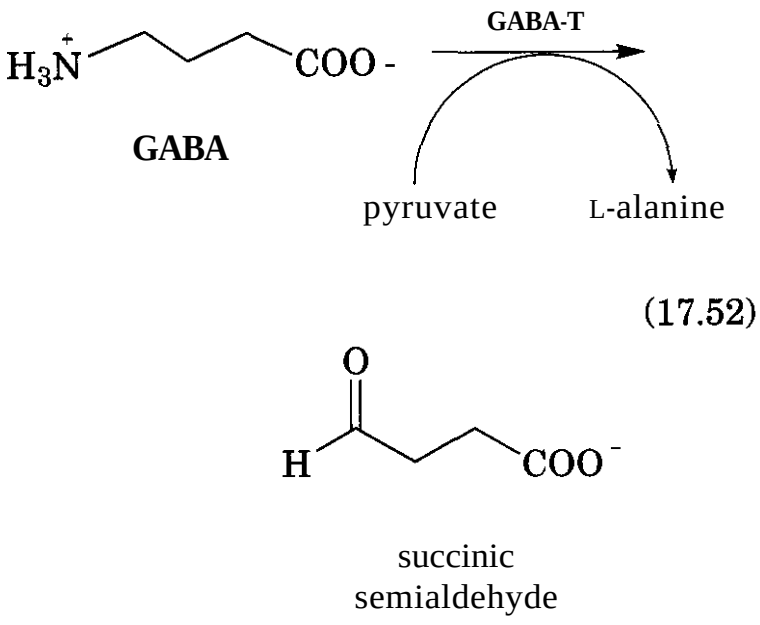


(17.51)

The first step involves Schiff base formation by the amino group of the substrate reading with pyridoxal phosphate to form an aldimine. This is followed by loss of a functional group (R_3, Equation 17.51), usually by abstraction by an active-site base, to form a resonance-stabilized carbocation.

y-Aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters in the mammalian central nervous system. A decrease in the concentration of GABA had been shown to lead to convulsions. Therefore it was suggested that inhibitors of GABA transaminase, the enzyme responsible for the breakdown of GABA, may act as antiepileptic agents, by providing an increase in the concentration of GABA in the brain.

GABA aminotransferase (GABA transaminase, GABA-T) catalyzes the conversion of y-aminobutyric acid to succinic semialdehyde with the subsequent transfer of an amino group to pyruvate (Equation 17.52).



(17.52)

succinic
semialdehyde

GABA-T is a PLP-dependent enzyme and a wide variety of mechanism-based inhibitors have been described for this enzyme (202, 203). These include inhibitors bearing an unsaturated moiety, a leaving group, as well as those forming a stable complex with the cofactor (202, 203). Vigabatrin (8), currently used as an antiepileptic drug, provides an excellent example of this approach. The proposed mechanism is shown in Fig. 17.34.

As with the normal mechanism of the enzyme, the inactivation starts with Schiff base formation with the enzyme-bound pyridoxal phosphate, followed by removal of an a-proton by an active-site base to form the reactive electrophilic intermediate (82). This then partitions between hydrolysis of the Schiff base linkage, resulting in the keto product (83) and enzyme reactivation, and Michael-type addition of an enzyme active-site nucleophile, resulting in a stable covalently bonded enzyme adduct (84).

Ornithine decarboxylase (ODC), another PLP-dependent enzyme, catalyzes the rate-limiting step in the biosynthesis of polyamines, i.e., the conversion of ornithine to putrescine (Equation 17.53).

The enzyme is a target for drugs against African sleeping sickness caused by *Trypano-*

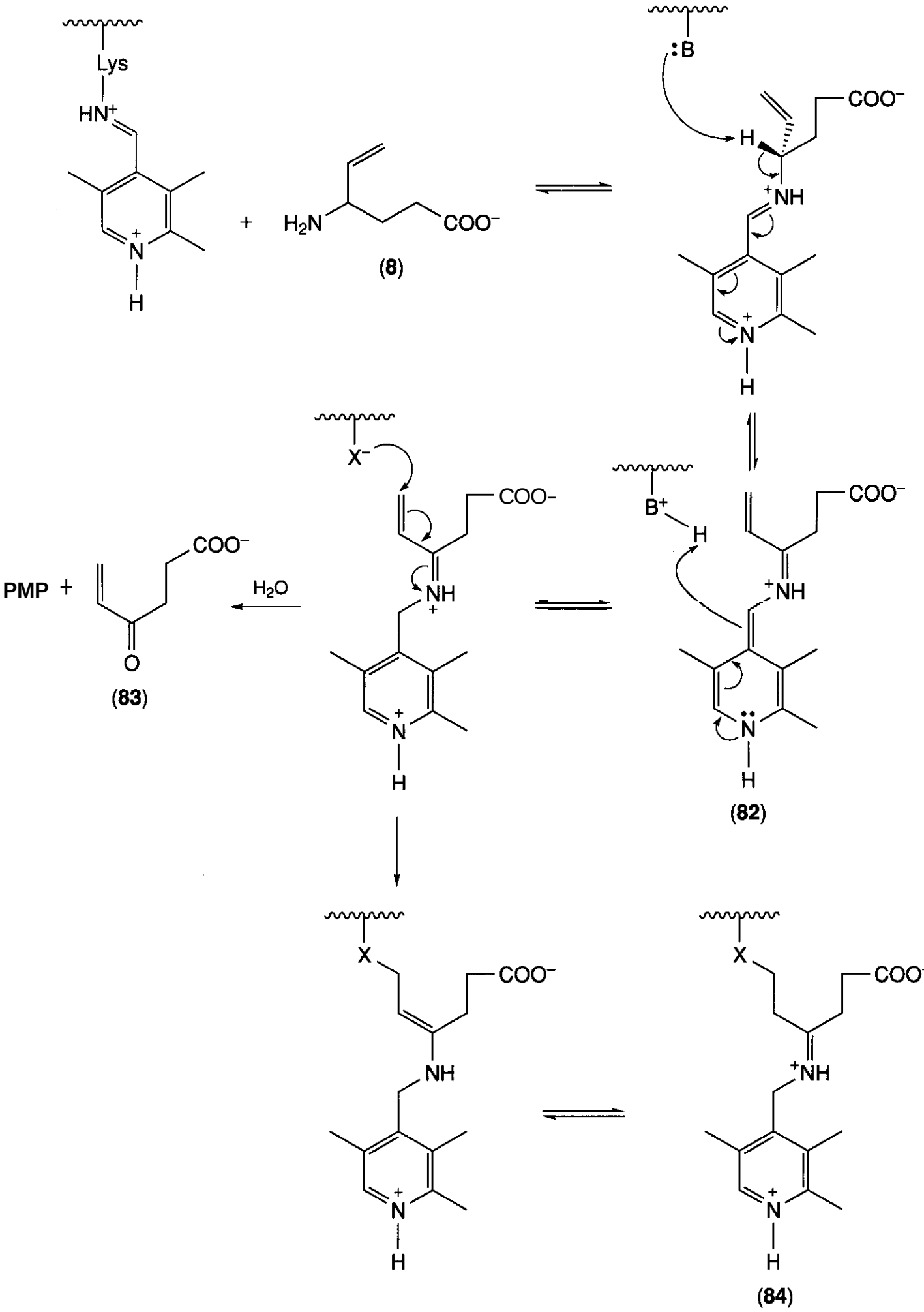
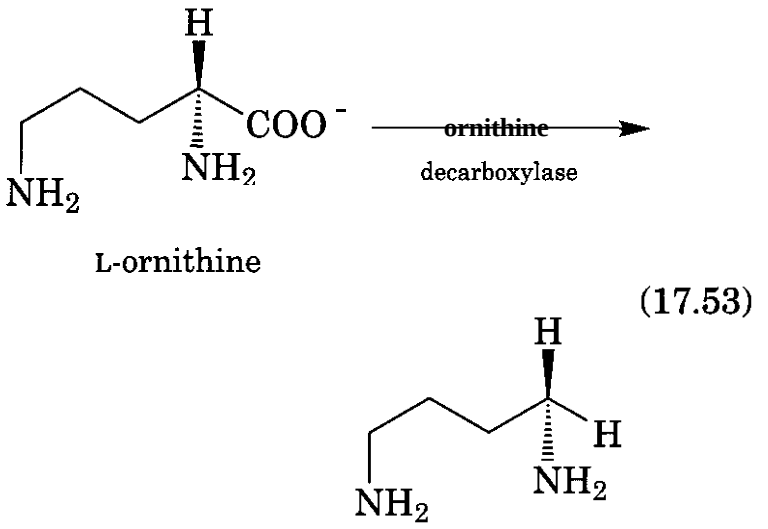
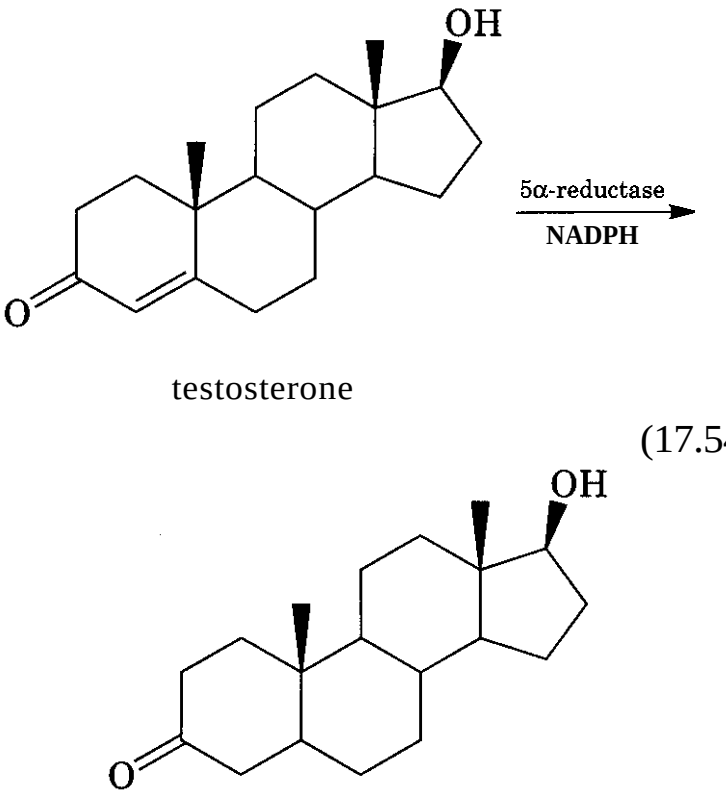


Figure 17.34. Inactivation of GABA transaminase by vigabatrin (PMP = pyridoxamine phosphate). For clarity, substituents on the pyridine ring are omitted.



(17.53)



(17.54)

soma brucei. One of the currently used drugs, eflomithine (a-difluoromethylomithine, DFMO) (**85**), is a mechanism-based inhibitor of ODC. The inactivation of ODC by DFMO involves the decarboxylation of DFMO by the enzyme, with subsequent stoichiometric binding of a reactive species to the enzyme (204). The proposed mechanism for inhibition of T. ***brucei*** ODC is outlined in Fig. 17.35.

DFMO initially forms a Schiff base with PLP (**86**), then, following decarboxylation, a fluoride ion is eliminated, thereby generating the **electrophilic** conjugated imine (87). Attack by the **nucleophilic** thiol group of **Cys360** and subsequent elimination of a second fluoride anion yields a second conjugated imine (88). The second imine then undergoes a **transaldimination** reaction with the amino group of **Lys69**. The enamine formed in this reaction (89) may then undergo an internal **cyclization** to yield a cyclic imine (**90**). This is the main product formed by the alkylation of ODC by DFMO (204). Recently, the X-ray structure of the DFMO-inactivated T. ***brucei*** ODC **K69A** mutant was solved (205). This structure was of the second conjugated imine (88) complexed with ODC and showed that, within a single active site, the decarboxylated DFMO bridges two subunits, forming a Schiff base with the PLP on one monomer and a covalent bond to **Cys360** on the second monomer.

The enzyme steroid **5α-reductase** is an NADPH-dependent enzyme that catalyzes the conversion of testosterone to **dihydrotestosterone**, a more potent androgen (Equation 17.54).

Dihydrotestosterone, rather than testosterone, had been implicated in endocrine disorders such as acne, enlargement of the prostate, and male pattern baldness, and it was suggested that **5α-reductase** was an attractive therapeutic target. Initially, finasteride (91) (Fig. 17.36) was developed as a potent reversible inhibitor of **5α-reductase** with a K_i in the low nanomolar range (206). Closer examination revealed that finasteride appeared to be a slow-binding, high-affinity inhibitor of the human prostate (type 2) **5α-reductase**, with a K_i of less than 1 n*M* (207). Finasteride is currently the drug of choice in the treatment of benign prostatic hyperplasia, and it is now thought that finasteride is, in fact, a mechanism-based inhibitor (208, **209**), which acts through an enzyme-bound **NADP-dihydrofinasteride adduct** (Fig. 17.36).

In this mechanism, reduction of finasteride (91) leads to the formation of an enolate (92) and the subsequent formation of an **adduct** with **$NADP^+$** (93) (where PADPR = **phos**phoadenonsine diphosphoribose). The dissociation constant for the enzyme-inhibitor complex is less than **10^{-13}** M, and the partition ratio for the enzyme-catalyzed formation of dihydrofinasteride (**94**) is less than 1.07 (208). Clearly, finasteride is an extremely efficient mechanism-based inhibitor. **As** shown in Fig. 17.36, the **$NADP^+$-finasteride adduct** will

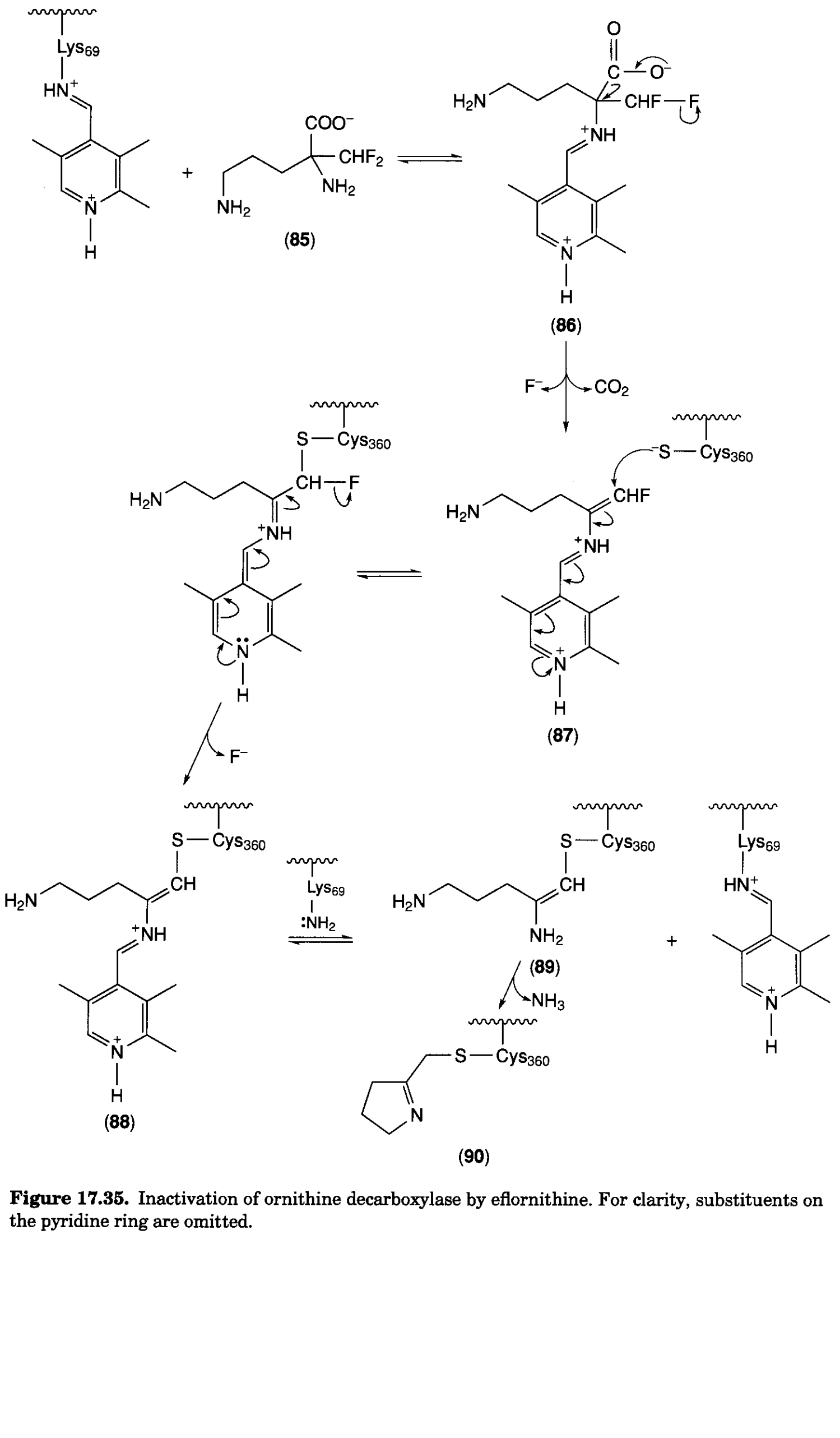


Figure 17.35. Inactivation of ornithine decarboxylase by eflornithine. For clarity, substituents on the pyridine ring are omitted.

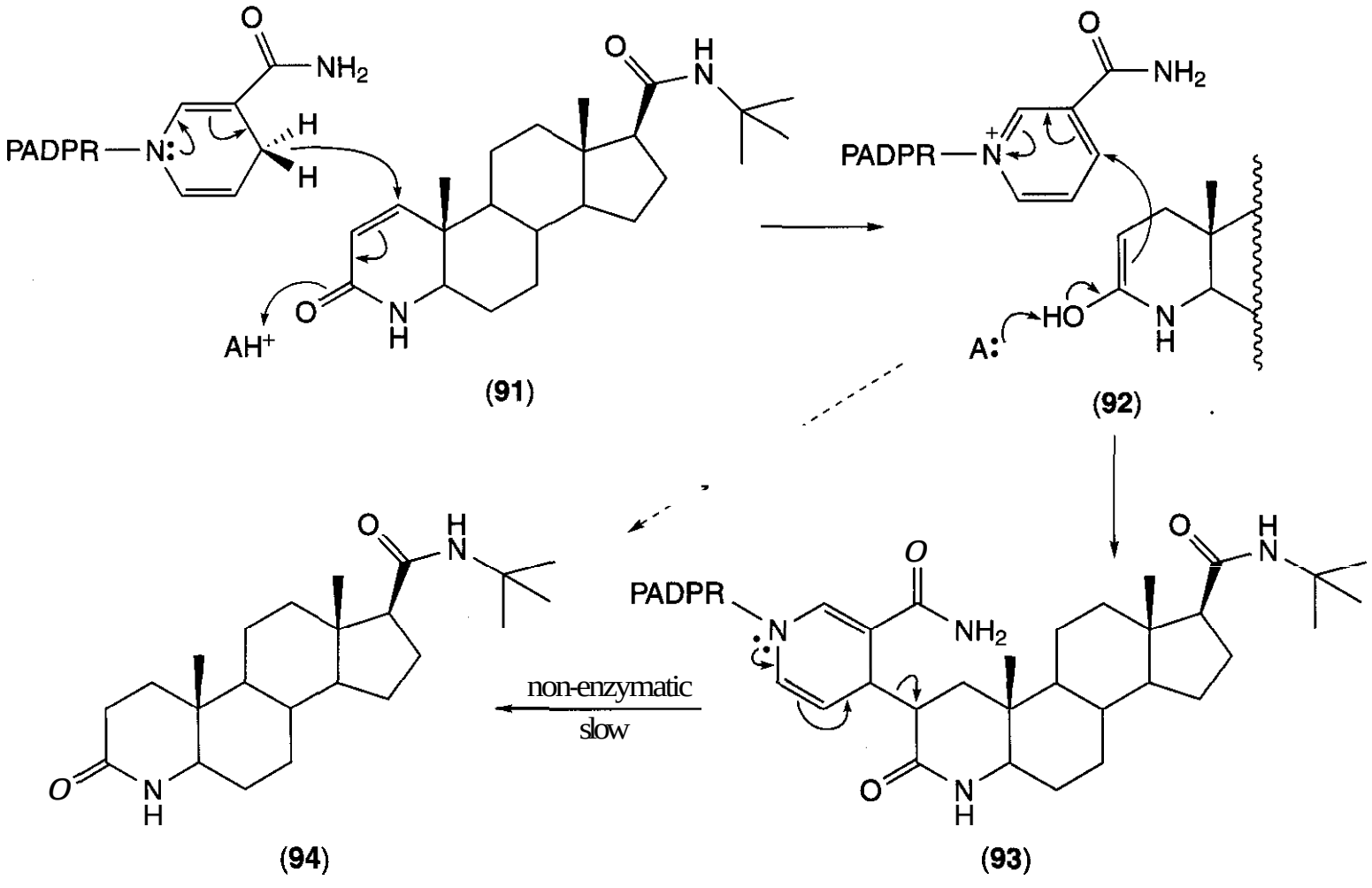


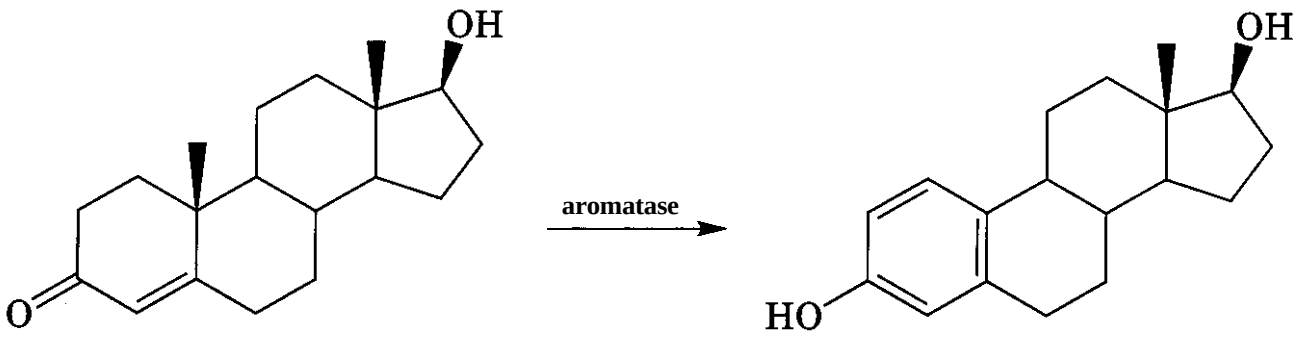
Figure 17.36. Inhibition of steroid 5α-reductase by finasteride (PADPR = phosphoadenosine diphosphoribose).

eventually dissociate and form dihydrofinasteride (**94**), although the half-life of 14 days also points to the effectiveness of finasteride as a steroid 5α-reductase inhibitor.

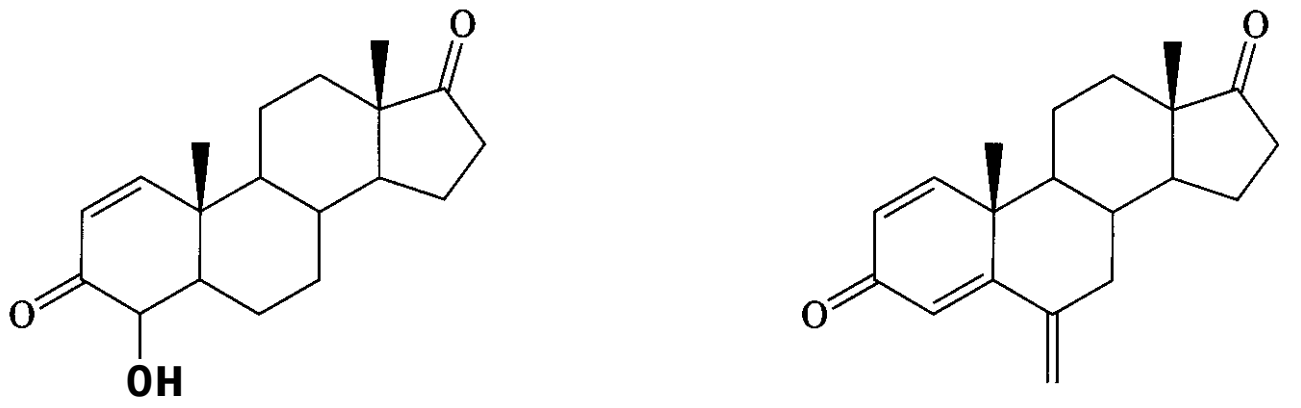
Enzymes involved in steroid biosynthesis have proved to be good targets, both for therapeutic intervention and for mechanism-based inactivators (2). Aromatase, for example, catalyzes the final, rate-limiting step in estrogen biosynthesis (Equation 17.55). Aromatase has proved susceptible to mechanism-based inhibitors such as formestane and exemestane. These are now both used in the treatment of breast cancer (210).

In the last decade there has been a considerable increase in the occurrence of antibiotic-resistant microbial pathogens. Vancomycin, one of the last resort antibiotics for treating some gram-positive bacterial infections, inhibits peptidoglycan synthesis by binding the terminal D-alanyl-D-alanine (D-Ala-D-Ala) dipeptide from pentapeptide precursors of ***Enterococcus*** cell walls. VanX is a zinc-dependent D-Ala-D-Ala dipeptidase (Equation 17.56), which has been implicated in high-level resistance to vancomycin (211, 212). As a consequence, VanX has become a prime drug target for overcoming vancomycin resistance and a number of transition-state analogs have been prepared (213,214).

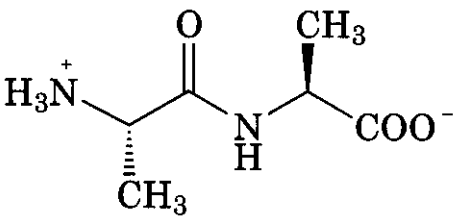
The enzyme was also shown to process dipeptides with bulky C-terminal amino groups (**213**) and, using this knowledge, a novel mechanism-based inhibitor was recently developed (215). Its mechanism is shown in Fig. 17.37. D-Ala-D-Gly($S\Phi p$-CHF_2)-OH (95) is a dipeptide-like analog of D-Ala-D-Ala and is readily accepted by VanX. Cleavage of the peptide bond and elimination of D-alanine results in the formation of the metastable 2-*p*-difluoromethylthioglycine (**96**), which spontaneously decomposes, yielding ammonia, glyoxylic acid, and p-difluoromethyl thiophenol (97). Elimination of a fluoride ion results in the electrophilic 4-thioquinone fluoromethide (**98**), which irreversibly alkylates the enzyme (99). Interestingly, the turnover of the analog was faster than that of D-Ala-D-Ala itself. However, the partition ratio of 7500 in-



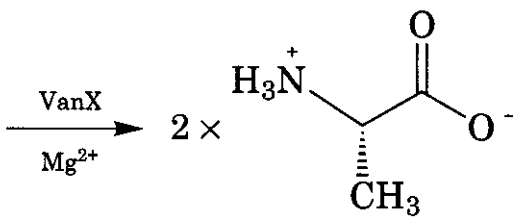
testosterone estradiol



formestane exemestane (17.55)



D-ala-D-ala (17.56)



dicated that one of the reactive intermediates must be relatively long-lived (215).

More detailed examples of approaches used to design mechanism-based inhibitors may be found in excellent reviews by Silverman (166, 167) and by Ator and Ortiz de Montellano (216).

3.4 Pseudoirreversible Inhibitors

Pseudoirreversible inhibitors are the least common of the covalently binding enzyme inhibitors. They have some features in common with both affinity labels (Section 3.2) and mechanism-based inhibitors (Section 3.3) but they have one distinguishing feature; that is, the covalent bond formed between the enzyme and the inhibitor is reversible (Equation 17.57). As with the affinity labels, initially they bind to the enzyme's active site in a non-covalent fashion to form an enzyme-inhibitor complex E . I but, unlike an affinity label, the pseudoirreversible inhibitor generally possesses unreactive functional groups. As with the mechanism-based inhibitor, the enzyme then starts the catalytic cycle and an active-site residue, usually one involved in covalent catalysis (60), reacts with the inhibitor, without producing a highly reactive species, and forms a covalent bond.

$$\mathrm{E} + \mathrm{I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{I} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E}-\mathrm{I}' \xrightarrow{k_3} \mathrm{E} + \mathrm{P} \quad (17.57)$$

The covalently bound inhibitor mimics the normal covalent reaction intermediate occurring during the normal reaction mechanism. However, the covalent adduct is far more stable, with half-lives on the order of several hours to days. The free enzyme may then, depending on the lability of the E-I' bond, be regenerated by hydrolysis or reversal of the covalent bond. The utility of a pseudoirrevers-

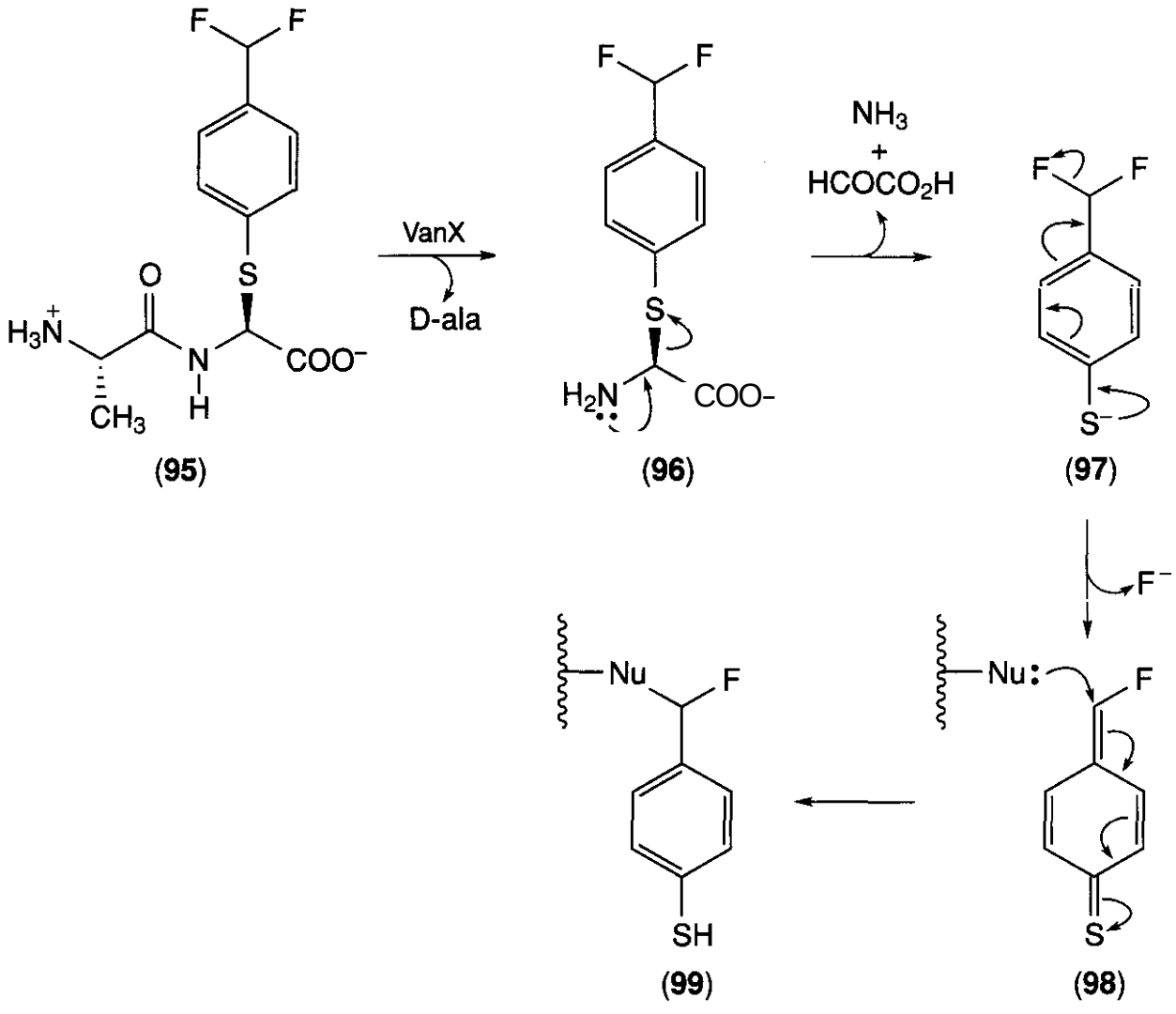


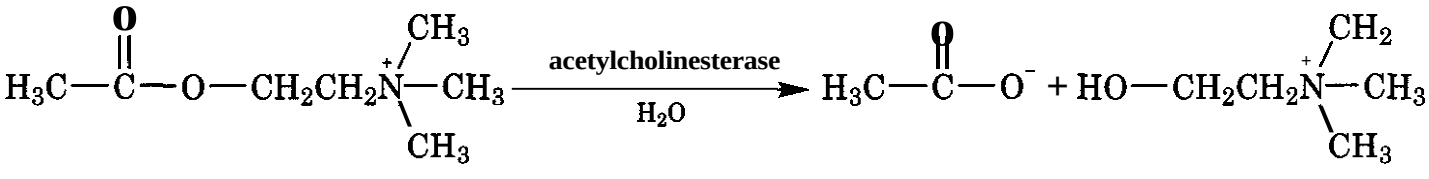
Figure 17.37. Mechanism-based inhibition of VanX.

ible inhibitor will be determined by a combination of the rate of formation of the covalent enzyme inhibitor adduct and the half-life for reactivation.

As may be expected, criteria for the study of pseudoirreversible inhibitors are very similar to those for both affinity labels and mechanism-based inhibitors. However, because of the inherent reversibility of pseudoirreversible inhibitors, it may be more difficult to obtain structural evidence for the covalent enzyme inhibitor adduct. Further, determination of the rate of reactivation and characterization of the products of the recovery process will also be of major importance in designating an inhibitor as pseudoirreversible.

Pseudoirreversible inhibitors can be broken into two classes, depending on how the active enzyme is regenerated. In the first class, exemplified by inhibitors of acetylcholinesterase, the enzyme is regenerated as the covalent E-I' bond is hydrolyzed (i.e., $k_3 \gg k_{-2}$). As shown in Equation 17.58 , acetylcholinesterase catalyzes the hydrolysis of acetylcholine, yielding choline and acetate.

Acetylcholine is a neurotransmitter that relays nerve impulses across the neuromuscular junction. Acetylcholinesterase (AcChE) rapidly breaks down acetylcholine, thereby lowering its concentration in the synaptic cleft and ensuring that nerve impulses are of a finite length. As shown in Fig. 17.38, a nucleophilic serine residue reacts with the substrate to form an acetyl-serine intermediate (100) with concomitant release of choline. This intermediate is then rapidly hydrolyzed by wa-



(17.58)

acetylcholine **acetate** **choline**

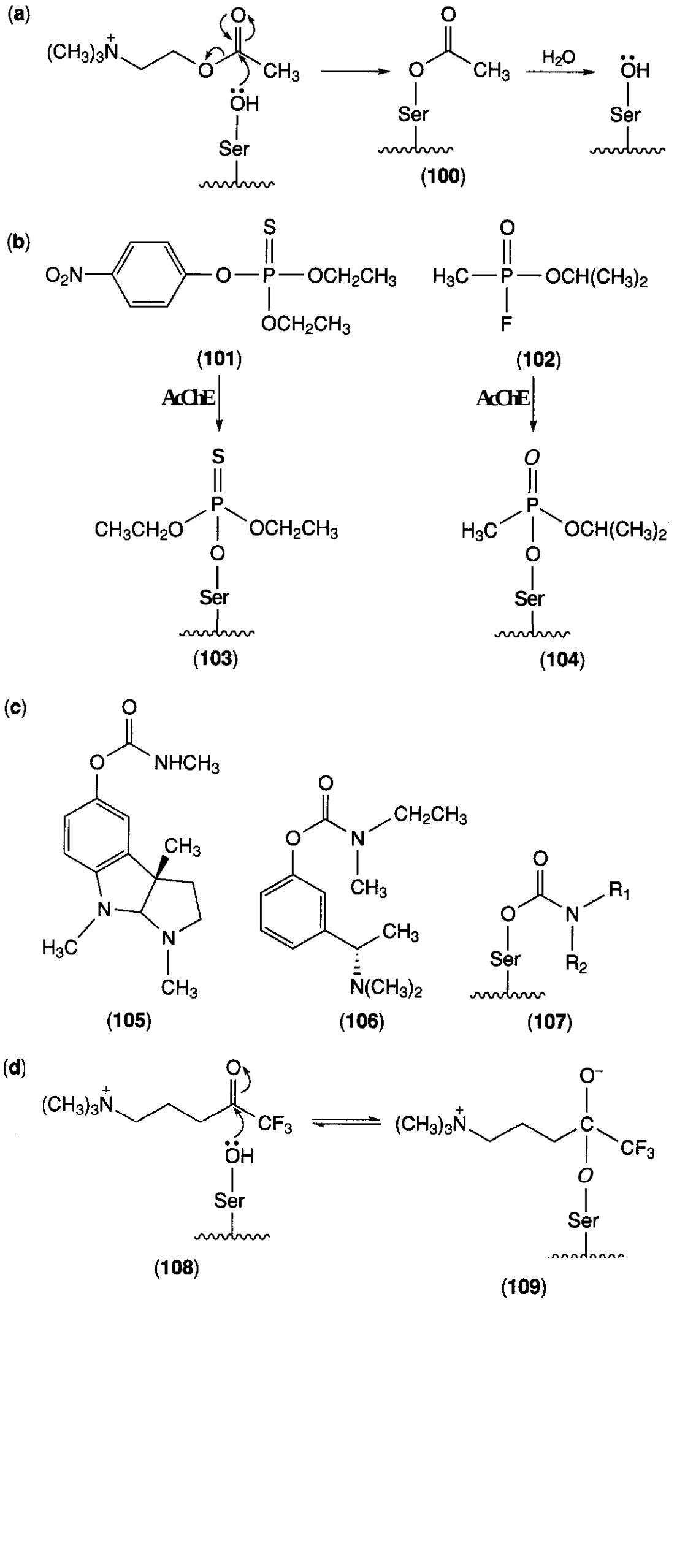


Figure 17.38. (a) Mechanism of reaction, (b) irreversible inhibitors, and (c,d) pseudoirreversible inhibitors of acetylcholinesterase.

ter, producing acetate and regenerated enzyme. Agents such as parathion (101) and sarin (102) have found utility as insecticides and nerve gases, respectively, because they react with the enzyme to form the active-site serine-phosphate esters, (**103**) and (**104**). These esters are hydrolyzed extremely slowly by water, making the inhibition effectively irreversible (i.e., both k_{-}, and $k_{}$ are very small), although the inhibition can be overcome with high concentrations of strong nucleophiles such as hydroxylamine.

More recently, it has been established that inhibitors of acetylcholinesterase may play a role in the memory enhancement in patients with Alzheimer's disease (217). Unlike (101) and (**102**), carbamate inhibitors such as physostigmine (105) and rivastigmine (**106**) are classified as pseudoirreversible inhibitors because they react with AcChE to form a carbamylated serine (107). By comparison with the serine-phosphate ester, the carbamylated serine is rapidly hydrolyzed, thereby regenerating AcChE. For example, reactivation of the physostigmine-inactivated enzyme is rapid, with a $t_{1/2}$ of less than 40 min (218). Rivastigmine, a more useful therapeutic agent, is considerably longer acting, with a half-life of more than 10 h (217, 219). Overall, for pseudoirreversible inhibitors of this type, the effectiveness and duration of the "irreversible" inhibition will be controlled by the chemical nature of the groups transferred to the active-site nucleophile, making it readily amenable to manipulation.

In pseudoirreversible inhibitors of the second class, the enzyme is regenerated by the inhibitor simply dissociating from the enzyme; that is, the binding is covalent but reversible ($k_{-2} \gg k_{}$). This class can also be exemplified by an AcChE inhibitor. For example, the trifluoromethyl ketone (**108**) binds to AcChE as a slow-binding inhibitor (Section 2.4.1) with a K_i value of 0.06 nM, and a k_{off} value of 6.7×10^{-6} s^{-1} (220). A linear correlation was observed between K_i values of a series of fluoromethyl ketones and the V_{max}/K_i value for the corresponding substrate (220). This suggests (127) that the tetrahedral adduct (**109**), in effect, mimics the transition state (or a high-energy intermediate), thereby accounting for the high affinity (Section 2.5.3). The affinity of the inhibitor for AcChE could be decreased (with a concomitant increase in the value of $k_{}$), by sequentially reducing the number of fluorine atoms into the methyl group adjacent to the ketone (220). Finally, it should be noted that the two classes of pseudoirreversible inhibitor can be differentiated by examining the decomposition products of the inhibition reaction. When hydrolysis is required for enzyme regeneration, cleavage products, such as substituted carbamates, will be in evidence. Conversely, the trifluoromethyl ketones will not be broken down by AcChE and no decomposition products will be observed.

4 CONCLUSIONS

Enzyme inhibitors have long played an important role in medicine, pharmacology, and basic research. The advances in DNA technology have enabled cloning and overexpression of large numbers of enzymes, and the approaches described in this chapter have already led to the development of novel therapeutic agents. However, in the postgenomics era, large numbers of new targets have been identified. Although the drug discovery process moves toward structure-based drug design as its prime tool, even with high-throughput crystallography, not all target proteins will be readily accessible. The evolution of algorithms that can predict enzyme function and mechanism will ensure that the rational design of enzyme inhibitors not only complements structure-based approaches but continues to play a stand-alone role in the discovery of novel therapeutics.

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