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Irreversible Inhibitors of Serine, Cysteine, and Threonine Proteases

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I. Introduction

Proteases or proteolytic enzymes form one of the largest and more important groups of enzymes. Proteases selectively catalyze the hydrolysis of peptide bonds and can be divided into four major classes (or groups): aspartic, serine, cysteine, and metalloproteases. Proteases are involved in numerous important physiological processes including protein turnover, digestion, blood coagulation and wound healing, fertilization, cell differentiation and growth, cell signaling, the immune response, and apoptosis. Uncontrolled, unregulated, or undesired proteolysis can lead to many disease states including emphysema, stroke, viral infections, cancer, Alzheimer's disease, inflammation, and arthritis. Protease inhibitors thus have considerable potential utility for therapeutic intervention in a variety of disease states.

II. Serine, Cysteine, and Threonine Proteases

In this review, we will discuss irreversible or covalent inhibitors of serine, cysteine, and threonine proteases. Serine, cysteine, and threonine proteases have many common active site features including an active site nucleophile and a general base, which are often the target of irreversible inhibitors. Thus far, this group includes the majority of proteolytic enzymes and many significant enzymes with involvement in human diseases. We will cover inhibitors commonly considered to be irreversible. This includes inhibitors that form "stable" covalent bonds with the enzyme. We will not include transition-state inhibitors such as peptide aldehydes, peptide α-ketoamides, and peptide trifluoromethyl ketones, which form a covalent tetrahedral adduct with serine, cysteine, and threonine proteases, because this adduct is usually in equilibrium with free enzyme and free inhibitor. In addition to transition-state inhibitors, there is clearly a large group of reversible inhibitors such as benzamidine inhibitors for trypsin-like enzymes, which form no covalent bonds with the enzyme. These reversible non-covalent inhibitors, although quite potent, will not be covered in this review. We will focus on irreversible inhibitors published since 1990, although, to be complete, we will also describe older work. In addition, we will pay particular attention to irreversible inhibitors where X-ray crystallographic structural information is available in the protein databank.

A. Families and Clans

Amino acid sequence data are now available for over 450 peptidases (endopeptidases and exopeptidases) from over 1400 organisms (bacteria, archaea, archezoa, protozoa, fungi, plants, animals, and vi-

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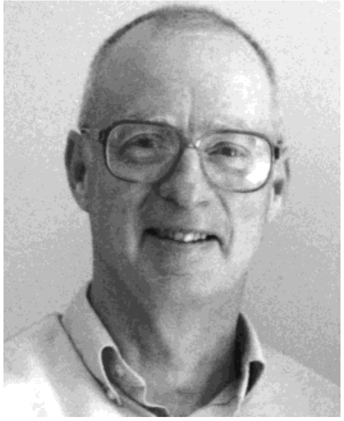

James C. Powers received his B.S. degree in chemistry from Wayne State University in 1959 and his Ph.D. degree in organic chemistry under the direction of George Büchi at the Massachusetts Institute of Technology in 1963. He taught organic chemistry at the University of California—Los Angeles from 1963 to 1967 as an Assistant Professor of Chemistry. He then studied biochemistry at the University of Washington under the direction of Philip Wilcox from 1967 to 1970. He has been in the School of Chemistry and Biochemistry at the Georgia Institute of Technolgy since 1970, where he rose through the ranks to become a Regents Professor of Chemistry and Biochemistry in 1987. In 1999, he received the Class of 1934 Distinguished Professor Award and in 2000, he received the Herty Award from the Georgia Section of the American Chemical Society. He has been married for 35 years to Christina M. Powers and has two children (Karen and David) and two grandchildren (Justin and Skylar). He enjoys hiking and photography and has climbed Mount Kilimanjaro (19340 ft) and the highest point in 48 of the 50 United States.

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Karen Ellis James was born in 1975 in Oxford, England. She moved to the United States in 1979 where she grew up in Atlanta, GA, and graduated from the Lovett School. She received her B.S. degree in chemistry and mathematics, graduating *magna cum laude* from Wake Forest University in 1998, where she conducted undergraduate research in the laboratory of Richard A. Manderville and participated in an NSF summer fellowship with James C. Powers at the Georgia Institute of Technology. She is currently a Ph.D. candidate (graduating December 2002) in biochemistry at the Georgia Institute of Technology under the direction of Dr. Powers. Her thesis research focuses on the design and synthesis of novel aza-peptide epoxide inhibitors for clan CD cysteine proteases.

ruses), and they have been organized into evolutionary families and clans by Rawlings and Barrett.[1,2] This effort led to the development of the MEROPS database (http://www.merops.co.uk), which now includes a frequently updated listing of all peptidase sequences.[3,4] Each new update of the database adds new members and families, but it is clear that the rate of discovery of new peptidases must slow in the upcoming postgenomic era. It is estimated that there may be as many as 700 distinct peptidases, but 550–650 is a more likely number.[4]

Table 1 lists some representative families and clans of serine, cysteine, and threonine peptidases. A family of peptidases contains a group of enzymes that shows evidence of their evolutionary relationship by their similar tertiary structures, by the order of catalytic residues in their sequences, or by common sequence motifs around the catalytic residues. Related families are grouped into a clan, which contains all of the current-day peptidases that arose from a single evolutionary origin. The designation of families follows the catalytic type, serine (S), cysteine (C), or threonine (T). Many of the clans are composed only of one catalytic type; for example, clan CA is composed of cysteine protease families. However, some of the clans contain families with two catalytic types and are grouped with other clans that contain two or more catalytic types. These mixed clans are designated with the letter “P”. For example, the clan PA(S) contains the S1 family with most of the

Table 1. Representative Families and Clans of Serine, Cysteine, and Threonine Peptidases (Total of 224 Families and 41 Clans at Present)

clan	family	representative family members
		Cysteine Proteases (58 Families, 119 Distinct Enzymes)
CA	C1	papain
	C2	calpain I (μ-calpain), calpain II (m-calpain)
CD	C11, C13, C14, C25, C50	clostripain, legumain, caspases, gingipain, separase
		Mixed (Cysteine, Serine, Threonine) Clans
PA(C)	C3	poliovirus picornain 3C protease
PA(S)	S1	chymotrypsin, trypsin, elastase, cathepsin G, granzymes
		Threonine Proteases (6 Families, 22 Distinct Enzymes)
PA(T)	T1	archaean proteasome, β-component
		Serine Proteases (51 Families, 137 Distinct Enzymes)
SB	S8	subtilisin Carlsberg
SC	S9, 10	prolyl oligopeptidase, carboxypeptidase Y
SE	S11, S12	D-Ala-D-Ala carboxypeptidases A and B

common serine proteases and the enzyme trypsin 2, which contains a histidine in place of the active site serine residue. In addition, the picornaviral 3C cysteine protease (family C3) has a serine protease fold[5] and is placed in clan PA(C). Threonine proteases are all placed in the mixed catalytic type clan PA(T).

B. Structures and Folds

It is clear from examination of the MEROPS database that X-ray crystal structures are not yet available for the majority of peptidase families in the MEROPS database. The determination of X-ray structures is lagging considerably behind sequence determinations, so it is hard to predict how many distinct protein folds will be observed in serine, cysteine, and threonine proteases. Protein structures are now analyzed in terms of their tertiary folding structures. One source of this information is the SCOP database (http://scop.mrc-lmb.cam.ac.uk/scop, mirror at http://scop.berkeley.edu).[6−10] Thus far, they have classified 947 superfamilies and 1557 families. Proteases seem to be distributed into all of the major classes of proteins [α-proteins, β-proteins, α- and β-proteins (α/β or α + β), multidomain proteins, membrane and cell surface proteins, and small proteins].

Prokaryotic and eukaryotic trypsin-like serine proteases, some viral serine proteases, and viral cysteine proteases with the trypsin-fold are classified as β-proteins. The proteasome subunits are α- + β-proteins composed mainly of antiparallel β-sheets with segregated α and β regions. The group of cysteine proteases with papain, cruzain, and cathepsin also has this structure. The subtilisins and caspases are members of the α/β group of proteins with parallel β-sheets (β-α-β units). The complement protease C1r is a member of the small protein group, which is usually dominated by disulfide bridges or metal ligand interactions or a heme moiety. The group of α-proteins contains the thermolysin and carboxypeptidase families and the DEATH domain, enzymes that are not covered in this review. The multidomain proteins (α and β) contain serpins and some carboxypeptidases and β-lactamases.

Although many serine proteases are classified as β-proteins, there are clearly major distinct families within this group. With serine proteases of the Ser-His type, there appear to be at least five distinct protein folds. These are the chymotrypsin/trypsin fold, the subtilisin fold (α,β-protein), the α/β-hydrolase fold, the Pro oligopeptidase fold, and the cytomegalovirus protease fold. One of the first comparisons of the members of the serine protease fold is due to James, who compared the three-dimensional structures of the bacterial serine proteases SGPA, SGPB, and α-lytic protease with those of the pancreatic enzymes α-chymotrypsin and elastase.[11] This comparison showed that approximately 60% (55−64%) of the α-carbon atom positions of the bacterial serine proteases were topologically equivalent to the α-carbon atom positions of the pancreatic enzymes. Many similar topological comparisons have since been made.[12,13]

Kraut was first to compare the active site residues of two serine proteases, chymotrypsin and subtilisin, which have dissimilar tertiary structures.[14,15] Although the tertiary structures of the two enzymes do not superimpose, the active site residues (Ser, His, and Asp) superimpose with a root mean square deviation of ∼1 Å.

The α/β-hydrolase fold represents another unique serine protease motif along with the subtilisin fold and is another example of convergent evolution. The structure of *Salmonella typhimurium* aspartyl dipeptidase (dipeptidase E, family S51, clan SN) reveals a strand-helix motif reminiscent of that found in the α/β-hydrolases such as serine carboxypeptidase (family S10), proline iminopeptidase, and acetylcholinesterase. Interestingly, the active site is composed of a Ser-His-Glu catalytic triad.[16] The proline iminopeptidase from *Xanthomonas campestris* is composed of two domains. The first and largest is very similar to the α/β-hydrolase fold found in yeast serine carboxypeptidase, and the second is placed on top of the larger domain and essentially consists of six helices.[17] This enzyme is a model for the Pro oligopeptidase folding family.

The other serine protease folds have major differences in their active site residues. The cytomegalovirus protease or assemblin (CMV protease, family S21) is a new serine protease fold[18,19] and has a catalytic triad composed of Ser 132, His 63, and His 157. The Ser-Lys group of serine proteases consists of *Escherichia coli* signal peptidase (SPase) and *E. coli* UmuD protease and has a Ser-Lys as a catalytic diad.[20] Despite a very low sequence identity, these functionally diverse enzymes share the same protein fold within their catalytic core. This complex fold is composed of several coiled β-sheets and contains an SH3-like barrel. The rhomboid protease is an intermembrane serine protease which has a Ser-His-Asn triad instead of the normal serine protease Ser-His-Asp triad.[21] This protease is highly specific, has an important role in intercellular signaling, and is sensitive to Tos-Phe-CH_2Cl and 3,4-dichloroisocoumarin.

With cysteine proteases, there is the papain/cathepsin B family of proteases and the caspase family. Both have quite different protein folds. Crystal structures are now available for several caspases and gingipain.[22] Legumain, caspases, clostripain, separase, and gingipains have been shown to belong to a new clan (CD) of cysteine proteases.[23,24] Structural differences between clans and families of proteases of the same class should be useful for the design of specificity into inhibitor structures.

The 20S proteasome is a 6500 amino acid protein with an active site N-terminal threonine (Thr 1). It plays a central role in protein degradation in eukaryotic cells. All proteasomes are composed of 28 subunits arranged in a cylindrical structure composed of four heptameric rings. The subunits range from 22 to 30 kDa, giving a total molecular weight of 700−750 kDa.[25] The proteasome was initially described as a multicatalytic protease by Orlowski due to several different catalytic activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase).[26] The outer rings of the proteasome are composed of α-subunits. The inner rings are composed of β-subunits that are catalytically active. It is thought that proteolysis takes place by threading protein substrates into the hollow core of the proteasome.

Another large protease fold is the tricorn protease from *Thermoplasma acidophilum*. The basic functional unit of tricorn is a homohexamer of the 121 kDa subunit, which can assemble further to form an isosahedral capsid with a molecular mass of 14.6 MKDa.[27,28] The active site is a Ser-His-Ser(Glu) tetrad and forms a covalent complex with Tos-Phe-CH_2Cl at Ser 965. The enzyme appears to have preferential dipeptidase and tripeptidase activity. The tricorn protease is downstream of the proteasome, which may channel cleavage products to the tricorn protease sitting as a cap on the top of the proteasome hollow core. The tricorn can also further channel the cleaved substrates to accessory amino peptidases. Thus, the three proteolytic components can act as a protein disassembly factory.[29]

C. Mechanism

The active site residues of serine, cysteine, and threonine proteases have many mechanistic features in common. Hydrolysis of a peptide bond is an energetically favorable reaction, but extremely slow.[30] The active site residues of serine, cysteine, and threonine proteases are shown in Figure 1. Each enzyme has an active site nucleophile and a basic residue, which can also function as a general acid in the catalytic mechanism.

The transition states for serine, cysteine, and threonine proteases all involve formation of a tetrahedral intermediate shown in Figure 2. The oxyanion of the tetrahedral intermediate is frequently stabilized by interaction with several hydrogen bond donors, which is commonly referred to as the oxyanion hole. The oxyanion hole of serine proteases is usually quite rigid and involves backbone peptide bond NH groups as hydrogen bond donors. Interaction with the oxyanion hole is usually essential for effective substrate hydrolysis. With cysteine proteases, the oxyanion hole does not seem to be as essential and is much more flexible at least in the case of the papain family. At present the nature of the oxyanion hole of the proteasome is not clear.

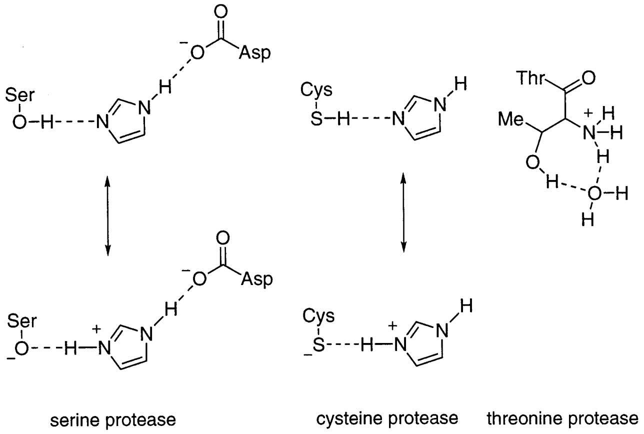


Figure 1. Active site residues of serine, cysteine, and threonine proteases.

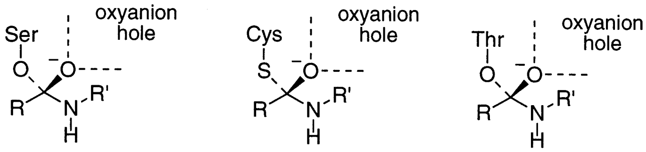


Figure 2. Transition states for the serine, cysteine, and threonine protease hydrolysis of peptide bonds.

Although the transition states for peptide bond hydrolysis within a protease group can be quite similar, frequently there are substantial differences. Papain and caspase-1 belong to different cysteine protease clans and have different folds, slightly different active site residues, and different oxyanion holes (Figure 3). The oxyanion hole in caspase-1 is very rigid and is formed from backbone residues and resembles the oxyanion hole in serine proteases, whereas the oxyanion hole in papain is much more flexible and is composed of one side-chain residue. Clearly, these active site differences can have considerable influence on inhibitor design because the papain/cathepsin family is inhibited very effectively by inhibitors such as epoxysuccinates, whereas the caspases are inert to epoxysuccinates such as E-64.

Other combinations of catalytic groups are clearly capable of peptide bond hydrolysis. The Ser-His-Asp triad of serine proteases can be replaced with Ser-His-Glu, Ser-His-His, or Ser-Lys in other members of the serine protease group.

Another group of recently described serine proteases are the serine carboxyl peptidases, which may bridge serine and aspartate proteases.[31] One new member is the serine carboxyl proteinase from *Pseudomonas* sp. 101 (PSCP), which has a superset of the subtilisin fold. An aldehyde inhibitor is covalently linked to the enzyme's serine residue in the X-ray structure. Thus, the structure of PSCP defines a novel family of serine-carboxyl proteinases (defined

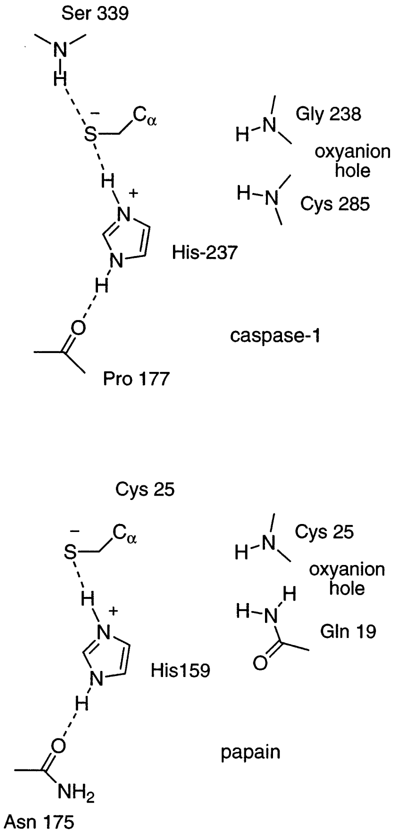


Figure 3. Active site residues and oxyanion hole of caspase-1 and papain.

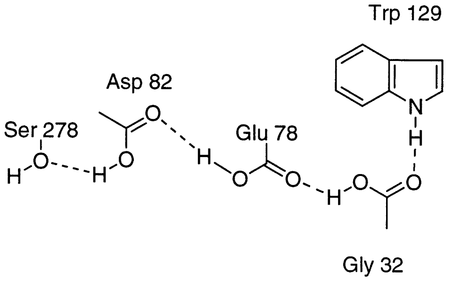


Figure 4. Active site residues of the serine carboxyl protease kumamolysin.

as MEROPS S53) with a unique catalytic triad consisting of Glu 80, Asp 84, and Ser 287.[31] Another example of this family is the serine carboxy protease kumamolysin.[32] This is built on the subtilisin fold and has a catalytic serine residue (Ser 278) hydrogen bonded to an Asp 82, which is hydrogen bonded to a Glu 78 (Figure 4), which enables the serine to attack at quite acidic pH values.[33,34] In addition, a Glu 32–Trp 129 hydrogen bonded pair may facilitate proton delocalization during peptide bond hydrolysis. The oxyanion hole is composed of Asp 164 and the backbone NH of Ser 278. Little is yet known of its mechanism, but it does seem to be inhibited by 3,4-dichloroisocoumarin, whereas 4-(2-aminoethyl)benzene sulfonyl fluoride does not inhibit the enzyme. It is unclear whether the chloromethyl ketone Ac-Ala-Ala-Phe-CH_2Cl is a reversible or an irreversible inhibitor.

D. Kinetics and Rate Constants

The rate of reaction of an irreversible inhibitor with a protease is typically measured using the incubation method. The enzyme is mixed with the inhibitor and allowed to incubate at a set of prescribed conditions. At various time intervals, an aliquot of this incubating inhibition mixture is removed, diluted into an assay solution containing a substrate for the protease, and assayed for residual enzymatic activity. The conditions in the assay mixture and the inhibition incubation mixture can be quite similar or quite different. The conditions in the inhibition incubation mixture control the rate of enzyme inhibition, whereas the conditions in the assay mixture determine the sensitivity of the assay for detection of residual enzyme activity.

Typically, inhibition kinetics are carried out using pseudo-first-order kinetics where the inhibitor concentration [I] is >10-fold higher than the enzyme concentration [E]. A plot of ln a (activity) against time gives the observed rate of inactivation k_{obs} using the equation $\ln a = -k_{obs}t$. With very potent irreversible inhibitors, rates are often too fast to measure using pseudo-first-order kinetics, and the inhibitor concentration is decreased until the concentrations of enzyme and inhibitor are of the same magnitude; then a second-order rate constant, k_{2nd}, can be determined using a second rate equation:

$$k_{2nd}t = [1/(i - e)]\ln[e(i - x)/i(e - x)]$$

where $e - x$ is the residual enzyme concentration.

If the inhibitor is very potent and binds in the active site, it is also possible to slow the reaction by incubating the inhibitor with the enzyme in the presence of a substrate or a competitive inhibitor. Inhibition rates can then be determined using various kinetic equations.

A more convenient method for measurement of the inhibition rate constant in the presence of substrates was introduced by Tian and Tsou.[35] This is referred to as the progress curve method and is suitable for measuring irreversible inhibition rates with fast inhibitors. The progress curve method also has the advantage that relatively few separate kinetic measurements, which consume valuable enzyme and substrate, are needed to measure inhibition rate constants. In this method, the inhibitor, substrate, and enzyme are incubated together, and the rate of substrate hydrolysis is measured continuously. Both the inhibitor and the substrate are competing for the same active site, and the observed inhibition rate is decreased. The active enzyme concentration is dropping as it reacts with the inhibitor, and a plot of substrate hydrolysis product versus time is hyperbolic as the amount of active enzyme is reduced. From this curve, the observed inhibition rate constant (k_{obs}) at a particular substrate concentration can be calculated. Measurement of k_{obs} at different substrate concentrations and extrapolation to zero substrate concentration give the apparent rate constant (k_{app}). This should be similar to k_{obs} determined by the incubation method if the inhibition rates are measured under pseudo-first-order conditions. If there is not a large excess of inhibitor relative to the enzyme concentration, then a second rate inhibition constant (k_{2nd}) could be determined.

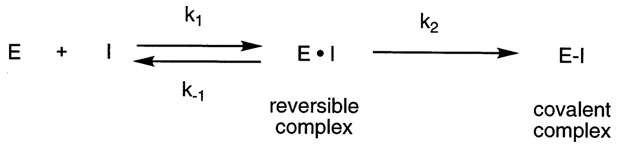


Figure 5. Irreversible inhibition kinetics.

The inactivation of a protease by an active-site-directed irreversible inhibitor usually proceeds by the rapid formation of a reversible enzyme–inhibitor complex (E·I, Figure 5). In a slower chemical step, a covalent bond is formed with the enzyme to generate the enzyme–inhibitor adduct (E–I).

When [I] > [E], the kinetics are described by the equation of Kitz and Wilson.[36]

$$k_{obs} = k_2/(1 + K_i/[I])$$

This equation can also be written in a linearized form.

$$1/k_{obs} = K_i/k_2[I] + 1/k_2$$

$$K_i = [E][I]/[E{\cdot}I] = k_1/k_{-1}$$

K_i is the dissociation constant of the enzyme–inhibitor complex, and k_2 is the maximum (or limiting) inhibition rate if the enzyme is saturated with inhibitor. This is a first-order rate constant. The ratio k_2/K_i is a second-order inhibition rate, similar to k_{cat}/K_M, and is the most commonly used parameter to report inhibition data.

If a series of experiments are carried out at different inhibitor concentrations, it is possible to measure k_2 and K_i from a plot of $1/k_{obs}$ versus 1/[I]. This is possible only if the inhibitor is forming an E·I complex and the inhibitor concentration used is below the point at which E is saturated; data points are obtained with I concentration in the range of K_i. If K_i > [I] or the enzyme is not forming an E·I complex, then this plot will go through the origin and $k_{obs}/[I] = k_2/K_i$ for an enzyme that forms E·I. However, if $K_i \approx [I]$, then this plot will yield k_2, K_i, and k_2/K_i.

It is fairly common to report IC_{50} values for inhibitors of all types in medicinal chemistry journals. This represents the inhibitor concentration necessary to effect 50% inhibition of the enzyme under the conditions of the enzyme assay. With an irreversible inhibitor, the IC_{50} value clearly depends on the time during which the enzyme is incubated with the irreversible inhibitor. The longer the incubation time, the lower the IC_{50} value. It is possible to make a rough estimate of the irreversible inhibition rate constant. If you assume that the IC_{50} (in molar) is approximately equal to the inhibitor concentration necessary to reduce the enzyme activity by 50% during the time of the incubation, then the assay time (t_{assay}) should be equal to the half-life $t_{1/2}$ for the pseudo-first-order inactivation rate.

$$k_{obs} = 0.693/t_{1/2} = 0.693/t_{assay}$$

$$k_{obs}/[I] = 0.693/[t_{assay} \times IC_{50}\ (\text{in M})]$$

Many inhibitors acylate (or sulfonylate or phosphonylate) the active site nucleophile to form an acyl enzyme intermediate. This acyl enzyme is frequently very stable, and the enzyme is essentially irreversibly inactivated. However, in some cases, the acyl enzyme will hydrolyze to regenerate active enzyme. The potencies of acylating inhibitors are frequently compared by the magnitude of their acylation rates k_{on} and their deacylation rates k_{off} (Figure 6).

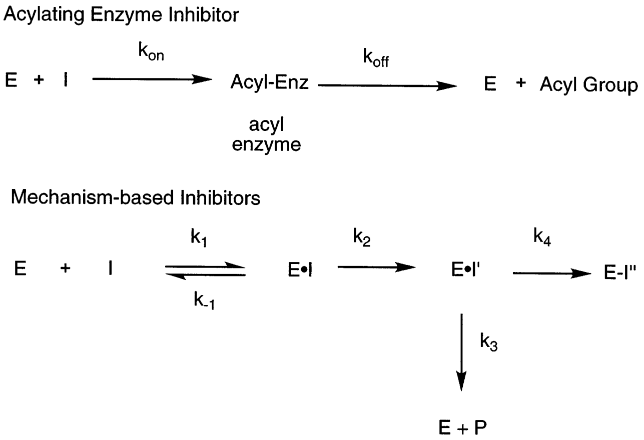


Figure 6. Acylation/deacylation and mechanism-based kinetics.

Mechanism-based inhibitors or enzyme-activated irreversible inhibitors often have several additional steps after formation of the E·I complex (Figure 6). Frequently, a latent complex (E·I′) is formed with the formation of a reactive group in the E·I′ complex. This E·I′ complex could be a simple reversible complex or an acyl enzyme. This complex can regenerate active enzyme and product by diffusion of the reactive inhibitor out of the active site (k_3) or by deacylation before a second covalent bond is formed with the enzyme. Alternatively, it could form a more stable irreversible complex (E–I″). Examples of mechanism-based inhibitors include isocoumarins, which frequently form two covalent bonds with serine proteases and β-lactams, which are also double-hit irreversible inhibitors. Indeed, multiple bond-forming pathways are frequently observed with β-lactam serine protease inhibitors, giving a more complex pathway than that shown in Figure 6. Mechanism-based irreversible inhibition is difficult to distinguish for other types of irreversible inhibitors by kinetics alone. More frequently, this type of inhibitor is only demonstrated following X-ray crystallographic studies or other structural studies (i.e., mass spectrometry or NMR).

Some inhibitors are quite reactive and may undergo decomposition during the inhibition reaction. Thus, a decreasing inhibition rate over time may indicate an unstable inhibitor. However, it is still possible to measure inhibition rates.[37,38]

A number of reviews of irreversible inhibition kinetics are available.[39,40] Mechanism-based inhibition kinetics have also been reviewed.[40–42]

E. Inhibitor Design

The first specific irreversible inhibitors for serine, cysteine, and threonine proteases were designed by taking a good substrate and attaching a reactive warhead to that substrate structure. The early warheads used were alkylating agents such as diazo

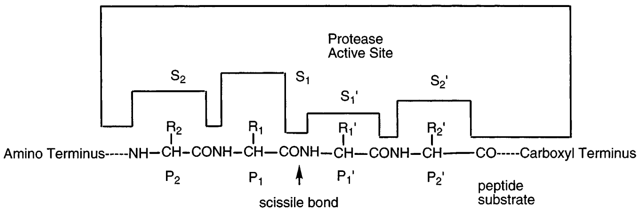


Figure 7. Subsite nomenclature used with proteolytic enzymes. The scissile bond is indicated with an arrow. Amino acid residues to the left of the scissile bond are numbered P1 (for peptide 1), P2, etc., with numbering increasing in the direction of the N-terminal residue of the substrate or inhibitor. Residues to the right of the scissile bond are numbered P1′, P2′, etc. The corresponding complementary regions of the enzyme's active site are numbered S1, S2, S1′, etc.

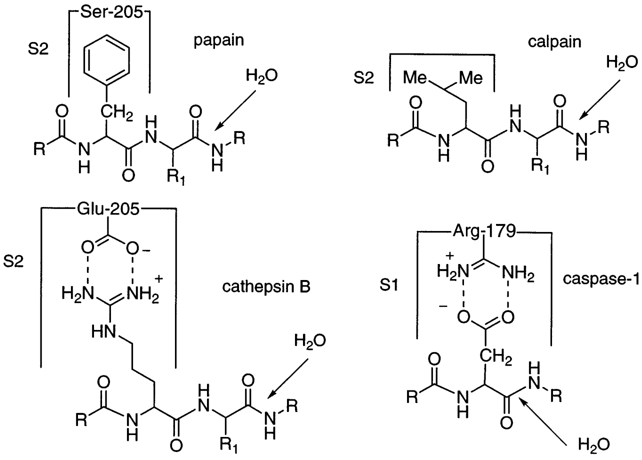


Figure 8. Cysteine protease binding sites.

compounds or haloketones. Subsequently a variety of other reactive warheads have been developed. The first step in designing an inhibitor for a new protease is frequently subsite mapping with a library of peptide substrates.[43] The nomenclature used to describe the binding of substrates and inhibitors to the active sites of proteases is shown in Figure 7.[44]

The primary specificity site shows considerable variation between individual proteases even within the same class or in the same clan. These differences are utilized in the design of specific inhibitors for a target protease. The binding sites of four cysteine proteases are shown in Figure 8. The primary specificities of papain, calpain, and cathepsin B are determined by the shape and electrostatic character of S2, whereas the specificity of the caspase family is determined by the interaction of the P1 Asp residues in substrates with Arg 179 in S1. The primary specificity of most serine proteases is also determined by S1. However, many proteases have extended substrate binding sites and require longer peptides or inhibitors for effective binding. Examples would be caspases, neutrophil elastases, and thrombin, among many others that require tri- or even tetrapeptides for effective substrate hydrolysis or for inhibitor potency.

Inhibitor design has now progressed far beyond the stage of simply attaching a warhead to the appropriate peptide sequence specific for the targeted proteases. Currently, there are two major approaches to the development of new protease inhibitors. The first involves rapid screening of libraries of small molecules already on hand or of newly synthesized combinatorial libraries. Several fairly interesting inhibitor structures have been discovered in this manner, but mass screening frequently reveals numerous uninteresting compounds. For example, screening for cysteine protease inhibitors often results in large numbers of nonspecific alkylating agents or oxidizing agents. The second major approach to inhibitor discovery is structure-based drug design using X-ray crystallography.[45,46] In the development of a new drug for a protease, dozens of X-ray structures of enzyme–inhibitor complexes are solved, most of which never appear in the literature. Medicinal chemists use the structural information to continuously improve their lead compounds. A notable example of the use of this technique is the recent work on the development of orally bioavailable inhibitors for the 3C protease, which are being tested against rhinoviruses. The structure-based drug design technique is likely to see many more applications in the future with the development of high-throughput crystallization and structure determination technologies.[47]

In this review, the various inhibitors are separated by their mechanism of inhibition. Thus, all of the alkylating agents are grouped together, followed by acylating agents, phosphonylating agents, and sulfonylating agents. Alkylating agents include widely studied fluoromethyl ketones, chloromethyl ketones, acyloxymethyl ketones, epoxides, aziridines, vinyl sulfones, and other Michael acceptors. Acylating agents include β-lactams, lactones, aza-peptides, and a variety of heterocyclic derivatives. Phosphonylating agents include peptide phosphonates and phosphonyl fluorides, whereas sulfonyl fluorides are the major group of sulfonylating agents. It is not always clear how to classify double-hit inhibitors, which frequently both alkylate and acylate the protease.

Recent Reviews. A variety of reviews have appeared in the literature. These include reviews on protease inhibitors,[48−50] bacterial proteases,[51,52] cysteine and serine protease inhibitors,[53] cysteine protease inhibitors,[54−58] serine protease inhibitors,[59,60] cathepsin inhibitors,[61,62] calpain inhibitors,[63,64] caspases,[65,66] granzymes,[67] rhinovirus 3C proteases inhibitors,[68−70] and proteasome inhibitors.[25,71−74]

III. Alkylating Agents

A. Halomethyl Ketones

Peptidyl chloromethyl ketones were among the first affinity labels developed for serine proteases and indeed were among the first active site-directed irreversible inhibitors reported for any enzyme.[75,76] Schoellmann and Shaw in the early 1960s developed Tos-Phe-CH_2Cl (TPCK) and Tos-Lys-CH_2Cl (TLCK) as specific inhibitors for the serine proteases chymotrypsin and trypsin, respectively. These inhibitors are now so widely used that they are discussed in elementary biochemistry textbooks. Chloromethyl ketone inhibitors irreversibly alkylate the active site histidine residue of serine proteases (Figure 9, X = Cl).

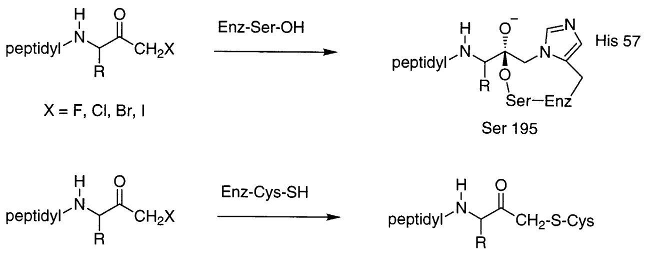


Figure 9. Inactivation of serine and cysteine proteases by peptidyl halomethyl ketone inhibitors.

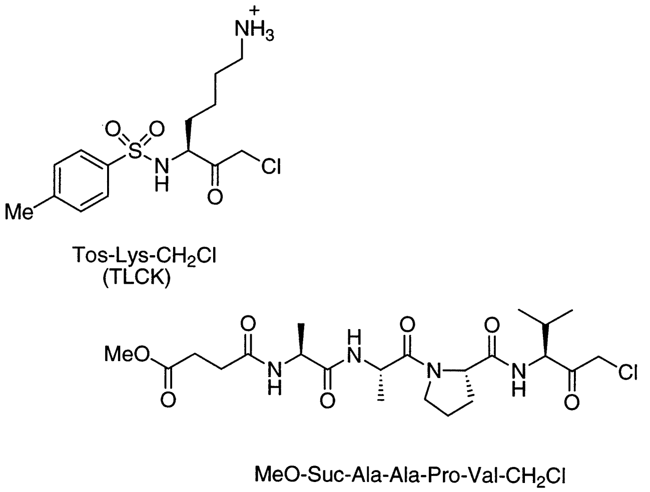


Figure 10. Nomenclature of halomethyl ketone inhibitors.

Early in their development, chloromethyl ketones were considered by some investigators to be histidine-specific reagents. However, numerous X-ray crystallographic structures have shown that chloromethyl ketone inhibitors are transition-state irreversible inhibitors. In the structure of the enzyme inhibitor adduct, the active site Ser 195 of the enzyme forms a tetrahedral adduct with the carbonyl group of the inhibitor, and the active site histidine is alkylated by the chloromethyl ketone functional group (Figure 9).

Peptidyl chloromethyl ketones were subsequently shown also to be potent inactivators of cysteine proteases. Their time-dependent inhibition results in alkylation of the active site cysteine residue to form an irreversible thioether adduct. Formation of this adduct may involve a thiohemiketal intermediate with the active site cysteine analogous to the hemiketal adduct involved in the serine protease inhibition mechanism.

The development of chloromethyl ketone inhibitors led to the investigation of analogous inhibitor structures with different leaving groups replacing the chlorine atom. Both bromomethyl and iodomethyl ketones have been synthesized and are typically more reactive but less stable in aqueous solutions. Replacement of the chlorine with carboxylates and sulfonates also gave reasonable inhibitor structures, which eventually led to the development of a major class of new inhibitors, acyloxymethyl ketones, which are described in the next section.

Peptide fluoromethyl ketones resisted synthesis for many years. They were postulated to be effective serine protease inhibitors as early as 1967; extensive attempts to synthesize these compounds occurred in the late 1960s and in the 1970s, but the required synthetic methods were not available. The first fluoromethyl ketone inhibitors were reported in the literature by Rasnick in 1985[77] and by Shaw's group in 1986.[78] Due to the inherent unreactivity of carbon—fluorine bonds, peptide fluoromethyl ketones were expected to be potent reversible transition state inhibitors for serine proteases. Indeed, trifluoromethyl ketone inhibitors were later developed and shown to be potent specific reversible inhibitors for serine proteases. However, once they were synthesized, peptide fluoromethyl ketones were shown to be highly reactive and selective irreversible inhibitors for cysteine proteases. They are poor irreversible inactivators for serine proteases.

Considerable specificity for individual serine and cysteine proteases can be obtained by altering the peptide sequence of the inhibitor. Fluoromethyl ketones are, in general, quite specific for cysteine proteases. Chloromethyl ketone inhibitors with the appropriate sequence have been developed as selective inhibitors for almost every serine protease described in the literature. These serine proteases include trypsin-like enzymes (plasmin, thrombin, kallikrein, and factor Xa), chymotrypsin-like proteases (cathepsin G and chymases), elastases (human neutrophil elastase and porcine pancreatic elastase), and many other serine proteases. However, chloromethyl ketones are so reactive they will also inhibit various cysteine proteases even though they are targeted primarily against a particular serine protease. Peptidyl chloromethyl ketones also inhibit a variety of cysteine proteases such as papain, cathepsins B, H, and L, calpains, and caspases. With these potent alkylating agents it is difficult to get absolute specificity with a particular cysteine protease. It should be noted that many investigators often claim specific inhibitors without actually demonstrating that the inhibitor does not react with the other potential target serine and cysteine proteases.

Nomenclature. Halomethyl ketones will be abbreviated RCO-AA-CH_2X, where AA is the amino acid residue and X^- is the leaving halide atom. Two examples are shown in Figure 10. The nomenclature for amino acid and peptide derivatives conforms to the Recommendations of the IUPAC—IUB Commission on Biochemical Nomenclature. Thus, the chloromethyl ketone derived from tosyl-L-lysine is abbreviated Tos-Lys-CH_2Cl instead of the more commonly used TLCK (Figure 10). An amino acid residue is represented by -AA-, for example, -Lys-. Chloromethyl ketones are really chloromethyl derivatives of amino acid residues, and some investigators have referred to these inhibitors as aminoacyl chloromethanes. This designation is proper but is not commonly used in the literature to designate these inhibitors. We will use the more common term, aminoacyl chloromethyl ketones. Sometimes, peptide chloromethyl ketones are abbreviated in the literature RCO-AA-CMK. This is clearly not a preferred abbreviation, and we will use RCO-AA-CH_2Cl.

Mechanism: Serine Proteases. The mechanism of inhibition of serine proteases by halomethyl ketones has been established by a variety of experi-

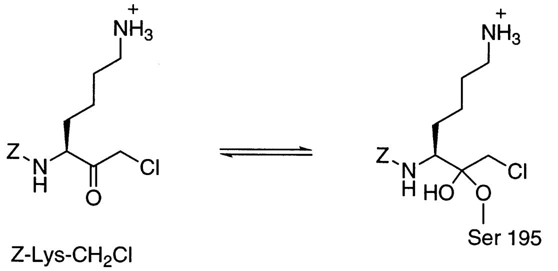


Figure 11. Formation of the covalent tetrahedral adduct in the active site of trypsin.

mental methods including kinetic studies, dilution and dialysis assays, solvent isotope effects, NMR studies, mass spectrometry, and X-ray crystallographic structural data. Early investigators showed that the halomethyl ketone inhibitors form an irreversible covalent adduct with the active site histidine residue of serine proteases by amino acid analysis.[78] Kraut and co-workers were the first investigators to demonstrate that a tetrahedral adduct is present in the serine protease chloromethyl ketone inhibitor complex.[79] Analysis of the crystal structures of subtilisin, which was alkylated with five different chloromethyl ketone inhibitors, revealed that all inhibitors formed a covalent bond between their methylene carbon and the nitrogen of the active site His 64 (57) and a second covalent bond with Ser 221 (195). The second covalent bond generates a tetrahedral adduct between the Oγ serine oxygen and the ketone carbonyl carbon.

A tetrahedral adduct has also been detected by ^{13}C NMR in a trypsin−chloromethyl ketone complex.[80] The carbonyl carbon of the chloromethyl ketone inhibitor Cbz-Lys-CH_2Cl was enriched with C-13 and has signals at 204.7 ppm in the ketone form and at 95.4 ppm in the hemiketal form (hydrate) as a result of the rapid equilibrium between the two forms. The carbonyl carbon of the covalently attached inhibitor Cbz-Lys-CH_2Cl gives a new signal at 98.0 ppm, which is associated with the covalent tetrahedral adduct (Figure 11).

Further evidence for the formation of the hemiketal and the ionized tetrahedral adduct was obtained by electrospray mass spectrometry. Porcine pancreatic elastase incubated with the chloromethyl ketone inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl gave mass shifts corresponding to the hemiketal (**2**, Figure 12), which still contains chlorine, and the covalent tetrahedral adduct (**4**, Figure 12).[81] Furthermore, the intensity of the peaks assigned to the tetrahedral covalent adduct (**4**) increased with the incubation time. Solely on the basis of the mass spectrum, the structure of the final adduct could be either a tetrahedral adduct (**4**) or a ketone (**5**).

The mechanism of inhibition of human leukocyte elastase with the chloromethyl ketone MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl has been studied using kinetics and solvent isotope effects. The data suggest that a Michaelis complex is formed initially, followed by the formation of a second complex, which accumulates. It is proposed that the second complex is the hemiketal formed from attack of the active site serine on the carbonyl carbon of the inhibitor (**2**). The hemiketal (**2**) is stable relative to the Michaelis complex, and it dissociates more slowly than it alkylates the active site histidine residue.[82] The inhibitor exists as a fully formed hemiketal in the rate-limiting transition state. The stability of the hemiketal arises from the utilization of the free energy that is released from the binding of the peptide portion of the inhibitor to the enzyme. Thus, the hemiketal is expected to be less stable for less specific chloromethyl ketone inhibitors.

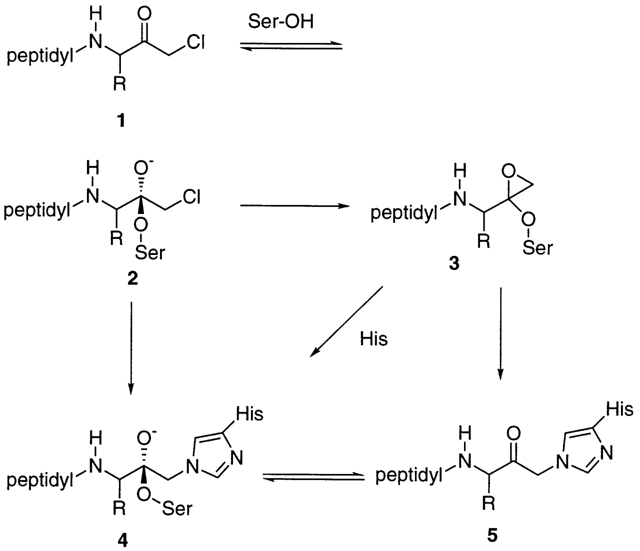


Figure 12. Proposed mechanisms of inhibition of serine proteases by peptidyl chloromethyl ketones.

Initially, two different mechanisms of inactivation of serine proteases by chloromethyl ketones have been proposed. Both mechanisms agreed on the formation of the hemiketal but differed in the mechanism of formation of the alkylated species. Poulos et al. proposed direct displacement of the chloride by the active site histidine, hence a single displacement mechanism (**2** → **4**).[79] On the other hand, Powers has suggested a double-displacement mechanism in which the hemiketal oxyanion displaces the chloride to give an epoxy ether intermediate (**2** → **3** → **4**, Figure 12).[83]

The mechanism of inhibition of chymotrypsin by the chloromethyl ketone Cbz-Ala-Gly-Phe-CH_2Cl and chloroethyl ketone Ac-Ala-Phe-CHCl-CH_3 has been studied by Abeles.[84] In addition to alkylating the enzyme, the chloromethyl ketone also undergoes hydrolysis to the corresponding hydroxymethyl ketone. With chymotrypsin methylated at N-3 of the active site histidine, only the hydrolysis reaction is observed. With methyl chymotrypsin an initial burst of free chloride is detected during the enzyme-catalyzed hydrolysis. The magnitude of the chloride burst is equivalent to 1:1 stoichiometry and indicates a rapid chloride releasing step, which gives an intermediate that is slowly converted to a hydroxyketone. The authors propose that this intermediate is the epoxy ether **3**. With the *S* isomer of the chloroethyl ketone Ac-Ala-Phe-CHCl-CH_3 the nonenzymatic hydrolysis proceeds with inversion of configuration, whereas the enzymatic hydrolysis results in retention of configuration. Retention of configuration is consistent with the initial formation of epoxy ether **3** (inversion of configuration) followed

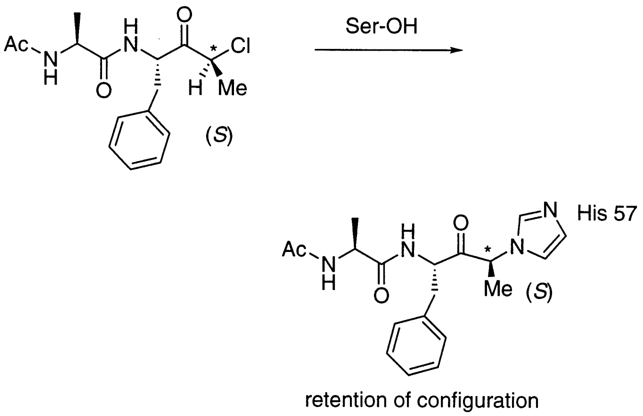


Figure 13. Retention of configuration at the chiral carbon of the peptide chloroethyl ketone inhibitor (2*S*)-Ac-Ala-Phe-$CH(CH_3)Cl$.

by formation of the hydroxy ether by another S_N2 reaction (inversion of configuration). Thus, the overall result of the double-displacement reaction is retention of configuration.

A crystal structure of γ-chymotrypsin inhibited by the chiral peptide α-chloroethyl ketone (2*S*)-Ac-Ala-Phe-$CH(CH_3)Cl$ has been determined. The peptide inhibitor alkylates the His 57 with retention of configuration at the chiral center (see Figure 13). The crystallographic result is consistent with a double-displacement mechanism. Thus, this mechanism involves the initial formation of an epoxy ether intermediate (**3**, Figure 12) followed by displacement by His 57 to form the final adduct (**5**, Figure 12). The bound inhibitor has an unusual conformation with the ketone carbonyl oriented away from the oxyanion hole. There is also no hydrogen bond with the P1 backbone carbonyl oxygen. The P1 side chain is poorly placed in the S1 pocket. This unique binding mode is probably due to the lack of a P3 residue, allowing a nonproductive mode of binding in the active site. In addition, the steric bulk of the methyl group of the chloromethyl ketone makes a normal binding mode unobtainable.[85]

Mechanism: Cysteine Proteases. Cysteine proteases also form an irreversible covalent adduct with halomethyl ketones. However, on the basis of the initial X-ray crystal structural data, it is the active site cysteine residue that is alkylated, forming a thioether bond.[86] Later crystallographic data with different cysteine proteases, such as cathepsin B, caspases-1, -3, and -8, cruzain, and gingipain R, confirmed the observation that the alkylated species is a thioether.

The mechanism of inactivation of cysteine proteases by halomethyl ketones is not clear. There are two possible mechanisms that could lead to the covalent thioether adduct (Figure 14). The first mechanism is the direct displacement of the halide group by the thiolate anion. The second mechanism involves a thiohemiketal (**8**) and a three-membered sulfonium intermediate (**9**). The intermediate structure then rearranges to give the final thioether adduct (**7**).

The crystal structure of caspase-3 inhibited by Ac-Asp-Val-Ala-Asp-CH_2F has been determined, and the structure of the adduct is the thioether **7** (Figure 14). The carbonyl oxygen of the inhibitor interacts with the oxyanion hole and forms a hydrogen bond with the amide proton of Gly 122.[87] These observations support either mechanism, but there is still no evidence of a possible three-membered sulfonium intermediate.

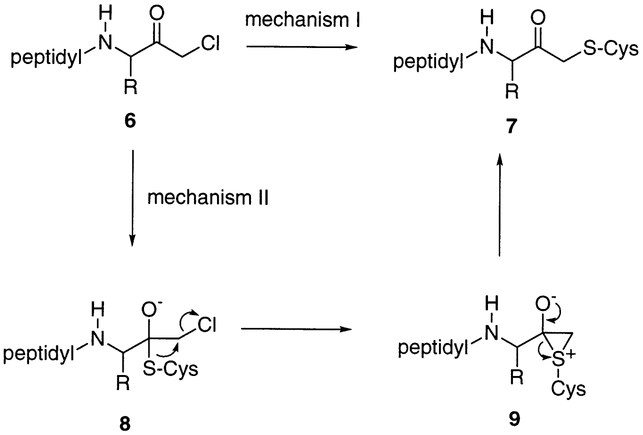


Figure 14. Proposed mechanisms of inhibition of cysteine proteases by peptidyl chloromethyl ketones.

Stability and Specificity. Due to the inherent chemical reactivity of the chloroketone functional group, the major disadvantage of peptidyl chloromethyl ketones is their lack of selectivity. They are reactive toward nontarget molecules such as nonproteolytic enzymes and biomolecules such as glutathione, which makes them unsuitable for many in vivo experiments. Nevertheless, chloromethyl ketones have been widely used in vivo and in animals.

Sortase is an example of an enzyme outside the peptidase group that is specifically inhibited by chloromethyl ketones. The peptidyl chloromethyl ketone analogue Cbz-Leu-Pro-Ala-Thr-CH_2Cl was found to be an irreversible inhibitor of recombinant sortase with a second-order rate constant of 883 M^{-1} s^{-1}.[88] This value is considerably smaller than those previously determined for the inactivation of cysteine proteases by chloromethyl ketone derivatives.

Peptidyl fluoromethyl ketones are not reactive toward bionucleophiles, where their rate of alkylation of glutathione was 0.2% of the rate with chloromethyl ketones.[89] They are also used in a variety of in vivo studies.

Crystal Structures: Serine Proteases. Peptidyl chloromethyl ketones have been useful for the mapping of interactions of peptide side chains with the various subsites of serine proteases. PDB codes of several X-ray crystal structures of serine proteases complexed with chloromethyl ketones are listed in Table 2.

One of the early examples includes the crystal structure of subtilisin inhibited by Phe-Ala-Ala-Lys-CH_2Cl (coordinates not in PDB). This crystal structure revealed that its hydrophobic S1 subsite can actually tolerate charged side chains.[79] The lysine side chain bends into the hydrophobic pocket with the methylene groups fitting into the S1 specificity cavity, whereas its amino group extends outward to interact with the side chain of Glu 156 on the surface of the enzyme. This illustrates the fact that some

Table 2. PDB Codes for X-ray Crystal Structures of Serine Proteases Inhibited with Chloromethyl Ketone Inhibitors

enzyme	inhibitor	PDB code	ref
achromobacter protease I	*p*-Tos-Lys-CH$_2$Cl	1ARC	737
chymase	Suc-Ala-Ala-Pro-Phe-CH$_2$Cl	1PJP	92
chymotrypsin	*N*-Ac-Ala-Phe-CH(CH$_3$)Cl	2GMT	85
chymotrypsin	Cbz-Gly-Gly-Phe-CH$_2$Cl	1DLK	738
coagulation factor IXa	D-Phe-Phe-Arg-CH$_2$Cl	1DAN	739
coagulation factor VIIa	1,5-Dns[a]-Glu-Gly-Arg-CH$_2$Cl	1CVW	91
coagulation factor VIIa	D-Phe-Phe-Arg-CH$_2$Cl	1DAN	740
coagulation factor Xa−trypsin chimera	D-Phe-Pro-Arg-CH$_2$Cl	1FXY	741
elastase (HNE)	MeO-Suc-Ala-Ala-Pro-Val-CH$_2$Cl	1PPG	93
elastase (HNE)	MeO-Suc-Ala-Ala-Pro-Ala-CH$_2$Cl	1HNE	742
plasminogen activator (*t*PA)	Dns-Glu-Gly-Arg-CH$_2$Cl	1BDA	743
plasminogen activator (*t*PA)	Glu-Gly-Arg-CH$_2$Cl	1A5I	743
plasminogen activator (*u*PA)	Glu-Gly-Arg-CH$_2$Cl	1LMW	744
proteinase K	MeO−Suc-Ala-Ala-Pro-Ala-CH$_2$Cl	3PRK	745
subtilisin DY	Cbz-Ala-Pro-Phe-CH$_2$Cl	1BH6	746
thrombin	D-Phe-Pro-Arg-CH$_2$Cl	1PPB	90
thrombin	D-Phe-Pro-Arg-CH$_2$Cl		12
thrombin	D-Phe-Pro-Arg-CH$_2$Cl	1ABJ	747
thrombin	D-Phe-Pro-Arg-CH$_2$Cl	1HAI	748
thrombin Y225F mutant	D-Phe-Pro-Arg-CH$_2$Cl	2THF	749
thrombin Y225I mutant	D-Phe-Pro-Arg-CH$_2$Cl	1B7X	749
thrombin Y225P mutant	D-Phe-Pro-Arg-CH$_2$Cl	1THP	749
thrombin complexed with (desamino Asp 55) hirudin (residues 55−65)	D-Phe-Pro-Arg-CH$_2$Cl	1DWE	750
thrombin complexed with DNA (5′-D(GpGpTpTpGpGpTpGpTpGpGpTpTpGpG)-3′)	D-Phe-Pro-Arg-CH$_2$Cl	1HUT	751
thrombin ternary complexed with hirudin (C-terminal fragment, residues 55−65)	D-Phe-Pro-Arg-CH$_2$Cl	1TMU	752
thrombin complexed with a receptor-based peptide Xa	D-Phe-Pro-Arg-CH$_2$Cl	1NRR	753
thrombin complexed with thrombomodulin	D-Phe-Pro-Arg-CH$_2$Cl	1HLT	753

[a] Dns = dansyl.

serine protease subsites can be much more accommodating than at first expected.

The *β*-sheet antiparallel hydrogen-bonding interaction between the peptide inhibitor and the extended substrate binding site of a serine protease was first observed in the structures of chymotrypsin and subtilisin inhibited by peptidyl chloromethyl ketones. This structural feature is a consistent part of the binding of serine proteases with a large variety of peptide inhibitors.

In the X-ray structure of human α-thrombin (a trypsin-like serine protease) the inhibitor D-Phe-Pro-Arg-CH$_2$Cl is bound to the active site Ser 195 and His 57, forming a hemiketal with the inhibitor P1 carbonyl carbon.[90] The inhibitor backbone forms the antiparallel *β*-sheet interaction with the peptide backbone of Ser 214-Gly 216 in the active site (Figure 15). In the S1 subsite, Arg is well accommodated via a salt bridge interaction with thrombin's S1 Asp 189. In contrast to other serine proteases, the subsites of thrombin appear to be deeper. The S2 subsite, consisting of side chains of Trp 215, Leu 99, His 57, Tyr 60A, and Trp 60D, is encapsulated and hydrophobic compared to trypsin. This cage-like subsite is closed by the D-Phe residue of the inhibitor.

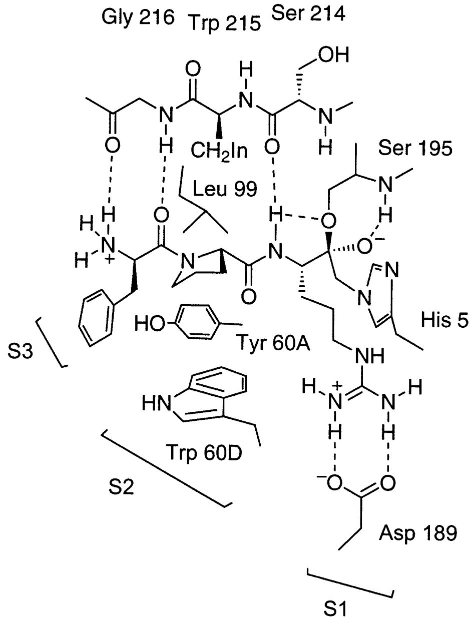


Figure 15. Structure of thrombin complexed with D-Phe-Pro-Arg-CH$_2$Cl.

Factor VIIa inhibited by 1,5-Dns-Gly-Gly-Arg-CH$_2$-Cl (1,5-Dns = 1,5-dansyl) exhibits similar interactions with the active site (Figure 16).[91] A covalent tetrahedral hemiketal adduct is formed with the Ser 195 and His 57 and the P1 carbonyl carbon. The P1 carbonyl oxygen reaches out to the oxyanion hole formed by the backbone NH bonds of Ser 195 and Gly 193. The inhibitor backbone makes favorable hydrogen-bonding interactions in an antiparallel *β*-sheet manner. As is the case with thrombin, the P1 Arg makes salt bridges with Asp 189.

In the crystal structure of human chymase (a chymotrypsin-like serine protease) in complex with the inhibitor Suc-Ala-Ala-Pro-Phe-CH$_2$Cl, the inhibitor is covalently bound to the Ser 195 Oγ and the His 57 N-2 at the carbonyl carbon of the P1 Phe residue (Figure 17).[92] The carbonyl oxygen of the P1

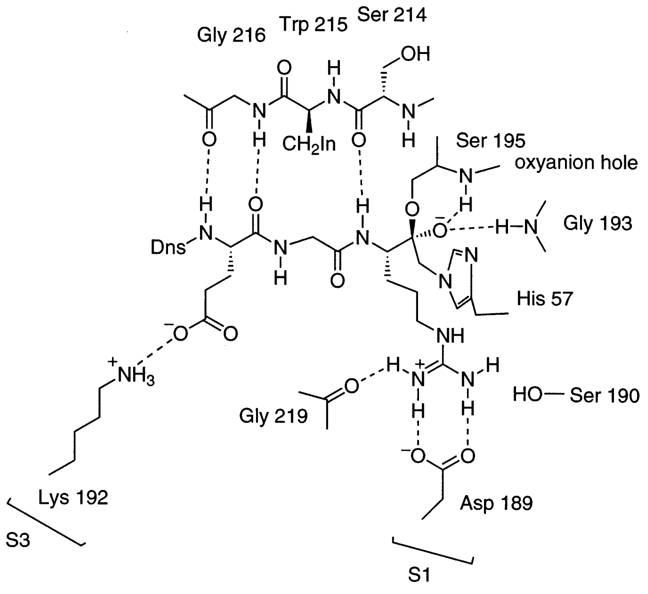


Figure 16. Structure of factor VIIa complexed with 1,5-Dns-Glu-Gly-Arg-CH_2Cl.

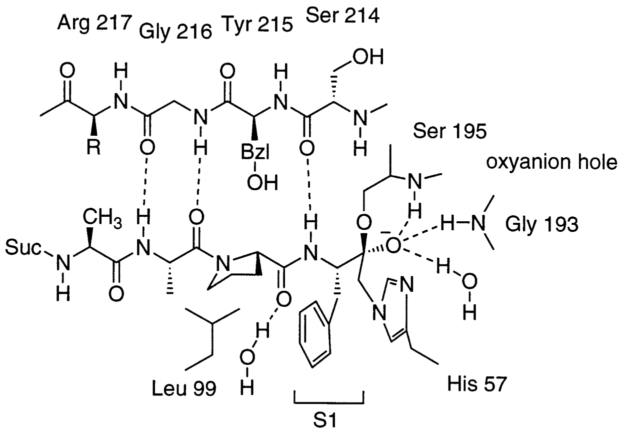


Figure 17. Structure of chymase complexed with Suc-Ala-Pro-Phe-CH_2Cl.

Phe makes hydrogen bonds with Gly 193 and Ser 195, forming the oxyanion hole. The inhibitor backbone makes an antiparallel β-sheet interaction with the Ser 214–Gly 216 residues. The P1 Phe ring is positioned between Phe 191 and Lys 192. The P2 Pro residue makes hydrophobic interactions with Leu 99 at S2.

Human leukocyte elastase in complex with the inhibitor MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl shows a clear preference for Val at P1 (Figure 18). The inhibitor backbone including the succinyl carbonyl forms an antiparallel β-sheet structure with the residues Ser 214–Gly 218. The Val P1 carbonyl carbon participates in the tetrahedral hemiketal adduct formation with Ser 195 Oγ and the His 57 nitrogen. The Val carbonyl oxygen extends into the oxyanion hole, making hydrogen bonds to the backbone NH of Ser 195 and Gly 193.[93]

Crystal Structures: Cysteine Proteases. Peptidyl chloromethyl ketones have also been used to investigate the subsite interactions of cysteine proteases. PDB codes of several cysteine proteases complexed with halomethyl ketones are listed in Table 3.

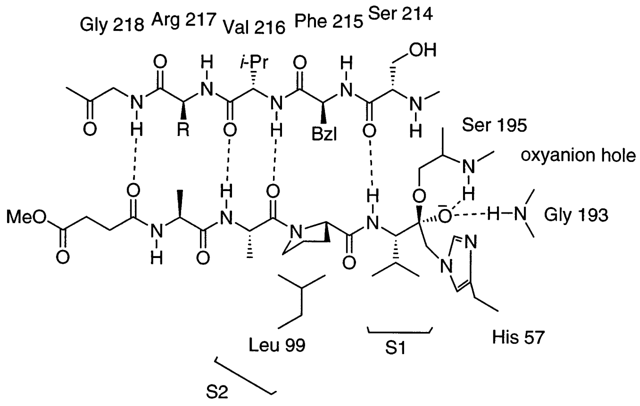


Figure 18. Structure of elastase complexed with MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl.

Table 3. PDB Codes for X-ray Crystal Structures of Cysteine Proteases Inhibited with Halomethyl Ketone Inhibitors

enzyme	inhibitor	PDB code	ref
caspase-3	Ac-Asp-Val-Ala-Asp-CH_2F	1CP3	87
caspase-8	Cbz-Glu-Val-Asp-$CHCl_2$	1QDU	94
cathepsin B	Cbz-Arg-Ser(*O*-Bzl)-CH_2Cl	1THE	754
cruzain	Bz-Tyr-Ala-CH_2F	1AIM	95
cruzain	Bz-Arg-Ala-CH_2F	2AIM	95
gingipain R	D-Phe-Phe-Arg-CH_2Cl	1CVR	22

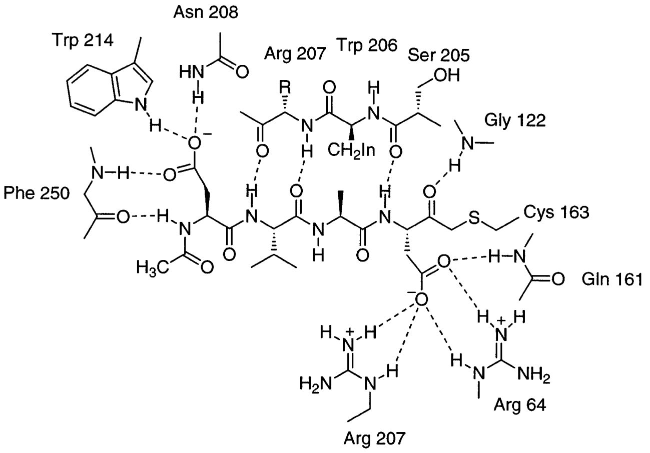


Figure 19. Structure of caspase-3 complexed with Ac-Asp-Val-Ala-Asp-CH_2F.

Early investigators analyzed the binding of various chloromethyl ketone inhibitors such as Cbz-Phe-Ala-CH_2Cl, Cbz-Gly-Phe-Gly-CH_2Cl, and Ac-Ala-Ala-Phe-Ala-CH_2Cl to papain.[86] All of the inhibitors' P1 methylene carbon formed a covalent adduct with the active site Cys 25 sulfur.

Caspase-3 forms a covalent thioether adduct between the active site Cys 163 sulfur and the inhibitor Ac-Asp-Val-Ala-Asp-CH_2F (Figure 19). The carbonyl carbon of the P1 Asp makes hydrogen bonds to the Gly 122 amide proton, which forms an oxyanion hole. The P1 Asp points into a deep pocket, where it makes salt bridges with Arg 64 and Arg 207 and a hydrogen bond with Gln 161. These interactions account for the absolute requirement for an Asp residue at the P1 position. The backbone of the inhibitor makes antiparallel hydrogen-bonding interactions with the backbone of the active site residues Ser 205–Arg 207. These hydrogen-bonding interactions are also seen

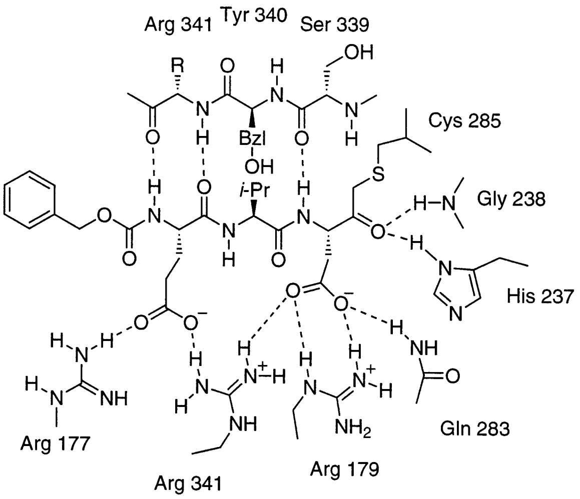


Figure 20. Structure of caspase-8 complexed with Cbz-Glu-Val-Asp-CH_2Cl.

with serine proteases. The S3 subsite is hydrophobic, and the P4 Asp interacts with the backbone of Phe 250 and the NH of Trp 214 and Asn 208 side chain. The interaction with the backbone of Phe 250 is one of the major factors in the substrate specificity of caspase-3.[87]

The crystal structure of caspase-8 complexed with Cbz-Glu-Val-Asp-CH_2Cl exhibits binding interactions similar to those seen with caspase-3. The active site Cys 285 sulfur atom forms a covalent thioether bond with the inhibitor (Figure 20). The carbonyl oxygen points into the oxyanion hole, making hydrogen bonds to Gly 238 and His 237. An antiparallel β-sheet interaction is observed between the inhibitor and the backbone peptide segment Ser 339−Arg 341. The absolute requirement of Asp at the P1 position is clear, because the Asp side chain forms salt bridges with Arg 179 and Arg 341 and a hydrogen bond with Gln 283. The S2 pocket is hydrophobic. The S3 and S4 pockets have the major influence on substrate specificity. The S3 Glu side chain interacts with Arg 177 and Arg 341. In contrast to caspase-3, the caspase-8 S4 subsite has Asn 342 and Trp 348 instead of the Phe 250 in caspase-3. Because there are no residues available that can make hydrogen bonds, caspase-8 prefers hydrophobic residues such as the Cbz group at the P4 position.[94]

Gingipain R is a cysteine protease with a caspase-like fold. Its crystal structure complexed with the inhibitor D-Phe-Phe-Arg-CH_2Cl (Figure 21) has interactions similar to those in caspases. The inhibitor is covalently bound to the active site Cys 244 sulfur, and the carbonyl oxygen of the P1 Arg makes hydrogen bonds to the backbone of Cys 244 and Gly 212, forming the oxyanion hole. The inhibitor backbone makes hydrogen bonds with the Gln 282−Trp 284 segment of the active site, forming a twisted β-sheet. The P1 Arg side chain extends into the S1 pocket, forming a salt bridge with Asp 163 and hydrogen bonds to peptide carbonyl carbons of Gly 210 and Trp 284, which explain the specificity for Arg at the P1 position.[22]

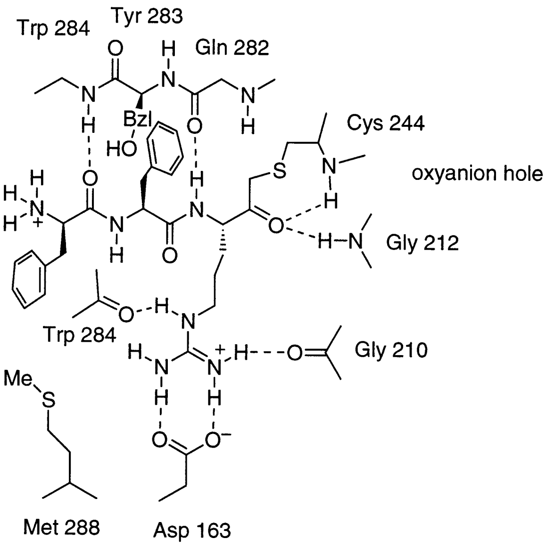


Figure 21. Structure of gingipain R complexed with D-Phe-Phe-Arg-CH_2Cl.

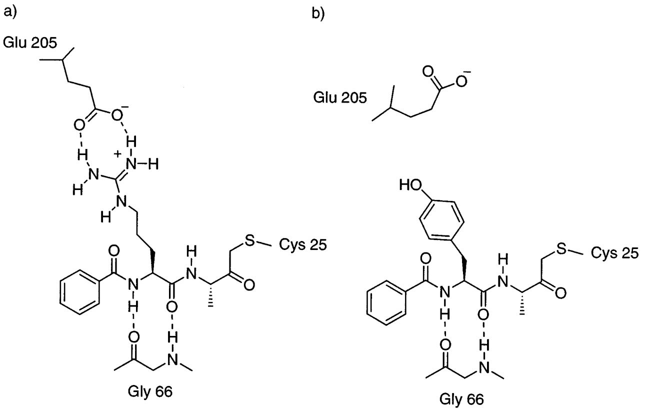


Figure 22. Structure of cruzain complexed with (a) Bz-Arg-Ala-CH_2F and (b) Bz-Tyr-Ala-CH_2F.

The crystal structures of cruzain, an essential cysteine protease from the parasite *Trypanosoma cruzi*, complexed with the inhibitors Bz-Tyr-Ala-CH_2F and Bz-Arg-Ala-CH_2F have been determined (Figure 22).[95] The inhibitor is covalently attached to the active site Cys 25 sulfur. The inhibitor backbone makes hydrogen bonds to the backbone of Gly 66. The Glu 205 at the base of the S2 pocket adopts different conformations according to the nature of the P2 residue. With the P2 Arg side chain, Glu 205 points into the pocket, forming a salt bridge with the positively charged guanidinium group (Figure 22a). With the Tyr at the P2 position, Glu 205 adopts a solvent-directed conformation and points out of the S2 pocket (Figure 22b). Kinetic data support this dual specificity at S2 and indicate that a P2 Phe is preferred over Arg by 15-fold at pH 6.0. Thus, the S2 subsite is an important specificity determinant for cruzain.

Structure—Activity Relationship: Serine Proteases. Chloromethyl ketone inhibitors have been developed and tested for inhibitory activity against trypsin-like, chymotrypsin-like serine proteases, elastases, and most other serine proteases. Kinetic constants for many other older peptide chloromethyl ketones have been reviewed by Powers.[59]

Table 4. Inactivation of Trypsin-like Serine Proteases by Peptidyl Halomethyl Ketones

	k_2/K_i (M^{-1} s^{-1})						
inhibitor	plasmin	plasma kallikrein	thrombin	urokinase	factor Xa	trypsin	ref
D-Phe-Pro-Arg-CH_2Cl	1000	800	9600000	1000	2300	3500000	755
D-Ile-Pro-Arg-CH_2Cl	2300		8100000	600	25000	3200	755
D-Ile-Phe-Arg-CH_2Cl	400	56000	400	<1	240	4200	756
D-Ile-Phe-Lys-CH_2Cl	77000	49000	2700	<1	1100	19000	756
Ile-Phe-Lys-CH_2Cl	5200	5100	130	<1	46	2100	756
Ile-Phe-Arg-CH_2Cl	130	8800	120	<1	88	950	756
Glu-Gly-Arg-CH_2Cl	100		60	7400	1300	107000	755
D-Val-Gly-Arg-CH_2Cl	260		86200	500	32500	548000	755
D-Phe-Pro-Arg-CH_2F	1.8	114	696		15	466	757
Bz-Phe-Arg-CH_2F	NI[a]	NI	200		NI	70	757

[a] NI = no inhibition.

Trypsin-like serine proteases prefer tripeptide inhibitors with Arg at P1. Quite potent, selective inhibitors for kallikrein, factor Xa, and thrombin have been reported (Table 4). Most of the second-order rate constants are in the range of 10^4 M^{-1} s^{-1}. However, some of the rates (thrombin and D-Phe-Pro-Arg-CH_2Cl) are close to diffusion controlled. Selective inhibitors for plasmin are hard to develop because of the broad specificity of this enzyme.

Chymotrypsin-like serine proteases prefer inhibitors with Phe at the P1 position. Their inhibition by chloromethyl ketones is quite slow. For example, chymotrypsin is inhibited by the tripeptidyl chloromethyl ketone inhibitor Boc-Gly-Leu-Phe-CH_2Cl with a second-order rate constant of 61 M^{-1} s^{-1}.[96] Another inhibitor, Suc-Pro-Leu-Phe-CH_2Cl, inhibits chymotrypsin with a second-order rate constant of 3.6 M^{-1} s^{-1}.[97] Chymases appear to be inactivated more rapidly by this class of inhibitor. The inhibitor Boc-Gly-Leu-Phe-CH_2Cl has a second-order rate constant of 173 M^{-1} s^{-1} for human skin chymase. Proteinase II (RMCP II) and cathepsin G are inactivated quite slowly by the same inhibitor, with second-order rate constants of 1.6 M^{-1} s^{-1} for RMCP II and 15 M^{-1} s^{-1} for cathepsin G.[96]

A derivative of the classical chloromethyl ketone derivative Cbz-Phe-CH_2Cl was found to be a potent chymase inhibitor with no inhibitory activity against human leukocyte cathepsin G.[98] This chloromethyl ketone 2-F-C_6H_4-CH_2CH_2CO-Phe-CH_2Cl has a CH_2 instead of the Cbz oxygen and an additional fluorine atom of the phenyl ring. It inhibits human chymase with an IC_{50} value of 0.36 μM, whereas no inhibitory activity is observed with cathepsin G at 1 mM. Cathepsin G is thought to have a role in the angiotensin II generation. Therefore, this inhibitor should be valuable for determining the physiological and pathological roles of chymases in the local generation of angiotensin II.

Several highly specific elastase chloromethyl ketone inhibitors have been developed (Table 5). The best inhibitor, MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, has a second-order rate constant of 1560 M^{-1} s^{-1}. This inhibitor is very specific as none of the chymotrypsin-like enzymes were inactivated by it.

Table 5. Inactivation of HNE and PPE by Peptidyl Chloromethyl Ketones[83]

	k_2/K_i (M^{-1} s^{-1})	
inhibitor	HNE[a]	PPE[b]
MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl	1560	55
Suc-Ala-Ala-Pro-Val-CH_2Cl	1400	73
Ac-Ala-Ala-Pro-Val-CH_2Cl	219	35
Ac-Ala-Ala-Pro-Ala-CH_2Cl	9.7	40
Ac-Ala-Ala-Pro-Ile-CH_2Cl	133	48

[a] HNE = human neutrophil elastase. [b] PPE = porcine pancreatic elastase.

Table 6. Inactivation of Cathepsin B by Peptidyl Halomethyl Ketones

inhibitor	k_2/K_i (M^{-1} s^{-1})	ref
Cbz-Phe-Ala-CH_2Cl	45300	77
Cbz-Phe-Ala-CH_2F	16200	77
Cbz-Phe-Phe-CH_2Cl	9000	78
Cbz-Phe-Phe-CH_2F	3700	78
Bz-Phe-Lys-CH_2F	105000	89
Ala-Phe-Lys-CH_2F	300000	89
Bz-Phe-Arg-CH_2F	390000	757
$PhCH_2OCOCH_2CH_2CO$-Phe-Ala-CH_2F	21000	99

Structure—Activity Relationship: Cysteine Proteases. The best peptidyl chloromethyl ketones for specific cysteine proteases frequently have the same amino acid sequence as a good peptide substrate. Several inhibitors for cathepsin B are listed in Table 6, and the best dipeptide chloromethyl ketone inhibitors have a Phe residue in the P2 position. The second-order rate constant k_2/K_i values are in the range of 10^4 M^{-1} s^{-1}. Peptidyl fluoromethyl ketones are as potent as chloromethyl ketones for the inhibition of cathepsin B. The first peptidyl fluoromethyl ketone, Cbz-Phe-Ala-CH_2F, has a Phe at P2 and is a good inactivator of cathepsin B with a k_2/K_i value of 16200 M^{-1} s^{-1}. The fluoromethyl ketone inhibitors with positively charged side chains such as Lys and Arg at the P1 position are potent inhibitors of cathepsin B, with second-order rate constants in the range of 10^5 M^{-1} s^{-1}. The best inhibitor is Bz-Phe-Arg-CH_2F, with a k_2/K_i value of 390000 M^{-1} s^{-1}. Potent dipeptidyl fluoromethyl ketones were obtained by variation of the N-terminal groups while the Phe-Ala sequence was kept constant.[99] The k_2/K_i values varied over 20-fold, suggesting that there is a significant contribution to inhibitory potency of the N-terminal part of the inhibitor. The inhibitor $PhCH_2OCOCH_2CH_2CO$-Phe-Ala-CH_2F was the most potent cathepsin B inhibitor in this series, with a second-order rate constant of 21000 M^{-1} s^{-1}.

Tripeptidyl chloromethyl ketones designed with appropriate peptide recognition sequences are potent

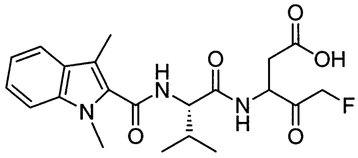
Table 7. Inactivation of Calpains by Peptidyl Chloromethyl Ketones[758]

inhibitor	ID_{50} (μM) calpain I	ID_{50} (μM) calpain II
Leu-Leu-Phe-CH_2Cl	0.20	0.19
Leu-Leu-Tyr-CH_2Cl	0.34	0.23
Leu-Leu-Lys-CH_2Cl	0.62	0.78
Tos-Phe-CH_2Cl	120	95
Tos-Lys-CH_2Cl	35	64
E-64	1.0	2.6

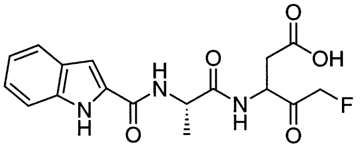
inhibitors of calpains I and II. The best inhibitor was Leu-Leu-Phe-CH_2Cl, having ID_{50} (the concentration of an inactivator necessary for 50% inactivation) values in the micromolar range and being 500−600-fold more potent than Tos-Phe-CH_2Cl and 4−5-fold more potent than the epoxysuccinate E-64 (Table 7).

Tripeptidyl fluoromethyl ketones were also tested with calpain I and cathepsin L. The inhibitor Cbz-Leu-Leu-Tyr-CH_2F was a better inhibitor of cathepsin L than calpain I, but this inhibitor was more effective against calpain I in intact platelets.[100] More potent and selective fluoromethyl ketone inhibitors of calpain I were developed by introducing different N-terminal groups on the Leu-Phe sequence.[101] The heterocyclic 1,2,3,4-tetrahydroisoquinolin-2-yl-carbonyl-Leu-Phe-CH_2F was one of the most potent and selective inhibitors ($k_2/K_i = 276000$ M^{-1} s^{-1}). It was selective for calpain I over cathepsins B and L by 36- and 6-fold, respectively. Inhibitors with a Boc N-terminal group were well tolerated and were more selective inhibitors for calpain I than cathepsins B and L. The inhibitor Boc-Leu-Phe-CH_2F preferred calpain I over cathepsin L >680-fold, and morpholino-4-sulfonyl-Leu-Phe-CH_2F was selective for calpain I >670-fold over cathepsin B. The tripeptidyl Cbz-Leu-Leu-Phe-CH_2F was also a very potent inhibitor ($k_2/K_i = 290000$ M^{-1} s^{-1}) but was not as selective as the other inhibitors mentioned.[101]

Many fluoromethyl ketone inhibitors for caspases, such as Cbz-Val-Ala-Asp-CH_2F, Cbz-Asp-Glu-Val-Asp-CH_2F, and Cbz-Tyr-Val-Ala-Asp-CH_2F are available from commercial sources (see Table 72). Although fluoromethyl ketones are widely used in biological studies, good irreversible inhibition rates with caspases and other cysteine proteases are not yet available in the literature for most of these compounds. Wu et al. reported that the fluoromethyl ketone derivatives IDN1965 and IDN1529 are potent inhibitors of caspases-1, -2, -3, -6, -8, and -9 (Figure 23).[94] Their design includes the reactive fluoromethyl ketone group, an Asp at P1, and a peptidomimetric moiety at P3 position. IDN1965 exhibits some selectivity for caspases-6, -8, and -9. The second-order rate constants are 2860, 1380, 13300, 17000, 21500, and 54700 M^{-1} s^{-1} for caspases-1, -2, -3, -6, -8, and -9, respectively. IDN1529, on the other hand, is a broad-spectrum caspase inhibitor with second-order rate constants of 47300, 30400, 21800, 14100, 70500, and 691700 M^{-1} s^{-1} for caspases-1, -2, -3, -6, -8, and -9, respectively. The rate constant k_2 for the covalent reaction that follows the non-covalent binding is highest for caspase-3 for both inhibitors (0.0128 s^{-1} for IDN1965 and 0.0042 s^{-1} for IDN1529). Although both inhibitors involve the same reactive warhead in their designs, their reactivity depends on the relative orientation of the reactive group at the active site.

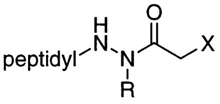


IDN1965



IDN1529

Figure 23. Fluoroketone inhibitors of caspases.



X = F, Cl, Br, I

Figure 24. Aza-peptide derivatives of peptide halomethyl ketones.

Aza-peptide Halomethyl Ketone Derivatives. Aza-peptide derivatives of peptide halomethyl ketones are derivatives in which the α-carbon has been replaced by a nitrogen (see Figure 24). They are diacyl hydrazides with one of the acyl groups having an α-halide, which is a potent alkylating agent. We will refer to these inhibitors as aza-peptide halomethyl ketones due to their resemblance to peptidyl halomethyl ketones.

Aza-peptide analogues of halomethyl ketones are potent inhibitors of papain, cathepsin B, calpains, caspase-1, and the 3C protease from human rhinovirus strain 1B (HRV 3C protease). No inhibitory activity could be detected toward trypsin and porcine pancreatic elastase. With cathepsin B, aza-peptide analogues of halomethyl ketones have second-order rate constants in the range of 10^2−10^5 M^{-1} s^{-1}.[102] For all of the inhibitors, the inhibitory potency increased in the order $X = I > Br > Cl$. The dipeptidyl aza-analogues Cbz-Tyr(I)-AGly-CH_2X ($X = Cl$, Br, I) are the most potent inhibitors in this series of inhibitors, and the second-order rate constants followed the order 306000 M^{-1} s^{-1} (I) > 267000 M^{-1} s^{-1} (Br) > 95700 M^{-1} s^{-1} (Cl). The Tyr(I) residue is preferred 30-fold over Phe at the P2 position. Although the S1 pocket of cathepsin B can accommodate an Ala side chain, a clear preference for Gly is observed at that position. One major factor for this preference is due to the conformation of 1,2-diacyl hydrazines. As seen in Figure 25, two internal hydrogen-bonding interactions within the molecule result in an almost planar central hydrazide structure and orthogonal positioning of the phenyl rings.

The dipeptide analogues with the azaglycine residue resemble simple 1,2-diacyl hydrazines and allow positioning of the side chain of the particular amino acid at the P2 position. Another factor for preference

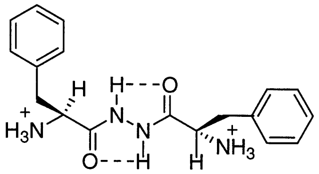


Figure 25. Conformation of a 1,2-diacyl hydrazine with two L-phenylalanine residues.

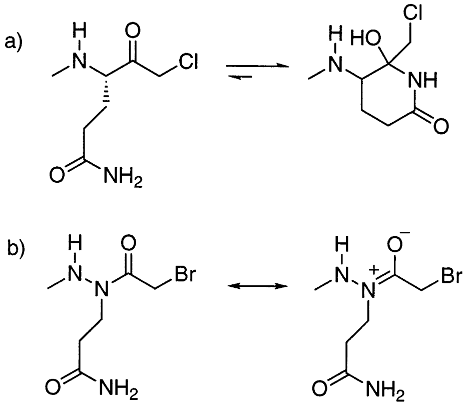


Figure 26. (a) Formation of a cyclic hemiaminal and (b) resonance conjugation of the nitrogen lone pair in the backbone.

Table 8. Inactivation of Human Rhinovirus 3C Protease by Aza-peptide Analogues of Bromomethyl Ketones[103]

	k_2/K_i[a] (M^{-1} s^{-1})			
inhibitor	HRV 3C	chymotrypsin	HNE	cathepsin B
Boc-Ala-AGln-CH_2Br	80	<1	<1	1
Boc-Val-AGln-CH_2Br	300	<1	<1	3
Boc-Phe-AGln-CH_2Br	310	<1	<1	18
Cbz-Val-AGln-CH_2Br	2460	<1	*b*	8
Cbz-Phe-AGln-CH_2Br	4800	1.8	1.1	18
Boc-Ile-Phe-AGln-CH_2Br	1540	<1		9
Boc-Ala-Ile-Phe-AGln-CH_2Br	23400	<1	10	7
Boc-Ile-Thr-Thr-AGln-CH_2Br	14500	<1	<1	4

[a] Compounds with values <1 showed little or no inhibition at 100 μM. [b] $IC_{50} = 54$ μM.

of Gly at the P1 position may be an additional hydrogen-bonding interaction with the enzyme's active site. Only Gly would be able to form this special hydrogen-bonding interaction.

Aza-peptide analogues of halomethyl ketones are less potent inhibitors of calpains I and II compared to cathepsin B.[102] The preferences for Tyr at the P1 position and for Leu at the P2 position are utilized in the aza-peptide analogue structures for calpains. The inhibitory potency of these compounds turned out to be low. Only the iodoacetyl derivative Cbz-Leu-Leu-ATyr-CH_2I displayed moderate reactivity (k_2/K_i = 450 M^{-1} s^{-1} for calpain II). Tripeptidyl aza analogues with Gly at the P1 position were more potent, with a second-order rate constant of 5000 M^{-1} s^{-1} for calpain II. The increase in potency might be due to the same interactions as in the case of cathepsin B (Figure 25).

HRV 3C protease is potently and selectively inhibited by aza-peptide analogues of bromomethyl ketones.[103] HRV 3C protease has a preference for the Ala-Ile sequence at the P4–P3 positions and for Gln residue at the P1 position. However, the synthesis of chloromethyl ketone inhibitors with a Glu residue at the P1 position was not successful because of the formation of a cyclic hemiaminal as shown in Figure 26a. This problem is solved by incorporating the aza functional group at the P1 position. The resonance conjugation of the nitrogen lone pair in the backbone would reduce the electrophilicity of the carbonyl carbon (Figure 26b). Hence, the potency of the inhibitors would be diminished.

By introducing the bromomethyl group, potent inhibitors for HRV 3C protease were obtained. The second-order rates of inhibition of aza-peptide analogues of bromomethyl ketones are in the range of $10-10^4$ M^{-1} s^{-1} (Table 8). Boc-Ala-Ile-Phe-AGln-CH_2Br is the best inhibitor with a k_2/K_i value of 23400 M^{-1} s^{-1}. Little or no inhibition was observed with chymotrypsin and elastase. Cathepsin B is only slightly inhibited, with second-order rate constants in the range of 1–10 M^{-1} s^{-1}. The LC-MS and tryptic digest analysis shows elimination of the bromide ion and covalent bond formation between the enzyme active site Cys 148 and the inhibitor.

Biological Studies. Peptidyl chloromethyl ketones have very little biological utility because of their potential toxicity that results from nonselective alkylation of cellular nucleophiles. However, peptidyl chloromethyl ketones have been useful tools in vivo to identify whether a particular serine protease inhibitor can have a therapeutic effect on a disease state or in an animal model. For example, neutrophil elastase inhibitors have been evaluated extensively in various emphysema animal models as potential therapeutic agents for treatment of human diseases. Emphysema can be induced in hamsters by intratracheal instillation of porcine pancreatic elastase (PPE) or human neutrophil elastase (HLE) and is ameliorated by intratracheal instillation of MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl (AAPV-CH_2Cl). One milligram of AAPV-CH_2Cl is given to hamsters 1 h before instillation of 300 or 360 μg of HLE or 1 or 4 h after instillation of 360 μg of HLE. The animals were studied for eight weeks after the treatment. The AAPV-CH_2Cl given 4 h after HLE did not ameliorate the emphysema. The AAPV-CH_2Cl given 1 h before HLE ameliorated the emphysema but not the bronchial secretory cell metaplasia. A molar ratio of instilled AAPV-CH_2Cl to HLE of 128 was required for 50% in vivo effectiveness in ameliorating emphysema. Clearance studies indicated that 6.9% of the instilled AAPV-CH_2Cl could still be lavaged from the lungs 1 h after instillation. These bioassays demonstrated the in vivo effectiveness of this chloromethyl ketone. Peptide chloromethyl ketones were the first class of compounds to be tested in animal models of emphysema and found to be effective, but the renal toxicity observed in these experiments prevented the further clinical use of chloromethyl ketones.

Thrombin is a key coagulation protease because it generates fibrin, which is cross-linked to form the thrombus matrix structure. In addition to mediating fibrin-rich venous thrombus formation, thrombin has a critical role in the activation of platelets during the formation of arterial thrombi.[104] The aggregation of the platelets enlarge the thrombus in a process that is resistant to heparin and aspirin but is effectively inhibited by low molecular weight synthetic thrombin inhibitors. The continuous infusion of the specific irreversible thrombin inhibitor D-Phe-Pro-Arg-CH_2Cl (PPACK, 100 nmol/kg/min) abolished platelet accumulation and occlusion of thrombogenic segments in baboon models of thrombosis. When PPACK was used as an anticoagulant for rabbit blood, clotting was prevented for at least 6 h at room temperature, but nearly all of the platelets agglutinated. Thus, PPACK cannot be used as an anticoagulant for rabbit blood.[105] PPACK was also used in a rat model of aspirin-insensitive arterial thrombosis.[106] Intravenous injection of PPACK (6 mg/kg) decreased thrombus weight by 90%. Reductions in thrombus weight were always associated with improvements in either average blood flow or vessel patency. The effect of PPACK on baboons subjected to carotid endarterectomy were evaluated to determine the relative antithrombotic efficacy and hemostatic safety of antithrombin therapy for vascular thrombus formation at sites of mechanical vascular injury.[107] The continuous intravenous injection of PPACK, 100 nmol/kg/min for 1 h, abolished acute carotid endarterectomy thrombosis for at least 48 h.

Abnormal bleeding is associated with the systemic administration of PPACK, and this can be reduced via local delivery.[108] Local delivery produced maximal inhibition of thrombosis without alterations in hemostasis in segments of thrombogenic vascular graft interposed in arteriovenous shunts in a porcine model. PPACK has been evaluated for its antithrombotic and hemostatic capacity in rabbits and compared to a specific factor Xa inhibitor, C921-78, and heparin.[109] At a maximally effective dose, only PPACK demonstrated dose-dependent thrombocytopenia. It is concluded that specific inhibition of factor Xa can be utilized for effective antithrombotic activity without any disruption of hemostatic parameters.

Serine protease inhibitors are very effective in suppressing cellular and humoral immune responses. The serine protease inhibitor Tos-Lys-CH_2Cl (TLCK), which is specific for trypsin-like enzymes, suppressed acute allograft rejection, suggesting a novel immunosuppressive strategy for treatment of acute organ rejection.[110] Tos-Phe-CH_2Cl (TPCK), an inhibitor of chymotrypsin-like serine protease, reduces hypoxic-ischemic brain injury in rat pups. Pretreatment with Tos-Phe-CH_2Cl in the newborn rat model of hypoxic-ischemic brain injury reduces DNA fragmentation, nitric oxide production, and brain injury.[111] Tos-Phe-CH_2Cl is also effective on post-traumatic brain injury and neuronal apoptosis. It prevents DNA fragmentation and apoptotic cell death in certain blood cell lines and reduces hippocampal damage caused by cerebral ischemia in rats.[112] Tos-Phe-CH_2Cl also prevents taxol-induced cell death of MCF-7 breast cancer cells.[113] Tos-Phe-CH_2Cl was effective on the MCF-7 cells phenotype, where an increase in the heat shock protein HSP27 content was observed.[114] It is hypothesized that a post-translational control on estrogen-regulated heat shock protein HSP27 levels by a serine protease might be operating in human mammary tumor cells.

The chloromethyl ketones Ac-Cys(dodecyl)-CH_2Cl and Ac-Cys(*trans*,*trans*-farnesyl)-CH_2Cl, which are probably inhibitors of a peptidase that cleaves farnesylated peptides, showed potent cytotoxicity against human B-lineage (Nalm-6) and T-lineage (Molt-3) acute lymphoblastic leukemia cell lines with IC_{50} values in the low micromolar range.[115] The *S*-alkyl chain length was a determinant of the antileukemic activity of these chloromethyl ketone compounds.[116] The undecyl and dodecyl derivatives are the most effective, with IC_{50} values of 1.7 and 2.0 μM against B-lineage leukemia cells, respectively. The hexyl derivative, on the other hand, is the best against T-lineage leukemic cells ($IC_{50} = 0.7\ \mu$M). The p-53-deficient Nalm-6 cell line was previously shown to be resistant to multiple chemotherapeutic agents such as alkylating agents, steroids, topoisomerase I inhibitors, topoisomerase II inhibitors, vincristine, and taxol. Therefore, the sensitivity of Nal-6 cells to the cysteine chloromethyl ketone derivatives is quite encouraging.

Fluoromethyl ketone inhibitors have been useful in early studies of the function of parasite proteases. *T. cruzi* is the causative agent of Chagas' disease, and cruzain, its major protease, is a possible target enzyme for chemotherapy. In an animal model of Chagas' disease, treatment with a peptide fluoromethyl ketone rescued mice from lethal infection.[117] Similarly, the orally administered Mu-Phe-Hph-CH_2F inhibitor of falcipain delayed the progression of murine malaria in mice.[118] Mu-Phe-Hph-CH_2F blocked parasite hemoglobin degradation and development at nanomolar concentrations. However, because of the potential toxicity of the fluoromethyl ketones, they could not be developed as drugs. The fluoromethyl ketone derivatives of amino acids can be metabolized to fluoroacetate, which enters the Krebs cycle and shuts down cellular ATP production.[119]

The fluoromethyl ketone inhibitor Cbz-Phe-Ala-CH_2F is a potent inhibitor of human cathepsin B and significantly decreased the severity of arthritis in rats.[120] This cathepsin B inhibitor was also found to prevent lipopolysaccharide-induced cytokine production of IL-1α, IL-1β, and tumor necrosis factor at the transcriptional level.[121] These results suggest that the previously observed therapeutic effects of Cbz-Phe-Ala-CH_2F are not due to cathepsin B inhibition alone but can also result from the inhibition of NF-κ-B-dependent gene expression.

The tetrapeptide chloromethyl ketone inhibitor Ac-Tyr-Val-Ala-Asp-CH_2Cl prevented cell death in neuronal cells by inhibiting cathepsin B.[122] This inhibitor is normally considered to be a caspase-1 inhibitor. This observation supports the role of cathepsin B in neuronal cell death. Inhibition of caspase-1-like activity by Ac-Tyr-Val-Ala-Asp-CH_2Cl induces a long-lasting neuroprotection in cerebral ischemia through apoptosis reduction and decrease of proinflammatory cytokines.[123]

Fluoro- and chloromethyl ketone inhibitors with P1 Asp have been used as tools for studying the mechanism of apoptosis and the intracellular signal cascade of cells in numerous studies.[124−137] The caspase-1 inhibitor Boc-Asp(OMe)-CH_2F has also been shown to inhibit apoptotic cell death for up to 48 h after a single application of 20 μM.[138] It was shown that a combination of the application of Boc-Asp(OMe)-CH_2F and systemic hypothermia is strongly effective against neuronal damage in the developing rat brain. A reduction of caspase-3 activity was observed as well.[139]

The broad-spectrum caspase inhibitor Cbz-Val-Ala-Asp-CH_2F was shown to be beneficial in brain ischemia.[140] Ischemic damage following 2 h of oxygen−glucose deprival (OGD) could be reduced by up to 56% with Cbz-Val-Ala-Asp-CH_2F.

In a mouse model of traumatic spinal cord injury it was demonstrated that both caspase-1 and caspase-3 are activated in neurons following the injury.[141] Caspase inhibition by Cbz-Val-Ala-Asp-CH_2F reduces post-traumatic lesion size and improves motor performance. Caspase inhibitors may be one of the agents to be used for the treatment of spinal cord injury.

The effect of inhibition of caspases on myocardial dysfunction following endotoxin treatment was investigated with Cbz-Val-Ala-Asp-CH_2F. Not only does it reduce caspase activities and nuclear apoptosis, but it also completely prevented endotoxin-induced myocardial dysfunction evaluated 4 h and even 14 h after endotoxin challenge.[142] These results suggest that inhibitors of caspases may have important therapeutic applications in sepsis.

Systemic lupus erythematosus (SLE) is a common, potentially fatal, non-organ-specific autoimmune disorder. Cbz-Val-Ala-Asp(OMe)-CH_2F was shown to be beneficial in the treatment of human SLE.[143] Daily administration of Cbz-Val-Ala-Asp(OMe)-CH_2F to female transgenic mice over a three week period resulted in significant amelioration of both glomerular and interstitial renal damage, independent of the effects on autoantibody levels of skin inflammation.

It has been speculated that peptidyl fluoromethyl ketones are metabolized in rodents to give the extremely toxic compound, fluoroacetate. Fluoroacetate formation has in fact been demonstrated following the administration of the cathepsin B inhibitor Cbz-Phe-Ala-CH_2F.[144]

B. Diazomethyl Ketones

Peptidyl diazomethyl ketones are irreversible inhibitors of cysteine proteases and inhibit the enzyme by irreversible alkylation of the active site thiol group (Figure 27). The diazomethyl ketone functional group was first observed to be an affinity label when Buchanan and co-workers showed that the antibiotic azaserine, a diazoacetyl derivative,[145] inhibited an enzyme in the purine biosynthesis pathway by alkylation of a cysteine residue. The acid protease pepsin was then observed to be inhibited by diazomethyl ketones in the presence of copper ion with the resulting esterification of an aspartate residue.[146] Two diazomethyl ketones, Cbz-Phe-CHN_2 and Cbz-Phe-Phe-CHN_2, were found to irreversibly inactivate papain, a cysteine protease.[147]

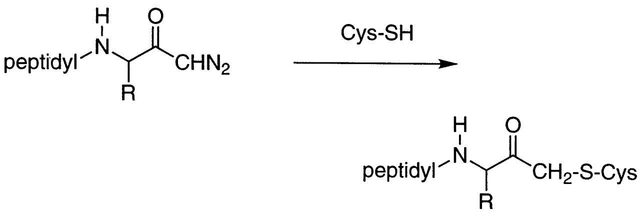


Figure 27. Inactivation of cysteine proteases by peptidyl diazomethyl ketones.

Mechanism. The inhibition mechanism of diazomethyl ketones is not yet completely understood, but it probably involves a proton transfer from the active site histidine to the methylene carbon of the inhibitor with the loss of N_2 and alkylation of the active site Cys residue (**10** → **13**). It is also possible that the active site cysteine adds to the carbonyl group of the inhibitor to give a tetrahedral adduct (**11**, Figure 28), which then rearranges to the stable thioether derivative (**13**, Figure 28).

Stability. Diazomethyl ketones are stable in the presence of dithiothreitol (DTT) and mercaptoethanol, which are necessary for accurate measurement of cysteine protease activity. Diazomethyl ketones are also cell permeable, which makes them suitable for use in vivo.[148] Experiments with various human tissues revealed that radiolabeled diazomethyl ketones mainly target cysteine proteases. They have been used to identify target cysteine proteases and investigate their roles in cells.[100]

Crystal Structures. A few peptidyl diazomethyl ketones have also been used for the investigation of subsite interactions with cysteine proteases. The crystal structure of glycyl endopeptidase inhibited with the diazomethyl ketone inhibitor Cbz-Leu-Val-Gly-CHN_2 is shown in Figure 29.[149] The inhibitor is covalently bound to the active site Cys 25. The S1 subsite consists of the side chains of Glu 23 and Arg 65, which allows small residues such as a Gly residue to be the P1 residue. This is consistent with the observed kinetic P1 specificity of that enzyme. The side chain of the P2 Val makes hydrophobic interactions with side chains of Val 133 and Ala 160. The backbone carbonyl and nitrogen of the S2 Val make a short antiparallel β-sheet interaction with Gly 66. The S3 subsite is defined by side chains of Tyr 61 and Tyr 67, forming a hydrophobic pocket. Both of these side chains are highly conserved in papain-like proteases. The phenyl ring of the Z protecting group makes hydrophobic interactions with the side chains of Ser 209 and Val 157. This is in contrast to the Z group binding mode in the papain−Cbz-Gly-Phe-Gly-CH_2Cl complex, which binds on the opposite side of the cleft.

Structure−Activity Relationships. Early in their development, diazomethyl ketones were thought to be specific inhibitors of cysteine proteases, because they did not inhibit other classes of proteases including serine proteases, metalloproteases, and aspartyl proteases.[150] However, it has been shown later that diazomethyl ketones slowly inactivate several serine proteases[151−153] and the proteasome.[154]

Diazomethyl ketone inhibitors have been developed for cysteine proteases such as papain, cathepsins B,

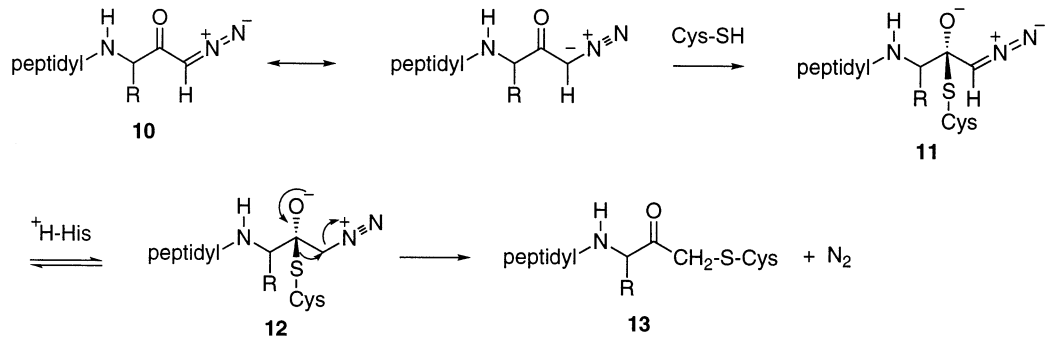


Figure 28. Proposed mechanism of inhibition of cysteine proteases by peptidyl diazomethyl ketones.

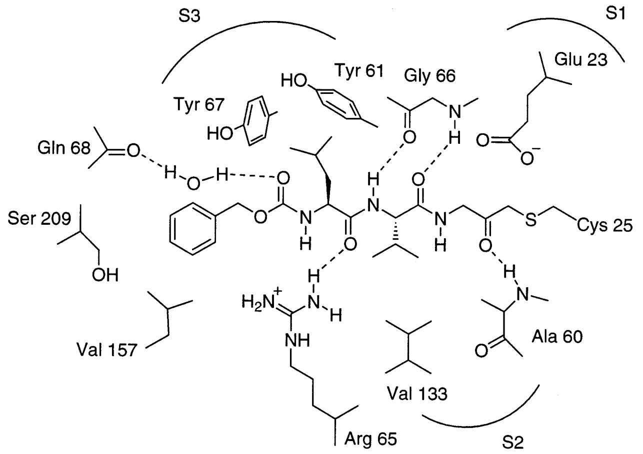


Figure 29. Structure of glycyl endopeptidase complexed with Cbz-Leu-Val-Gly-CHN_2.

C, H, L, and S, calpain, streptopain, and clostripain.[54,155–159] The peptide chain provides specificity for each enzyme, and the irreversible second-order rate constants are in the range of 10^3–10^6 M^{-1} s^{-1} (Table 9). The inhibitor Cbz-Phe-Ala-CHN_2 was effective in inactivating papain, cathepsins B and L, and streptopain, but it was ineffective toward calpain, probably because calpain prefers small alkyl residues in S2. The inhibitor Cbz-Ala-Phe-Ala-CHN_2, with a peptide sequence specific for streptopain, was a good inactivator of that enzyme and a moderate inactivator of cathepsin B. Cbz-Tyr-Ala-CHN_2 was a potent inactivator of cathepsins B and L. The iodinated inhibitor Cbz-Tyr(I)-Ala-CHN_2 was even more potent, with a second-order rate constant of 1128000 M^{-1} s^{-1}. The iodinated inhibitor was useful in radioiodination studies. The tripeptide inhibitor Cbz-Leu-Leu-Tyr-CHN_2 was a potent inhibitor of calpain I (k_2/K_i = 113000/230000 M^{-1} s^{-1}). Because cathepsins B, L, and S also have preference for the aromatic side chain at the P1 position, those enzymes are also inhibited potently by this inhibitor. The compound Cbz-Phe-Arg-CHN_2, which has a P1 Arg, a preferred residue for clostripain, inhibited clostripain potently with a second-order rate constant of 86000 M^{-1} s^{-1}. It also inhibited cathepsin B effectively.

The diazomethyl ketone analogue Cbz-Leu-Pro-Ala-Thr-CHN_2 is found to be a time-dependent irreversible inhibitor of recombinant sortase with a second-order rate constant of 367 M^{-1} s^{-1}.[88] Sortase is a transpeptidase with some peptidase activity. The biotinylated peptidyl diazomethyl ketone analogue, biotinyl-Ahx-Leu-Pro-Ala-Thr-CHN_2, can be used as an affinity label to detect the presence of wild-type sortase in crude cell lysates prepared from *Staphylococcus aureus*.

C. Acyloxymethyl Ketones and Related Activated Ketones

Halomethyl ketones were originally conceived as affinity labels for serine proteases and incorporated a peptide-targeting sequence and a reactive functional group to covalently react with the active site of the target protease.[77,78] However, halomethyl ketones have limited clinical utility due to the inherent chemical reactivity of the halomethyl ketone functional group. Acyloxymethyl ketones were designed by Allen Krantz and his research group at Syntex Canada as clinically useful halomethyl ketone analogues. He termed this approach the "quiescent nucleofuge strategy".[160,161] Ideally, he hoped that the acyloxymethyl ketone moiety would be reactive toward the active site nucleophile of the target enzyme but unreactive (quiescent) toward other biomolecules.

Table 9. Inactivation of Cysteine Proteases by Peptidyl Diazomethyl Ketones

	k_2/K_i[a] (M^{-1} s^{-1})					
inhibitor	papain	cathepsin B	cathepsin L	calpain I	streptopain	clostripain
Cbz-Phe-Ala-CHN_2[759]	35000	1100/1250	620000/136000[157]	<10	7666	<0.005
Cbz-Ala-Phe-Ala-CHN_2[155]		1250/1175			29500	
Cbz-Tyr-Ala-CHN_2[156]		1800	120000	<10		
Cbz-Tyr(I)-Ala-CHN_2[157]		27800	1128000	<10		
Cbz-Leu-Val-Gly-CHN_2[158]	600000	3300/10640[158]	118000		102	
Cbz-Leu-Leu-Tyr-CHN_2		1300	1500000	113000/230000[157]		
Cbz-Phe-Tyr(*t*Bu)-CHN_2[159]		10	200000	NI[b]		
Cbz-Phe-Tyr(OBzl)-CHN_2[759]		300000				
Cbz-Phe-Arg-CHN_2[151]		45000				86000

[a] Values separted by a slash indicate different values in the literature. [b] NI = no inhibition.

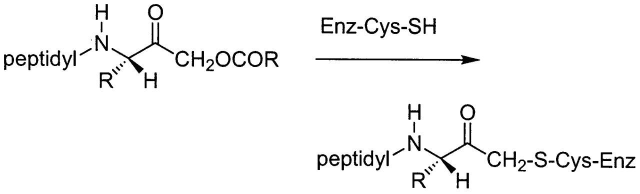


Figure 30. Inactivation of cysteine proteases by peptidyl acyloxymethyl ketones.

Peptide acyloxymethyl ketones inhibit cysteine proteases by alkylating the active site cysteine residue to form a thioether ketone (Figure 30). Acyloxymethyl ketones are time-dependent inhibitors of cathepsins B, L, and S, calpains, caspases, and other cysteine proteases.[162] The inhibitory potency of acyloxymethyl ketones is based on the affinity of the enzyme for the peptide portion of the inhibitor and the nature of the leaving group. A wide variety of peptides and leaving groups have been incorporated into acyloxymethyl ketone structures, which has allowed control of the selectivity and reactivity toward different cysteine proteases. These inhibitors are quite selective toward cysteine proteases. They do not show time-dependent inhibitory activity toward other classes of proteases, particularly serine proteases. Acyloxymethyl ketones are relatively inert toward bionucleophiles such as glutathione; thus, they are suitable for in vivo studies and development as clinically useful drugs.

Following the development of acyloxymethyl ketones a variety of activated ketones with various leaving groups have been described in the literature. The various inhibitor structures are shown in Table 10. The leaving groups include aromatic carboxylates in arylacyloxymethyl ketones, phenols in aryloxymethyl ketones, heterocyclic derivatives in pyrazolyloxymethyl, benzotriazolyloxymethyl and tetronoyloxymethyl ketones, diphenylphosphinic acid in diphenylphosphinyloxymethyl ketones, sulfonylamines in sulfonylaminomethyl ketones, and acylamines in acylaminomethyl ketones.

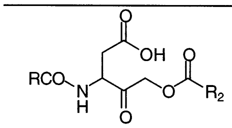
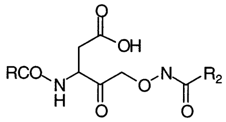
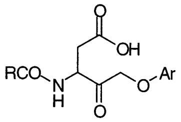
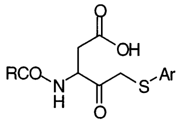
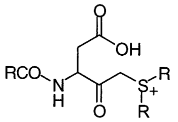
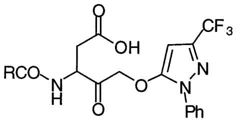
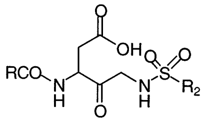
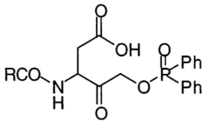
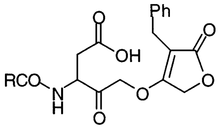
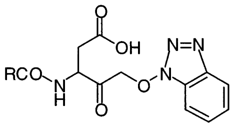
Nomenclature. Acyloxymethyl ketones will be abbreviated RCO-AA-CH_2OCOAr, where AA is the amino acid residue and $-OCOAr$ is the aryl carboxylate leaving group. Thus, the first structure in Table 10 is abbreviated RCO-Asp-CH_2OCOR_2. The structures of the other activated ketones are designated in a similar fashion.

Mechanism. The mechanism of inhibition of cysteine proteases by acyloxymethyl ketones was investigated by dialysis, dilution assays, and NMR characterization of the stable enzyme adduct as a thioether derivative. The dialysis experiments and dilution assays established the formation of a covalent bond between the enzyme and the inhibitor. The dilution experiments with cathepsin B exhibited a good correlation between the k_2 value obtained from the continuous rate assay and the rate of irreversible inactivation. The incubation of cathepsin B with various acyloxymethyl ketone inhibitors resulted mainly in no recovery of the enzyme activity over a 24 h dialysis period.[161] Following the inhibition of caspase-1 with the inhibitor Ac-Tyr-Val-Ala-Asp-CH_2-OCO-2,6-$(CF_3)_2$-Ph, there was no recovery of the enzyme activity upon addition of saturating levels of substrate.[163] NMR studies were performed with the inhibitors Cbz-Phe-Gly-CH_2OCO-2,4,6-Me_3-Ph and Cbz-Phe-Gly-CH_2OCO-$CH_2C_6F_5$, which were labeled with ^{13}C at the ketone carbonyl and methylene carbon. These inhibitors form an irreversible covalent adduct with papain, releasing, respectively, mesitoic acid (2,4,6-trimethylbenzoic acid) and pentafluorophenol.[164] In both cases, the same covalent adduct was formed, resulting in ^{13}C NMR signals at 214.7 ppm ($\mathit{C}OCH_2$) and 38.1 ppm ($CO\mathit{C}H_2$), which are consistent with thioether formation. An identical spectrum was observed from the adduct formed from the analogous ^{13}C-labeled chloromethyl ketone Cbz-Phe-Gly-CH_2Cl.[164] The thioether structure of chloromethyl ketone adducts has previously been established by X-ray crystallography (see previous section on chloromethyl ketones). The formation of a thioether ketone with the active site Cys 285 with caspase-1 was demonstrated by mass spectrometry and sequence analysis of the inactivated derivative. Caspase-1 inactivated with Ac-Tyr-Val-Ala-Asp-CH_2-OCO-2,6-Me_2-Ph was analyzed by HPLC-ESI-MS. The molecular mass of the p20 subunit of the inactivated enzyme was 20349.6 Da, which is in excellent agreement with the predicted mass of the thioether ketone of 20349.7 Da. Analysis of the tryptic digest confirmed inactivation at Cys 285.[163]

Brady and co-workers analyzed the kinetic behavior of a large number of activated ketones (>600) with caspase-1. The inhibitors evaluated included nine different classes of activated ketones [acyloxymethyl ketones, aryloxymethyl ketones, arylthiomethyl ketones, alkylthiomethyl ketones, acylaminooxymethyl ketones, sulfonylaminomethyl ketones, α-ketoamides, α-(1-phenyl-3-trifluoromethylpyrazol-5-yl)oxymethyl ketones (PTP), and aliphatic ketones].[165,166] Three types of kinetic behavior were observed between activated ketones and caspase-1. These were reversible, irreversible, and bimodal (Figure 31). Irreversible inhibition involved formation of a reversible E·I complex followed by rapid formation of a covalent thioether complex. Bimodal inhibition involved reversible inhibition followed by a slow irreversible inactivation of the enzyme at a rate that saturates with increasing inhibitor concentration. The mechanism of inhibition could not be predicted according to the nature of the leaving group in the various types of activated ketones. For the various types of inhibitors shown in Table 10, the numbers of inhibitors which were reversible, irreversible and bimodal are listed.

Crystal structures of 22 inhibitors, from eight different ketone classes, complexed with caspase-1, showed thiohemiketal formation between the active site Cys and the ketone carbonyl. The inhibitors adopted two different conformations, where the Cys 285 S-C-C_α'-LG (LG = leaving group) dihedral angle was either approximately 60° or 180°. Most of the activated ketones with the 180° conformation were able to undergo S_N2 displacement and exhibited bimodal inactivation. With the bimodal inhibitors the His 237 Nδ is positioned in close proximity to the thiohemiketal oxygen. When irreversible inhibition

Table 10. Activated Ketones and Their Kinetic Behavior

structure	name	enzyme	kinetic behavior
	acyloxymethyl ketones	caspase-1	118 reversible, 166 bimodal, 17 irreversible
		cathepsins B, L, S	
		calpain I	
	acylaminooxymethyl ketones	caspase-1	14 reversible, 7 bimodal
	aryloxymethyl ketones	caspase-1	7 reversible, 16 bimodal, 1 irreversible
	arylthiomethyl ketones	caspase-1	27 reversible, 3 bimodal
	sulfonium methyl ketones	cathepsin B calpain I	
	pyrazolyloxymethyl ketones (PTP)	caspase-1	4 reversible, 27 bimodal
		cathepsin B	
		calpain I	
	sulfonylaminomethyl ketones	caspase-1	63 reversible, 5 bimodal
	diphenylphosphinyloxymethyl ketones (DPP)	caspase-1 cathepsin B calpain I	
	tetronoylmethyl ketones	caspase-1	
	benzotriazolyloxymethyl ketones (BTA)	cathepsin B, L calpain I	

is observed, hydrogen bonding occurs between His 237 Nδ and the ketone/thiohemiketal oxygen.

On the basis of these observations, Brady and coworkers proposed the mechanism shown in Figure 32. The active site residues in caspase-1 exist as a thiolate/imidazolium ion pair, by analogy to papain. The His 237 imidazolium ring is in position to polarize the ketone carbonyl group. The Cys 285

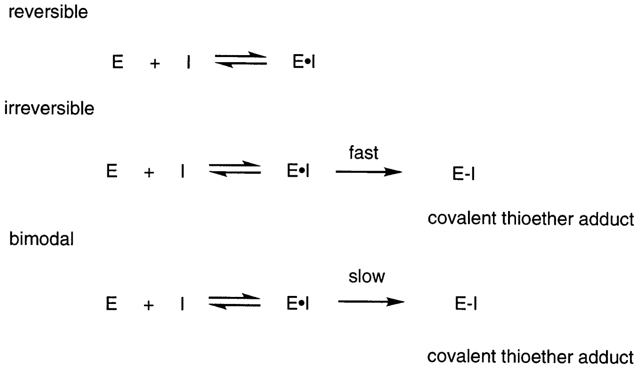


Figure 31. Kinetic behavior of activated ketones with cysteine proteases.

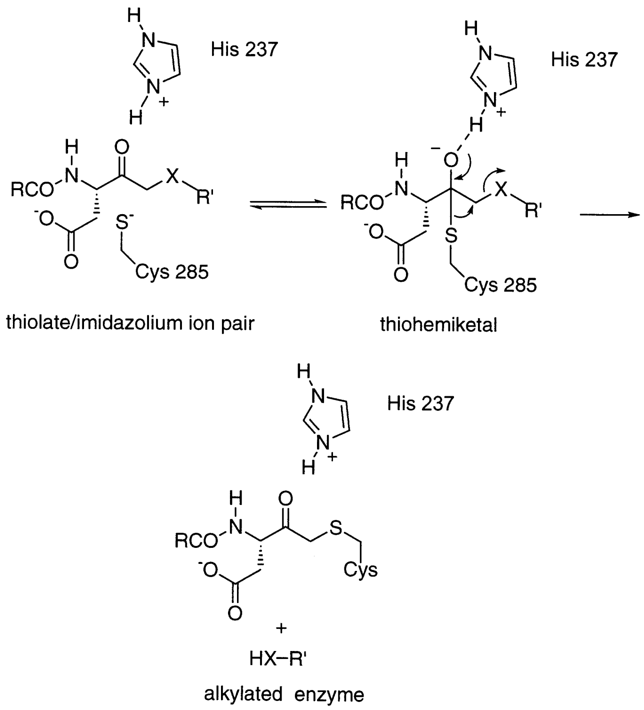


Figure 32. Proposed mechanism of inhibition of cysteine proteases by activated ketones.

thiolate attacks the carbonyl carbon, leading to the thiohemiketal complex. Depending on the nature of the leaving group, S_N2 displacement can take place, resulting in either irreversible inhibition or bimodal inhibition. A water molecule is positioned via a hydrogen bond with Gly 238 close to the leaving group. This probably transfers a proton to the leaving group. General acid catalysis could provide an explanation for the independence of the inhibition rates on the pK_a values of the leaving group. This is probably a general mechanism of inhibition of cysteine proteases by activated ketones.

Structure—Activity Relationships. In the original paper by Krantz a series of acyloxymethyl ketone inhibitors of the general structure Cbz-AA_2-AA_1-CH_2-OCOAr were shown to be irreversible inhibitors of cathepsin B. The best inhibitors were based on the Cbz-Phe-Ala sequence, which is complementary to the S1 and S2 subsites of cathepsin B. The most effective inhibitors were Cbz-Phe-Ala-CH_2OCO-2,6-$(CF_3)_2$-Ph and Cbz-Phe-Lys-CH_2OCO-2,6-$(CF_3)_2$-Ph, with rate constants (k_2/K_i) in the range of 1600000 to > 2000000 M^{-1} s^{-1}.

Table 11. Inhibition of Cathepsin B by Peptidyl Acyloxymethyl Ketones (Cbz-Phe-Ala-CH_2OCO-R)[760]

R	pK_a	$k_2/K_i{}^a$ (M^{-1} s^{-1})
2,6-$(CF_3)_2$-Ph	0.58	1600000
2,6-Cl_2-Ph	1.59	690000
C_6F_5	1.48	520000
2,6-F_2-Ph	2.24	26000
2-CF_3-Ph	2.49	17000
2,4,6-$(Me)_3$Ph	3.45	14000^b
4-NO_2-Ph	3.43	610
4-F-Ph	4.15	290
4-Me-Ph	4.37	260^b
Ph	4.20	90^b
4-OMe-Ph	4.50	NA^c
Me	4.76	140
CCH_3	5.03	330^b
$CH(CH_2CH_3)_2$	4.73	70
CH_2OCH_3	3.57	240

[a] The inhibition constants were measured in 100 mM phosphate, 1.25 mM EDTA, and 1 mM dithiothreitol, pH 6.0 and 25 °C. Bovine spleen cathepsin B was used. [b] Second-order rate constants k_2/K_i were determined at one inhibitor concentration near the solubility limit. [c] Not time dependent over 10–20 min near the solubility limit.

Krantz et al. evaluated the effect of the leaving group (OCOR) on the inhibition of cathepsin B in the acyloxymethyl ketones Cbz-Phe-Ala-CH_2OCOR (Table 11).[161] The inhibitory potency was dependent on the nature of the carboxylate leaving group, which had pK_a values ranging from 0.6 to 5.0. Inhibitors having better leaving groups with lower pK_a values inactivated the enzyme more rapidly. The second-order inhibition rate constants k_2/K_i for the inhibition reaction span a range of 6 orders of magnitude (Table 11).

The inhibitors were quite specific and did not inhibit serine proteases. For example, Cbz-Phe-Lys-CH_2OCO-2,4,6-Me_3-Ph, a powerful inactivator of cathepsin B, binds to trypsin with a $K_i = 17$ μM without irreversibly inactivating trypsin. Two inhibitors with peptide sequences complementary to the active site of human leukocyte elastase did not show significant time-dependent activity ($k_2/K_i < 15$ M^{-1} s^{-1}). Krantz also demonstrated that the acyloxymethyl ketone inhibitor did not exhibit time-dependent activity against either aspartate proteases or metalloproteases. Thus, acyloxymethyl ketones appear to be quite selective toward cysteine proteases.

Acyloxymethyl ketone inhibitors were then extended to cathepsins L and S.[162] The irreversible inhibition data are shown in Table 12. Again, the rate of inactivation was strongly dependent on both the nature of the peptide moiety and the leaving group of the inhibitor. Replacement of alanine at the P1 position with a basic residue in Cbz-Phe-X-2,4,6-Me_3-Ph resulted in a 20-fold increase in k_2/K_i value for cathepsin L and a 250-fold increase for cathepsin S. This observation is in accordance with the respective substrate specificities of these enzymes.[167,168] Acyloxymethyl ketone inhibitors containing *S*-benzylcysteine or *O*-benzylserine at the P1 position displayed unusual specificity. They were 7- and 80-fold better inactivators of cathepsin L than of cathepsin S. As was the case with cathepsin B, the inactivation rate

Table 12. Inactivation of Cathepsins B, L, and S by Peptidyl Acyloxymethyl Ketones[a]

	k_2/K_i (M^{-1} s^{-1})		
inhibitor	cathepsin B	cathepsin L	cathepsin S
Cbz-Phe-Lys-CH_2OCO-2,4,6-Me_3-Ph	230000	71000	120000
Cbz-Phe-Ser(Bzl)-CH_2OCO-2,6-$(CF_3)_2$-Ph		4290000	52000
Cbz-Phe-Cys(Bzl)-CH_2OCO-2,6-$(CF_3)_2$-Ph		10700000	1550000[a]
Cbz-Phe-Ala-CH_2OCO-2,4,6-Me_3-Ph	14000	4200	500
Cbz-Phe-Ala-CH_2OCO-2,6-$(CF_3)_2$-Ph	1600000	332000	364000
Cbz-Phe-Ala-CH_2OCO-2,6-Cl_2-Ph	690000	143000	686000
Cbz-Phe-Ala-CH_2OCO-2,6-Me_2-4-CO_2Me-Ph	58000	3600	42000
Cbz-Phe-Ala-CH_2OCO-4-NO_2-Ph	610	44000	3300

[a] Human cathepsin S was expressed and purified. Cathepsin L was purified from the lysosomal fraction of rat liver. The kinetic experiments were performed with a constant enzyme concentration of 50 mM sodium acetate buffer (pH 5.5) for cathepsin L (0.7 nM) and in a 50 mM potassium phosphate buffer containing 0.01% Triton X-10 (pH 6.5) for cathepsin S (2.3 nM). Substrates used were Cbz-Phe-Arg-AMC (1 μM) for cathepsin L and Cbz-Val-Val-Arg-AMC (10 μM) for cathepsin S.[162]

Table 13. Inhibition of Caspase-1 by Peptidyl Acyloxymethyl Ketones ($PhCH_2CH_2CO$-Val-Ala-Asp-CH_2OCO−Ar)[761]

Ar	pK_a	k_2/K_i (M^{-1} s^{-1})	ref
2,6-$(CF_3)_2$-Ph	0.58	900000	161
2,6-$(OH)_2$-Ph	1.22	710000	762
2,6-$(Me)_2$-Ph	3.35	1200000	163
Ph	4.20	280000	762
C_6F_5	5.53	1100000	160
4-NO_2-Ph	7.16	1300000	762

was strongly dependent on the pK_a of the leaving group.

Peptide acyloxymethyl ketones with a P1 Asp residue and an appropriate peptide recognition sequence (e.g., Ac-Tyr-Val-Ala-Asp-CH_2OCOAr) are potent, irreversible inhibitors of caspase-1 (interleukin 1β-converting enzyme, ICE).[163] The second-order inhibition rates of a series of tripeptide inhibitors $PhCH_2CH_2CO$-Val-Ala-Asp-CH_2OCO-Ar have k_2/K_i values as high as 10^6 M^{-1} s^{-1} with caspase-1 (Table 13). In contrast to the cathepsins (cathepsins B, L, and S), the inhibition rates were not dependent on the pK_a values of the leaving group. Many inhibitors with leaving group pK_a values in the range of 0.58−7.2 had similar inhibition rates of approximately 10^6 M^{-1} s^{-1}. With caspase-1, the single amino acid derivative Cbz-Asp-CH_2OCO-2,6-Cl_2-Ph is a relatively strong inhibitor. With other inhibitors containing different leaving groups, a recognition sequence of at least two amino acids is required to obtain k_2/K_i values of 10^3 with caspase-1.[169]

The inhibition rate constants with caspase-1 are directly proportional to the reaction macroviscosity, which was measured in glycerol solutions. This led to the proposal that the rate-determining step in inactivation is a diffusion-controlled reaction. These Asp acyloxymethyl ketone inhibitors are quite potent and selective toward caspase-1, probably because of the strict requirement for Asp at P1. In contrast to cysteine proteases, such as calpain, it appears that caspase-1 has the ability to accommodate relatively bulky arylacyloxy leaving groups. It should be noted that these inhibitors have not yet been tested with other caspases, and it is highly likely that they will exhibit cross-reactivity. The selectivity of a tetrapeptide biotinylated derivative was tested with supernatants from THP-1 cells, where caspase-1 comprises <0.001% of the total protein. Under these conditions 99.99% of the caspase-1 was inactivated and there was no detectable labeling any other proteases.[163]

A large number of other activated Asp-containing methyl ketones such as pyrazolyloxy (PTP),[170] diphenylphosphinyl (DPP),[171] and tetranoate and tetramoate methyl ketones[172] were synthesized and tested as inhibitors of caspase-1 (Table 10). Like acyloxymethyl ketones, these activated ketone derivatives are irreversible inhibitors, with k_2/K_i values in the range of 10^3−10^5 M^{-1} s^{-1}. The DPP derivatives with appropriate sequence also inhibited other cysteine proteases potently. For example, Cbz-Phe-Ala-CH_2-DPP inhibited cathepsin B and Cbz-Leu-Phe-CH_2-DPP inhibited calpain I potently. However, the PTP derivatives with Cbz-Phe-Ala and Cbz-Leu-Phe sequences were ineffective toward cathepsin B and calpain I. Thus, The PTP derivative is considered a caspase-1 specific inhibitor (Table 14).

Extended subsite interactions (S′−P′) have been utilized for improving the potency and selectivity of acyloxymethyl ketones.[173] Two representative extended acyloxymethyl ketone inhibitors are shown in Figure 33. The first has an Ac-Phe-Gly sequence attached to a Cbz-Leu leaving group. It is a moderate inhibitor of papain and cathepsin B. The second has an Fmoc-Asp sequence with an Ac-D-Val-D-Pro leaving group, which is a slow inhibitor of caspase-1. The corresponding acyloxymethyl ketones with an acetoxy leaving group were much poorer inhibitors. For example, Cbz-Phe-Gly-CH_2OCOMe had a k_2/K_i of 2.5 M^{-1} s^{-1} with papain and was a slow binding inhibitor with cathepsin B. The Fmoc-Asp-CH_2OCOMe showed no inhibition of caspase-1. The D-amino acid residues were introduced in the amino acid or dipeptide leaving group to prevent hydrolysis of the ester linkage by other proteases for in vivo studies.

Acyloxymethyl ketones with the appropriate peptide recognition sequence are relatively weak inactivators of calpain I. For example, Cbz-Leu-Leu-CH_2OCO-2,6-$(CF_3)_2$-Ph shows no time-dependent inhibition toward calpain I, whereas it is a potent inhibitor of cathepsin B with a k_2/K_i value of 270000 M^{-1} s^{-1}.[174] Cbz-D-Ala-Leu-Phe-CH_2OCO-2,6-F_2-Ph is the only acyloxymethyl ketone inhibitor that is potent ($k_2/K_i = 31000$ M^{-1} s^{-1}) and has a 100-fold selectivity for calpain I over cathepsins B and L.[175] There are several reasons for the poor potency of acyloxymethyl ketones toward calpain I. Calpain I cannot tolerate the steric bulk of the carboxylate leaving group as

Table 14. Inhibition of Caspase-1, Cathepsin B, and Calpain I by Activated Methyl Ketones[169–172]

	k_2/K_i (M^{-1} s^{-1})		
inhibitor[a]	caspase-1[b]	cathepsin B[c]	calpain I
Cbz-Asp-CH_2-DPP	11800		
Cbz-Val-Asp-CH_2-DPP	50200	<500	<500
Cbz-Val-Ala-Asp-CH_2-DPP	117000	1700	
Cbz-Val-Ala-Asp-CH_2-DPP	230000		
Cbz-Phe-Ala-CH_2-DPP	<500	400000	
Cbz-Leu-Phe-CH_2-DPP	<500	<500	10000
Cbz-Val-Ala-Asp-CH_2-PTP	280000		
Cbz-Val-Ala-D-Asp-CH_2-PTP	288000		
Cbz-Phe-Ala-CH_2-PTP		<500	<500
Cbz-Leu-Phe-CH_2-PTP		<500	<500
Cbz-Asp-CH_2-2,6-Cl_2-Ph	7100	<100	
Cbz-Val-Asp-CH_2-2,6-Cl_2-Ph	41000	380	
Cbz-Val-Ala-Asp-CH_2−2,6-Cl_2-Ph	406000	2250	
Cbz-Asp-CH_2-R	2850		
Cbz-Val-Asp-CH_2-R	21200		
Cbz-Val-Ala-Asp-CH_2-R	252000		
Cbz-Val-Ala-AAsp-CH_2DPP	19400		
Cbz-Val-Ala-AAsp-CH_2PTP	700		
Cbz-Val-Ala-AAsp-2,6-Cl_2-Ph	470		
Cbz-Val-Ala-AAsp-CH_2-R	120		
Ac-Tyr-Val-Ala-Asp-CHN_2	16500		

[a] DPP = diphenyl phosphinyloxyl; PTP = pyrazolyloxy; R = tetronoyl; AAsp = aza-aspartate. [b] Caspase-1 was partially purified from THP.1 cells. The caspase-1 assay contained 10 mM HEPES (pH 7.5), 25% glycerol, 1 mM dithiothreitol (DTT), and 10 μM Suc-Tyr-Val-Ala-Asp-AMC. Progress curves were obtained at 37 °C over 30 min.[169] [c] Bovine spleen cathepsin B was used and assayed with Cbz-Phe-Arg-AMC (AMC = amino methylcoumarin, pH 6.5, 25 °C).[191] [d] Human erythrocyte calpain I was assayed with Cbz-Leu-Arg-MNA (MNA = methoxynaphthylamide, pH 7.5, 8 °C).[175]

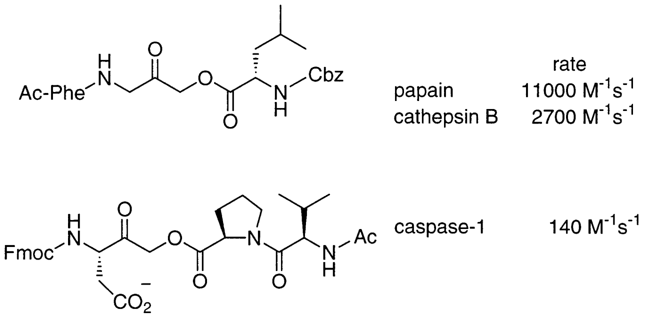


Figure 33. Peptide acyloxymethyl ketone inhibitors with extended side chains. In the caspase-1 inhibitor, the leaving group is Ac-D-Val-D-Pro.

easily as the lysosomal proteases such as cathepsins B and L. With calpain I, there is a requirement for an additional active site residue to participate in the expulsion of the carboxylate leaving group for potent inhibition.[174] Tripathyl et al. developed peptidyl benzotriazoloxymethyl ketones, which are potent inhibitors of calpain I.[176] The inhibitors Cbz-Leu-Phe-CH_2-OR (R = benzotriazolyl) and Cbz-Leu-Leu-CH_2-OR (R = benzotriazolyl) have second-order rate constants of 320000 and 175000 M^{-1} s^{-1}. They also inhibit cathepsin B potently, with second-order rate constants of 118000 and 19400 M^{-1} s^{-1}. The tripeptidyl inhibitor Cbz-Leu-Leu-Phe-CH_2-OR (R = benzotriazolyl) is a better inhibitor than its dipeptidyl analogues (k_2/K_i = 524000 M^{-1} s^{-1}). The inhibitor Cbz-Leu-Phe-CH_2-R (R = benzotriazolyl) is found to be a poor inhibitor of calpain I. With this finding it is claimed that the N−O bond might play a role in sterically favorable binding of the bulky leaving group at the active site of calpain I. When more crystallographic structural data become available, the active site properties of calpain I will be better understood.

To increase the potency and stability of the Asp-containing activated methyl ketone inhibitors such as pyrazolyloxy (PTP),[170] diphenylphosphinyl (DPP),[171] and tetranoate and tetramoate methyl ketones,[172] the corresponding aza-amino acid analogues have been developed.[177] The activated methyl ketone inhibitors with D-stereochemistry at the P1 position are equally as potent as the L-amino acid analogues (Table 14).[178] The reason for the equal potency of D- and L-amino acids could not be investigated because of the potential problem of epimerization of the inhibitors' Asp at the P1 position. The substitution of α-CH with N would result in nonchiral analogues, and epimerization would no longer be a complication in evaluating the kinetic data. However, the exact effect of this substitution on inhibitory potency could not be predicted.

An *N*-methyl scan experiment with the inhibitor Cbz-Val-Ala-Asp-CH_2-PTP revealed the importance of the amido functional group at the P1 and P3 positions that is utilized in making hydrogen-bonding interactions with the caspase-1 active site. Because the acidity of the hydrazide NH group is higher than that of an amido NH group, it would result in a stronger hydrogen-bonding interaction. Hence, incorporation of the aza-amino acid residue would result in more potent inhibition due to better hydrogen bonding.

The rates of inhibition of caspase-1 by the aza-Asp analogues are 100−1000-fold slower than those of their methyl ketone parents (Table 14).[177] The decrease in inhibitory potency for the aza-Asp analogues is probably due to an unfavorable structural modification of the inhibitor at the active site resulting from the incorporation of the aza group. However, the rates are still comparable to the rates of the tetrapeptide diazomethyl ketone inhibitor Ac-Tyr-Val-Ala-Asp-CHN_2 (16500 M^{-1} s^{-1}).

Peptidyl Sulfonium Salts. In contrast to acyloxymethyl ketones, several activated ketones have been shown to be potent inactivators of calpain I. The sulfonium methyl ketones are among the most potent and selective inhibitors of calpain I. The inhibitor Cbz-Leu-Leu-Phe-$CH_2S^+(Me)_2Br^-$ has a k_2/K_i value as high as 200000 M^{-1} s^{-1}.[174] Another inhibitor, Cbz-Leu-Phe-DPP, inactivated calpain I potently and selectively with a k_2/K_i value of 10000 M^{-1} s^{-1}, whereas cathepsin B has a k_2/K_i value of only <500 M^{-1} s^{-1}.[171] Certain benzotriazolyloxymethyl ketones are also potent and specific calpain I inhibitors and are shown in Table 14.[176]

Peptidyl sulfonium salts are potent inactivators of papain, cathepsin B, and calpain I (Table 15).[174,179] Peptidyl sulfonium salts are the most potent class of inhibitors for calpain among activated ketones, whereas for lysosomal cysteine proteases such as papain and cathepsin B, the inhibitor reactivity follows the descending order of acyloxymethyl ketones ≫ fluoromethyl ketones > sulfonium methyl

Table 15. Inactivation of Cysteine Proteases by Peptidyl Sulfonium Salts[174,179,763]

	k_2/K_i (M^{-1} s^{-1})			
inhibitor	papain	cathepsin B	calpain I	clostripain
Cbz-Phe-$CH_2S^+(CH_3)_2$	32			
Cbz-Phe-Ala-$CH_2S^+(CH_3)_2$	4700	5800	1100	
Cbz-Val-Phe-$CH_2S^+(CH_3)_2$		1200	3500	
Cbz-Leu-Leu-Phe-$CH_2S^+(CH_3)_2$		3500	>200000	
Cbz-Phe-$CH_2S^+(CH_3)$-CH_2Ph	233			
Cbz-Lys-$CH_2S^+(CH_3)_2$				167000
Cbz-Phe-Lys-$CH_2S^+(CH_3)$-CH_2Ph				107000

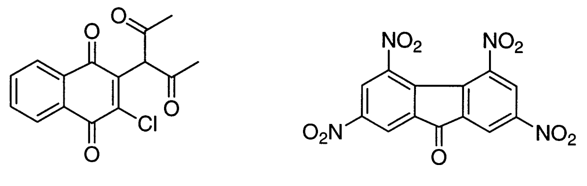
ketones > diazomethyl ketones. In general, sulfonium methyl ketones are more effective inhibitors for cysteine proteases than for serine proteases.

In terms of binding affinities, the affinity of the inhibitor Cbz-Lys-$CH_2S^+(CH_3)_2$ for clostripain is in the nanomolar range, whereas for trypsin-like serine proteases the affinity for the dipeptide inhibitor Cbz-Phe-Lys-$CH_2S^+(CH_3)$-CH_2Ph is in the range of 10 μM. The kinetic behaviors of trypsin-like serine proteases, such as plasma kallikrein, plasmin, and thrombin, differ toward the peptidyl sulfonium salts. The inhibitor Cbz-Phe-Lys-$CH_2S^+(CH_3)_2$ is an irreversible inhibitor with second-order rate constants in the range of 1−10 M^{-1} s^{-1}. On the other hand, the inhibitor Cbz-Phe-Lys-$CH_2S^+(CH_3)$-CH_2Ph acts as a slow binding reversible inhibitor toward these serine proteases.

The inhibition of serine and cysteine proteases by peptidyl sulfonium salts is pH dependent. As the pH is increased, the second-order rate constant for inhibition increased. At physiological pH values peptidyl sulfonium salts may exist as an ylide structure by losing a proton, which may be the active form of the inhibitor. The exact reaction mechanism is unknown, but it is probably mechanism-based rather than an alkylation by a single displacement reaction.

Stability. Some acyloxymethyl ketones are quite stable, but it is not clear whether this class of cysteine protease inhibitors is sufficiently stable for therapeutic use. The cathepsin B inhibitor Cbz-Phe-Ala-CH_2OCO-2,4,6-Me_3-Ph was stable to the in vitro cathepsin B assay conditions ($t_{1/2}$ > 24 h, pH 6.0, 25 °C, 1 mM dithiothreitol), in methanol, and methanol containing catalytic CF_3COOH.[180] However, in a basic solution (95% aqueous MeOH, 4 mM K_2CO_3), it underwent epimerization to a mixture of the L,L and L,D diastereomers. Another cathepsin B inhibitor, Cbz-Phe-Lys-CH_2OCO-2,4,6-Me_3-Ph, which contains a basic side chain, was more susceptible to epimerization and underwent rapid epimerization in neat ethanol and under basic conditions. This compound was quite stable to human plasma ($t_{1/2} \gg 33$ h) and rat plasma ($t_{1/2} = 1.6$ h) in an aqueous suspension of carboxymethyl cellulose, which was used as the dosing vehicle. This inhibitor was also stable when incubated at a 2 μM concentration for at least 90 min in the presence of the bionucleophile glutathione (3−20 nM) in human plasma (37 °C).[180]

Cbz-Leu-Phe-CH_2-OR (R = benzotriazolyl) has a half-life of 5 min at neutral pH. The fluoromethyl ketone Cbz-Leu-Phe-CH_2F has a half-life of 60 min under the same conditions. Due to the poor aqueous stability, peptidyl benzotriazoloxymethyl ketones have limited therapeutic utility.



14 **15**

Figure 34. Miscellaneous ketone-based inhibitors.

The aza-Asp analogues exhibit greater stability toward bionucleophiles such as DTT and glutathione. The inhibitor Cbz-Val-Ala-AAsp-CH_2PTP has a half-life of 3.5 h in a caspase-1 assay buffer containing glutathione at 70 °C, whereas Cbz-Val-Ala-Asp-CH_2-PTP has a half-life of 0.96 h under the same conditions. In addition, the inhibitor Cbz-Val-Ala-AAsp-CH_2PTP stayed 89% intact after 120 h in a caspase-1 assay buffer containing DTT at 25 °C.

Biological Studies. The acyloxymethyl ketone inhibitors have been tested for their in vivo activity for liver cathepsin B inhibition in rats. The liver cathepsin B activity is measured ex vivo for the inhibitor Cbz-Phe-Lys-CH_2OCO-2,4,6-Me_3-Ph, which has been shown to be quite effective with ED_{50} values of 18 mg/kg when administered orally and 5.0 mg/kg when administered intraperitoneally after 4−5 h. An ED_{50} value of 2.4 mg/kg is obtained with subcutaneous administration at 24 h postdose for liver cathepsin B inhibition, which is measured ex vivo.[180] There was no correlation found between in vitro cathepsin B inactivation rates and the in vivo inhibitory activity; hence, the differences in bioavailability and metabolism might be important.

Acyloxymethyl ketone inhibitors have also been tested for their caspase-1 activity in vivo. They have been shown to inhibit interleukin-1β release from monocytes.[181] For example, the inhibitor Cbz-Val-Ala-Asp-CH_2OCO-2,6-Cl_2-Ph inhibits interleukin-1β release with an IC_{50} value of 2 μM, whereas its ethyl aspartyl ester analogue, Cbz-Val-Ala-Asp(OEt)-CH_2-OCO-2,6-Cl_2-Ph, has an IC_{50} value of 0.5 μM. The inhibitor Cbz-Val-Ala-Asp(OEt)-CH_2OCO-2,6-Cl_2-Ph also inhibits apoptosis with an IC_{50} value of ~1.0 μM. The effective inhibition of apoptosis is most likely the result of the inhibition of other caspases by this inhibitor.[182,183]

Miscellaneous Ketone-Based Inhibitors. Several 2,3-disubstituted-1,4-naphthoquinone structures have been found to exhibit inhibitory activity against the serine protease from human cytomegalovirus (hCMV).[184] Compound **14** inhibited hCMV irreversibly with an IC_{50} value of 0.8 μM (Figure 34). An analysis of the enzyme−inhibitor complex by tryptic digest and sequencing by MS/MS identified the amino acid residue Cys 202 as the covalent attachment point of the inhibitor. This led to the conclusion that the enzyme has a specific recognition site for this inhibitor away from the active site. Several other 2,3-disubstituted-1,4-naphthoquinone structures have been tested with serine proteases such as thrombin and human neutrophil elastase (HNE). No inhibition was observed against thrombin, whereas HNE was only moderately inhibited. These compounds also reacted with the cellular nucleophile glutathione; hence, they have limited chemical stability.

Compound **15** has been identified as an inhibitor for human adenovirus cysteine protease (hAVCP) using the computer docking program EUDOC and in silico screening of a chemical database with this enzyme (Figure 34).[185] Compound **15**, 2,4,5,7-tetranitro-9-fluorenone, irreversibly inhibits hAVCP with a second-order rate constant of 1940 M^{-1} s^{-1}. The mechanism of inhibition involves nucleophilic aromatic substitution of the nitro group by the active site Cys 122 residue. Compound **15** also inhibited papain with a second-order rate constant of 108 M^{-1} s^{-1}. According to the docking study, the 18-fold decrease in potency with papain is due to the lack of favorable hydrogen bonds in the papain−inhibitor complex, which are observed with the hAVCP−inhibitor complex. To further evaluate the selectivity of compound **15**, studies with serine proteases such as chymotrypsin and cellular nucleophiles such as glutathione must be completed.

D. Epoxides

1. Epoxysuccinyl Peptides

The first epoxysuccinyl peptide discovered was E-64, a natural inhibitor, which was initially isolated from *Aspergillus japonicus* by Hanada et al. in 1978.[186] The chemical structure was determined by optical rotation, NMR, IR, MS, elemental analysis, and amino acid analysis to be *N*-(*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl)agmatine (Figure 35).[187] Hanada and co-workers showed that E-64 would inactivate the plant cysteine proteases papain, ficin, and bromelain.[186,188]

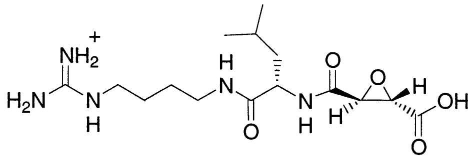


Figure 35. Chemical structure of E-64.

Once the E-64 structure was elucidated, the research groups of Katunuma, Barrett, and others discovered E-64's inhibitory potency toward a large number of other cysteine proteases (Table 16). E-64 inhibits papain, ficin, bromelain,[188] cathepsins B,[189,190] H,[191] F,[192] K,[193,194] L,[191,195,196] O,[197] S,[198] V,[199] and X,[200] calpain,[201−203] calpain II,[204] cruzain,[205] and other cysteine proteases.[206,207] Streptococcal cysteine protease, dipeptidyl peptidase, and clostripain, a trypsin-like cysteine protease, are slowly inhibited by E-64.[191,208]

Unlike many other microbial inhibitors, E-64 is a potent and specific irreversible inhibitor of cysteine proteases and is used as a diagnostic reagent for identification of cysteine proteases. The compound E-64 does not inhibit serine proteases, aspartic proteases, or metalloproteases.[188,191] However, not all cysteine proteases are inhibited by E-64. Examples of noninhibited cysteine proteases are legumain and caspases.[209,210] Caspases and legumain are members of the CD clan of cysteine proteases, whereas papain, cathepsins, and calpains are members of clan CA. Table 16 lists those enzymes that are inactivated by E-64 and those which are not inactivated.

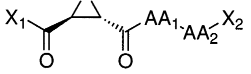
Once the inhibitory potency of E-64 toward cysteine proteases was recognized, many investigators including Katunuma and investigators at the Taisho Pharmaceutical Co. systematically synthesized a large number of derivatives targeted at specific cysteine proteases, such as cathepsins B and L. Additional peptidyl epoxysuccinates derivatives have been discovered in natural products. Common epoxysuccinate derivatives are listed in Table 17.

Since its discovery, E-64 has been used as an active site titrant and a diagnostic tool for cysteine proteases. E-64 derivatives have been proposed as possible therapeutic agents and have been utilized in a variety of biological studies. Recently, E-64 derivatives labeled with biotin, radiolabeled iodine, and fluorescent dyes, such as rhodamine B, have been

Table 16. Inhibition of Cysteine Proteases by E-64

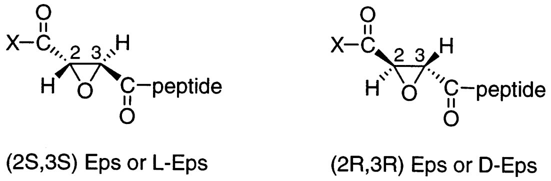
enzymes inactivated	rate (M^{-1} s^{-1})	ref	enzymes not inactivated	ref
ficin	0.084 (ID_{50})	186	trypsin	186
fruit bromelain	0.110 (ID_{50})	186	α-chymotrypsin	186
stem bromelain	0.025 (ID_{50})	186	kallikrein	186
papain	0.104 (ID_{50})	186	pepsin	186
cathepsin B	89400	191	plasmin	186
cathepsin H	4000	191	elastase	186
cathepsin L	96250	191	mold acid protease	186
cathepsin K	1.8 nM (K_i)	194	LDH	186
cathepsin S	99000	198	thermolysin	191
cathepsin X	775	200	collagenase	191
cathepsin O	>100 μM (IC_{50})	192	clostripain	191
cathepsin F		197	caspase 1 (ICE)	209
cathepsin V	>0.1 μM (IC_{50})	199	legumain	210
DPPI	100	191, 208		
streptococcal proteinase	624	191		
papaya proteinase IV	58000	207		
calpain II	7500	204		
bleomycin hydrolase	>160 μM (IC_{50})	206		
cruzain	70600	205		
vignain	32500	210		

Table 17. Common Epoxysuccinate Derivatives



derivative	X_1	AA_1	AA_2[a]	X_2
E-64	HO	Leu		Agm[b]
E-64a	HO	Leu		$NH(CH_2)_4NH_2$
E-64c (Ep-475)	HO	Leu		$NH(CH_2)_2CH(CH_3)_2$
E-64d (EST, loxistatin)	EtO	Leu		$NH(CH_2)_2CH(CH_3)_2$
circinamide	HO	Leu		$N((CH_2)_4NH_2)_2$
cathestatin C	HO	Tyr		$NH(CH_2)_5NH_2$
CA028	HO	Ile	Pro	OH
CA030	EtO	Ile	Pro	OH
CA074	*n*Pr-NH	Ile	Pro	OH
CLIK148	2-(2-pyridyl)-ethyl-NH	Phe		$N(CH_3)_2$

[a] AA_2 is the amino acid residue proline or no AA residue. [b] Agm = agmatine (1-amino-4-guanidinobutane).



(2S,3S) Eps or L-Eps

(2R,3R) Eps or D-Eps

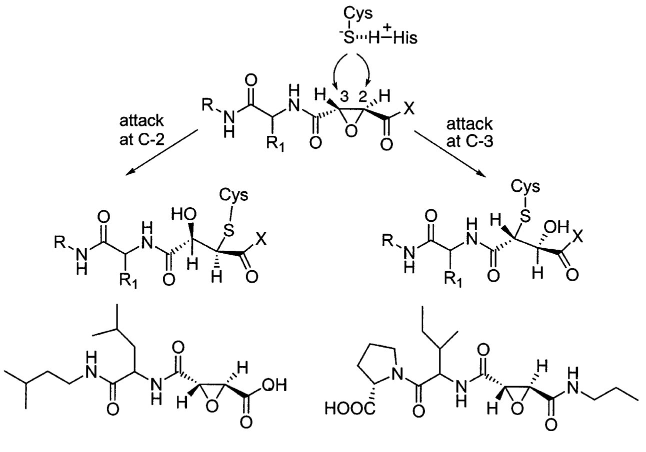
Figure 36. Stereochemistry of *trans*-epoxysuccinates.

developed as affinity labels for cysteine proteases.[211−213] The derivatives have proven to be useful for studies of the biological function and histochemical mapping of cysteine proteases.

One advantage of epoxysuccinyl peptide inhibitors is their stability under physiological conditions toward simple thiols. In addition, although they have limited selectivity toward different cysteine proteases, their reactivity toward cysteine proteases and not other proteases, along with their chemical unreactivity, makes this class of inhibitors useful as pharmaceutical agents.[202] Epoxysuccinate derivatives have been included in previous reviews of cysteine proteases.[48,57,214−217]

Nomenclature and Stereochemistry. The stereochemistry of the epoxysuccinyl moiety of E-64 was determined to have the 2*S*,3*S*-configuration, whereas the amino acid residues of the peptidyl part of the inhibitor have the L-configuration. Katunuma et al. named an early generation of epoxysuccinyl inhibitors CA-030 and CA-070, where the "CA" represents cathepsin.[218,219] Some recently synthesized inhibitors from Taisho Pharmaceuticals have been named CLIK (cathepsin L inhibitor Katunuma) and CSIK (cathepsin S inhibitor Katunuma).[220]

There are four structural isomers at the epoxide moiety, two trans isomers (2*S*,3*S* and 2*R*,3*R*) and two cis isomers (2*R*,3*S* and 2*S*,3*R*). The two trans isomers are shown in the Figure 36. Older papers refer to the 2*S*,3*S* epoxysuccinate as L-*trans*-epoxysuccinate and to the (2*R*,3*R*) as D-*trans*-epoxysuccinate.[187,221] E-64 has the 2*S*,3*S*-*trans*-configuration. To be consistent, in this review *trans*-epoxysuccinates are designated (2*S*,3*S*)-Eps and (2*R*,3*R*)-Eps, respectively. On the epoxide ring, C-2 is assigned to the carbon next to the carboxylic acid end of the Eps moiety, whereas C-3 is the carbon close to carbonyl which acylates the peptide. If the carboxylic acid is esterified or converted into an amide derivative, the numbering remains the same. If the epoxysuccinate inhibitor contains two peptides attached to both carboxyls, we number the epoxide such that the C-2 of the epoxide is located at the P side of the active site, whereas the C-3 is on the P′ side. The epoxide ring numbering for all inhibitors is shown in Figure 36.



E-64-c with papain

CA-074 with cathepsin B

Figure 37. Mechanism of inhibition of cysteine proteases by epoxysuccinates. The active site cysteine nucleophilically attacks either C-2 or C-3, opening the reactive three-membered ring and alkylating the enzyme.

Mechanism. E-64 and analogues inhibit cysteine proteases by S-alkylation of the active site cysteine, which results in the opening of the epoxide ring.[222−224] Peptidyl epoxysuccinates can inhibit the enzyme by forming a thioether bond via a nucleophilic attack at C-2 or C-3 of the epoxide ring by the active site cysteine residue. Attack occurs at either C-2 or C-3 depending on the orientation of the epoxysuccinate in the active site. For example, the attack occurs at C-2 when papain is inhibited by E-64c or at C-3 when cathepsin B is inhibited by CA-074 (Figure 37). Knowledge of how the epoxysuccinate derivative binds is important for determining whether it is the C-2 or C-3 carbon of the oxirane ring that is attacked. The only technique used thus far to determine the structure and stereochemistry of the adducts has been X-ray crystallography and ^{13}C NMR.[222]

The stereochemistry of the enzyme inhibitor adduct has undergone an inversion in configuration at the reaction site due to a nucleophilic attack by the active site thiolate in an S_N2 reaction. For example, E-64, which has the 2*S*,3*S*-configuration before the nucleophilic attack, will become 2*R*,3*R* after the covalent bond between the cysteine residue and C-2 of the oxirane ring is formed. The 2*S* to 2*R* conversion is the result of nucleophilic attack by the Cys thiolate, whereas 3*S* to 3*R* conversion is due to a change in the ranking order of substituents. In only a few cases has X-ray crystallographic analysis been able to unambiguously assign the stereochemistry in the enzyme inhibitor adducts.

Initially, it was postulated that when E-64 inhibits papain, the oxirane ring would be protonated by His 159, which is analogous to the mechanism of inactivation of papain by peptidyl aldehydes.[215] However, the crystal structure of papain inhibited by E-64

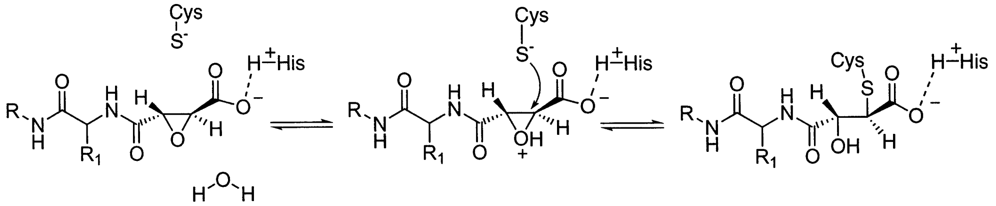


Figure 38. Proposed mechanism of inhibition of cysteine proteases by epoxysuccinates.

Table 18. PDB Codes for X-ray Crystal Structures of Cysteine Proteases and Epoxysuccinyl-Based Inhibitors

enzyme	inhibitor	PDB code	ref
actinidin	E-64	1AEC	238
caricain D158E	E-64	1MEG	237
caricain D158E	E-64c	1EUX	*a*
cathepsin B	CA-030	1CSB	243
cathepsin B	CA-074	1QDQ	231
cathepsin B	E-64c	1ITO	241
cathepsin K	E-64	1ATK	233
cathepsin L	E-64	N/A	234
papain	E-64	N/A	223
papain	E-64c	1PE6	240
papain	E-64c	1PPP	239
papain	HO-Eps-Ile-Pro	N/A	242
papain	HO-Eps-Ile-NEt$_2$	N/A	242
papain	CLIK 148	1CVZ	246
staphopain	E−64	1CV8	764

[a] Theoretical model in PDB by S, Bhattacharya, A. Pal, A. Bera, S. Chakraborty, and A. Banerjee.

suggests that the epoxide ring might be protonated by water (Figure 38) due to the distance (5.5 Å) between the His 159 and the resulting hydroxyl group.[223] The rate-limiting step in the enzyme inactivation is the rate with which the epoxysuccinyl docks in the active site preceding the formation of the covalent complex.[225]

Epoxysuccinate derivatives such as E-64c with a carboxyl group are more potent than those where the carboxyl group is replaced, suggesting an interaction with His 159 (see discussion in the SAR section). Thus, the role of His 159 is different in the epoxide inhibition reaction than in the substrate hydrolysis. Further mechanistic studies confirmed that His 159 is not necessary for protonation of the epoxide and that water is the predominant source of protons.[226,227]

Crystal Structures and Binding Modes. Over a dozen X-ray structures of epoxysuccinate derivatives bound to cysteine proteases have been reported. The structures currently available in the protein data bank (PDB) are listed in Table 18.

Three general binding modes have been observed or proposed (Figure 39). In the most commonly observed binding mode, the epoxysuccinate binds in the S subsites.[228−230] However, in contrast to the substrate binding mode (N-terminal to C-terminal, Figure 39a), the peptide chain of the inhibitor is oriented in the reverse direction (C-terminal to N-terminal, Figure 39b). In the second binding mode, the inhibitor is located in the S′ subsites (Figure 39c). Newly developed inhibitors, which extend in both directions, are proposed to bind with both the S and S′ subsites (Figure 39d). One crystal structure is available so far for a P and P′ extended inhibitor (papain−CLIK148, 1CVZ).

Nucleophilic attack by the active site Cys is dictated by the binding mode of the peptide portion of the inhibitor. Binding in the S subsites leads to C-2 attack (Figure 39b), whereas binding in the S′ subsites leads to C-3 attack (Figure 39c). So far, the latter binding mode has been observed only with cathepsin B. As a general rule, the covalent bond with the cysteine residue will be formed at the oxirane carbon that possesses the P′ substituent.[231]

S Subsite Binding. Initially, it was proposed that E-64 derivatives bound in the S′ subsites of the cysteine proteases papain and cathepsins. However, X-ray structures with papain (1PE6, 1PPP, and 1CVZ), actinidin (1AE6), the mutant D158E of caricain (papaya protease omega, 1MEG, 1EUX), cathepsin B (1ITO), cathepsin K (1ATK), and cathepsin L demonstrated that binding instead involved primarily the S subsites. In papain, the carboxylate group at C-2 position in E-64 orients the epoxide for alkylation by Cys 25 by means of a hydrogen-bonding interaction with His 159 and the oxyanion hole (Gln 19).[227] As a result, E-64 and several analogous inhibitors bind in S subsites (Figure 39b).

E-64 and E-64c frequently form a short parallel β-sheet hydrogen-bonding interaction with the peptide backbone NH and carbonyl of Gly 66. This is observed in the complexes papain−E-64, papain−E64c (form I, 1PE6), cathepsin L−E-64, cathepsin K−E-64 (1ATK), and several other structures.[223,232−234] The parallel β-sheet is formed by two hydrogen bond interactions between the epoxysuccinate amide carbonyl oxygen and the peptide backbone NH of Gly 66 and between the NH of the inhibitor C-terminal amide and the backbone carbonyl of the Gly 66 (Figures 40 and 42). In contrast, other types of inhibitors, such as peptide aldehydes, and substrates form an antiparallel β-sheet interaction with Gly 66.[235,236] Several inhibitor structures do not have an appropriate C-terminal hydrogen bonding group and do not interact with the backbone carbonyl of Gly 66.

In addition to the β-sheet interaction, there are additional hydrogen bonds or electrostatic interactions that stabilize and orient the inhibitor in the active site of cysteine proteases. In particular, there is an interaction between the E-64 amide and the backbone carbonyl of Asp 158 (papain, cathepsin K, and cathepsin L). In addition, there is often an electrostatic interaction or a hydrogen bond between Asp 158 and the hydroxyl group formed in the E-64 inhibition reaction. This Asp 158 is equivalent to Asp 161 in actinidin, which also forms an electrostatic interaction with Asn 66. X-ray structures of E-64 with papain, actinidin, caricain D158E, cathepsin L, and cathepsin K show the same overall hydrogen bonding pattern.[223,237,238]

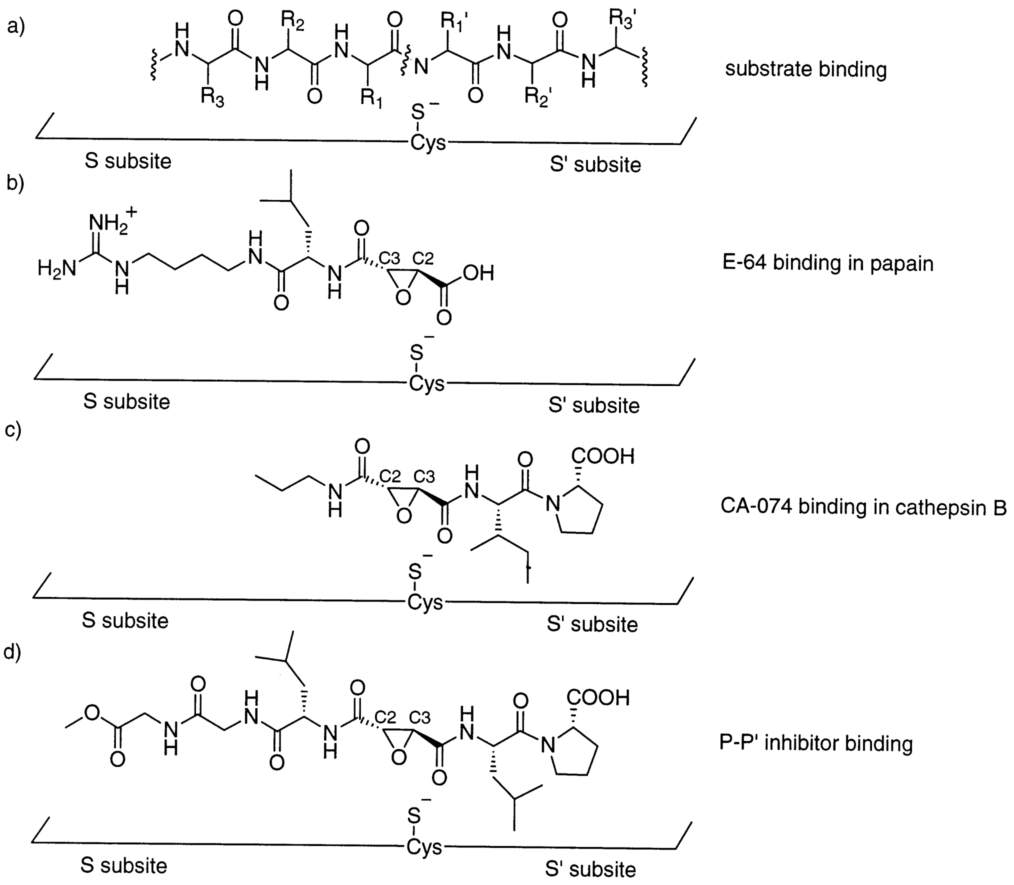


Figure 39. Schematic representation of the binding modes of peptidyl epoxysuccinates: (a) normal binding mode with a peptide substrate; (b) binding mode of E-64 to papain; (c) binding mode of CA-074 to cathepsin B; (d) proposed binding mode of MeO-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH to cathepsin B. Arrow indicates the reversed peptide chain. Cys-S^- represents the side-chain thiolate of the active site cysteine residue.

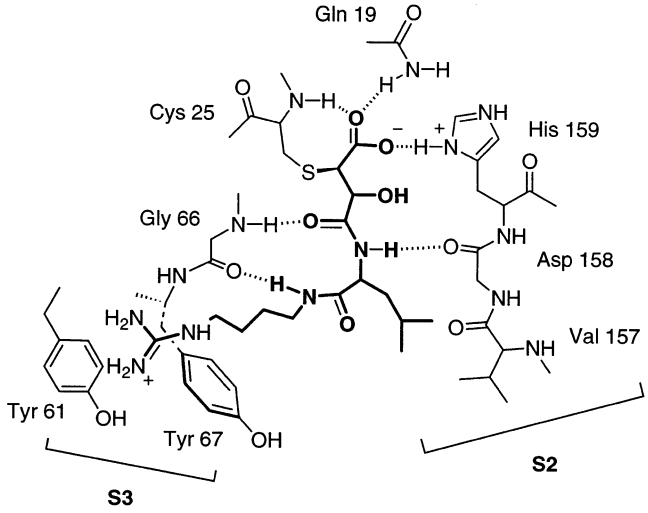
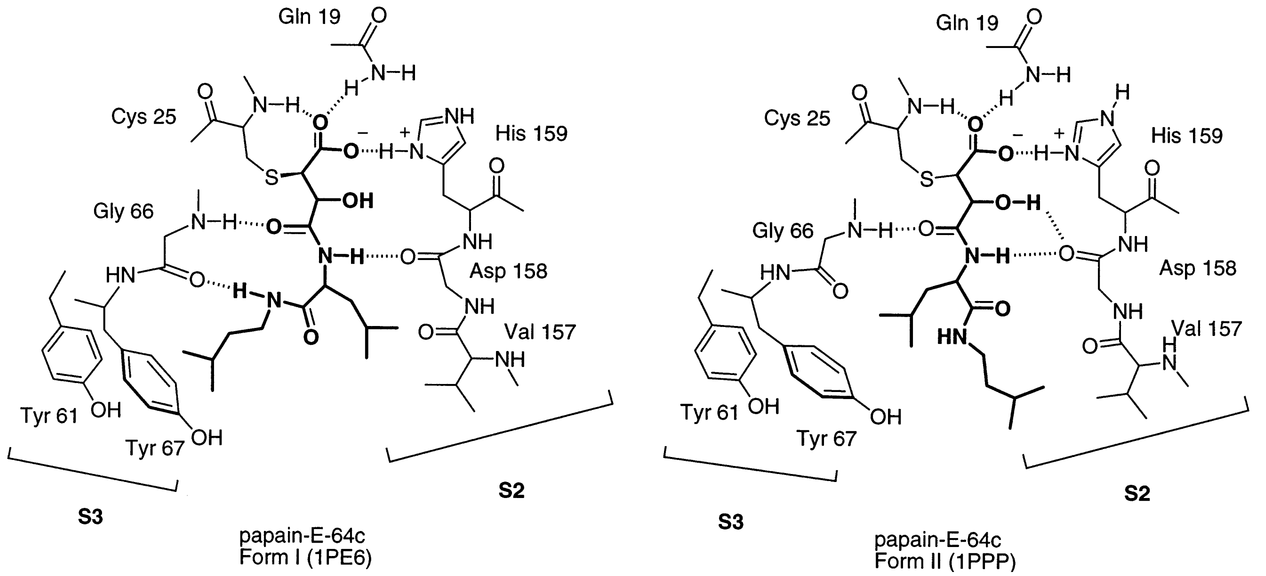


Figure 40. S subsite binding mode of E-64 in papain–E-64 complex.

The N-terminal carboxyl group of the epoxysuccinate moiety forms three hydrogen bonds with the backbone amide NH of the catalytic Cys 25, the Nδ of His 159, and the side chain NH of Gln 19. The NH of Cys 25 and the side chain NH of Gln 19 form the oxyanion hole of papain family cysteine proteases (Figure 40). The side chains of the amino acid residues or C-terminal groups in epoxysuccinate derivatives make a variety of interactions with the various binding pockets in the active sites of cysteine proteases. However, within related families of cysteine proteases, which have matching backbone tertiary structures, there are many similarities in the binding mode of E-64 and its derivatives to individual enzymes in the family.

There are two major polymorphous forms of the papain–E-64c complex where there are significant differences in the binding mode of the leucine and isoamylamide side chains of the inhibitor moiety.[239] The hydrogen bonds, electrostatic interactions with the carboxylic group of the succinate moiety, and the S1 subsite interactions are nearly the same in the two structures. The orientations of leucyl side chain and isoamylamide moiety are rotated in opposite directions in each form (Figure 41). Form I of the papain–E-64c complex (PDB code 1PE6) has the leucyl side chain in the S2 pocket and the terminal isoamylamide group in the S3 pocket,[240] whereas form II (PDB code 1PPP) shows reverse orientation.[239] Due to the reverse orientation in the S2 pocket, form II of the papain–E-64c complex does not make a parallel β-sheet interaction with the enzyme, and there is only one hydrogen bond between E-64c and Gly 66. This β-sheet is a characteristic of many complexes that utilize the S subsite binding mode (Figure 39b).

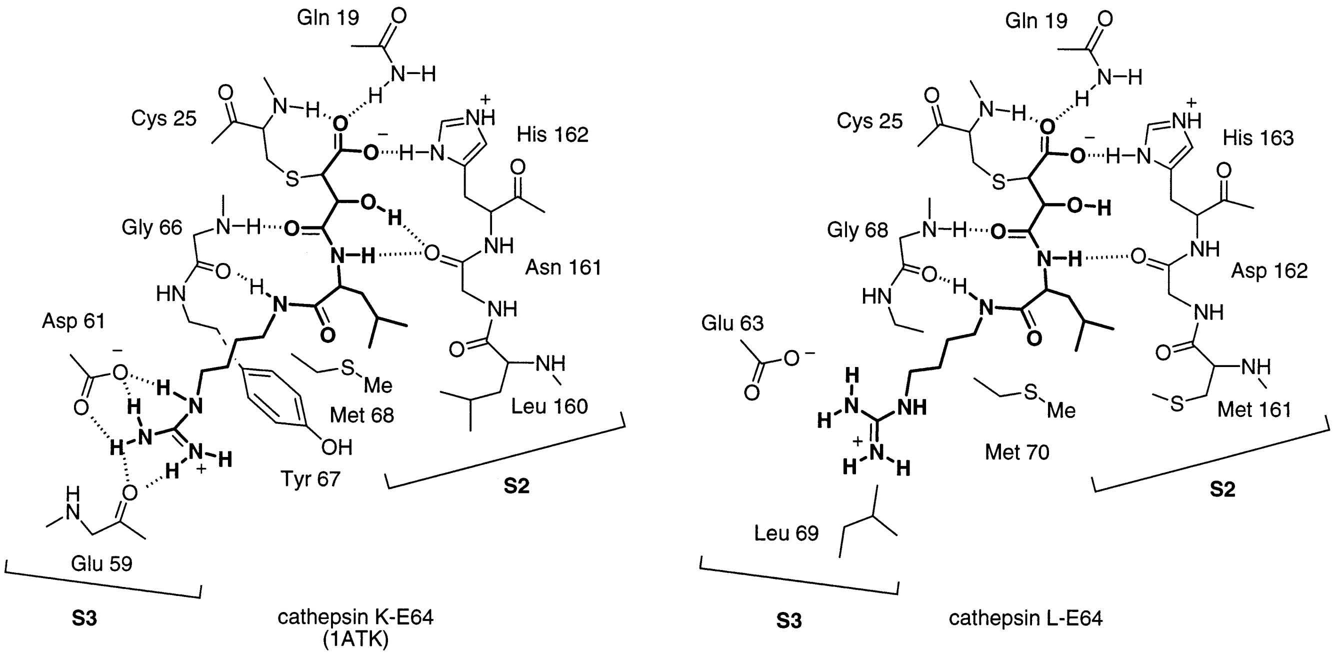
In papain and actinidin, the E-64 leucyl side binds in the S2 subsite, which is equivalent to the binding of E-64c to papain in form I. The leucyl side chain is located at the entrance to the hydrophobic S2 pocket.



papain-E-64c Form I (1PE6)

papain-E-64c Form II (1PPP)

Figure 41. Binding of E-64c with papain (form I and form II).



cathepsin K-E64 (1ATK)

cathepsin L-E64

Figure 42. S subsite binding mode of E-64 in cathepsin K (1ATK) and cathepsin L.

This subsite is formed by Val 133, Val 157, Asp 158, and Ala 160 (papain) or Met 211, Ile 160, Ala 136, and Ile 70 (actinidin).

The 4-guanidinobutyl group of E-64 binds to papain with the guanidinium group of agmatine stacked against the aromatic rings of Tyr 61 and Tyr 67 (S3). In papain, the S3 subsite consists of Gly 66 and Tyr 61, whereas Tyr 67 is found at the boundary of the S2 and S3 pockets. The orientation of the 4-guanidinobutyl group is different in the actinidin–E-64 complex. There is one weak interaction in the actinidin complex between one of the guanidinium NHs and the amide carbonyl oxygen of the Asn 88 amide. In many E-64 derivatives, such as E-64c, the agmatine group is replaced with other substituents, and yet inhibitory activity is maintained. This indicates that interactions in the S3 subsite are not essential for inhibition but can be used for increased reactivity and specificity.

The interaction of E-64 with cathepsin L or K is significantly different from that with papain.[234] The β-sheet interaction is still observed, but the leucyl group is buried more deeply in the S2 hydrophobic subsite. The S2 subsite is formed by Met 70, Ala 135, Met 161, and Asp 162 for cathepsin L and by Met 68, Ala 134, and Leu 160 for cathepsin K. In cathepsin K there are three strong hydrogen bond interactions between the guanidinium group of E-64 and the S3 subsite, whereas in cathepsin L there is only one weak interaction between the guanidinium NH and the side-chain oxygen of Glu 63 (Figure 42). These interactions are not possible in papain because the Tyr 67 aromatic ring blocks the entrance to the S3 pocket.

The X-ray structure of the E-64c–cathepsin B complex (1ITO) was recently reported.[241] The carboxylate group at C-2 makes hydrogen bonds with the side chain of Gln 23 and the amide nitrogen of

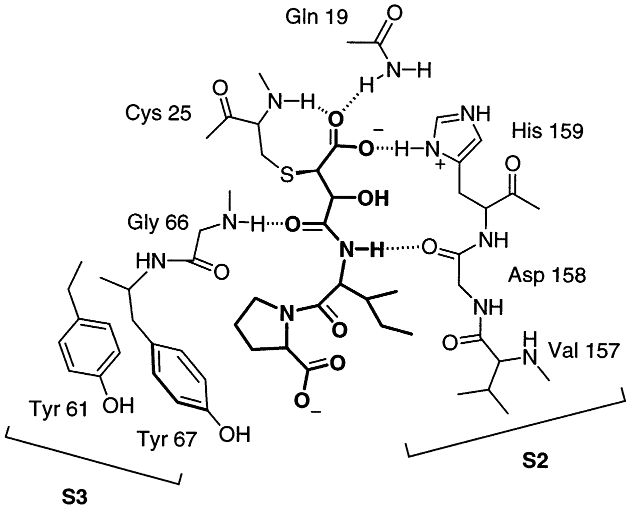


Figure 43. Active site of the papain−CA-028 complex.

Cys 29 in the oxyanion hole. There is one hydrogen bond between the epoxysuccinate amide carbonyl oxygen and the peptide backbone of Gly 74 and an electrostatic interaction between the NH of the inhibitor C-terminal amide and the backbone carbonyl of Gly 74. Therefore, the *β*-sheet hydrogen pattern seen with papain and other cathepsins is not present. The hydrophobic S2 pocket interacts with the Leu residue, whereas the terminal isoamyl moiety is locked in the S3 pocket by the hydrophobic interactions with Phe 75. As expected, E-64c binds in the S subsite of cathepsin B, opposite to the binding of CA-074, which lies in the S′ subsite (see section below).

The crystal structure of the “CA” inhibitor HO-(2*S*,3*S*)-Eps-Ile-Pro-OH (CA-028) with papain reveals a binding mode similar to that of E-64 derivatives (Figure 43).[242] Nucleophilic attack occurs at the C-2 atom, and the Ile-Pro residues interact with the S1 and S2 subsites. This interaction is more energetically favored in papain than the interaction with the S1′ and S2′ subsites (observed with CA-030 and cathepsin B). Due to the proline residue at P2, the parallel *β*-sheet is not present.

S′ Subsite Binding. The S′ subsite binding mode (Figure 39c) has been observed with cathepsin B and several CA inhibitors. Both of the epoxysuccinate analogues, *n*Pr-NH−(2*S*,3*S*)-Eps-Ile-Pro-OH (CA-074) and EtO-(2*S*,3*S*)-Eps-Ile-Pro-OH (CA-030), occupy the S′ subsites of cathepsin B in the X-ray structures. Attack of the cysteine thiolate occurs at the C-3 atom of the oxirane ring. This is in contrast to the S subsite binding mode, where the thiolate attack occurs at C-2. Refinement of the crystal structure of the complex of bovine cathepsin B with CA-074 shows the chirality change from 2*S*,3*S* to 3*R*,3*R*. This chirality change occurs both with S subsite binding (Figure 39b) followed by C-2 attack and with S′ subsite binding followed by C-3 attack (Figure 39c).

In cathepsin B, the amino acid residues in dipeptide inhibitors are positioned in the S′ subsites due to the interaction between the C-terminal carboxylate of the peptide and two histidine residues in the insertion loop (His 110 and His 111) of cathepsin B (Figure 44).[243]

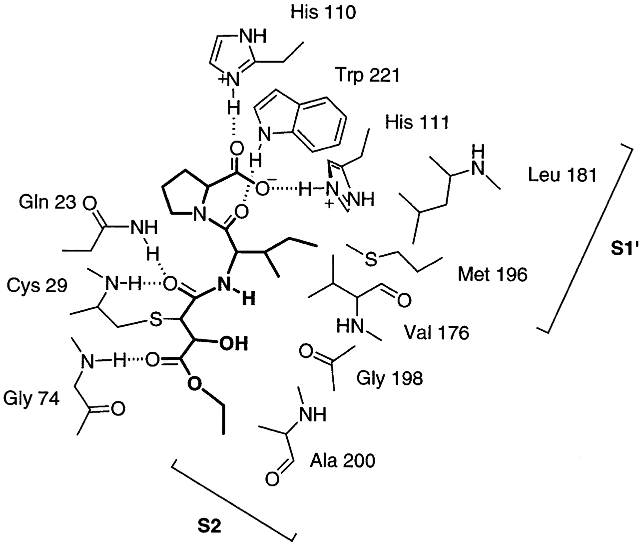


Figure 44. S′ subsite binding of CA-030 in cathepsin B (1CBS).

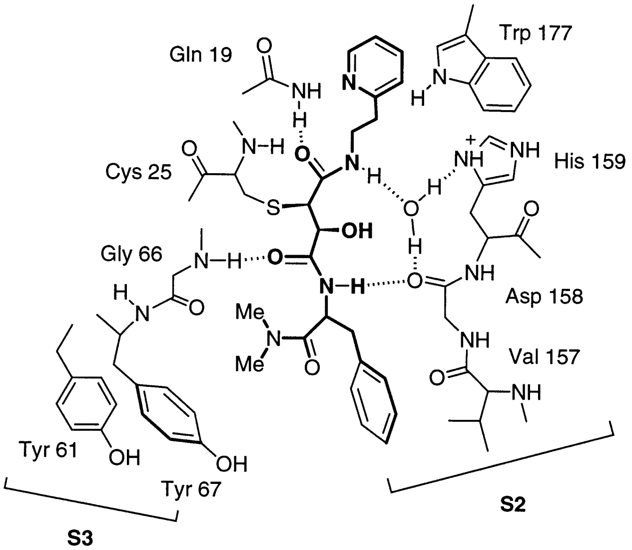


Figure 45. Epoxysuccinate derivative CLIK 148 binds to both S and S′ subsites of papain (1CVZ).

S and S′ Subsites Binding Mode. Recently, bispeptidyl derivatives of epoxysuccinates have been synthesized.[244] The inhibitors have different peptide chains on both carboxyl groups of the epoxysuccinate moiety. Thus, they have the ability to interact with both the S and S′ subsites of cysteine proteases. Thus far, S and S′ subsite inhibitors have been designed for cathepsins B and L.

CLIK 148, designed on the basis of SAR studies, uses both S and S′ subsites for binding, inhibits cathepsin L, and does not inhibit other cathepsins.[245] The crystal structure of CLIK 148 with papain shows the inhibitor binding in the reverse substrate mode, similar to E-64, and extending into the S′ subsite (Figure 45).[246] In the S′ subsite there is hydrophobic stacking between the aromatic rings of the pyridine ring of CLIK 148 and Trp 177. There is also an interaction between the nitrogen atom of the pyridine ring and the Trp 177 NH. Only two hydrogen bond interactions are observed between the epoxysuccinate carbonyl group, the backbone amide NH of the

Table 19. Inhibition of Cysteine Proteases by Epoxysuccinate Derivatives

	rate (M^{-1} s^{-1})					
inhibitor	papain	cathepsin B	cathepsin H	cathepsin L	cathepsin S	ref
HO-(2*S*,3*S*)-Eps-Leu-Agm (E-64)	638000	89400	4000	96300	99000[c]	191[a]
HO-(2*R*,3*R*)-Eps-Leu-Agm	60900	1900	65	2700		191
HO-(2*S*,3*S*)-Eps-Leu-NH-$(CH_2)_7$-NH_2	874000	339000	2070	142000		191
HO-(2*S*,3*S*)-Eps-Leu-NH-$(CH_2)_2$-$CH(CH_3)_2$	357000	298000	2018	206000		191
HO-(2*S*,3*S*)-Eps-D-Leu-NH-$(CH_2)_2$-$CH(CH_3)_2$	32500	3790	28			191
*n*PrNH-(2*S*,3*S*)-Eps-Ile-Pro-OH	112000	<10	20	<10		256
*n*PrNH-(2*S*,3*S*)-Eps-Ile-Pro-OMe	70	210	180	85		256
HO-(2*S*,3*S*)-Eps-Leu-OBzl	52500	24700		791000	171000	198, 251[b,c]
HO-(2*S*,3*S*)-Eps-Leu-NHBzl	90000	37600		4200000	542000	198, 251
HO-(2*S*,3*S*)-Eps-Phe-NHBzl	43000	9000		27800000	501000	198, 251
HO-(2*S*,3*S*)-Eps-D-Phe-NHBzl	3700	670		117000	118000	198, 251
HO-(2*S*,3*S*)-Eps-Leu-Tyr(I_2)-NH-iC_5H_{11}	29000	105000				253[d]
HO-(2*R*,3*R*)-Eps-Leu-Tyr(I_2)-NH-iC_5H_{11}	3500	750				253
EtO-(2*S*,3*S*)-Eps-Leu-Pro-OBzl	176000	8700				250
*i*BuNH-(2*S*,3*S*)-Eps-Leu-Pro-OBzl	2070	206				250
*i*BuNH-(2*S*,3*S*)-Eps-Leu-Pro-OH	558	52000				250
EtO-(2*S*,3*S*)-Eps-Leu-Pro-OH	6130	44400		170		252[e]
EtO-(2*R*,3*R*)-Eps-Leu-Pro-OH	56	567000		26		252
HO-(2*R*,3*R*)-Eps-Leu-Pro-OH	3270	270		74		252
*n*PrNH-(2*R*,3*R*)-Eps-Leu-Pro-OH	5	29400		46		252
*n*PrNH-(2*S*,3*S*)-Eps-Leu-Pro-OH	103	153000		22		252

[a] Apparent second-order rates were measured at 40 °C and pH 6.8 for papain, at 40 °C and pH 6.0 for human cathepsin B, and at 40 °C and pH 5.5 for cathepsin L. [b] k_{inact}/K_i values were obtained at 25 °C and pH 6.5 for papain and at 25 °C and pH 6.0 for cathepsin B. [c] Second-order rate constants were measured at 22 °C and pH 5.5 for cathepsin L and at 22 °C and pH 6.5 for cathepsin S. [d] Pseudo-first-order conditions at 25 °C and pH 6.8 for both papain and bovine spleen cathepsin B. [e] k_2/K_i values were determined at 30 °C and pH 5.5 for papain, human cathepsin B, and cathepsin L.

catalytic Cys 25, and the side chain NH of Gln 19. The hydrogen bond with the N^{δ} of His 159 is not present, but a water molecule situated next to His 159 is involved in hydrogen bonding in this complex. The water molecule forms three hydrogen bonds with His 159, Asp 158, and the NH of CLIK 148. There is only one hydrogen bond between the backbone NH of Gly 66 and the epoxysuccinate carbonyl group of the CLIK 148; hence, the β-sheet is not formed. As expected, the phenylalanine residue is located in the S2 subsite. As indicated above, CLIK 148 shows the same binding mode as E-64, but the specificity for cathepsin L arises due to interactions with the N-terminal pyridine. This is the first E-64 derivative that uses both the S and S′ subsite binding modes to achieve greater selectivity.

Using the S and S′ subsite approach, Schaschke et al. designed dipeptide epoxysuccinate inhibitors selective for cathepsin B. The inhibitor MeO-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH was obtained by elongation of the CA-030-like fragment HO-(2*S*,3*S*)-EPS-Leu-Pro with the cathepsin B propeptide derived fragment Gly←Gly←Leu (the arrow ← represents a reversed amino acid residue). Modeling studies have revealed that the dipeptidyl epoxysuccinate inhibitor occupies the S subsite, whereas the terminal glycine residue is located on the surface of the enzyme. The inhibitor inhibits cathepsin B with a k_2/K_i value of 1520000 M^{-1} s^{-1}, and it is 1260-fold more selective for cathepsin B than cathepsin L (Table 25). This is one of the most potent inhibitors for cathepsin B yet described, and it is likely that the inhibitor binds both in the S and S′ subsites. The proposed binding mode is shown in Figure 38d.

Structure—Activity Relationships. Many peptidyl epoxysuccinate derivatives were designed and synthesized following the isolation and characterization of E-64 in 1978 by Hanada and co-workers. This class of inhibitors has the advantage of being specific for cysteine proteases. Using structure−activity relationships, researchers have developed derivatives that are selective toward certain cysteine proteases, such as cathepsins B, L, and S and cruzain. In general, SAR studies revealed the importance of the epoxide moiety, the 2*S*,3*S*-stereochemistry of the epoxide ring, and the L-configurations of the amino acid at P1 (except for cruzain). Modifying the substituents on the carboxylic group of the epoxysuccinate and the P1 and P2 residues generated many inhibitors.

One of the first studies showed that the presence of the epoxysuccinyl group is necessary for inhibition.[188] E-64 is >1000 times more potent than HO-Fum-Leu-NH$(CH_2)_2$-$CH(CH_3)_2$ (DC-11), where the epoxide ring was replaced by a double bond.[191] Aziridines, in which the oxygen of the oxirane ring is replaced by a nitrogen, are generally much poorer inhibitors of cysteine proteases. Thiirane derivatives, where a sulfur atom substitutes for the oxygen in the epoxide moiety, have been recently synthesized and tested against papain.[247] Cyclopropyl analogues have also been recently synthesized, but biological data are not available.[248]

All active inhibitors have the *trans*-configuration at the epoxide ring, and the 2*S*,3*S*-configuration is 10−1000 times more potent than the corresponding 2*R*,3*R*-configuration (Table 19).[191,249,250] Also, the 2*S*,3*S* isomer of E-64 ($k_2 = 7500$ M^{-1} s^{-1}) inactivates calpain II ~7-fold better than the 2*R*,3*R* isomer ($k_2 = 1070$ M^{-1} s^{-1}).[204] The analogues that contain L-amino acids are 10−100-fold more reactive than the corresponding D-amino acid derivatives (see Table 19).[191,198,250,251] This has led to the nearly exclusive study of inhibitors with the 2*S*,3*S*-configuration and

Table 20. Inactivation of Calpain II by Epoxysuccinyl Peptides

inhibitor	k_2[a] (M^{-1} s^{-1})
HO-(2*S*,3*S*)-Eps-Leu-NH-$(CH_2)_4$-NH-Z	23340
HO-(2*S*,3*S*)-Eps-Leu-Agm[a] (E-64)	7500
HO-(2*R*,3*R*)-Eps-Leu-Agm	1070
HO-(2*S*,3*S*)-Eps-Leu-NH-CH_2-$CH(CH_3)_2$	7450
HO-(2*S*,3*S*)-Eps-Leu-NH-$(CH_2)_7$-NH_2	4990

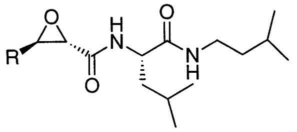
[a] Apparent second-order rate constants were determined at 22 °C and pH 7.5. [b] Agm = agmatine (1-amino-4-guanidinobutane).

L-amino acids. However, recent research has shown that some inhibitors in the 2*R*,3*R*-configuration also have high activity (see the section on dipeptidyl epoxysuccinates and Table 25)[252] and that certain D-amino acids can be used to obtain selective inhibition of cruzain (Table 22).[205]

Substitution of the charged guanidine group with an isopentyl or heptylamine did not affect the inhibitory potency for papain, cathepsin B, and cathepsin L. However, increasing the side-chain length from four to seven, as shown in HO-(2*S*,3*S*)-Eps-Leu-NH-$[CH_2]_4$-NH_2 ($k_2 = 2790$ M^{-1} s^{-1}) and HO-(2*S*,3*S*)-Eps-Leu-NH-$[CH_2]_7$-NH_2 ($k_2 = 4990$ M^{-1} s^{-1}), increased the rates of inactivation of calpain (Table 20).[204] Derivatives containing a P2 phenylalanine, monoiodotyrosine, and diiodotyrosine were better inhibitors for cathepsin B rather than for papain and calpain.[250,253] Replacement of leucine with isoleucine at P1 and placing a proline residue at P2 result in excellent inhibitors of cathepsin B (Table 21).[219] Phenylalanine at the P1 position yields inhibitors that are selective for cathepsin L,[198] whereas D-homotyrosine at P1 resulted in specific inhibitors for cruzain (Table 22).[205]

Analogues of E-64c in which the free carboxylate has been replaced by a hydroxamic acid, amide, methyl ketone, hydroxyl, or an ester show that the rates of inhibition of papain and cathepsin B follow the order $CO_2H > CONHOH > CONH_2 > COR > CO_2R > CH_2OH > H$ as shown in Table 23. The results show that the electrostatic attraction between the carboxylate of the inhibitor and the protonated His 159 of papain make possible the docking of the inhibitor in the active site of the enzyme.

Table 23. Inhibition of Papain and Cathepsin B by Carboxylate Derivatives of E-64c



R	papain[a] k_i (M^{-1} s^{-1})	cathepsin B[b] k_{obs}/[I] (M^{-1} s^{-1})
HOOC	290000[c]	175000
HONHCO	100000	
NH_2CO	6800	150
CH_3CO	2300	30[d]
CH_3CH_2OCO	770	11
$HOCH_2$	4	NI[e]
H	3[d]	NI

[a] Meara and Rich, 1996.[227] Assays conducted at 30 °C and pH 6.8 for papain. [b] Bihovsky et al., 1993.[225] The k_{obs} value was obtained at equimolar concentrations of inhibitor and enzyme, at 25 °C and pH 6.0 for cathepsin B. [c] Meara and Rich, 1995.[226] [d] Mixture of diastereomers. [e] NI = no inhibition.

A different series of compounds, in which an ester or an amide replaces the free carboxylate, have been explored for potency and selectivity with cysteine proteases. In one series of epoxysuccinate derivatives, the ester inhibited calpains in lysed and intact cells, whereas the amide inhibited calpain only in lysed cells.[254] Short-chain amide and ester derivatives of Eps-Leu-Pro-OH and Eps-Ile-Pro-OH are poorer in-

Table 21. IC_{50} Values of Epoxysuccinyl Peptide Inhibitors for Cathepsins B, L, and H and Calpain II[a]

inhibitor	IC_{50} (nM) cathepsin B	cathepsin L	cathepsin H	calpain II	ratio cathepsin L/ cathepsin B	ratio cathepsin H/ cathepsin B
HO-(2*S*,3*S*)-Eps-Ile-Pro-OH	30.4	530	15000	82000	17	500
EtO-(2*S*,3*S*)-Eps-Pro-Pro-OH	25	47000	1000000	200000	188	40000
EtO-(2*S*,3*S*)-Eps-Thr-Ile-OH	13.5	540000	1000000	200000	40000	74000
EtO-(2*S*,3*S*)-Eps-Ile-Pro-OH	2.28	32000	240000	200000	14000	105000
cC_6H_{11}O-(2*S*,3*S*)-Eps-Ile-Pro-OH	1.11	5600	19000	200000	5000	17000
*n*PrNH-(2*S*,3*S*)-Eps-Ile-Pro-OH	2.24	172000	420000	200000	77000	188000
*i*PrNH-(2*S*,3*S*)-Eps-Ile-Pro-OH	4.64	260000	1000000	200000	56000	216000
EtO-(2*S*,3*S*)-Eps-Ile-Pro-NH_2	5600	18000	112000	200000	3	20
HO-(2*S*,3*S*)-Eps-Leu-NH-$(CH_2)_2$-$CH(CH_3)_2$	3.36	0.09	1640	3000	0.03	488

[a] Activities of cathepsins B, H, and L were assayed at pH 5.5 and 37 °C. Calpain II was assayed at pH 7.5 and 30 °C. *n*Pr = *n*-propyl; *i*Pr = isopropyl, *c*C_6H_{11} = cyclohexyl.

Table 22. Inactivation of Cruzain and Rhodesain by E-64 Analogues[205,259]

inhibitor	k_{inact}/K_i[a] (M^{-1} s^{-1}) cruzain	rhodesain	papain	cathepsin B
HO-(2*S*,3*S*)-Eps-Leu-NH-$(CH_2)_2$-$CH(CH_3)_2$ (E-64c)	70600	92200	200000	52300
HO-(2*S*,3*S*)-Eps-Hph-NH-$(CH_2)_2$-$CH(CH_3)_2$[b]	65000	57750	12200	7000
HO-(2*S*,3*S*)-Eps-Hph-NH-$(CH_2)_4$-OH	91500		39000	32500
BzlONH-(2*S*,3*S*)-Eps-D-HTyr-NH-$(CH_2)_2$-$CH(CH_3)_2$[b]	441600	34000	1450	1790
BzlONH-(2*S*,3*S*)-Eps-D-Hph-NH-$(CH_2)_2$-$CH(CH_3)_2$	98500	93700		
BzlONH-(2*S*,3*S*)-Eps-D-Hph-C_6H_5	179700			

[a] Data were determined at room temperature and pH 5.5 for cruzain, papain, and bovine cathepsin B. [b] Hph = homophenylalanine; HTyr = homotyrosine.

Table 24. Inhibition of Cathepsins X, B, and L by E-64 Derivatives

inhibitor	$k_{\text{inact}}/K_{\text{i}}$ (M^{-1} s^{-1}) cat. X	cat. B	cat. L
*n*PrNH-(2*S*,3*S*)-Eps-Ile-OH	225	33	21
*n*PrNH-(2*S*,3*S*)-Eps-Leu-Pro-OH (CA-074)	4	136000	31
HO-(2*S*,3*S*)-Eps-Leu-Agm (E-64)	775	55700	37700

[a] Second-order rate constants were measured at 25 °C and pH 5.0.

hibitors of papain, cathepsin L, and cathepsin H than the corresponding carboxylates as shown in Tables 19 and 21.[219,250,252,255] However, short-chain amide derivatives such as *n*Pr-NH−(2*S*,3*S*)-Eps-Ile-Pro-OH (CA-074) are more active and selective toward cathepsin B ($k_{\text{inact}}/K_{\text{i}}$ = 136500 M^{-1} s^{-1} at pH 5.0).[200,219] Modifications at the N-terminus of CA-074, where a 4-hydroxyphenol moiety is added to the pre-existing *n*-propyl group, resulted in a compound (MB-074) that is still specific for cathepsin B, but is 5-fold less potent.[213] Esterification of the free carboxy terminal of the proline residue to a methyl ester in all CA-074 analogues resulted in loss of potency and selectivity toward cathepsin B.[256] Recently, a one amino acid CA-074 derivative, *n*Pr-NH−(2*S*,3*S*)-Eps-Ile-OH, was designed to inhibit cathepsin X.[200,257] This is a specific, but not very potent, inhibitor for cathepsin X, with a 10-fold preference over cathepsins B and L (Table 24).

Dipeptidyl epoxysuccinate derivatives have been successfully designed as selective inactivators of cathepsin B.[244,252] These inhibitors mimic the reverse peptide binding in the S subsites as well as the normal substrate binding mode in the S′ subsites. The first inhibitor contained a peptide moiety, ornithylagmatine, as a replacement for the ester group of EtO-Eps-Leu-Pro-OH.[252] In the second generation, the ornithylagmatine moiety was replaced with the tripeptide Leu-Gly-Gly-OMe, which was selected to position the inhibitor in the antisubstrate orientation at P2−P4.[244] These inhibitors were tailored for cathepsin B, because the tripeptide moiety represents the propeptide activation sequence of cathepsin B (residues 46−48). The second-order rate constants of inactivation of papain, cathepsin B, and cathepsin L are shown in Table 25.

Other E-64 derivatives that selectively inhibit cathepsin L have been developed by Katunuma et al.[220,258] The CLIK series (cathepsin L inhibitor Katunuma) contains the common structural feature *N*-*trans*-Eps-Phe-NMe$_2$, which is essential for selective inhibition of cathepsin L. The 2*S*,3*S*-stereochemistry at the epoxide moiety is required for inhibition. CLIKs inhibit cathepsin L at a concentration of 10^{-7} M in vitro, whereas no inhibition of cathepsin B, C, S, or K was observed.[220] However, second-rate inhibition constants are not available; therefore, the efficiency of CLIKs cannot be compared with that of other E-64 derivatives.

Recently, analogues of E-64 have been designed as highly selective inhibitors of cruzain and rhodesain, two parasitic cysteine proteases.[205,259] This series of inhibitors is less potent than vinyl sulfone inhibitors. The most potent inhibitors of cruzain have a D-HTyr or D-Hph residue at P2 and an *O*-benzyl hydroxamate at the P′ site (Table 22). The epoxysuccinyl derivative BzlONH-(2*S*,3*S*)-Eps-D-HTyr-NH-(CH$_2$)$_2$-CH(CH$_3$)$_2$ is 13-fold more potent for cruzain than rhodesain and ∼300-fold more selective for cruzain versus cathepsin B and papain. Most of the compounds show little or no activity in vitro when tested against J744 cells infected with *T. cruzi* in tissue culture.[205]

Neutral epoxysuccinyl analogues of E-64 can be membrane permeable. This is achieved by replacing the carboxylate of the epoxide moiety with esters or amides, whereas the agmatine group is replaced by alkyl groups. Modifications of the peptidyl portion, especially the leucine side chain in E-64, resulted in tighter binding in the S2 site, which is the major specificity determinant for cysteine proteases. As a result of SAR studies, very potent and specific epoxysuccinate inhibitors of selected cysteine proteases are now available.

Biological Studies. Many biological studies were published shortly after it was discovered that E-64 and its derivatives specifically inhibit cysteine proteases. Epoxysuccinates are very useful in vivo due to their the potent inhibitory activity, stability and permeability into cells and tissues. Using radiolabeled E-64 derivatives, it was shown that peptidyl epoxysuccinates are incorporated into the lysosomes, where they bind and inactivate the target cysteine proteases. The most biologically studied epoxysuccinyl inhibitors are E-64, E-64c, E-64d (also known as aloxistatin, loxistatin, EST, and Ep 453), CA-074, and CA-030.

E-64 derivatives selectively inhibit lysosomal protein degradation in isolated hepatocytes.[260] Early studies show the effects of E-64 and analogues on cathepsins B and L in vivo and in vitro.[190,261] Both isomers of E-64, containing the D- and L-leucine

Table 25. Inactivation of Cysteine Proteases by Epoxysuccinyl Dipeptides[a]

inhibitor	papain k_2/K_{i} (M^{-1} s^{-1})	cathepsin B k_2/K_{i} (M^{-1} s^{-1})	cathepsin L k_2/K_{i} (M^{-1} s^{-1})	ratio cathepsin B/ papain	ratio cathepsin B/ cathepsin L	ref
Agm←Orn←(2*S*,3*S*)-Eps-Leu-Pro-OH[b,c,d]	6220	197000	250	32	790	252
Agm←Orn←(2*R*,3*R*)-Eps-Leu-Pro-OH	225	63300	26	281	2440	252
MeO-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH	14800	1520000	1200	103	1260	244
MeO-Gly←Gly←Leu←(2*R*,3*R*)-Eps-Leu-Pro-OH	870	215000	269	247	798	244
HO-(2*S*,3*S*)-Eps-Leu-Agm (E-64)	869000	81400	43800	0.09	1.9	252

[a] Second-order rate constants were determined at 30 °C and pH 5.5 for papain, human cathepsin B, and cathepsin L. [b] Agm = agmatine (1-amino-4-guanidinobutane). [c] Orn = ornithine. [d] Arrows indicate that the peptide chain runs from right to left.

residues, inhibit cathepsins B and L in vivo, but the D-isomer is less potent. The derivative E-64c showed strong inhibitory activity in vivo against cathepsins B and L.[190] E-64c was first injected subcutaneously in the pectoral muscle of dystrophic chickens and inhibited cathepsins B and H in a dose-dependent manner.[262] It is effective in animal models of muscular dystrophy when it is administered intraperitoneally and has no effect when given orally as it is not absorbed well from the intestines.[249]

E-64d, the ethyl ester of E-64c, is the most intensively examined epoxysuccinyl derivative in biological systems. The ester moiety of E-64d is hydrolyzed during permeation through the intestinal membrane and is converted to E-64c in the blood.[249] At one time, E-64d was considered to be a promising prodrug for the treatment of muscular dystrophy.[263] Clinical trials were conducted in Japan, but development stopped in the early 1990s due to its low efficacy,[264] side effects, such as hepatic injury in rats,[265] and teratogenic effects on rat embryogenesis.[266,267] Pharmacokinetic studies were performed in healthy adult male volunteers, who were orally administered 100 mg three times a day for one day or seven consecutive days.[268] The drug was detected as E-64c in urine and serum, and no accumulation was observed after repetitive administration. About 30% of the dose was recovered in the urine. No changes in subjective and objective symptoms or clinical tests were observed.

The inhibitor E-64d is more effective at inhibiting cathepsins B and L when it is given orally. Oral administration of 100 mg/kg of body weight strongly inhibits cathepsin B and L activities in the skeletal heart muscle, heart, and liver of hamsters.[249] In dystrophic mice, E-64 and E-64d increased the locomotor activity, survival time, and serum creatine phosphokinase activity but did not increase the weight of the mice.[269] In cultured rat macrophages, E-64d inhibited the processing of the single-chain forms of cathepsins B, H, and L to the two chain forms, which demonstrated that the intracellular processing is accomplished by cysteine proteases in the lysosomes.[270] E-64d is also an effective inhibitor of proteoglycan degradation of interleukin-1 stimulated bovine septum cartilage, whereas E-64 and E-64c showed no inhibition at concentrations up to 100 μM.[209]

The epoxysuccinate E-64d has been tested for its ability to inhibit calpain activity in vivo. In intact platelets, some inhibition was observed at the lowest concentration tested (20 μg/mL), and complete inhibition was obtained at 50 μg/mL.[271] Pectoralis muscle cells and myoblasts treated with E-64d display morphological changes, delays in myofibril assembly, and reduced ability to fuse to form multinucleated myotubes.[272,273] Recently, it was shown that E-64d prevents both calpain up-regulation and apoptosis in the lesion and penumbra following spinal cord injury in rats.[274,275] E-64d has been associated with attenuation of parathyroid hormone in osteoblasts,[276,277] prevention of calcium ionophore cataracts,[278] antiviral activity on foot-and-mouth-disease virus,[279] inhibition of coronavirus protein processing,[280] and many other biological and physiological processes.

The inhibitor CA-074 inactivates cathepsin B only in vivo, whereas the derivative CA-030 shows no specificity, because the ethyl ester at the epoxysuccinyl moiety is hydrolyzed by esterases to produce the nonspecific inhibitor CA-028.[218] The compound CA-074 was designed to be resistant to esterases; it contains an amide group instead of an ester. The dose−responses and time courses of the inhibitory effects of CA-074 and E-64c in vivo and their roles in intralysosomal protein degradation in vivo and in vitro have been determined.[281] CA-074 administered intraperitoneal to rats at a dose of 4 mg/100 g inhibited cathepsin B activity in the kidney and liver for 4 h. In vivo studies of liver lysosomes using FITC-labeled asialofetuin showed that CA-074 selectively inhibited liver cathepsin B and that E-64c was a general inhibitor of cysteine proteases. However, the intralysosomal degradation of FITC-labeled asialofetuin was incomplete.[281]

Mice infected with *Leishmania major* have been treated with CA-074 and CLIK-148. The mice treated with the cathepsin B inhibitor CA-074 showed resistance against infection with *L. major*, and a change of immune response from Th2 to Th1 occurred.[282] It appears that cathepsin B may have a role in antigen presentations.[282−284] Treatment with the cathepsin L inhibitor, CLIK-148, modulates the processing of soluble *L. major* antigen in antigen-presenting cells and exacerbates the infection.[285] Other effects of the treatment with CA-074, such as antigen processing in ovalbumin-immunized mice[286] and degradation of exogenous polypeptidase,[287] suggest a direct role for cathepsin B in antigen presentation. However, it was recently shown that CA-074b, where the *n*-propylamino group of CA-074 is changed to an isobutylamino group, is unable to penetrate cells, which implies that the effects on antigen presentation are not due to the inhibition of cathepsin B.[213]

The epoxysuccinyl inhibitors, E-64c and CA-074, have been used to explore the mechanism of ischemic neuronal death in monkeys.[288,289] There is an increase in the activity of cathepsins B and L in monkeys undergoing a complete 20 min whole brain ischemia. After intravenous administration of CA-074 or E-64c, approximately 67 and 84% of neurons of the hippocampus were saved from delayed neuronal death on day 5 in eight monkeys in this model of brain ischemia. Therefore, peptidyl epoxysuccinate inhibitors have potential therapeutic uses in the treatment of ischemic injuries to the human central nervous system.

Epoxysuccinyl inhibitors of cathepsin L have been studied in a variety of biological processes in vitro and in vivo. Administration of E-64 decreases proteinuria, a major manifestation of glomerular disease in an experimental model of glomerulonephritis in rats.[290,291] Osteoclastic bone resorption is inhibited by E-64 both in vivo and in vitro.[292−294] The immunoreactivity for intracellular cathepsin L is very strong in osteoclasts pretreated with E-64. In contrast, when osteoclasts are treated with E-64, only a few immunoreaction intracellular products are found and the extracellular release of cathepsin L is inhibited.[295] Selective inhibition of cathepsin L was tested in vivo

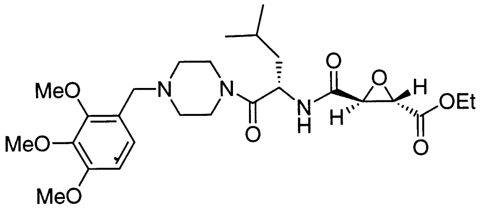


Figure 46. Chemical structure of NCO-700, an epoxysuccinate with a piperazine ring.

by intraperitoneal injection of CLIK-148, -181, and -195 in mice hepatic lysosomes. At 45 and 120 min after administration of 3 mg/kg of inhibitor, the activities of cathepsin L were inhibited, whereas cathepsin B was not. CLIK-195 inhibits cathepsin L in a dose-dependent manner. As a result, CLIK-148, -181, and -195 were shown to permeate the lysosomes and to selectively inhibit hepatic cathepsin L in vivo.[220] The epoxysuccinyl inhibitors, CLIK-148 and E-64, have also been used to explore the effects of proteolytic activity on cell invasion and metastasis in ras-transformed human breast epithelial cells.[296] CLIK-148 also has antihypercalcemic and antimetastatic effects in vivo and significantly prevents bone pit formations induced by TNF-α or M-CSF in vitro.[258,297] Oral treatment of tumor-bearing animals with CLIK-148 significantly reduced the serum calcium levels, therefore preventing malignant hypercalcemia. Direct cancer metastasis of colon tumor and distant bone metastasis are also reduced by treatment with CLIK-148. The inhibitor CLIK-148 has also proven to be effective against osteoporosis.

The ester of a piperazine-containing epoxide, NCO-700 (Figure 46), is an inhibitor of calpain[298] and is being evaluated for use in blocking proteolysis of myocardial proteins associated with myocardial infarction.[299,300] In a number of animal studies, this compound is well tolerated, is nontoxic, and shows a protective effect on myocardial tissue following ischemic injury.[298,301−304] Recently, NCO-700 was shown to possess anticancer properties when tested in vitro and in vivo against human breast and prostate tumors.[305]

Highly selective affinity labels for cathepsin B, based on the dipeptidyl epoxysuccinate inhibitor MeO-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH, have been attached to a spacer-functionalized conjugate of β-cyclodextrin.[211,306] The inhibitor−cyclodextrin conjugate represents a selective drug carrier system, which is fully water-soluble. However, neither the inhibitor nor the cyclodextrin epoxysuccinate derivative is cell-permeable at concentrations necessary for complete inhibition of cathepsin B when tested in MCF-7 breast cancer cells, fibroblasts, and HaCat-cells.[306] Using a similar approach, two epoxysuccinyl affinity labels attached to rhodamine B and biotin were synthesized and characterized (Table 26). Affinity blot analysis using the biotinylated inhibitor permits a selective and sensitive non-radioactive detection of cathepsin B in biological systems. Both conjugates are non-cell-permeable, potent, and selective inhibitors of cathepsin B.[211]

To target the intracellular cathepsin B, the same dipeptidyl epoxysuccinate inhibitor was covalently attached to a cell-penetrating peptide.[307] The C-terminal heptapeptide of penetratin was shown to be efficient for cell membrane translocation.[308] The heptapeptide of penetratin, in which Met-54 is exchanged for a Nle, attached to the parent epoxysuccinate selectively inhibits intracellular cathepsin B, with a second-order rate inhibition constant of 6100100 M^{-1} s^{-1} (Table 26). Membrane permeability studies with MCF-7 cells show effective cell membrane translocation of the inhibitor. Incubation of MCF-7 cells with 0.01 μM conjugate blocks 60% of the intracellular cathepsin B, whereas a concentration of 0.3 μM results in complete inactivation. The non-cell-permeable and cell-permeable conjugates represent useful tools to investigate the biological roles of cathepsin B.

Radioiodine-labeled epoxysuccinyl peptide analogues of CA-074 have been developed and synthesized using solid phase peptide chemistry by Bogyo et al. (Figure 47). The *n*-propyl group of CA-074 was replaced with a tryptamine moiety to allow labeling with ^{125}I.[212] The new non-cell-permeable inhibitor (MB-074) has a low affinity for cathepsin B (k_2/K_i = 2750 M^{-1} s^{-1}) and shows selectivity for cathepsin B over cruzain.[213] Other labeled compounds, such as [^{125}I]-JPM-565, [^{125}I]-MB-074, DCG-03, and DCG-04 (Figure 47), are based on the structures of E-64 and JPM-565.[309] The cathepsin B-specific label [^{125}I]-MB-074 was used to show that cathepsin activity is stimulated during placenta-derived cytotrophoblasts.[213] These new reagents can be used to localize cysteine proteases in biological systems.

Natural Products. Since the discovery of E-64, other naturally occurring epoxysuccinate inhibitors have been discovered as metabolites of different fungal strains (Table 27). Estatins,[310] AM4299s,[311] cathestatins,[312,313] kojistatin A,[314] CPI's,[315] TCM-52's,[316] WF14861,[317] and WF1865s[318] are inhibitors

Table 26. Second-Order Rate Constants for Inactivation of Cysteine Proteases by Epoxysuccinyl Dipeptide Derivatives

inhibitor	cathepsin B k_2/K_i (M^{-1} s^{-1})	cathepsin L k_2/K_i (M^{-1} s^{-1})	ratio cathepsin B/ cathepsin L	ref
β-CD-εAhx-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH[a]	1050000	1204	2672	306
rhodamine B-NH-$(CH_2)_6$-NH-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH	1530000	323	4736	211
biotinyl-NH-$(CH_2)_6$-NH-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH	1726000	256	6742	211
penetratin HP-CO-$(CH_2)_5$-NH-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH	6100000	3590	1699	307
MeO-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH	1520000	1200	1262	244
HO-(2*S*,3*S*)-Eps-Leu-Agm (E-64)	81400	43800	1.9	252

[a] Arrows indicate that the peptide chain runs from right to left. β-CD = β-cyclodextrin; εAhx = ε-aminohexanoic acid; penetratin HP = H_2N←Lys←Lys←Trp←Lys←Nle←Arg←Arg.

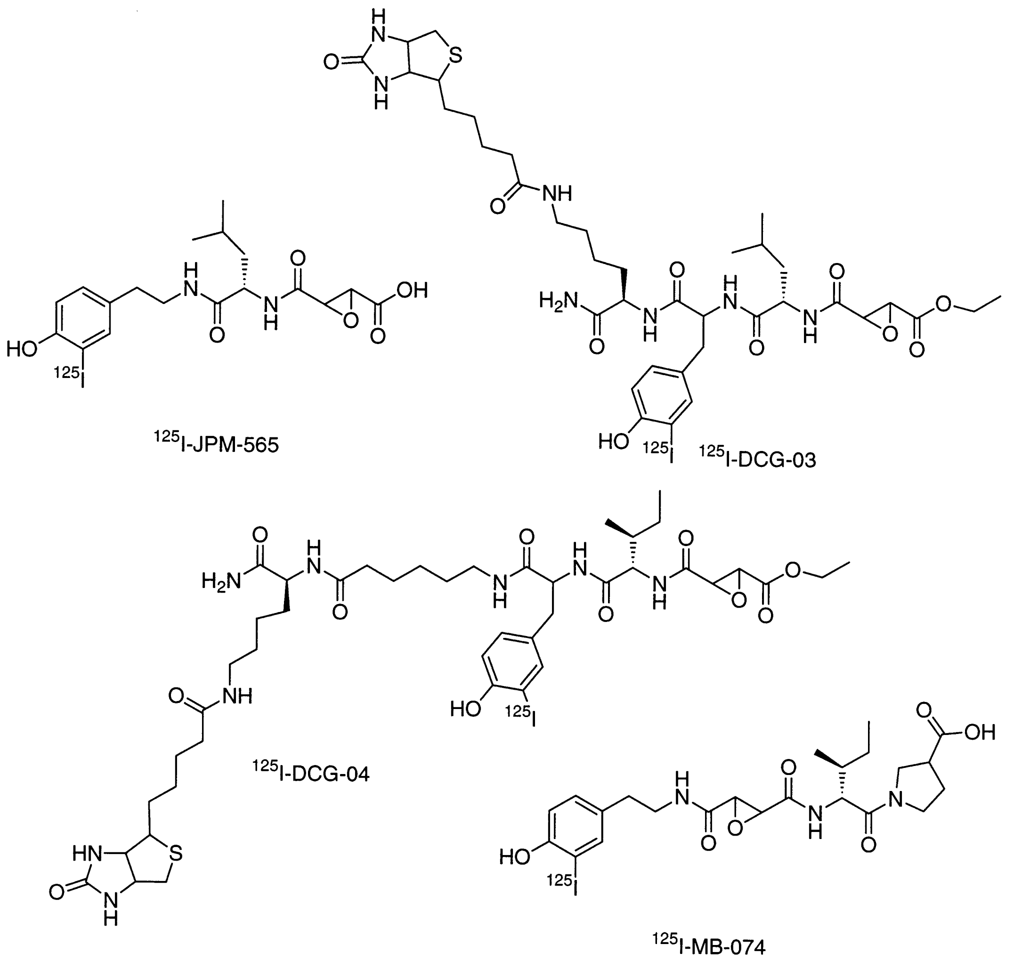


Figure 47. Structure of epoxysuccinyl radiolabeled probes.

of papain, cathepsins B and L, ficin, and bromelain. These compounds do not inhibit serine proteases, aspartic proteases, or metalloproteases, but WF1865s slowly inhibits bovine trypsin with an IC_{50} value of 2.2×10^{-4} M. All of these inhibitors have a *trans*-epoxysuccinyl group, a bulky amino acid, such as leucine, isoleucine, phenylalanine, or tyrosine, and an aminoalkyl moiety, such as spermidine, agmatine, putrescine, or 1*H*-imidazole-2-ylamine. Estatins, cathestatins, and WF14861 are more selective toward cathepsin L than cathepsin B, a selectivity that is not seen with E-64. Esterification of the carboxylate function and replacement of the charged residues with uncharged alkyl groups can increase cell permeability of these compounds (Table 27). The esters are less potent (100−1000-fold less) than the free acids, but the rapid hydrolysis of these esters into their active forms makes them encouraging as prodrugs.[262] The in vivo efficacies WF14865A and B were studied in a low-calcium-diet-fed mouse model. Subcutaneous injections of 10 mg/kg lowered the plasma calcium levels to 60 and 68%, respectively, of the initial level after 6 h.[318] Natural products WF1865s do not show any antimicrobial activity against bacteria and fungi and have low toxicity in a pregnant mouse model.

2. α,β-Epoxyketone Derivatives of Peptides

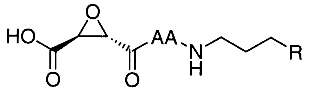
The first peptidyl α,β-epoxyketones described in the literature are the natural microbial products epoxomicin and eponemycin (Figure 48).[319,320] Epoxomicin, an α,β-epoxyketone, is a natural microbial metabolite, which was first isolated from *Actinomyces* due to its in vivo antitumor activity against murine B16 melanoma tumors.[320] Despite this potent activity, the mechanism of biological action has not been elucidated.

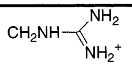
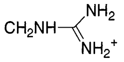
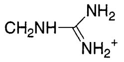
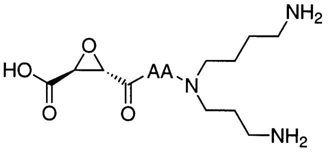
Spaltenstein and co-workers, in search of a selective proteasome inhibitor, first reported in 1996 enzyme inhibitors that inhibited the proteasome. The tripeptide α,β-epoxyketone Cbz-Ile-Ile-Phe-(2*R*)-EP inhibits the chymotrypsin-like activity of the 20S proteasome at low nanomolar concentrations (IC_{50} = 5 nM).[321] Peptidyl α,β-epoxyketones act as covalent irreversible inhibitors via alkylation of the enzyme by the reactive epoxide function.

The structure of a simple α,β-epoxyketone derivative is shown in Figure 49. In this review, the epoxide moiety is referred to as EP. It should be noted that these derivatives are more correctly termed aminoacyl epoxides or peptidyl epoxides. The term peptidyl α,β-epoxyketones is not correct nomenclature and thus should more ideally be referred to as α,β-epoxyketone derivatives of peptides. However, because they are commonly called peptidyl α,β-epoxyketones, this nomenclature is used here.

Shortly after the discovery of this new class of compounds that inhibited the proteasome, the search began to identify other inhibitors of the proteasome

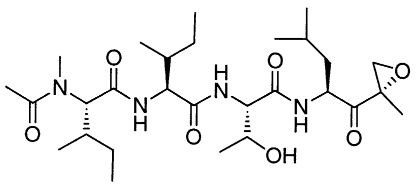
Table 27. IC_{50} Values for Inactivation of Cysteine Proteases by Epoxysuccinate Natural Products



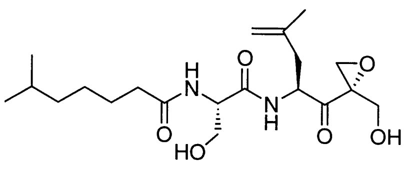
inhibitor			IC_{50} (nM)					
name	AA	R	papain	cathepsin B	cathepsin L	calpain II	calpain I	ref
E-64	Leu	 $CH_2NH{-}C(NH_2)NH_2^+$	36	18	50		41000	186
estatin A	Phe	 $CH_2NH{-}C(NH_2)NH_2^+$	130	270	4			310
estatin B	Tyr	 $CH_2NH{-}C(NH_2)NH_2^+$	180	320	6			310
AM4299 A	Leu	CH_2CH_2OH	88	73	390			311
AM4299 B	Leu	$CH(NH_2)COOH$	280	130	1000			311
cathestatin A	Phe	CH_2NH_2	360	260	7			312
cathestatin B	Tyr	CH_2NH_2	230	280	9			312
cathestatin C	Tyr	$(CH_2)_2NH_2$						313
kojistatin A	Ile	$CH_2NH(CH_2)_4NH_2$	8.2	1.8	4.8			314
CP-2	Ile	CH_2NH_2	8.4	1.4	9.4			315
CP-3	Ile	$CH_2)_2NH_2$	3.7	0.8	4.0			315
TMC-52 A	Tyr	$NH(CH_2)_4NH_2$	44	320	13	52000	64000	316
TMC-52 B	Tyr	$CH_2NH(CH_2)_3NH_2$	7	200	10	37000	44000	316
TMC-52 C	Phe	$NH(CH_2)_4NH_2$	88	460	10	78000	92000	316
TMC-52 D	Phe	$CH_2NH(CH_2)_3NH_2$	49	280	6	93000	90000	316
WF14865A	Ile	1*H*-2-imidazolylamine	650	8.4	64		18000	318
WF14865B	Leu	1*H*-2-imidazolylamine	390	13	72		2000	318
								
CP-1	Tyr		0.9	9.4	0.3			315
CP-5	Phe		2.6	10.3	0.3			315
WF14861	Tyr		850	16	1.1		95000	317

that incorporated the α,*β*-epoxyketone's pharmacophore. The research group of Craig Crews turned its attention to the previously isolated natural products and tested eponemycin and epoxomicin for activity against the 20S proteasome. Both compounds contain a linear peptide α,*β*-epoxyketone and are potent and selective inhibitors of the 20S proteasome in vitro and in vivo.[322,323] This suggested that other α,*β*-epoxyketones, such as epoxomicin and its derivatives, could target and inactivate the proteasome and could be used as molecular probes and therapeutic agents.

Epoxomicin potently inhibits primarily the chymotrypsin-like activity of the 20S proteasome with a value of $k_{obs}/[I] = 35400\ M^{-1}\ s^{-1}$, whereas the trypsin-like and peptidyl-glutamyl peptide hydrolyzing (PGPH) activities are inhibited at rates 100- and 1000-fold slower.[323] Epoxomicin, in contrast to other inhibitors, such as peptide aldehydes and vinyl sulfones, selectively inhibits the proteasome and does not inhibit the cysteine proteases, papain, cathepsin B, and calpain or the serine proteases, such as chymotrypsin and trypsin, at concentrations up to 50 μM.[324] Subsequently, it was shown that α,*β*-epoxyketone inhibitors inactivate the cysteine protease cruzain.[325]

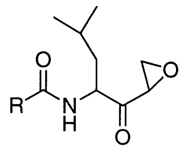


epoxomicin



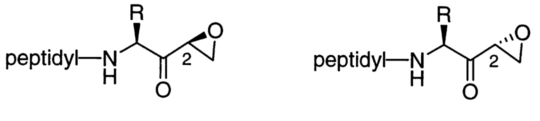
eponemycin

Figure 48. Epoxomicin and eponemycin, linear peptide α,β-epoxyketone natural products.



RCO-Leu-EP

Figure 49. Structure of an α,β-epoxyketone derivative.



2S epoxy ketone

2R epoxy ketone

Figure 50. Stereochemistry of the α,β-epoxyketones.

Stereochemistry. There are two stereoisomers at the C-2 of the epoxyketone moiety, the 2*R*- and 2*S*-isomers (Figure 50). The two isomers of epoxomicin have different inhibitory activities against the 20S proteasome. The naturally occurring 2*R*-isomer is 100 times more potent than the 2*S*-isomer.[324] So far, all natural products and synthetic epoxyketone compounds that show inhibitory activity against the proteasome have the 2*R*-configuration at the epoxide moiety. Interestingly, potent inhibitors of the cysteine protease cruzain show the opposite 2*S*-stereochemistry.[325]

Mechanism. The mechanism of inhibition of the 20S proteasome by epoxomicin involves the N-terminal threonine, which is a unique characteristic of the active site of the proteasome. Epoxomicin inhibits the 20S proteasome by alkylation of the active site threonine residue (Thr 1) of the catalytic subunits, forming an irreversible morpholine derivative. The presence of a six-membered morpholine ring was discovered by X-ray crystallography of the 20S proteasome inhibited by epoxomicin (PDB code 1G65).[326] On the basis of the X-ray structure, Groll and co-workers proposed that the morpholine derivative resulted from the reaction between the epoxyketone moiety and the hydroxyl and amino groups of the N-terminal Thr 1 (Figure 51).

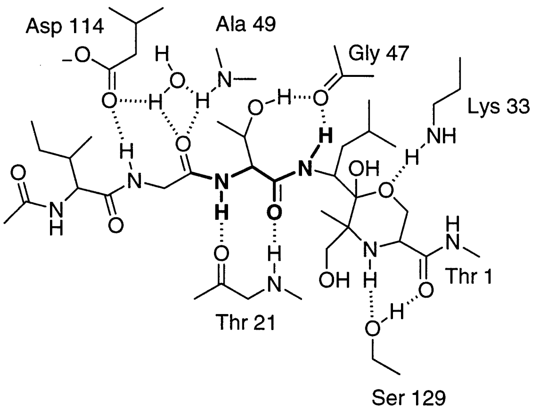


Figure 51. Schematic drawing of the active site of proteasome inhibited by epoxomicin.

The mechanism of formation of the morpholine derivative in the proteasome−epoxomicin complex is a two-step process (Figure 52).[326] The first step involves the formation of a the tetrahedral adduct or hemiketal between the threonine hydroxy group and the ketone carbonyl of epoxomicin. The OH group of Thr 1 is activated by the N-terminal amino group[327] or by an adjacent water molecule that acts as a base. The water molecule forms a bridge between the nucleophilic hydroxyl of the Thr side chain and the N-terminal amino group. This structure is observed in the native structure of the 20S proteasome, which does not contain an inhibitor, and other related enzymes, such as penicillin acylase.[328,329]

In the second step, the morpholino adduct is formed by an intermolecular cyclization reaction. The N-terminal amino group of Thr 1 opens the epoxide ring by means of an intramolecular displacement and inversion of the configuration of the C-2 carbon of the epoxide. The stereochemistry of the C-2 carbon of the epoxide ring changes from 2*R* in epoxomicin to 2*S* in the morpholino ring of the enzyme inhibitor adduct. The nucleophilic attack occurs at the more hindered C-2 of the epoxide, resulting in favorable 6 *exo-tat* ring closure. Attack at the less hindered C-1 methylene of the epoxide would result in an unfavorable 7 *endo-tet* ring closure.[326] The formation of the morpholine ring could explain why epoxomicin selectively inhibits the proteasome and not other proteases.

The mechanism of inhibition of cruzain by peptidyl α,β-epoxyketones is not yet proven but must involve another mechanism. Due to structural similarities between the α,β-epoxyketones and epoxysuccinates, it is most likely that the thiol group of the active cysteine residue of cruzain attacks at the C-2 of the epoxyketone. Also, the fact that the inhibitors which have the 2*S*-configuration at the epoxy moiety are more potent[325] suggests that the C-2 carbon is involved in the nucleophilic attack. In general, epoxysuccinates with the *S*-configuration at the epoxide moiety are more reactive with cysteine proteases.

Structure—Activity Relationships. Recently, several α,β-epoxyketones have been synthesized to determine the effect of the peptide length, amino acid subsite preferences, steric bulk, and N-terminal blocking group on the rate of inhibition of specific proteasome activities. The first α,β-epoxyketone tripeptide to show anti-proteasome activity was Cbz-Ile-Ile-Phe-(2*R*)-EP, which has an IC_{50} value of 5

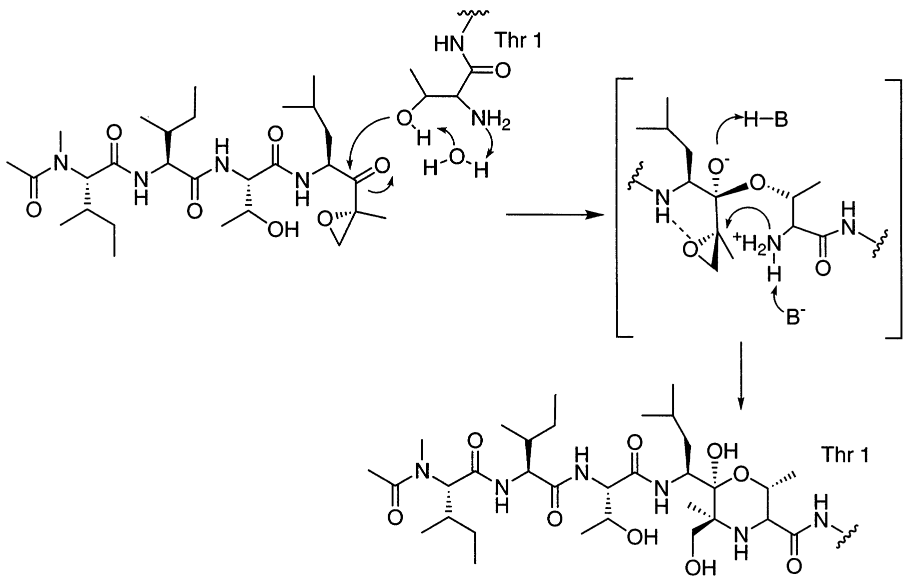
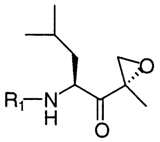


Figure 52. Proposed mechanism of proteasome inactivation by epoxomicin based on the crystal structure.

Table 28. Potency of Peptidyl α,β-Epoxyketones as Inhibitors of the 20S Proteasome



R_1	k_{obs}/[I]			ref
	ChT[a]	T[b]	PGPH[c]	
Ac-Leu-Leu-Leu	14000 (50–150 nM)	NI[f]	9.2 (100–160 μM)	330
Ac-Leu-Leu	780 (1–2.5 μM)	5.1 (100–150 μM)	120 (8–12 μM)	330
Ac-Hph-Leu-Leu[d]	63000 (40–80 nM)	5.4 (120–150 μM)	5.4 (120–150 μM)	330
(YU101) Ac-Hph-Leu-Phe	166000 (5–12 nM)	7.1 (80–130 μM)	21 (80–150 μM)	330
Hph-Leu-Phe	5200 (0.11 μM)	580 (0.5–5 μM)	11 (10–150 μM)	330
(YU102) Ac-Gly-Pro-Phe	5 (50–150 μM)	NI	254 (4–25 μM)	73
Ac-Nle-Pro-Phe[e]	1.9 (100–150 μM)	NI	115 (10–40 μM)	73
isooctanoyl-Pro-Phe	190 (1–2 μM)	NI	194 (10–40 μM)	73
pyrazinecarbonyl-Pro-Phe	520 (1–2 μM)	3.7 (125–150 μM)	23 (40–80 μM)	73
Ac-Ile-Val	5 (100–150 μM)	6.7 (100–150 μM)	14.6 (50–125 μM)	73
inhibitor				
eponemycin	114 (8–10 μM)	17 (100–150 μM)	217 (8–50 μM)	73
epoxomicin	37000 (30–80 μM)	79 (8–12 μM)	37 (50–100 μM)	330
dihydroeponemycin	66 (20–60 μM)	4.4 (125–500 μM)	60 (12.5–50 μM)	322

[a] ChT = chymotrypsin-like activity. [b] T = trypsin-like activity. [c] PGPH = peptidyl-glutamyl peptide hydrolyzing activity. [d] Hph = homophenylalanine. [e] Nle = norleucine. [f] NI = no inhibition.

nM.[321] Derivatives, based on the peptide moiety of the epoxomicin, that have the amino acid Leu at P1 provided the best peptidyl glutamyl peptide hydrolyzing (PGPH) selectivity. Hydrophobic residues at the P2–P4 positions are important for strong inhibition of the chymotrypsin-like activity.[330] Also, the presence of a residue at the P4 position is important for inactivation of the chymotrypsin-like activity by α,β-epoxyketones, which have a leucine side chain at P1 (Table 28).

The inhibitors with the Pro-Phe-Leu tripeptide sequence (P3–P1) do not inhibit the trypsin-like activity of the proteasome, regardless of the nature of the P4 residue. At the amino terminal of the inhibitor, substitution of an acetyl group with a bulky aromatic group increases inhibitory potency for the chymotrypsin-like activity, whereas the inhibitors become less PGPH selective. Most of the compounds that are more selective toward the PGPH activity of the proteasome (YU101 and YU102) possess an acetyl group at the N-terminus. Epoxyketones YU102 and Ac-Nle-Pro-Phe-Leu-(Me)EP inhibit selectively the PGPH activity 50–60-fold more potently than the chymotrypsin-like activity. YU102, at 8 μM concentrations, inhibits only the PGPH activity, whereas the natural product dihydroeponemycin inhibits both the PGPH and chymotrypsin-like activity at the same concentration. Epoxomicin and YU102 exhibit time-dependent inhibition kinetics, which point to modification of the catalytic Thr 1 residue of the proteasome.

Kim and co-workers synthesized and characterized chimeric epoxomicin/dihydroeponemycin inhibitors to evaluate subunit binding specificity, potency, and antiproliferative activities.[331] Epoxomicin and dihydroeponemycin possess different structural features in their left-hand, central, and right-hand fragments.

Table 29. Inhibition of Cruzain by Peptidyl α,β-Epoxyketones

inhibitor	k_{inact}/K_i (M^{-1} s^{-1})	IC_{50} (μM)
Cbz-Phe-Ala-(2*S*)-EP	128200	0.08
Cbz-Phe-Ala-(2*R*)-EP	NA	1
Cbz-Phe-Hph[a]-(2*S*)-EP	330000	0.01
Cbz-Phe-Hph-(2*R*)-EP	NA	>1

[a] Hph = homophenylalanine.

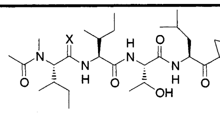
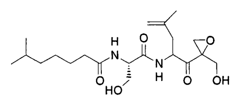
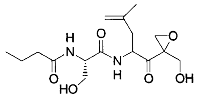
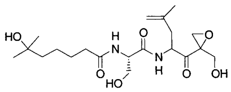
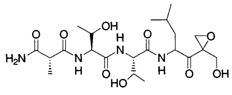
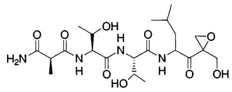
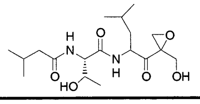
Both compounds are linear peptides that contain the α,β-epoxyketone's pharmacophore. In general, substitution of the N-terminal fragment [N-Ac-(N-Me)-Ile-Ile] with an isooctanoic acid moiety resulted in a 300−500-fold decrease in k_{obs}/[I] values for inhibition of the chymotrypsin-like activity and a 75-fold decrease in k_{obs}/[I] values for inhibition of the trypsin-like activity. Therefore, the chymotrypsin- and trypsin-like activities are strongly influenced by the P4−S4 interactions. There was no major effect on the potency against the PGPH activity, suggesting that the subunit responsible for this activity has no strong preference for peptide inhibitor length.

Peptidyl α,β-epoxyketones are potent, irreversible inhibitors of cruzain (Table 29).[325] The inhibitors combine a portion of the epoxide moiety of the epoxysuccinyl inhibitor E-64c and the dipeptide sequence of the dipeptidyl fluoromethyl ketone, Cbz-Phe-Ala-CH_2F. The second-order rate constant for inhibition of cruzain by Cbz-Phe-Hph-(2*S*)-EP is 4-fold greater than that of E-64c (70600 M^{-1} s^{-1}, Table 22).[205] Compounds that contain 2*S*-stereochemistry at the epoxide moiety are more active than the 2*R*-epimers, as seen by the IC_{50} values listed in Table 29. Therefore, α,β-epoxyketones could be explored in the future as inhibitors for other cysteine proteases.

Biological Studies. Epoxomicin and dihydroeponemycin, an eponemycin analogue,[319] are potent antitumor agents, and biotinylated affinity derivatives demonstrate that the intracellular target is the 20S proteasome.[323,324] Epoxomicin binds to four proteasome catalytic subunits, X, LMP7, MECL1, and Z,[323,324] whereas dihydroeponemycin selectively binds to the β catalytic subunits LMP2 and LMP7.[322] Additional in vitro labeling studies, using biotinylated derivatives of chimeric epoxomicin/dihydroeponemycin inhibitors, suggest that the binding specificities are due to the differences at the amino terminal left-hand fragments of the epoxides, which correspond to P3−P4 residues [N-Ac-(N-Me)Ile-Ile versus isooctanoyl groups].[331]

Epoxomicin successfully inhibits NF-κB activation in vitro and possesses anti-inflammatory activity in vivo.[323] In vivo studies demonstrated that p53 levels increased by 30-fold when human umbilical vein endothelial cells (HUVECs) were incubated with epoxomicin for 6 h. Incubation of cells for longer than 48 h results in >95% cellular apoptosis. Also, epoxomicin effectively inhibits NF-κB-mediated proinflammatory signaling by preventing IκBα degradation and activation of NF-κB binding activity.[323] The anti-inflammatory effects were studied in vivo in the picryl chloride mouse model with contact sensitivity (CS), a cutaneous inflammatory disease. Treatment with a nontoxic dose of epoxomicin (0.58 mg/kg/day) reduced the CS response by 44%, whereas a dose 5 times higher inhibited the inflammatory response by 95%.[323]

Table 30. Inhibition of the Proteasome Activity by α,β-Epoxyketones from Natural Products

inhibitor	structure	IC_{50} (μM) ChT[a]	 T[b]	 PGPH[c]	ref
epoxomicin					320
eponemycin		1.1	7.4	5.4	332
TMC-86A		5.1	51	3.7	332
TMC-86B		1.1	250	31	332
TMC-89A		1.1	0.4	7.2	334
TMC-89B		1.1	0.5	71	334
TMC-96		2.9	36	3.5	332

[a] ChT = chymotrypsin-like activity. [b] T = trypsin-like activity. [c] PGPH = peptidyl-glutamyl peptide hydrolyzing activity.

Dihydroeponemycin-mediated proteasome inactivation induces a spindle-like cellular morphological change and apoptosis.[322] Incubation of bovine aortic endothelial cells (BAECs) with 4 μM dihydroeponemycin resulted in spindle-like morphological changes never reported before in non-neuronal cells.[322] DNA fragmentation occurs in >95% of cells incubated with dihydroeponemycin over the course of 48 h. The results indicated that this inhibitor inactivates cellular processes mediated by the 20S proteasome.

Other peptidyl derivatives were tested for their ability to arrest DNA synthesis and antiproliferation activities. Compounds designed by Myung et al.,[73] which possessed similar k_{obs}/[I] values for the inhibition of the PGPH activity but different chymotrypsin-like inhibitory activities (see Table 30), were tested in vivo to measure the inhibition of bovine aortic endothelial (BAE) cell proliferation.[73] One class of compounds, including isooctanoyl-Pro-Phe-Leu-(Me)-EP and pyrazinecarbonyl-Pro-Phe-Leu-(Me)EP, inhibited DNA synthesis with IC_{50} values of ~0.5 μM.

Other compounds, such as YU102 or Ac-Nle-Pro-Phe-Leu-(Me)EP, required 100-fold higher concentrations to inhibit with IC_{50} values of ~50 μM. These results imply that selective inhibition of PGPH activity is not sufficient to inhibit intracellular mediated degradation, as reflected by the lack of proliferation inhibition in BAE cells. The same compounds were tested for antiproliferative activity in a short-lived green fluorescent protein (GFP) assay.[73] Epoxomicin was the best inhibitor of the GFP degradation with low nanomolar concentrations. The same trend was observed with α,β-epoxyketones specific for the PGPH activity [Y102 and Ac-Nle-Pro-Phe-Leu-(Me)EP]. These compounds accumulated Ub^{G76V}−GFP at concentrations ~50-fold higher than α,β-epoxyketones, which have a higher rate of inhibition of the chymotrypsin-like activity. Therefore, the PGPH activity is not essential in proteasome-mediated protein degradation, whereas chymotrypsin activity degrades proteins independently of the PGPH activity.

Natural Products. Since the discovery of the inhibitory potency of eponemycin and epoxomicin for the proteasome, other natural products that contain the α,β-epoxyketone pharmacophore have been isolated (Table 30). These newly isolated linear peptide natural products were discovered solely on the basis of their proteasome inhibitory properties by screening from microbial metabolites. New natural product proteasome inhibitors include TMC-86A and TMC-86B isolated from *Streptomyces* sp. TC 1084, TMC-96 isolated from *Saccharothrix* sp. TC 1094,[332,333] and TMC-89A and -B isolated from *Streptomyces* sp. TC 1087.[334] These new members of the α,β-epoxyketone class do not inhibit calpain II, cathepsin L, or trypsin at 100 μM concentrations. Eponemycin and epoxomicin were originally isolated on the basis of strong in vitro cytotoxicities against various tumor cell lines.[319,320] However, the second-generation natural products are less cytotoxic against tumor cells of human colon carcinoma (HCT-116) and murine melanoma (B-16) by 100−2000-fold. It is likely that studies with these new α,β-epoxyketones isolated from natural products will increase our understanding of the biological roles of the 20S proteasome.

3. α-Aminoalkyl Epoxide Inhibitors

Peptidyl epoxide inhibitors are a relatively new family of irreversible inhibitors for cysteine proteases that are stable to neutral and basic conditions.[335,336] The peptide sequences in these inhibitors were designed to match a good substrate sequence of the cysteine proteases, papain, cathepsin B, and clostripain. Peptidyl epoxides are ineffective toward serine proteases even when substrate-like sequences for chymotrypsin, subtilisin, elastase, and trypsin are used.[337,338] Mechanistically, these compounds are weak electrophiles that become highly electrophilic when protonated and take advantage of the initial/simultaneous protonation step displayed by cysteine proteases. Peptidyl epoxides have been reviewed by Albeck.[339]

Stereochemistry. Peptidyl epoxides have two isomers at the C-2 carbon of the epoxide ring (Figure 53). The *erythro*-isomer is the 2*S*-stereoisomer, whereas the *threo*-isomer represents the 2*R*-stereoisomer. For the purposes of this review the epoxide moiety in an α-amino alkyl epoxide will be referred to as Ep. The C-2 position of the epoxide is equivalent to the position of the P1 carbonyl group of a peptide substrate. The correct abbreviation would be peptidyl-NHCH(R_1)Ep, but we referred to them using the amino acid abbreviation. Thus, we will call them peptidyl-AA-Ep. The stereochemistry at the P1 amino acid side chain (R_1) can be either D or L. Peptidyl epoxides are stereoselective toward cysteine proteases. The 2*S*-isomer of the epoxide ring and the L-isomer of the amino acid residue at P1 are better inhibitors of papain and cathepsin B.

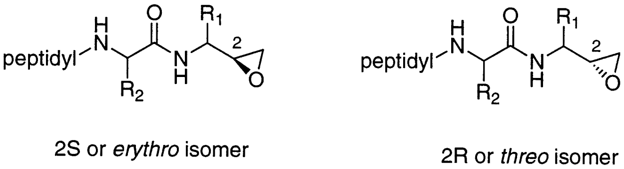


Figure 53. Stereochemistry of the peptidyl epoxides.

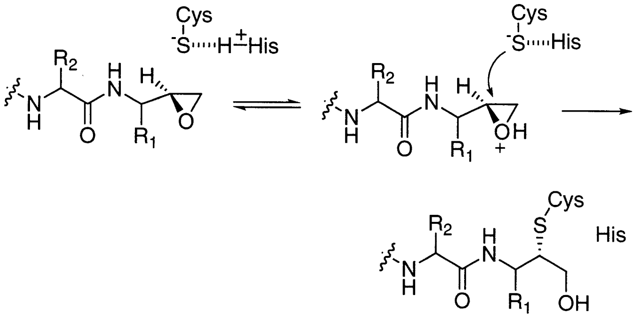


Figure 54. Mechanism of inactivation of cysteine proteases by peptidyl epoxides.

Mechanism. The mechanism involves opening of the epoxide ring and formation of a thioether bond with the enzyme via a nucleophilic attack at C-2 or C-3 on the epoxide ring by the active site cysteine residue, resulting in inhibition of the enzyme (Figure 54).[338] Peptidyl epoxides are mechanistically different from the epoxysuccinyl derivatives, which are activated epoxides. Using ^{13}C radiolabeled peptidyl epoxides and NMR, it was shown that the attack of cysteine occurs at C-2 and that the regioselectivity of the alkylation takes place on the epoxide "exo" carbon (Figure 54).[339] The epoxides are thought first to bind to the enzyme to form a Michaelis-type complex, aligning the epoxide near the active site.[337] Inhibition of an enzyme with a radioactive epoxide inhibitor resulted in one molar equivalent of inhibitor being retained with the enzyme and loss of all enzymatic activity, indicating that the peptidyl epoxides form irreversible covalent complexes by alkylating the active site cysteine residue.[338] Mass spectroscopy was used to confirm the alkylation site of the active-site cysteine by peptidyl epoxides.[339]

Structure—Activity Relationships. A variety of structures have been reported with epoxides replacing the carboxyl group of amino acid or peptide derivatives. They can be directed toward various cysteine proteases by varying the amino acid sequence of the epoxides.[337,338] The 2*R*,*threo*-configuration at the epoxide moiety is not active toward

Table 31. Inactivation of Cysteine Proteases by Peptidyl Epoxides

enzyme	inhibitor	$k_{obs}/[I]$ ($M^{-1} s^{-1}$)	k_2/K_i ($M^{-1} s^{-1}$)	ref
papain	Cbz-Phe-Ep[a]		6.03	335
	Cbz-Phe-Ala-Ep		2.08	337
	Cbz-Ala-Ala-Phe-Ep		1.75	337
	Cbz-Gly-Leu-Phe-Ep		1.56	337
	Cbz-Phe-Thr(Bzl)-Ep		17	337
cathepsin B	Ac-Phe-Gly-Ep	0.058		341
	$PhOCH_2OCO$-Gly-Ep	0.018		341
	Cbz-Phe-Ep		3.95	335
	Cbz-Phe-Thr(Bzl)-Ep		333	337
	Cbz-Phe-Ala-Ep		1.94	337
clostripain	Cbz-Phe-Ala-Ep		0.04	337
	Cbz-Phe-Thr(Bzl)-Ep		0.46	337

[a] Ep = epoxide.

cysteine proteases, but the 2*S*,*erythro*-configuration is found in inhibitors. Also, the natural L-isomer is preferred at the P1 position, which makes this class of compounds stereoselective toward cysteine proteases.[337] Although the actual inhibitory potency of current peptidyl epoxides is poor, it is likely that they can be improved (Table 31).

Incubation of *erythro*-peptidyl epoxides with papain, cathepsin B, and clostripain shows time- and concentration-dependent inhibition (Table 31).[337,340] Cathepsin B is inhibited by Cbz-Phe-Thr(Bzl)-Ep 2000-fold more rapidly than by Cbz-Ala-Ala-Ala-Ep. There is also a degree of selectivity among cysteine proteases. The compound Cbz-Phe-Thr(Bzl)-Ep inactivates cathepsin B 20 times more rapidly than it inactivates papain and 725 times more rapidly than clostripain.[337] Epoxides derived from alkyl amides and esters are weak inhibitors of papain and cathepsin B.[341] Peptidyl epoxides inactivated chymotrypsin, subtilisin, elastase, or trypsin upon long incubation (1−3 h) at high concentration (0.5−5 μM).[337]

The stability of peptidyl epoxides was studied under a variety of conditions. Epoxides are stable under neutral and basic conditions.[336] Peptidyl epoxides are stable in the presence of nucleophiles, such as thiols. The half-life of a radiolabeled inhibitor (2 h at room temperature) in human serum was measured using ^{13}C NMR.[339] As a result, peptidyl epoxides may be appropriate for in vivo biological applications.

E. Aziridine Derivatives of Amino Acids and Peptides

Aziridinyl peptide inhibitors are aza-analogues of epoxysuccinyl peptide inhibitors and are susceptible to ring opening by nucleophiles such as the epoxysuccinyl peptide inhibitors.[342,343] This new class of inhibitors contains a reactive aziridine-2,3-dicarboxylic acid or aziridine-2-carboxylic acid moiety attached to a peptide or amino acid residue.[342,343] Aziridines have been tested against several types of proteases, including serine proteases, aspartate proteases, and metalloproteases, but were found to selectively inhibit cysteine proteases.[342,344] In fact, aziridines, such as aziridine-2-carboxylates and aziridine-2,3-dicarboxylates, are hydrolyzed by serine proteases.[345,346]

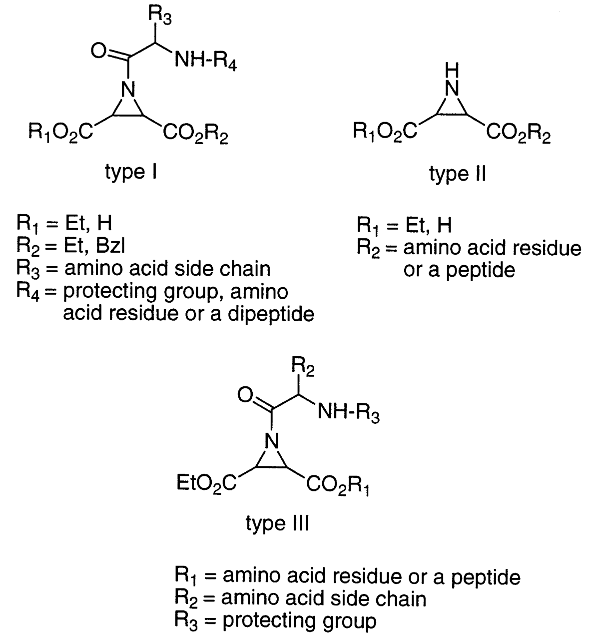


Figure 55. Types of aziridine-containing peptides.

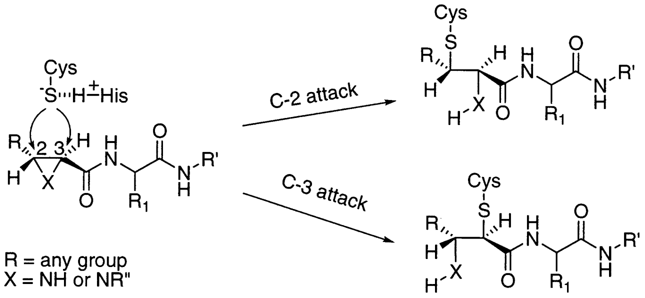


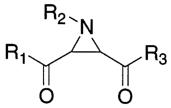
Figure 56. Mechanism of inhibition of cysteine proteases by aziridinyl peptides. The active site cysteine nucleophilically attacks either C-2 or C-3, opening the reactive three-membered ring and alkylating the enzyme.

These compounds are irreversible inhibitors of the cysteine proteases papain, cathepsins B, L, and H.

Three types of aziridine inhibitors of cysteine proteases have been described (Figure 55). Unlike the epoxysuccinyl analogues, substituents can also be placed at the heteroatom of the aziridine ring.[343] Type I are N-acylated aziridines with the aziridine ring on the C-terminus of the peptide or amino acid. Type II consist of N-unsubstituted aziridines with the aziridine ring at the N-terminus of the molecule and are aza-analogues of epoxysuccinyl peptides. Type III represent N-acylated derivatives with the aziridine moiety within the middle of the peptide chain.[343] The unsubstituted aziridine ring will be abbreviated "Azi."

Mechanism. The aziridine's ring is activated for opening by nucleophilic attack either by protonation or by acylation of the heteroatom on the ring.[343] Nucleophilic attack of the cysteine residue can occur either at C-2 or at C-3 (Figure 56). The proposed mechanism of inhibition of cysteine proteases by aziridines is similar to that proposed for epoxysuccinyl derivatives. However, it is known that aziridines inhibit most effectively at low pH values.[347] This increased inhibitory reactivity is due to the protonation of the aziridine nitrogen possibly by the

Table 32. Aziridine Derivatives



enzyme	R_1	R_2 (*N*-acyl group)	R_3	configuration	type	k_2/K_i (M^{-1} s^{-1})	ref
papain	EtO	H	Leu-OBzl	*R*,*R*	II	19	344
	HO	H	Pro-OBzl	*S*,*S* + *R*,*R*	II	127	348
	EtO	Boc-Phe-Ala	OEt	*S*,*S*	I	23	348
	EtO	H	Leu-*i*Am	*R*,*R*	II	55	347
	HO	H	Leu-*i*Am	*S*,*S*	II	1800	347
	HO	H	Leu-*i*Am	*R*,*R*	II	1440	347
cathepsin B	EtO	Boc-Phe	Leu-Pro-OBzl	*R*,*R*	III	32	343
	EtO	H	Leu-OBzl	*R*,*R*	II	27	343
	EtO	Boc-Phe	OH	*S*,*S* + *R*,*R*	I	21	344
	EtO	Boc-Phe	Leu-Pro-OH	*S*,*S*	III	114	348
	EtO	Boc-Phe	Leu-Pro-OH	*R*,*R*	III	109	348
cathepsin L	EtO	H	Leu-OBzl	*R*,*R*	I	271	343
	EtO	Boc-Phe	Leu-Pro-OBzl	*R*,*R*	III	98	343
	EtO	Boc-Gly-Leu	OBzl	*S*,*S* + *R*,*R*	I	54	344
	HO	H	Pro-OBzl	*S*,*S* + *R*,*R*	I	115	348
	HO	Boc-Phe	OH	*S*,*S* + *R*,*R*	I	635	348
	HO	Boc-Gly-Leu	OEt	*S*,*S* + *R*,*R*	I	183	348

active site histidine residue. The protonated aziridine can also interact with the oxyanion hole of cysteine proteases with energetically favorable hydrogen bonding.[343] The oxyanion hole of the papain family of cysteine proteases contains a Gln residue, which can act as either a hydrogen bond donor or acceptor, in contrast to serine proteases where the oxyanion hole acts as a donor.

Although both the aziridine and the epoxysuccinyl may have similar chemical reactivities, differences exist between the two classes of inhibitors. Ready protonation of the nitrogen of type II aziridines is one difference between aziridines and their epoxysuccinyl analogues.[344] A second difference is the hydrogen-bonding abilities of the two classes of inhibitors. Aziridines are H-bond donors, whereas the epoxysuccinyl inhibitors are H-bond acceptors. These differences suggest that the two classes of inhibitors may have different binding modes and possibly variable interactions with cysteine proteases.[344] Last, unlike most epoxysuccinyl inhibitors, the *R*,*R*-configuration of the aziridine ring is preferred for inhibition in both types II and III aziridine inhibitors, whereas the type I aziridine inhibitors with the *S*,*S*-configuration are better inhibitors.[343]

Structure—Activity Relationships. Aziridines irreversibly inhibit papain, cathepsin B and cathepsin L, whereas inhibition of cathepsin H is not time-dependent.[344] Examples of aziridine inhibitors for cathepsins B and L are shown in Table 32.

Type I inhibitors are more active with the *S*,*S*-configuration of the aziridine ring and are expected to bind in substrate-like binding modes (see Figure 39 for a similar binding mode with the epoxysuccinates). With a few exceptions, type II inhibitors are the most powerful of the peptidyl aziridine inhibitors against papain and cathepsins, particularly at low pH values, and are comparable to their epoxysuccinyl peptide analogues in potency at pH ~4.[343,347] For example, HO-(2*S*,3*S*)-Azi-Leu-*i*Am (*i*Am = isoamyl amide) inhibits papain with a rate of k_{inact}/K_i of 1.8 × 10^3 M^{-1} s^{-1} at pH 6.5 and with a rate of k_{inact}/K_i of 6.9 × 10^4 M^{-1} s^{-1} at pH 4.0, which represents an increase of 300-fold in the inhibition rate at lower pH.[347]

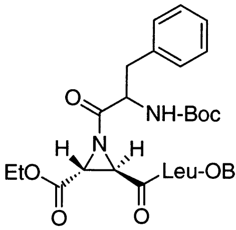


Figure 57. Structure of type III aziridine Boc-Phe-(*R*,*R*)-(EtO)-Azi-Leu-OBzl.

Type III aziridines, particularly bis-peptide derivatives, have increased selectivity and potency for cathepsin B (with Boc-Phe linked to the aziridinyl nitrogen, see Figure 57).[247] The increased selectivity for cathepsin B is thought to be due to favorable interactions of the Boc-Phe with the S2 subsite of the enzyme and hydrogen bonding of the free carboxylic acid at the C-terminus with the enzyme's occluding loop.[348] Type III aziridine derivatives have also been tested for non-time-dependent inhibition with calpains I and II and cathepsin H. The best aziridine derivative, Boc-Phe-(*R*,*R*)-(EtO)-Azi-Leu-OBzl (Figure 57), has K_i values of 19 and 42 μM for calpains I and II, respectively, and a K_i of 137 μM for cathepsin H.[344] In addition, those type II and III inhibitors with a carboxylic acid at one or both ends of the inhibitor have even greater potency toward cathepsins compared to other cysteine proteases.[348]

Natural Products. Miraziridine is an aziridinesuccinyl derivative isolated from the marine sponge *Theonella mirabilis*.[349] This rare natural product inhibits cathepsin B with an IC_{50} value of 1.4 μg/mL. Characterization of this compound reveals the absolute stereochemistry of aziridine to be 2*R*,3*R* (Figure 58). Miraziridine contains a vinylogous arginine residue, which was not previously reported in a natural product,[349] and an aziridine-2,3-dicarboxylic acid, which has previously been isolated from *Streptomyces*.[350]

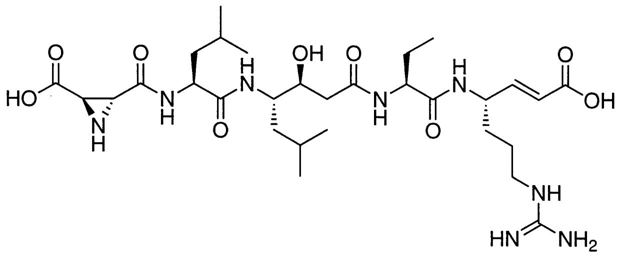


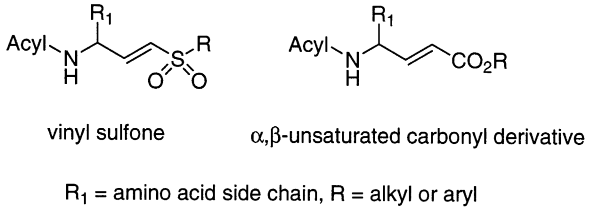
Figure 58. Strucuture of miraziridine.

F. Vinyl Sulfones and Other Michael Acceptors

Peptide and amino acid derivatives that contain a Michael acceptor are specific irreversible cysteine protease inhibitors. This class of inhibitors includes vinyl sulfones, α,*β*-unsaturated carbonyl derivatives, and miscellaneous derivatives (Figure 59). They inhibit cysteine proteases by forming covalent bonds with the active site thiol of cysteine proteases. These cysteine protease inhibitors are very successful and have second-order inhibition rates as high as 14000000 M^{-1} s^{-1}.

One of the first Michael acceptors described in the literature is the fumarate derivative of E-64c, HO-Fum-Leu-NH$(CH_2)_2CH(CH_3)_2$ (Fum = *trans*-COCH=CH−CO−). This inhibitor contains an α,*β*-unsaturated carboxyl moiety and was found to be an irreversible inhibitor of cathepsin B (k_{app} = 625 M^{-1} s^{-1}), cathepsin H (k_{app} = 11 M^{-1} s^{-1}), and cathepsin L (k_{app} = 2272 M^{-1} s^{-1}).[191] Hanzlik and co-workers were the first to design a series of amino acid derived Michael acceptor inhibitors for cysteine proteases. They showed that various vinyl sulfone and α,*β*-unsaturated carbonyl derivatives of phenylalanine are inhibitors of papain and DPPI (Figure 60).[351,352] These compounds are active-site-directed irreversible inhibitors or affinity-labeling reagents for papain. The Michael acceptor moiety is essential for activity as the saturated and decarboxylated derivatives of Ac-Phe-NH-CH_2-CH=CH-COOMe were less potent papain inhibitors with K_i values of 19.5 and 9.2 mM, respectively.[352,353] Some of these phenylalanine derivatives are also weak competitive inhibitors for the serine protease chymotrypsin (K_i ~ 30 mM) and the metalloprotease leucine aminopeptidase (K_i ~ 23−60 mM).[351,352]

Hanzlik and co-workers then incorporated a range of Michael acceptor electrophiles into dipeptide analogues and studied their effects on papain.[353] The most reactive derivatives with papain were an unsaturated ester and a simple vinyl sulfone. These vinyl sulfone cysteine protease inhibitors were later developed into a class of inhibitors for a variety of other cysteine proteases by Palmer and co-workers.[354] They designed potent inhibitors against disease-associated cysteine proteases, such as cathepsins B, L, S, and K (also called cathepsin O2), calpains, and cruzain.[354] This class of irreversible inhibitors, which contain a double bond activated by an electron-withdrawing sulfone functional group, was subsequently shown also to inhibit the rhinovirus 3C protease and cathepsin V.[355,356] Vinyl sulfones do not irreversibly inhibit serine proteases, although some derivatives act as weak competitive inhibitors.[354] Peptidyl vinyl sulfones and other Michael acceptors are stable, are unreactive toward nucleophiles, and need the catalytic machinery of the cysteine proteases for activation. Vinyl sulfones are less reactive toward nucleophiles than vinyl ketones or vinyl esters, which is an advantage for in vivo studies. In addition, vinyl sulfones can be manipulated on both the P and P′ side of the molecule, allowing for greater selectivity and reactivity toward target enzymes. Thus, most of the Michael acceptors, which have been studied, are vinyl sulfones.

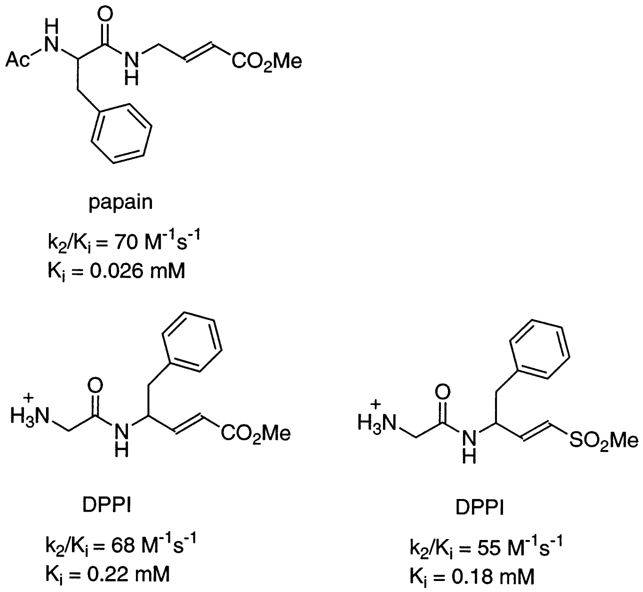


vinyl sulfone

α,β-unsaturated carbonyl derivative

R_1 = amino acid side chain, R = alkyl or aryl

Figure 59. Structure of vinyl sulfones and α,*β*-unsaturated carbonyl derivatives.



papain

k_2/K_i = 70 $M^{-1}s^{-1}$
K_i = 0.026 mM

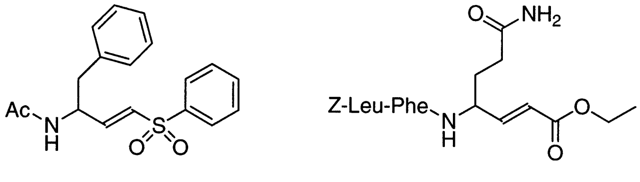
DPPI

k_2/K_i = 68 $M^{-1}s^{-1}$
K_i = 0.22 mM

DPPI

k_2/K_i = 55 $M^{-1}s^{-1}$
K_i = 0.18 mM

Figure 60. Phenylalanine derivatives that inhibit papain and DPPI.



Ac-Phe-VS-Ph

Z-Leu-Phe-Gln[CH=CH]-CO_2Et

Figure 61. Nomenclature of vinyl sulfones and α,*β*-unsaturated ester derivatives.

Nomenclature. The nomenclature for designating vinyl sulfone and α,*β*-unsaturated carbonyl inhibitors is illustrated in Figure 61. The vinyl sulfone functional group will be abbreviated "VS". Thus, a vinyl sulfone derivative of an amino acid will be abbreviated AA-VS-R, where R is the substituent on the sulfone functional group.

The amino acid attached to the double bond in the α,*β*-unsaturated carbonyl derivative (Michael accep-

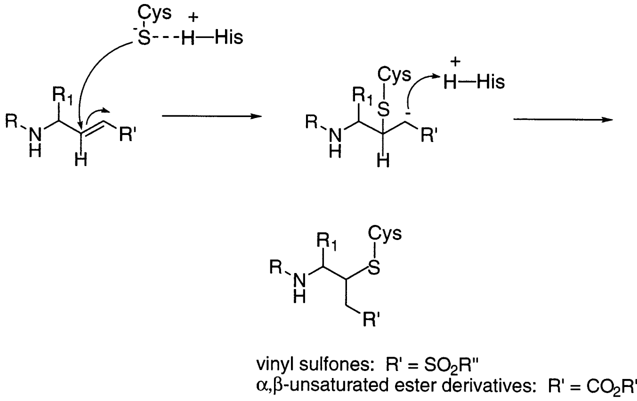


Figure 62. Mechanism of inhibition of cysteine proteases by vinyl sulfones and α,β-unsaturated ester derivatives.

Table 33. Inhibition of Papain by Ac-Phe-NH-CH$_2$-CH=CH-R

R	k_2/K_i (M^{-1} s^{-1})	R	k_2/K_i (M^{-1} s^{-1})
COOCH$_3$	26.1	CN	1.7
SO$_2$CH$_3$	18.7	CONH$_2$	1.1
COOH	5.0	PhNO$_2$	

tor) will be abbreviated -AA[CH=CH]-R, where [CH=CH] replaces the carboxyl terminus of an amino acid and R is the substituent on the double bond.

Mechanism. The mechanism of inhibition of cysteine proteases by vinyl sulfones, α,β-unsaturated esters, and other α,β-unsaturated carbonyl derivatives proceeds via a Michael addition with an attack on the β-carbon by the active site cysteine residue followed by protonation of the α-carbon to form the thioether derivative (Figure 62).[351]

Evidence of this mechanism was obtained using a halogenated α,β-unsaturated ester derivative in which a Michael addition would release a chloride ion (Figure 63).[357] A stoichiometric amount of the chloride ion was released upon inhibition of papain.[357] The inactivated derivative was stable and did not regain any enzyme activity after removal of excess inhibitor.[354]

The chemical reactivity of the Michael acceptor moiety has a direct effect on the inhibition rate with papain (Table 33).[353] In general, those compounds with good electron-withdrawing groups on the Michael acceptor have increased rates in simple Michael additions, along with higher rates of inhibition of papain. In this series of compounds, there was relatively little difference in enzyme recognition and binding upon changing the electrophilic group. The exception was the carboxylic acid derivative, which has higher reactivity than expected. Hanzlik et al., hypothesized that the hydrogen bonding or ion pairing of the carboxylate of Ac-Phe-NH-CH$_2$-CH=CH-COO$^-$ with His-159 in the active site of papain results in a greater ability to activate the olefinic bond for a Michael addition in the enzyme's active site than is observed with this compound in solution.[353] The nitrophenyl derivative (Table 33, R = PhNO$_2$) was not a time-dependent inhibitor and was a simple competitive inhibitor of papain (K_i = 0.06 mM). It is possible that this is due to an interaction between the nitrophenyl moiety of the inhibitor with the hydrophobic S1′ subsite of papain.

On the basis of molecular modeling studies with cysteine proteases, Rasnick has presented a hypothesis to explain the high reactivity of vinyl sulfone inhibitors with cysteine proteases and the failure of vinyl sulfones to inactivate serine proteases.[55] He concluded that the protonated form of the active site histidine is the determining factor in the selective inactivation of cysteine proteases by vinyl sulfones. The active site histidines of serine and cysteine proteases have opposite roles during catalysis. In cysteine proteases, the histidine is protonated and acts as a general acid, whereas in serine proteases, the histidine is unprotonated and acts as a general base. His modeling showed hydrogen bonding between the protonated histidine of a cysteine protease and one of the vinyl sulfone oxygens. This hydrogen bond polarizes the vinyl group, which gives the β-vinyl carbon a positive charge, and promotes nucleophilic attack by the active site thiolate. The other sulfone oxygen can participate in hydrogen bonding with the active site glutamine, which is part of the oxyanion hole (Figure 64). The large difference in pK_a values of the active site histidine and the α-carbon of the inhibitor forces the reaction to be irreversible, because deprotonation of the enzyme–inhibitor product is unlikely.

Specificity. The vinyl sulfones are specific for cysteine proteases because MeO-Suc-Ala-Ala-Pro-Val-VS-Ph incubated with human leukocyte elastase neither inhibited nor bound to this serine protease. The vinyl sulfones are also stable toward circulating thiols such as glutathione, as the second-order rate constant for Mu-Phe-Lys-VS-Ph (Mu = 4-morpholinecarbonyl) with glutathione was only 5.5×10^{-4} M^{-1} s^{-1}.[354] Michael acceptors are also 283000 times more reactive with papain than with the simple thiol 3-mercaptopropionate.[353]

Stereochemistry. Both the *cis* and *trans* derivatives of the nonhalogenated α,β-unsaturated carboxyl (acids or esters) inhibitors are selective competitive or irreversible inhibitors of papain and cathepsin B.[357] However, only the *trans*-configurations of the halogenated α,β-unsaturated carboxyl derivatives

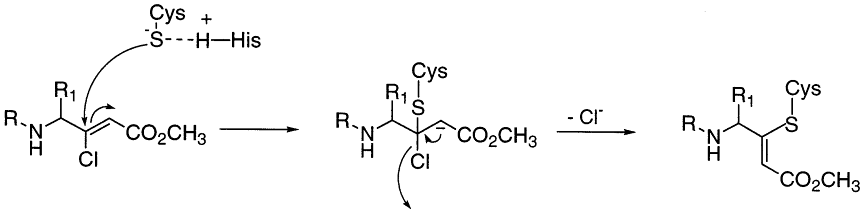


Figure 63. Evidence of inhibition mechanism using a halogenated α,β-unsaturated ester derivative.

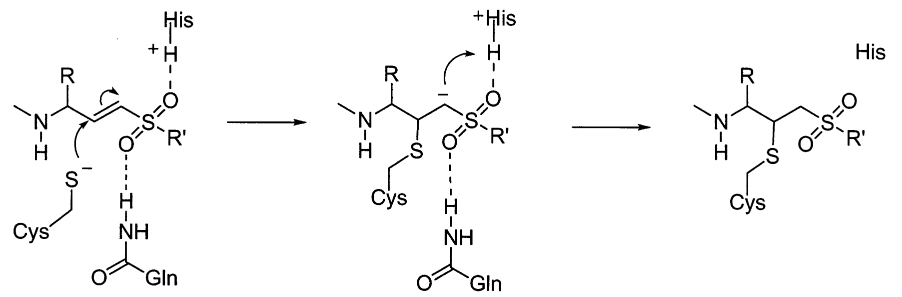
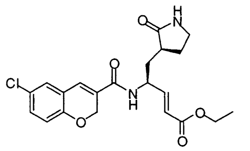
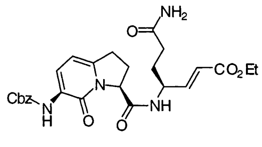
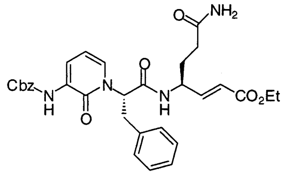


Figure 64. Hydrogen bonding between the histidine and glutamine of a cysteine protease and the vinyl sulfone oxygens.

Table 34. PDB Codes for Enzymes Complexed with Vinyl Sulfones and Michael Acceptors[a]

enzyme	inhibitor	PDB Code	ref
cruzain	Mu-Phe-Hph-VS-Ph	1F29	364
cruzain	Cbz-Phe-Hph-VS-CH_2-Ph	1F2A	364
cruzain	Cbz-Phe-Hph-VS-ONp	1F2B	364
cruzain	N-Me-Pip-CO-Phe-Hph-VS-NH-O-CH_2-Ph	1F2C	364
cathepsin K	N-Pip-CO-Leu-Hph-VS-Ph (APC3328)	1MEM	359
cathepsin V	N-Me-Pip-CO-Phe-Hph-VS-Ph (APC3316)	1FHO	356
cathepsin S	Mu-Phe-Hph-VS-Ph (APC2848)		361
rhinovirus 3C protease	Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et		355
rhinovirus 3C protease	PhCH_2-S-CO-Leu-Phe-Gln[CH=CH]-CO_2Et		363
rhinovirus 3C protease	Michael Acceptor AG7088	1CQQ	362
rhinovirus 3C protease			380
rhinovirus 3C protease			381
rhinovirus 3C protease			379
proteasome	Ac-PRLN-VS-CH_3		396
proteasome	NLVS	1KYI	397

[a] Mu = 4-morpholinecarbonyl; Pip = piperazinyl; Np = nitrophenyl.

were found to be irreversible inhibitors of papain and cathepsin B, possibly a result of the bulky chlorine substituent at the β-position.[357] The *cis*-configuration of the α,β-unsaturated halogen derivative (Ac-Phe-NH-CH_2-CCl=CH-COOMe) was a competitive inhibitor of papain and cathepsin B (K_i values of >5 and 3 mM, respectively) and of the serine protease α-lytic protease (Ac-Phe-NH-CH_2-CCl=CH-COOMe, K_i = 2.78 mM). Ester derivatives interact with cathepsin B and papain more readily than the carboxylate derivatives.[357] In addition, the nonhalogen unsaturated derivative (*trans*-Ac-Phe-NHCH_2-CH=CH-COOMe, $k_2/K_i = 20\ M^{-1}\ s^{-1}$ for cathepsin B) seems to inactivate much better than the halogenated derivative (*trans*-Ac-Phe-NHCH_2-CCl=CH-COOMe, $k_2/K_i = 1.7\ M^{-1}\ s^{-1}$ for cathepsin B).[357]

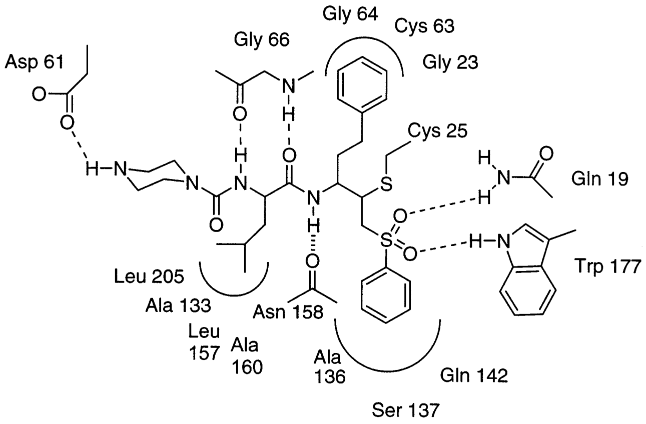


Figure 65. Structure of cathepsin K complexed with APC3328 (*N*-piperazinyl-CO-Leu-Hph-VS-Ph).

Crystal Structures. PDB codes for crystal structures of protease complexes with vinyl sulfones and Michael acceptors can be found in Table 34. The binding of vinyl sulfone inhibitors to cysteine proteases closely mimics substrate binding from P1 to P3. This binding mode is very similar to that observed with other peptide inhibitors, including peptide aldehydes and chloromethyl ketones. However, many epoxysuccinate derivatives bind in the reverse direction. Vinyl sulfones, in contrast to peptide aldehydes and chloromethyl ketones, can also extend into the prime side of the binding cleft. Vinyl sulfones have a polarized yet inert double bond as well as the sulfonyl unit, which provides hydrogen bond acceptors. In general, with cysteine proteases, vinyl sulfones can hydrogen bond with the Gln 19 side chain and the protonated histidine in the active site, which would align the inhibitor for nucleophilic attack by the active site cysteine residue (Figure 64).[354] Nucleophilic attack by the active site Cys 25 occurs on the *si* face of the inhibitor at the vinyl carbon nearest the side chain; this is equivalent to the carbonyl carbon of a P1 residue in a substrate.

The first crystal structure of a cysteine protease containing a vinyl sulfone inhibitor was cathepsin K complexed with APC3328 (*N*-piperazinyl-CO-Leu-Hph-VS-Ph, Hph = homophenylalanine, K_i = 11.5 nM, second-order rate constant = 5.7×10^6 M^{-1} s^{-1}).[358,359] The inhibitor forms a short section of antiparallel β-sheet with the peptide backbone (Gly 66) of the enzyme (Figure 65). An additional hydrogen bond is observed with the carbonyl oxygen of Asn 158. The vinyl sulfone moiety is positioned on the prime side of the substrate binding cleft with the phenyl ring of the vinyl sulfone in the P1′ pocket, which is composed of Asn 158, Trp 177, and Ala 136. One of the sulfone oxygens hydrogen bonds to Trp 177 and Gln 19. The phenyl residue of the vinyl sulfone in APC3328 does not make optimal interactions with the prime site residues, and structural alteration in this region of the inhibitor could lead to improvement in potency.[359]

A complex of cathepsin S with the vinyl sulfone inhibitor APC2848 (Mu-Phe-Hph-VS-Ph, k_{inact}/K_i = 5×10^6 M^{-1} s^{-1}, Mu = 4-morpholinecarbonyl) was refined with more difficulty.[360,361] As with cathepsin K, the inhibitor occupies four binding pockets in cathepsin S and has similar hydrogen-bonding interactions with the enzyme (Figure 66).

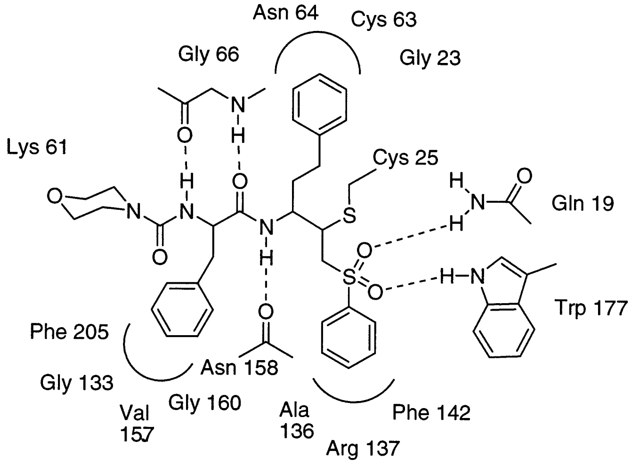


Figure 66. Structure of cathepsin S complexed with the vinyl sulfone inhibitor APC2848 (Mu-Phe-Hph-VS-Ph).

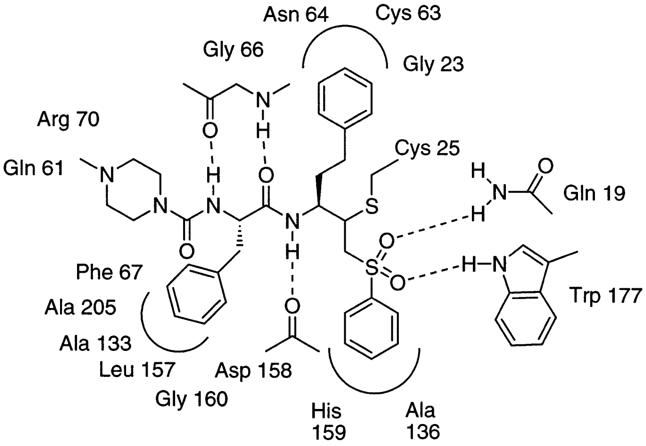


Figure 67. Structure of cathepsin V complexed with APC3316 (*N*-Me-Pip-CO-Phe-Hph-VS-Ph).

The crystal structure of cathepsin V complexed with APC3316 (N-Me-Pip-CO-Phe-Hph-VS-Ph) has been determined.[356] This crystal structure is very similar to those of cathepsins S and K described above (Figure 67). The residues Arg 70, Gly 59, and Gln 61 define the S3 pocket. The presence of Arg 70 at S3 is interesting and unique to cathepsin V compared to related enzymes. Inhibitors with residues that could interact with the Arg could be selective for cathepsin V. The sulfone oxygens interact with the Gln 19 and Trp 177, and the phenyl ring binds in a pocket formed by Trp 177, His 159, Asp 158, and Ala 136.

Due to the interest in the design of inhibitors for the rhinovirus 3C protease for treatment of the common cold, several crystal structures of Michael acceptor inhibitors have been determined and used to design more potent inhibitors. The catalytic machinery in 3C proteases, which is Cys 147, His 40, and Glu 71, closely resembles that of trypsin-like serine proteases (Ser, His, and Asp), suggesting that these enzymes are mechanistically related although they use different nucleophiles.[362] The first crystal structure contains the vinyl ester inhibitor Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et (k_{obs}/[I] = 25000 M^{-1} s^{-1}).[355,362] The overall structure is quite similar to

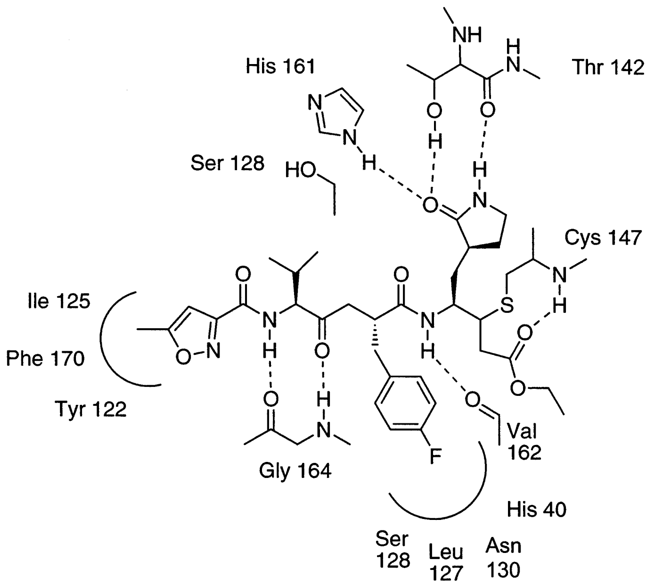


Figure 68. Structure of rhinovirus 3C protease complexed with the inhibitor AG7088.

those observed with cathepsins K, V, and S. Because the inhibitor is an α,*β*-unsaturated ester derivative and not a vinyl sulfone, the structure lacks the sulfone oxygen interactions with the Trp NH and Gln, and instead has a hydrogen bond between the carbonyl of the Michael acceptor moiety and the backbone amide of the active site Cys 147. The inhibitor's glutamine amide forms many important hydrogen bonds with His 161 and Thr 142. The leucine residue at P3 is solvent exposed, suggesting that a variety of amino acids could be tolerated at this position. The P2 backbone amide forms a hydrogen bond with the hydroxyl of Ser 128. A hydrophobic pocket on the surface of the enzyme contains the N-terminal Cbz protecting group, but no optimal interactions are observed. It was determined by analyzing the crystal structure that modifications could be made at the ethyl ester and α-position of the Michael acceptor to make more potent compounds.

That crystal structure led to improvements in inhibitors for the human rhinovirus 3C protease, and a crystal structure was obtained that contained the inhibitor Ph-CH$_2$-S-CO-Leu-Phe-Gln[CH=CH]-CO$_2$-Et (k_{obs}/[I] = 280000 M^{-1} s^{-1}).[363] The sulfur atom at the N-terminal of the inhibitor is buried more deeply into the S4 binding pocket than the Cbz's oxygen was in the previous crystal structure. It was concluded that the optimized recognition by the 3C protease of the thiocarbamate moiety at the N terminus of the inhibitor improved its potency.

Another rhinovirus 3C protease inhibitor, AG7088 (k_{obs}/[I] = 1470000 M^{-1} s^{-1}), that has potent antiviral activity against multiple human rhinovirus serotypes was discovered, and a crystal structure of it complexed with the serotype 2 3C protease was determined.[362] AG7088 has a cyclic Gln at P1 and binds very similarly to Cbz-Leu-Phe-Gln[CH=CH]-CO$_2$Et, but its N-terminal protecting group is oriented differently in the S4 binding pocket (Figure 68). Replacing the P2 backbone amide with a methylene moiety in AG7088 allows the Ser 128 to interact preferentially with the bulk solvent. The five-membered lactam ring at P1 makes three hydrogen bonds with the protease S1 subsite similar to the glutamine residue, but the hydrogen bond between the lactam amide NH and the backbone carbonyl of Thr 142 is longer and the geometry less favorable. The lactam CH$_2$ groups pack against the P3 valine side chain, stabilizing the active conformer in solution, and make van der Waals contacts with the backbone atoms of residues 143 and 144.[362] In this structure, the NH in the peptide bond of residues 144−145 points toward the oxyanion hole and may hydrogen bond to the carbonyl oxygen of the Michael acceptor in the transition state.

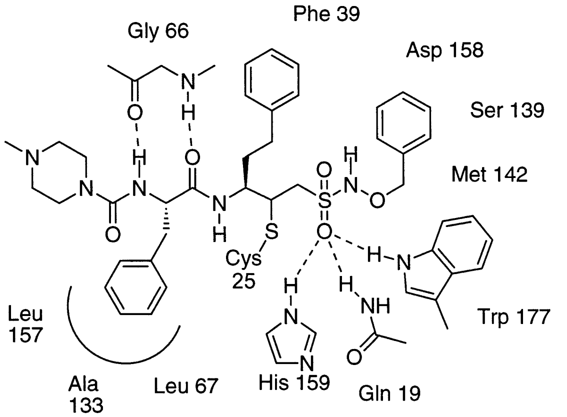


Figure 69. Schematic of the vinyl sulfonamide *N*-Me-Pip-CO-Phe-Hph-VS-NH-O-CH$_2$-Ph bound to cruzain.

Crystal structures of cruzain bound to a variety of vinyl sulfone, sulfonate, and sulfonamide inhibitors were determined in order to probe the aromatic-rich P1′ region of this enzyme.[364] Vinyl sulfonamides are designated peptidyl-VS-NH-R, where R = alkyl or aryl, whereas vinyl sulfonate esters are designated peptidyl-VS-O-R, where R = aryl. The active site cleft contains the catalytic triad, Cys 25, His 159, and Asn 175, as well as the conserved Trp 177. In the prime side region, there is a large open surface bound by Trp 177. The four crystal structures contain the inhibitors Mu-Phe-Hph-VS-Ph (k_{inact}/K_i = 319000 M^{-1} s^{-1}, Mu = 4-morpholinecarbonyl, Hph = homophenylalanine), Cbz-Phe-Hph-VS-CH$_2$-Ph (k_{assoc} = 1965000 M^{-1} s^{-1}), Cbz-Phe-Hph-VS-ONp (k_{assoc} = 4330000 M^{-1} s^{-1}, Np = nitrophenyl), and N-Me-Pip-CO-Phe-Hph-VS-NH-O-CH$_2$-Ph (k_{assoc} = 6500000 M^{-1} s^{-1}). A schematic of N-Me-Pip-CO-Phe-Hph-VS-NH-O-CH$_2$-Ph bound to cruzain can be seen in Figure 69. Analysis of the kinetic data for these compounds indicates that their potency is directly related to the length of the spacer between the sulfonyl unit and phenyl ring, as well as to the electronic nature of the spacer atoms.[364]

Cathepsin K has an active site region similar to that of cruzain. In crystal structures of cathepsin K and inhibitors containing P1′ phenyl rings, constructive *π*−*π* stacking interactions between the phenyl ring of the inhibitor and Trp 177 were observed. Brinen et al. hypothesized that the phenyl ring on the vinyl sulfone moiety would have aromatic interactions with Trp 177 of cruzain.[364]

There are some interactions common to all four cruzain crystal structures. The hydrophobic interactions in the S2 binding site between the inhibitors' Phe residue and Leu 67, Ala 133, and Leu 157 of cruzain help anchor the inhibitor. Another is the multiple hydrogen bonds in the S1′ region between a sulfonyl oxygen and Gln 19, His 159, and Trp 177 of cruzain. A hydrogen bond between the backbone nitrogen of Phe on the inhibitor and the backbone carbonyl of Gly 66 of cruzain is observed in all of the crystal structures. The inhibitors' Hph residue extends into the solvent and, therefore, does not form any constructive interactions with cruzain.

The first crystal structure containing Mu-Phe-Hph-VS-Ph lacks any spacer between the vinyl sulfone moiety and the phenyl ring. The phenyl ring is positioned almost perpendicularly to the six-membered ring of Trp 177, having an end-on aromatic interaction with the indole ring of Trp 177, and points toward Met 142.[364] The second crystal structure contains Cbz-Phe-Hph-VS-CH_2-Ph, an inhibitor of greater length and additional degrees of freedom due to the methylene spacer. The structure shows the vinyl sulfone phenyl ring bent backward away from Trp 177 and toward the Hph phenyl ring, forming an end-on aromatic interaction and almost cyclizing the inhibitor molecule. The third crystal structure with the vinyl sulfonate ester Cbz-Phe-Hph-ONp shows the nitrophenyl ring pointing toward the side-chain of Met 142. As with the second crystal structure, the nitrophenyl ring does not extend toward the aromatic rich region of cruzain, as hypothesized, but is almost parallel with the Hph phenyl ring. A hydroxylamine unit is the linker in the vinyl sulfonamide derivative N-Me-Pip-CO-Phe-Hph-VS-NH-ONp as seen in the fourth crystal structure (Figure 69). As with the other structures, the P1′ *O*-benzyl hydroxylamino unit curves away from cruzain and sits on a shelf formed by Ser 139, Met 142, and Asp 158. The Hph phenyl ring has weak contacts with Phe 39 of cruzain.

The crystal structures with cruzain showed results that differed from what was hypothesized after the cathepsin K structures were viewed. The three hydrogen bonds at the sulfonyl oxygen lock the inhibitor in place and are strong enough to outweigh other potential interactions. To position the vinyl sulfone phenyl ring toward Trp 177, the inhibitor would have to adopt a highly strained conformation.[364]

Structure—Activity Relationships: Cathepsin Family. The first vinyl sulfone inhibitors reported had broad reactivity toward many cysteine proteases and had relatively little selectivity. Cathepsin S was the most easily inactivated enzyme followed by cathepsin L, cruzain, cathepsin K (also called cathepsin O2), and cathepsin B, whereas the calpains were resistant to most vinyl sulfone inhibitors (Table 35).[354] To prepare more reactive human cathepsin K inhibitors, Brömme et al. synthesized a series of peptidyl vinyl sulfones with variations at the P2 residue.[360] However, most of the inhibitors were also very reactive with other cathepsins. The general structure of the inhibitors was Mu-AA-Hph-VS-Ph, with AA being Gly, Ala, Val, Leu, Ile, Ahx (aminohexanoic acid), Met, $MetO_2$, and Phe. Cathepsins K, L, and S all preferred leucine in the P2 position ($k_{inact}/K_i = 727000$, 325000, and 14600000 $M^{-1}\ s^{-1}$, respectively). Cathepsin K accepted Phe very poorly at the P2 position ($k_{inact}/K_i = 6900\ M^{-1}\ s^{-1}$). Brömme et al. concluded that the S2 pocket of cathepsin K seems to be more spatially restricted than cathepsins S and L, as cathepsin S is more tolerant and accepts the hydrophobic unbranched residues such as Met, $MetO_2$, and Ahx. The presence of Gly 133, Gly 160, and Phe 205 in the S2 pocket of cathepsin S probably accounts for the more spacious binding site and preference for hydrophobic side chains.[361]

Table 35. Kinetics for Inhibition of Cathepsins, Cruzain, and Calpains by Vinyl Sulfones

	k_{inact}/K_i ($M^{-1}\ s^{-1}$)		
enzyme	Mu-Np2-Hph-VS-2Np[a]	Mu-Leu-Hph-VS-Ph	Cbz-Leu-Leu-Tyr-VS-Ph
cathepsin S[b]	56000000	26300000	
cathepsin L[c]	9200000	387000	
cathepsin K[d]	<300[h]	772700	
cathepsin B[e]	420000	4250	
cruzain[f]	1040000	620000	
calpain I (II)[g]			24300[h] (6400[h])

[a] Mu = 4-morpholinecarbonyl; Hph = homophenylalanine; Np = nitrophenyl. [b] Conditions: 50 mM phosphate buffer, pH 6.0, 2.5 mM EDTA, 2.5 mM DTT, substrate was Cbz-Arg-Arg-AMC. [c] Conditions: 50 mM acetate buffer, pH 5.5, 2.5 mM EDTA, 2.5 mM DTT, substrate was Cbz-Phe-Arg-AMC. [d] Conditions: 50 mM phosphate buffer, pH 6.5, 2.5 mM EDTA, 2.5 mM DTT, substrate was Cbz-Val-Val-Arg-AMC. [e] 50 mM phosphate buffer, pH 6.5, 2.5 mM EDTA, 2.5 mM DTT, substrate was Cbz-Phe-Arg-AMC. [f] Conditions: 50 mM acetate buffer, pH 5.5, 2.5 mM EDTA, 2.5 mM DTT, substrate was Cbz-Phe-Arg-AMC. [g] Conditions: 50 mM Tris buffer, pH 7.5, 5 mM Ca^{2+}, 2.5 mM DTT, substrate was Suc-Leu-Tyr-AMC. [h] $k_{obs}/[I]$ ($M^{-1}\ s^{-1}$).

Unsaturated aldehydes have been shown to be cysteine protease inhibitors. One explanation for the poor degradation of oxidized macromolecules in macrophages is that lipid oxidation products, generated during the oxidation of low-density lipoprotein, inactivate the lysosomal cysteine proteases. Crabb et al. have performed some research to support this hypothesis.[365] Treatment of macrophages with the lipid peroxidation product 4-hydroxy-2-nonenal reduces cathepsin B activity. By tandem mass spectrometry, Crabb et al. show that cathepsin B is inactivated by covalent interaction (generation of Michael adducts) between the unsaturated aldehyde 4-hydroxy-2-nonenal and Cys 29 and His 150 of the enzyme.[365]

Korver et al. identified peptide vinyl sulfone inhibitors of DPPI that are suitable for use in short-term cell culture and have both the in vivo and in vitro potential to block activation of natural substrates such as progranzymes.[366] These vinyl sulfones, which inhibit intracellular DPPI activity, were nontoxic, stable at pH 5.5, reactive with purified DPPI, nonreactive with granule serine proteases, and only slightly reactive with lysosomal cathepsins. Inhibition data can be found in Tables 36 and 37. The best inhibitor was Leu-Phe-VS-CH_3, which had an IC_{50} of 4.7 μM for intracellular DPPI inhibition. Compounds with charged amino acid residues (Glu) tended to be less effective, suggesting that it is harder for these compounds to cross the cellular membranes.

Table 36. Inhibition of DPPI and Related Cysteine Proteases by Dipeptide Vinyl Sulfones

	$k_{obs}/[I]$ (M^{-1} s^{-1})		
inhibitor	intracellular DPPI[a]	cathepsin B[b]	cathepsin L[c]
Leu-Phe-VS-CH_3	210 (4.7)	NI[d]	106
Val-Phe-VS-Ph	1600 (6)	0.1	21
Val-Phe-VS-CH_3	340 (11)	0.3	21
Gly-Phe-VS-CH_3	72 (21)	1.3	1.0
Thr-Glu-VS-Ph	2900 (45)		
Glu-Glu-VS-Ph	710 (>250)		

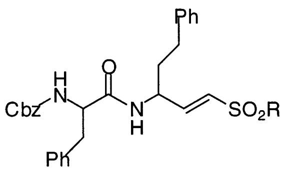
[a] Conditions: 50 mM NaAc, 30 mM NaCl, pH 5.5, 1 mM EDTA, 1 mM DTT, 23 °C, substrate was Gly-Phe-AMC. IC_{50} values (μM) are given in parentheses. [b] Conditions: 50 mM NaAc, 30 mM NaCl, pH 6.0, 1 mM EDTA, 1 mM DTT, substrate was Cbz-Arg-Arg-AMC. [c] Conditions: 50 mM Mes, 2.5 mM EDTA, 1 mM DTT, 0.01% Brij, pH 5.5, substrate was Cbz-Phe-Arg-AMC. [d] NI = no inhibition.

Table 37. Amount of Granzyme Activity Remaining after Treatment with Vinyl Sulfones

inhibitor	tryptase (granzymes A, K) (%)	chymase (%)	metase (%)
Leu-Phe-VS-CH_3	98	96	98
Gly-Phe-VS-Ph	97	93	96

Cruzain. Roush and co-workers have carried out extensive SAR studies with vinyl sulfone inhibitors of cruzain to develop new therapies for Chagas' disease. Cruzain is the major cysteine protease of *T. cruzi*, the causative agent of Chagas' disease. Initially, Roush et al. expanded on Palmer's work[354] with Mu-Phe-Hph-VS-Ph ($k_{inact}/K_i = 203000$ M^{-1} s^{-1}) and made a number of structural variants to design potent and selective inhibitors of cruzain.[367] The crystal structure of cathepsin K with the vinyl sulfone APC3328 revealed that the phenyl residue of the vinyl sulfone unit did not make optimal interactions with prime site residues.[359] Roush et al. synthesized vinyl sulfonamides and vinyl sulfonate esters to enhance the prime site interactions. These inhibitors have the structure Cbz-Phe-Hph-VS-R, where R = Ph, CH_2Ph, CH_2CH_2Ph, NHPh, and OPh, and were found to be time-dependent inhibitors of cruzain, papain, and cathepsin B (Table 38). Vinyl sulfonamides are designated peptidyl-VS-NH-R, where R = alkyl or aryl, whereas vinyl sulfonate esters are designated peptidyl-VS-O-R, where R = aryl. Roush discovered the most potent cruzain inhibitor reported to date, Cbz-Phe-Hph-VS-OPh ($k_{assoc} = 16800000$ M^{-1} s^{-1}, Table 39), which has the preferred one-atom oxygen spacer between the sulfonyl unit and phenyl ring.[367,368]

Table 38. Second-Order Rate Constants for Inhibition of Cruzain and Other Cysteine Proteases by Cbz-Phe-Hph-VS-R



R	cruzain (M^{-1} s^{-1})	papain (M^{-1} s^{-1})	cathepsin B (M^{-1} s^{-1})
Ph	634000[b]	7000[a]	<2000
CH_2Ph	1956000[a]		
CH_2CH_2Ph	149000[a]		
NHPh	289000[a]		
OPh	14280000[a]	325000[b]	112200[b]

[a] k_{assoc}. [b] k_{inact}/K_i.

Table 39. Inhibition of Cruzain by Second-Generation Vinyl Sulfonamide Inhibitors

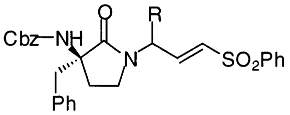
inhibitor[a]	k_{assoc} (M^{-1} s^{-1})	survival of *T. cruzi*-treated macrophages (days)
Mu-Phe-Hph-VS-Ph	181000	>24
Cbz-Phe-Hph-VS-OPh	16800000	toxic
Mu-Phe-Hph-VS-OPh	10200000	toxic
Mu-Phe-Hph-VS-NHPh	4920000	9
Mu-Phe-Hph-VS-CH_2Ph	1670000	20
Mu-Phe-Hph-VS-NH-OCH_2Ph	2270000	>24
N-Me-Pip-CO-Phe-Hph-VS-NH-OCH_2Ph	6480000	>24

[a] Mu = 4-morpholinecarbonyl; Pip = piperazinyl.

Unfortunately, none of the above vinyl sulfonate ester and vinyl sulfonamide inhibitors were active against *T. cruzi* in tissue culture assays.[368] In view of these results, Roush et al. designed and synthesized second-generation vinyl sulfonamide inhibitors, with the help of crystal structures and previous results from the McKerrow group (Mu-Phe-Hph-VS-Ph), that would have improved in vitro activity.[117,364] Even though inhibitors containing a P3 morpholinyl carbonyl or *N*-methylpiperazinyl carbonyl residue instead of the Cbz had increased in vivo activity and oral bioavailability,[117] the vinyl sulfonate ester inhibitor Mu-Phe-Hph-VS-OPh and vinyl sulfonamide inhibitor Mu-Phe-Hph-VS-NHPh still did not show significant activity in cell culture assay, whereas Mu-Phe-Hph-VS-CH_2Ph did (Table 38).[368] Using the knowledge of the sulfonyl oxygen hydrogen bonds seen in the crystal structures and the assumption that binding constants and reactivity as Michael acceptors should both influence the potency of these inhibitors, Roush et al. designed *N*-sulfonyl hydroxylamine derivatives as potential inhibitor structures. It was proposed that these compounds would have electronic properties at the sulfonyl group between sulfonate esters and sulfonamides and should have improved solubility characteristics. As a result, the vinyl sulfonamide inhibitors Mu-Phe-Hph-VS-NH-O-CH_2-Ph and N-Me-Pip-CO-Phe-Hph-VS-NH-O-CH_2-Ph were shown to be potent irreversible inhibitors of cruzain (k_{assoc} = 2270000 and 6480000 M^{-1} s^{-1}, respectively) and excellent inhibitors of *T. cruzi* in the J774 macrophage cell culture assays (Table 39).[368]

Using a crystal structure of cruzain bound to the fluoroketone Cbz-Phe-Ala-CH_2F, Scheidt et al. hoped to design and synthesize more potent, conformationally constrained cruzain inhibitors.[369] He expanded on the Palmer inhibitor Mu-Np2-Hph-VS-2Np ($k_{inact}/K_i = 1040000$ M^{-1} s^{-1}, Table 35, Mu = 4-morpholinecarbonyl).[354] A γ-lactam or pyrrolidinone isostere was used to fix the bioactive P1−P2 conformation observed in the crystal structure. The inhibitors synthesized contained a P1−P2 pyrrolidinone unit and a phenyl vinyl sulfone moiety, as work at the University of California−San Francisco revealed that naphthyl vinyl sulfones were toxic to mammalian cells.[369] The pyrrolidinone inhibitor (Table 40) with R = CH_2CH_2Ph was a weak, reversible inhibitor of

Table 40. Kinetics of Unconstrained and Pyrrolidinone-Containing Vinyl Sulfone Inhibitors for Cruzain



	k_{inact}/K_i (M^{-1} s^{-1})			
inhibitor	cruzain[a]	cathepsin B[b]	papain[b]	leishmania cpB[b]
R = Me	(>10)			
R = CH_2CH_2Ph	(2.0)			
Cbz-Phe-Ala-VS-Ph	93800 (0.03)	500	14600	4700
Cbz-Phe-Hph-VS-Ph	634000 (0.001)	<2000	7000	

[a] Buffer was 100 mM NaAc, pH 5.5, 10 mM DTT; substrate was Cbz-Phe-Arg-AMC. IC_{50} values (μM) are given in parentheses. [b] Buffer was 100 mM NaAc, pH 5.5, 10 mM DTT, 1 mM EDTA, and 0.1% Triton-X100; substrate was Cbz-Phe-Arg-AMC.

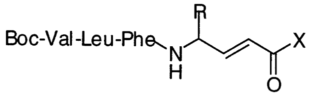
cruzain ($IC_{50} = 2$ μM), whereas its unconstrained analogue Cbz-Phe-Hph-VS-Ph was a good selective inhibitor of cruzain ($k_{inact}/K_i = 634000$ M^{-1} s^{-1}) with respect to mammalian cathepsin B ($k_{inact}/K_i = <2000$ M^{-1} s^{-1}). It was concluded that the added ethylene unit of the pyrrolidinone moiety must impose steric problems, preventing the inhibitors from having optimal binding in cruzain's active site. Overall, Scheidt's pyrrolidinone-containing vinyl sulfones were significantly less active against cruzain when compared to their unconstrained analogues (Table 40).

T. cruzi contains a number of other proteases in addition to cruzain. Joyeau et al. designed a vinyl sulfone inhibitor for the prolyl-specific Tc80 proteinase, a serine protease, present in *T. cruzi*.[370] The inhibitor Cbz-Leu-Gly-Pro-VS-Ph exhibited only weak inhibition of the Tc80 peptidase ($K_i = 35000$ nM; $IC_{50} = >10000$ nM) even after a long preincubation period.

Rhodesain. Rhodesain is the major cysteine protease of *Trypanosoma brucei rhodesiense. T. brucei rhodesiense* is the causative agent of sleeping sickness in humans. Differences between rhodesain and the related trypanosome protease cruzain were revealed using peptidomimetic vinyl sulfone inhibitors.[259] It is suggested that both enzymes have extracellular functions as they maintain significant activity and stability up to pH 8.0, unlike some mammalian cathepsins. Both enzymes react similarly with a variety of vinyl sulfone inhibitors having the sequence Cbz-Phe-Hph-VS-R. For rhodesain, the order of potency with the various R substituents was found to be OPh > 2-pyridyl > CH_2Ph > Ph > CH_2CH_2Ph > OEt > NHPh, with Cbz-Phe-Hph-VS-OPh being the most potent inhibitor ($k_{assoc} = 5808000$ M^{-1} s^{-1}).[259]

Rhinovirus 3C Protease. Considerable effort has been devoted to the development of specific inhibitors for the rhinovirus 3C protease.[68] This pathogenic RNA virus is the major cause of the common cold in humans, and a "cure" would diminish considerable human suffering. The rhinovirus 3C protease cleaves viral precursor polyproteins into structural and enzymatic proteins, which are essential for viral replication. The 3C protease has a unique specificity for Gln at P1, which has made the design of specific inhibitors practical.

Table 41. Rhinovirus 3C Protease: Enzyme Inhibition and Antiviral Activity of Peptidyl Michael Acceptors

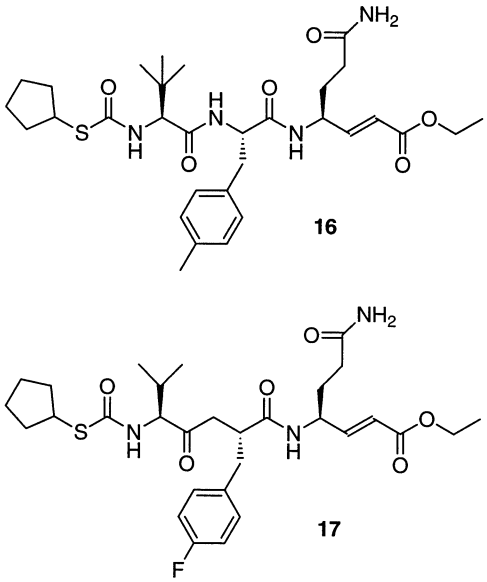


R	X	% enzyme inhibition	enzyme inhibition[a] IC_{50} (μM)	plaque reduction IC_{50} (mg/mL)
$CH_2CH_2CONH_2$	OMe	100	0.25	0.74
$CH_2CH_2CONH_2$	OEt	100	0.13	0.41
$CH_2CH_2CONH_2$	OH	43	17.7	>10
$CH_2CH_2SO_2Me$	OMe	48	13.6	>10

[a] Conditions: 50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA; substrate was EALFQ-*p*NA.

The first Michael acceptor inhibitors for the human rhinovirus 3C protease (HRV 3C protease) designed by Kong et al. were peptidyl Michael acceptors based on the substrate cleavage site.[371] They synthesized peptidyl derivatives of vinylogous glutamine or methionine sulfoxide (e.g., Boc-Val-Leu-Phe-Gln[CH=CH]-CO_2R and Boc-Val-Leu-Phe-Met(O_2)[CH=CH]-CO_2R) and evaluated them as inhibitors of the serotype 14 HRV 3C protease and as inhibitors of viral replication in cell culture in vitro (plaque reduction). These tetra- and pentapeptide Michael acceptors inhibited the 3C protease with sub-micromolar IC_{50} values and exhibited a very low level of cytotoxicity (Table 41). The inhibitor Boc-Val-Leu-Phe-Gln[CH=CH]-CO_2Et was an effective active site titrant for 3C protease. Inhibitors lacking the P4 residue or containing vinylogous methionine sulfone esters had reduced to negligible activity against 3C protease. Inhibitors derived from hydrolysis of the vinylogous glutamine ester also showed decreased inhibitory activity.

Dragovich et al. also investigated peptidyl Michael acceptors as inhibitors of the human rhinovirus 3C protease.[355] These irreversible inhibitors have $k_{obs}/[I]$ values ranging from 100 to 600000 M^{-1} s^{-1} and exhibit antiviral activity against HRV-14 infected H1−HeLa cells (EC_{50} values approaching 0.5 μM). Analysis of a crystal structure of Cbz-Leu-Phe-Gln-[CH=CH]-CO_2Et with HRV-2 3C protease suggested additional substitution could be tolerated at the α-position of the Michael acceptor and in the vicinity of the ethyl ester. Compounds were synthesized that maintained the Cbz-Leu-Phe-Gln[CH=CH] backbone but varied the Michael acceptor moiety.[355,362] Ester-derived Michael acceptors were good inhibitors with $k_{obs}/[I]$ values of 3000−40000 M^{-1} s^{-1}. Amide-containing Michael acceptors had reduced activity against the 3C protease and poorer antiviral activity. Aliphatic and aryl α,β-unsaturated ketones showed excellent inhibitory activity ($k_{obs}/[I]$ values between 120000 and 500000 M^{-1} s^{-1}) but were toxic and had reduced antiviral activity ($EC_{50} > 2$ μM). Nitriles, oximes, phosphonates, vinyl sulfones, and vinyl heterocycles had weak to negligible inhibitory activity ($k_{obs}/[I] < 600$ M^{-1} s^{-1}). Acyl lactam, acyl oxazolidinone, and acyl urea Michael acceptors were potent inhibitors but are inactivated by nonenzymatic thiols. The *trans*-α,β-unsaturated esters were the preferred Michael acceptors because of their good inhibitory



16

17

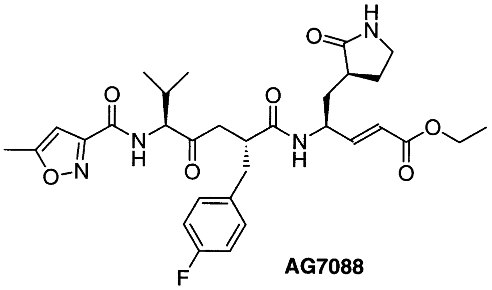
Figure 70. Structures of rhinovirus inhibitors **16** and **17**.

and antiviral activity, stability toward nonenzymatic thiols, low cellular toxicity, and ease of synthesis.[362]

Dragovich et al. continued their studies by synthesizing more inhibitors that varied the amino acids in the peptidyl binding determinant.[363] Although the P1 glutamine was essential for potent inhibition, some modifications could be made at the P2, P3, and P4 positions. It was found that additional functionality at the 4-position of the P2 phenyl ring could increase $k_{obs}/[I]$ values. The best modifications were incorporated into one compound to give **16** (Figure 70), which exhibited a $k_{obs}/[I]$ of 800000 M^{-1} s^{-1} and an EC_{50} of 0.056 μM with HRV 3C protease serotype 14.

Dragovich et al. then replaced the solvent-exposed backbone P2−P3 amide moiety in the peptide portion of Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et with a ketomethylene isostere to yield a compound with less peptidic character. This compound showed slightly reduced inhibitory activity ($k_{obs}/[I] = 17400$ M^{-1} s^{-1}) compared to Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et ($k_{obs}/[I] = 25000$ M^{-1} s^{-1}) but showed improved antiviral properties.[372] Because the P3 leucine is solvent exposed, a wide variety of substituents was tolerated at this position. The crystal structure of Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et revealed a gap between the carbamate oxygen and the side chain of Phe 170. Replacing this oxygen with a larger, more easily polarized sulfur atom resulted in increased inhibitory activity ($k_{obs}/[I] = 280000$ M^{-1} s^{-1}) and improved antiviral properties ($EC_{50} = 0.27$ μM).[355,362,363] Compound **17** (Figure 70) proved to be the superior antiviral agent, being potent with a variety of rhinovirus serotypes with EC_{90} values of <0.25 μM.

There was concern that the N-terminal thiocarbamate moiety (Figure 70) might undergo facile in vivo metabolism. Therefore, Dragovich et al. further modified some irreversible human rhinovirus 3C protease inhibitors by optimization of the N-terminal amide moiety.[373] This resulted in a tripeptide Michael acceptor containing an N-terminal 5-methylisoxazole-3-carboxamide, which exhibited potent, irreversible anti-3C protease activity ($k_{obs}/[I] = 260000$ M^{-1} s^{-1} with serotype 14) and broad-spectrum antirhinoviral properties (average $EC_{50} = 0.47$ μM against four different serotypes).



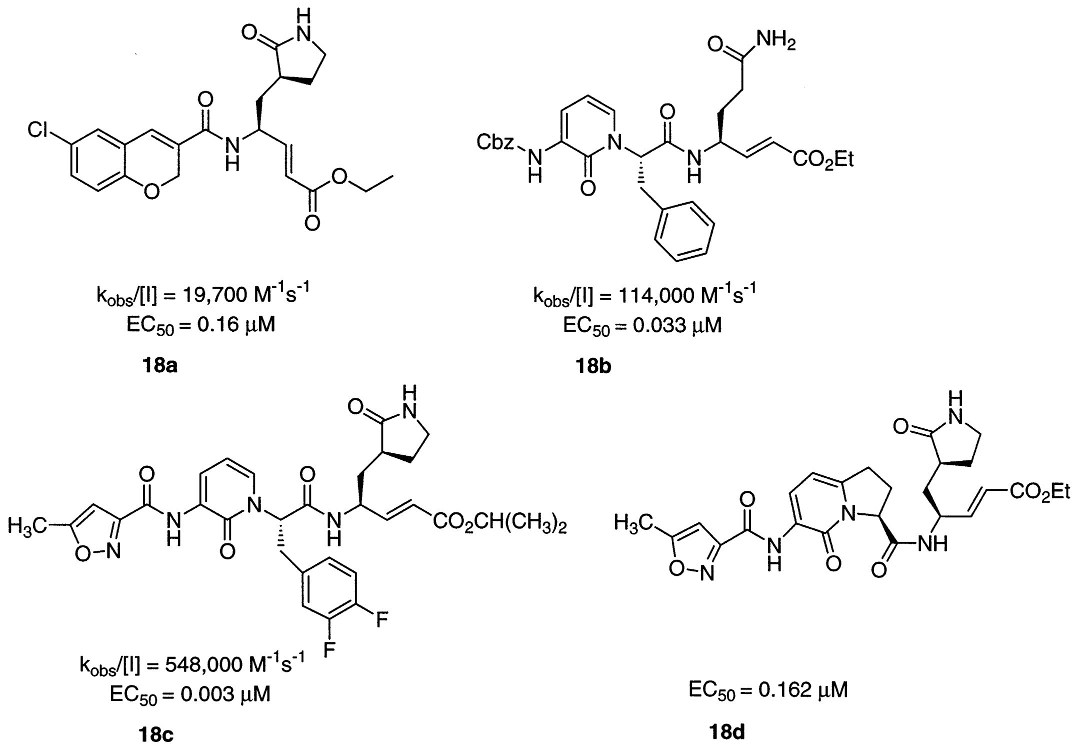
AG7088

Figure 71. Structure of the 3C protease inhibitor AG7088, which is in clinical trials.

To further remove the peptidic characteristics of the inhibitors, Dragovich et al. introduced methyl groups along the amide backbone of the typical 3C protease inhibitor Cbz-Leu-Phe-Gln[CH=CH]-CO_2-Et.[374] The *N*-methyl amino acids were not tolerated at the P1 or P3 position, as expected from X-ray analysis, because the inhibitor backbone amide NHs form hydrogen bonds with the enzyme. However, as seen with the ketomethylene substitution at the P2−P3 amide moiety,[372] inhibitors containing P2 *N*-methyl amino acids were highly active 3C protease inhibitors ($k_{obs}/[I]$ values up to 610000 M^{-1} s^{-1} with serotype 14) and effective in vitro antirhinoviral agents (EC_{50} values approaching 0.03 μM with serotype 14).[374]

The P2−P3 amide bond was also replaced with an ester by Webber et al. to create irreversible depsipeptidyl human rhinovirus 3C protease inhibitors.[375] When compared to their amide and ketomethylene counterparts, these compounds showed reduced human rhinovirus serotype 14 3C protease activity and comparable or improved antiviral activity but were the least stable in vitro. It was shown that the hydrogen bond seen in crystal structures between the P2−P3 amide NH and Ser 128 of the enzyme is not optimal with a P2−P3 ester.

Compounds were also synthesized by Dragovich et al. and examined where the P1 glutamine residue was replaced with a lactam moiety.[376] A crystal structure showed that the glutamine NH was in the *cis*-conformation when bound to enzyme. Incorporating a P1 lactam moiety into the inhibitor design would force the *cis*-amide geometry for glutamine, and using the *S*-stereochemistry at the lactam α-carbon would correctly position the side chain for hydrogen bonding.[362] The lactam should bind more tightly to the 3C protease as its rigid side chain would lose less conformational entropy on binding than the flexible glutamine side chain. The resulting compounds exhibit increased 3C protease inhibitory activity, have improved antirhinoviral properties, and are selective for HRV 3C protease over other serine and cysteine proteases. Combination of all the beneficial modifications resulted in the inhibitor AG7088 (Figure 71, ruprintrivir, $k_{obs}/[I] = 1470000$ M^{-1} s^{-1} for the serotype 14 HRV 3C protease; $EC_{90} \sim 0.10$ μM for 48 different HRV serotypes; $EC_{50} \sim 0.023$ μM for serotype 14), which is undergoing evaluation in



$k_{obs}/[I] = 19{,}700\ M^{-1}s^{-1}$
$EC_{50} = 0.16\ \mu M$

18a

$k_{obs}/[I] = 114{,}000\ M^{-1}s^{-1}$
$EC_{50} = 0.033\ \mu M$

18b

$k_{obs}/[I] = 548{,}000\ M^{-1}s^{-1}$
$EC_{50} = 0.003\ \mu M$

18c

$EC_{50} = 0.162\ \mu M$

18d

Figure 72. Structures of nonpeptidic inhibitors of rhinovirus 3C protease.

phase I clinical studies with humans to explore its potential as a nasally delivered antirhinoviral agent.[362,377−379]

Continuing in the efforts to develop low molecular weight, nonpeptidic human rhinovirus 3C protease inhibitors, Johnson et al. attempted to replace the P2−P4 portion of Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et with a smaller, nonpeptidic substituent while maintaining the P1-Michael acceptor moiety.[380] All of the active compounds contain planar, hydrophobic, aromatic moieties and are predicted to occupy only the S2 pocket of human rhinovirus 3C protease. The best compounds, such as **18a** (Figure 72), are nontoxic and have rates of inactivation comparable to those of the lead compound Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et but have increased antiviral activity. A crystal structure of **18a** with human rhinovirus serotype 2 3C protease shows the bulky bicyclic ring binding deeply in the S2 pocket.[380] Unfortunately, broad-spectrum activity may be a problem as the different serotypes have high variability at S2.

Dragovich et al. then focused on developing orally bioavailable 3C protease inhibitors for treatment of the common cold and other picornaviral infections. Nothing with sufficient oral bioavailability was discovered during the research that led to the development of AG7088, so Dragovich et al. sought inhibitors with nonpeptidic chemical structures distinct from earlier studies.[379] The crystal structure of Cbz-Leu-Phe-Gln[CH=CH]-CO_2Et with human rhinovirus serotype 2 3C protease suggested that the P3 amino acid could be replaced with a 3-amino-2-pyridone moiety and still maintain key interactions. This led to extensive structure−activity studies of 2-pyridone-containing 3C protease inhibitors. A crystal structure of one of the compounds (**18b**, Figure 72) with the human rhinovirus 3C protease serotype 2 showed Ser 128 positioned within van der Waals contact distance adjacent to the pyridone ring.[379] As seen in other crystal structures, an antiparallel β-sheet hydrogen-bonding interaction was seen between Gly 164 and the pyridone moiety of the inhibitor. To prevent enzyme-mediated hydrolysis of the ethyl ester on the Michael acceptor, it was replaced with an isopropyl ester to yield compound **18c** (Figure 72).[379] This compound had improved stability in human liver and human plasma but had worse anti-3C protease and antiviral potency compared to the ethyl ester derivative. However, it did have good antirhinoviral potency against 15 human rhinovirus serotypes in cell culture and good bioavailability and pharmacokinetic properties after oral administration in the dog ($F = 48\%$).

In continuation of the above research, Dragovich et al. synthesized and evaluated bicyclic 2-pyridone-containing human rhinovirus 3C protease inhibitors.[381] By properly optimizing bicyclic 2-pyridone-containing inhibitors, potent, broad-spectrum antirhinoviral agents were obtained. One such compound, **18d**, is shown in Figure 72. In a crystal structure of one of the compounds and human rhinovirus 3C protease serotype 2, there were no appreciable contacts between the inhibitor and the S2 binding subsite of the enzyme, and the remaining interactions were similar to those observed in other crystal structures.[381]

Biological Studies. Vinyl sulfone inhibitors have considerable potential for use as drugs. Potent vinyl sulfone inhibitors of cruzain are a novel chemotherapy for Chagas' disease. Engel et al. designed vinyl sulfone inhibitors that were able to rescue mice from a lethal *T. cruzi* infection and were less toxic than the fluoromethyl ketone inhibitors.[117] The inhibitor Mu-Phe-Hph-VS-Ph (Mu = 4-morpholinecarbonyl) was the most effective compound in vitro and

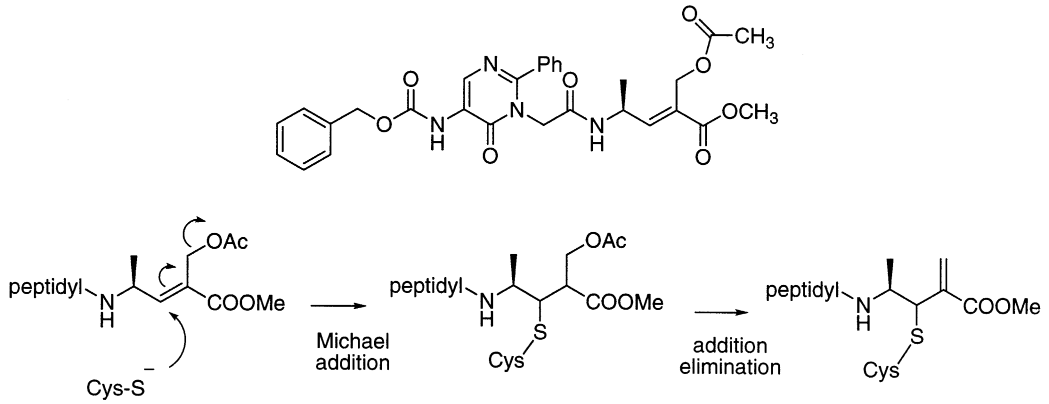


Figure 73. Pyrimidinyl antimalarial agent and its mechanism of action.

exhibited 100% growth inhibition of the intracellular cycle of *T. cruzi*. It not only blocked the intracellular development of *T. cruzi* but eventually eliminated all parasites. The inhibitor Mu-Phe-Hph-VS-Ph also extended the lifetime of J744 cells infected with *T. cruzi* for >28 days at 10 μM inhibitor concentration.[205] The compounds Mu-Phe-Hph-VS-Ph and N-Me-Pip-CO-Phe-Hph-VS-Ph (K11777), the more water-soluble derivative, were also tested in a mouse model of acute Chagas' disease and disrupted the life cycle of *T. cruzi* in vivo.[117,382,383]

Various species of the protozoan *Leishmania* cause a parasitic infection known as leishmaniasis. The targets for chemotherapy are the enzymes required for parasite growth and virulence, a family of cathepsin L-like (cpL) and cathepsin B-like (cpB) cysteine proteases found in all species of *Leishmania*. Selzer et al. has found a psuedopeptide substrate analogue (K11002, Mu-Phe-Hph-VS-Ph) that is an irreversible inhibitor of the cysteine proteases in *Leishmania* (k_{inact}/K_i = 107000 M^{-1} s^{-1}, *L. major* cpB) while maintaining selectivity versus homologous host enzymes (k_{inact}/K_i = 1400 M^{-1} s^{-1}, mammalian cathepsin B).[384] This inhibitor kills *Leishmania* parasites in vitro at concentrations that do not affect mammalian host cells.

Cathepsin K is a valuable therapeutic target for the treatment of diseases with excessive bone resorption. Xia et al. demonstrated for the first time that selective inhibition of cathepsin K with vinyl sulfone inhibitors interferes with bone resorption.[385] The inhibitor Mu-Leu-Hph-VS-Ph is a potent cathepsin K inhibitor (k_{inact}/K_i = 727000 M^{-1} s^{-1}).[354] This inhibitor reduced bone resorption by 80% in a dose-dependent manner at a concentration of 10^{-7} M (IC_{50} = 0.05 μM).[385]

The papain family cysteine protease falcipain, from the human malaria parasite *Plasmodium falciparum*, is required for the degradation of hemoglobin by erythrocytic malaria parasites and, as a result, is a novel target for antimalarial drugs. Rosenthal et al. demonstrated that vinyl sulfones inhibited falcipain and blocked parasite hemoglobin degradation and development at nanomolar concentrations.[386] The compound Mu-Leu-Hph-VS-Ph (IC_{50} = 0.003 μM, Mu = 4-morpholinecarbonyl) was the optimal vinyl sulfone inhibitor for falcipain and had decreased activity with the murine malaria parasite *P. vinckei* (IC_{50} = 0.2 μM), a falcipain analogue. Olson et al. then evaluated the in vivo efficacy of orally administered peptidyl falcipain inhibitors.[118] Two analogues of Mu-Leu-Hph-VS-Ph, N-Me-Pip-CO-Leu-Hph-VS-Ph (IC_{50} = 5 nM) and N-Me-Pip-CO-Leu-Hph-VS-Np2 (IC_{50} = 2 nM), were synthesized to improve aqueous solubility and bioavailability. Both were found to be potent falcipain inhibitors and inhibited hemoglobin degradation, development, and metabolic activity in cultured *P. falciparum* parasites. When administered orally twice a day for 4 days, the compound N-Me-Pip-CO-Leu-Hph-VS-Np2 cured 40% of mice from malaria. Thus, peptidyl inhibitors of falcipain have the potential to be antimalarial chemotherapeutic agents.

A new, potential antimalarial drug target is falcipain-2, a cysteine protease and essential hemoglobinase of *P. falciparum*. A homology model for falcipain-2 has been developed by Sabnis et al. and validated by docking known vinyl sulfone inhibitors.[387] Singh et al. tested the falcipain-2 inhibitors Mu-Leu-Hph-VS-Ph, N-Me-Pip-CO-Leu-Hph-VS-Ph, and N-Me-Pip-CO-Leu-Hph-VS-Np2 against five strains of *P. falciparum*, and all inhibitors strongly blocked hemoglobin degradation at a 1 nM concentration in all strains.[388] The results suggest that there is no cross-resistance between cysteine protease inhibitors and other commonly used antimalarial agents in parasites that are now circulating. Thus, falcipain-2 remains a promising chemotherapeutic target.

A series of pyrimidinyl peptidomimetics have good antimalarial activity in parasite-bearing mice, and one of the best inhibitors is shown in Figure 73.[389] This Michael acceptor has an interesting mechanism of action. Once the active site cysteine has added to the unsaturated ester, it can eliminate to form a new unsaturated ester covalently linked to enzyme. This new Michael acceptor could react with another active site nucleophile such as the His, or it may just stabilize the initial adduct. It would be appropriate to carry out additional mechanistic studies with this group of inhibitors to learn if they are indeed double-hit inhibitors.

Cysteine proteases are involved in tissue destruction in the joints of animals with arthritis. Papain-like cysteine protease activity, probably cathepsins, is up-regulated in inflamed joint tissue from rats with

Table 42. Inhibition of Proteasome Catalytic Activities by Various Vinyl Sulfones[a]

	k_{obs}/[I] (M^{-1} s^{-1})			
inhibitor	ChT[b]	T[c]	PGPH[d]	cathepsin B
NLVS (**19**)	13500	10	24[d]	191[e]
Cbz-Leu-Leu-Leu-VS-CH_3	29	8	5	
Leu-Leu-Leu-VS-CH_3	2.7	11	0.21	
Leu-Leu-Leu-Leu-VS-CH_3	240	1500	29	

[a] Conditions: 20 mM Hepes, pH 8.0, 0.5 mM EDTA, and 0.01% SDS at 37 °C. Substrates were Suc-LLVY-AMC for chymotrypsin-like activity, Boc-LRR-AMC for trypsin-like activity, and Ac−YVAD-AMC for PGPH activity. [b] ChT = chymotrypsin-like activity. [c] T = trypsin-like activity. [d] PGPH = peptidyl-glutamyl peptide hydrolyzing activity. [e] Bogyo et al., 1998.[392] [f] Meng et al., 1999.[323]

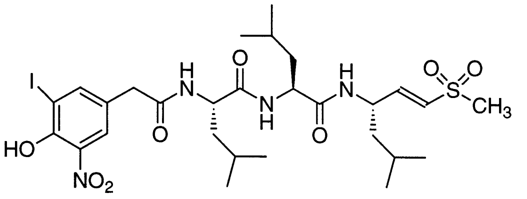


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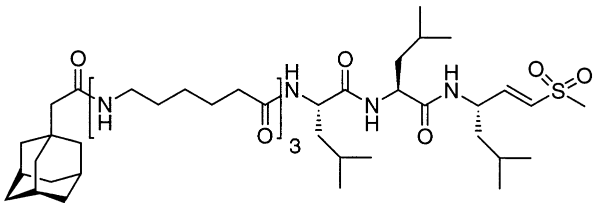
Figure 74. Structure of the proteasome inhibitor NLVS (**19**), nitrophenol-Leu-Leu-Leu-VS−CH_3.

induced arthritis, and this activity correlates positively with the severity of joint destruction and degree of inflammation.[390] Biroc et al. showed that vinyl sulfone inhibitors decreased enzyme activity in vitro and in vivo in the joints of rats with induced arthritis.[390] Oral administration of Mu-Leu-Hph-VS-Ph (2.2 mg/kg/day for four weeks) was effective in the animal arthritis model and reduced the signs of inflammation and tissue destruction.

Vinyl Sulfone Inhibitors of the Proteasome. The proteasome is a 700-kDa multicatalytic protease complex responsible for the degradation of many cellular proteins. The proteasome contains an active site threonine residue and has various proteolytic activities such as chymotrypsin-like activity, trypsin-like activity, and peptidyl-glutamyl peptide hydrolyzing activity (PGPH).

In 1997, Bogyo and co-workers unexpectedly discovered that peptide vinyl sulfones were a new class of irreversible, active site-directed inhibitors of the proteasome.[391] The vinyl sulfone inhibitor Cbz-Leu-Leu-Leu-VS-CH_3 inhibits the trypsin-like activity, the chymotrypsin-like activity, and the peptidylglutamyl peptidase activity of the proteasome both in vitro and in vivo (Table 42). The tripeptide Leu-Leu-Leu vinyl sulfone, ArCH_2CO-Leu-Leu-Leu-VS (NLVS, **19**, Figure 74), can permeate cells and can be used to inhibit proteasomes in living cells (Table 42).[322,323] It was concluded that a ^{125}I derivative of NLVS (**19**) labels at least five distinct β-subunits of the proteasome.

Bogyo et al. analyzed the substrate binding and specificity of the proteasome using various tripeptide and tetrapeptide vinyl sulfones.[392] Tripeptide vinyl sulfones capped with a bulky acyl group containing a nitrophenyl (NLVS) or benzyloxy carbonyl group (Cbz-Leu-Leu-Leu-VS-CH_3) are potent proteasome inhibitors, but removal of the cap to create free amino tripeptide vinyl sulfones results in severely diminished binding and inhibition (Table 42). However, N-terminal unblocked tetrapeptide vinyl sulfones are decent proteasome inhibitors (Table 42). These data suggest that the proteasome has a minimal length requirement for substrate binding and hydrolysis.



Ada-Ahx$_3$-LLL-VS

Figure 75. Structure of proteasome inhibitor Ada-Ahx$_3$-LLL-VS.

Vinyl sulfone inhibitors also modify the active site threonine of the *Escherichia coli* HslV homologue and *Rhodococcus* proteasome.[391,393] Inhibition of the *Rhodococcus* proteasome by Cbz-Leu-Leu-Leu-VS-CH_3 ($k_{inact}/K_i = 150\ M^{-1}\ s^{-1}$) results in a shifted retention time for the β-subunit upon separation from the α-subunit by reverse-phase HPLC, suggesting that the vinyl sulfone covalently reacts with the β-subunit.[393] Prior treatment of the proteasome with *clasto*-lactacystin β-lactone (see β-lactam section) prevents this modification. The above result combined with analysis of the tryptic digest of the vinyl sulfone-modified β-subunits suggests that the vinyl sulfone inhibits the proteasome by modification of the Oγ of Thr 1.

Kessler et al. have designed vinyl sulfone inhibitors with N-terminal extensions that are potent proteasome inhibitors.[394] Introduction of different substituents at the N-terminus (P4) of the trileucine vinyl sulfone core results in inhibitors with a preference for the β5 (NLVS, ZL$_3$VS) and β2 (YL$_3$VS) catalytic subunits of the proteasome. Kessler's new inhibitors consist of the core trileucine vinyl sulfone moiety and are extended at the N-terminus by aminohexanoic acid spacers (Ahx) and N-terminal caps of different size such as Ac, Z (Cbz), and adamantanylacetyl Ada. These extended peptide vinyl sulfones have enhanced inhibition in living cells, and unlike all other inhibitors to date, they target all of the individual active subunits (β1, β2, and β5) with comparable affinity.[394] The most potent inhibitor, Ada-Ahx$_3$-LLL-VS (Figure 75), is cell-permeable and can be modified to yield radiolabels and affinity labels. The data suggest considerable overlap between the individual catalytic activities of the proteasome.

Nazif and Bogyo have reported the preparation of a peptide vinyl sulfone library for the proteasome with an asparagine vinyl sulfone at P1.[395] Their results emphasized the importance of the P2−P4 subsites for directing substrate processing and led to the design of inhibitors such as Ac-PRLN-VS that specifically target the Z (β2, trypsin-like) subunit of the proteasome. One inhibitor, NIP-LLN-VS, which is NLVS with asparagine instead of the P1 leucine, targets all of the active sites of the proteasome.

Groll et al. also emphasize the importance of binding interactions distal to the active site threonine

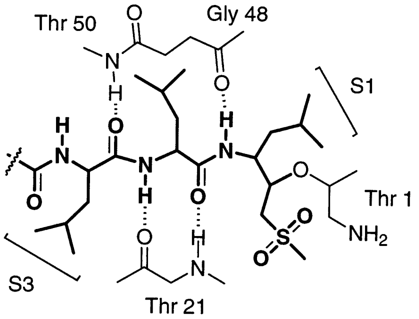


Figure 76. Schematic of NLVS bound to *H. influenzae* HslUV.

in controlling the substrate specificity of the proteasome.[396] Two inhibitors, which vary only in their P3–P4 residues, have very different specificities toward the catalytic subunits of the proteasome. The crystal structure of the β2-selective inhibitor (Ac-PRLN-VS) bound to the yeast 20S proteasome core particle is compared to that of the general inhibitor (Ac-YLLN-VS), which modifies all three catalytic subunits of the proteasome (β1, β2, and β5).[396] Having a positively charged basic residue at P3 and a proline or tyrosine at P4 results in specificity for the β2 subunit, whereas placing an aliphatic leucine residue at P3 abolishes the selectivity of the inhibitor.

In the crystal structure of Ac-PRLN-VS, the P3 arginine projects into a deep acidic pocket of the proteasome and the nitrogen atoms hydrogen bond to Asp 28 and Cys 118 in the S3 pocket.[396] These interactions probably determine the selectivity of the trypsin-like active site. The proline at P4 sits in a small S4 pocket and causes the N-terminal moiety of the inhibitor to bend away from the inner cavity. It is proposed that the presence of bulky hydrophobic groups at the P4 position might result in an inhibitor that does not bind to β2. The vinyl sulfone oxygens form hydrogen bonds with backbone amides of the enzyme and the free amino terminus of Thr 1.

Sousa et al. solved the crystal structure of NLVS bound to *H. influenzae* HslUV, a prokaryotic mimic of the eukaryotic proteasome (PDB code 1KYI).[397] A schematic of the enzyme–inhibitor complex is shown in Figure 76. The peptide backbone of NLVS is sandwiched between the peptide backbone of two loops of HslUV, resulting in two short sections of antiparallel β-sheets. Two of the leucine side chains bind loosely in the S1 and S3 pockets of the protease. On the basis of the amino acid residues at the roof of the pockets, Sousa et al. suggests that the S1 and S3 pockets should have hydrophobic or acidic and hydrophobic or basic binding preferences, respectively.[397]

Mechanism. The vinyl sulfone inactivates the proteasome by covalent modification of the N-terminal threonine of the catalytically active β subunits (Figure 77).[391]

Biological Studies. Glas and co-workers studied the inactivation of the proteasome by vinyl sulfone inhibitors in mammalian cells.[398] When EL-4 lymphoma cells are maintained in the presence of the vinyl sulfone inhibitor NLVS, they have drastically reduced proteasome activity and die after 24–48 h. However, after prolonged exposure to NLVS, a small number of EL-4 cells, termed adapted cells, recover and grow. Glas et al. concluded that the proteasomes can be replaced functionally by other protease activities. Demasi and co-workers discovered that the proteasome undergoes enhanced glutathiolation (a process in which glutathione forms direct adducts with cellular proteins) during exposure to vinyl sulfone inhibitors.[399] They propose that the binding of the inhibitor causes conformational modification of the proteasome, which may result in partial opening of the α- and β -ring structures, allowing increased thiolation of the exposed cysteine residues.

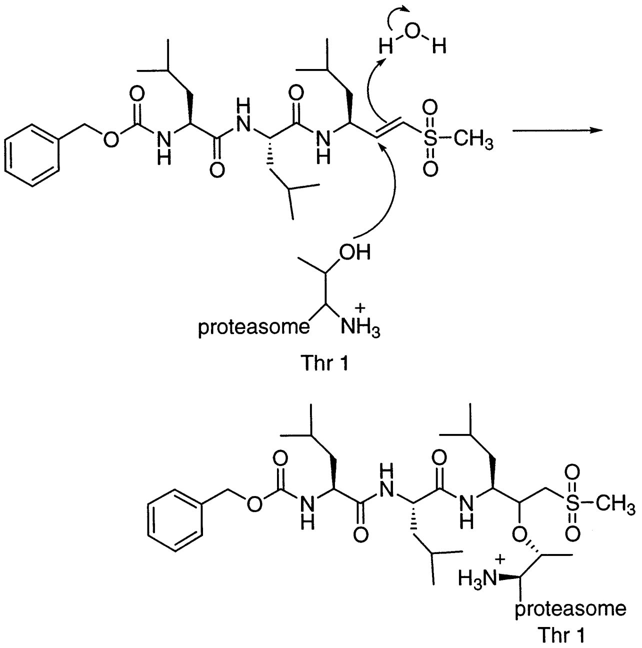


Figure 77. Mechanism of inhibition of the proteasome by vinyl sulfones.

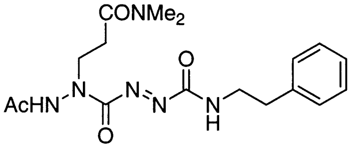


Figure 78. Azodicarboxamide inhibitor of hepatitis A virus.

G. Azodicarboxamides

Azodicarboxamides, the products of the oxidation of bishydrazides, are potent, irreversible inhibitors of hepatitis A virus and human rhinovirus 3C enzymes with IC_{50} values in the low micromolar range.[400] It is proposed that azodicarboxamides inhibit the enzymes by covalent modification of the active site thiol via Michael addition to the azo moiety to yield a covalent complex, which is seen in electrospray mass spectrometry. One such azodicarboxamide is shown in Figure 78 ($IC_{50} = 10\ \mu M$, $k_{inact}/K_i = 594\ M^{-1}\ s^{-1}$ for hepatitis A virus, and $IC_{50} = 12\ \mu M$ for human rhinovirus).

IV. Acylating Agents

A. Aza-peptides

Aza-peptides are peptides with an aza-amino acid residue that has its α-carbon replaced with a nitrogen

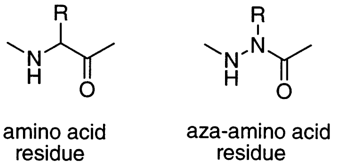


Figure 79. Structure of a normal amino acid residue and an aza-amino acid residue.

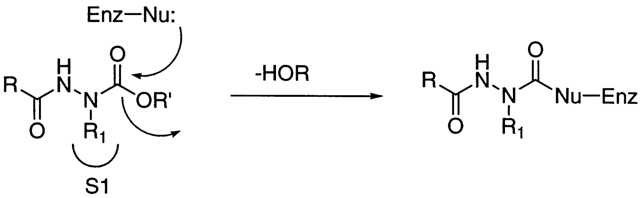


Figure 80. Mechanism of inhibition of cysteine (Nu = cysteine thiol) and serine (Nu = serine hydroxyl) proteases by aza-peptide inhibitors.

atom (Figure 79). This manipulation yields a structure that has a planar α-nitrogen and is intermediate in configuration between D- and L-amino acids. The nitrogen substitution has little effect on the overall polarity of the molecule and on the relative spacing of the side-chain residues, but should increase the stability of the aza-peptide to proteases. Replacing the α-carbon of P1 with nitrogen decreases the electrophilicity of the P1 carbonyl and changes the geometry of the α-position from tetrahedral to trigonal or planar. As a result, some aza-peptides are more resistant to enzymatic hydrolysis than their normal peptide analogues. The P1 amino acid also might be held more rigidly due to strong binding of the side chain by the enzyme, resulting in an unfavorable orientation of the carbonyl group in the active site.

Aza-peptide esters were originally designed as active site titrants and inhibitors for serine proteases[401] but were later found to inhibit cysteine proteases.[402] Serine proteases are inhibited by aza-peptides esters that have good leaving groups at the C-terminus, but not by aza-peptides esterified with alkyl residues or poor leaving groups (such as ethoxide). However, both types of aza-peptides inhibit cysteine proteases.[403] If a chromogenic leaving group such as nitrophenol is used, then the aza-peptide esters are good active site titrants for a variety of serine proteases.[404]

Nomenclature. Aza-amino acids will be designated in a peptide sequence by a four-letter abbreviation starting with the letter "A" followed by the normal three-letter amino acid code. For example, aza-phenylalanine is abbreviated APhe. Aza-peptide esters are also called peptidyl carbazates.

Mechanism. It is believed that aza-peptides inhibit cysteine and serine proteases by a direct acylation mechanism. The first step in the inhibition of cysteine and serine proteases by aza-peptides is nucleophilic attack by the active site thiol or hydroxyl on the aza-peptide carbonyl. This results in the formation of a covalent, stoichiometric, acyl (carbazyl) enzyme intermediate (see Figure 80). A simple ester derivative is formed with normal peptide substrates, whereas aza-peptides carbamoylate (carbazoylate) the active site nucleophiles. Irreversible inhibition occurs with aza-peptides as a result of the poor electrophilicity, trigonal geometry, and slow hydrolysis of the acyl carbonyl group. The carbazate carbonyl carbon is less electropositive than a simple ester because of the resonance effect of the adjacent nitrogen, allowing it to be less susceptible to nucleophilic attack by water (deacylation).[405] In the presence of added nucleophiles, faster reactivation or deacylation occurs.

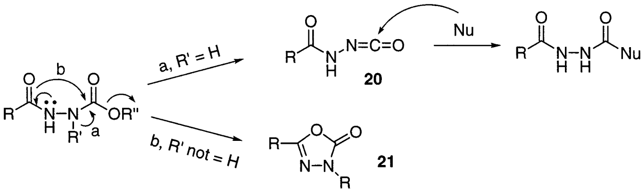


Figure 81. Possible mechanisms for enzyme inactivation and nitrophenol release (R″ = nitrophenyl, Np).

Aza-peptides that have a nitrophenol ester moiety inactivate papain rapidly but also decompose rapidly in solution to release nitrophenol. Two possible mechanisms besides direct acylation could give rise to this inactivation and nitrophenol release.[403] The intramolecular cyclization pathway (Figure 81, pathway b) gives rise to an oxadiazolone (**21**, Figure 81), which is unreactive toward basic hydrolysis and cannot acylate the enzyme. An alternative is the isocyanate (E1cb) mechanism (Figure 81, pathway a). The peptidyl isocyanate (**20**, Figure 81) could inactivate proteases by carbamoylating the active site residue. Xing and Hanzlik propose that both aza-glycine and aza-alanine derivatives inactivate papain by direct acylation.[403] Therefore, the formation of isocyanates in solution is not involved in enzyme inactivation, and the release of nitrophenol is a result of intramolecular cyclization to form the noninhibitory oxadiazolone derivative (**21**, Figure 81).

Structure. The complex Ac-NH-N(CH_2-Ph-F)-CO_2-Enz (Enz = α-chymotrypsin) and an analogue, which contains a deuterated fluorophenyl ring, were analyzed by NMR to learn about conformational dynamics at the active site of acylated chymotrypsin. The results indicate that the phenyl ring is immobilized probably by occupation of the S1 binding site of chymotrypsin.[406,407] The acylated enzyme complex is resistant to denaturation near the active site.

Structure—Activity Relationships: Serine Proteases. Aza-peptides that have a P1 aza-amino acid residue and a good leaving group (such as phenol) inhibit serine proteases, but those with poor leaving groups or alkyl groups do not. For example, an aza-peptide ethyl ester inhibited chymotrypsin with a rate of 0.019 M^{-1} s^{-1}.[408] Unfortunately, the compounds with the good leaving groups also undergo undesired hydrolytic reactions in solution, having half-lives on the order of a few minutes.[401] This makes the prospects of using aza-peptide phenyl esters in vivo for the treatment of diseases very poor.

Chymotrypsin. Kurtz and Niemann reported that Ac-APhe-OEt, an aza-analogue of the good chymotrypsin substrate Ac-Phe-OEt, was not a substrate of α-chymotrypsin but a poor competitive inhibitor. This suggests that the compound binds at the active

Table 43. Inhibition of Human Leukocyte Elastase and Cathepsin G by Aza-peptide Esters

inhibitor	$k_{obs}/[I]$ (M^{-1} s^{-1}) cathepsin G[a]	$k_{obs}/[I]$ (M^{-1} s^{-1}) HLE[b]
Ac-Ala-Ala-ANva-OPh	44	343
Ac-Ala-Ala-ANva-OCH_2CF_3	2.2	4.8
Ac-Ala-Ala-ANva-OEt	NI[c]	NI
Ac-Ala-Ala-ANle-OPh	71	32.4
Ac-Ala-Ala-ANle-OCH_2CF_3	9.8	1.5
Ac-Ala-Ala-ANle-OEt	0.64	NI

[a] Conditions were pH 6.0, 0.1 M citrate, 5% acetonitrile, 25 °C, 0.17 μM cathepsin G, with Boc-Tyr-ONp (Np = nitrophenyl) as substrate. [b] Conditions were pH 6.0, 0.1 M citrate, 5% acetonitrile, 25 °C, 0.3 μM elastase, with Boc-Ala-ONp (Np = nitrophenyl) as substrate. [c] NI = no inhibition.

Table 44. Inhibition of HLE and PPE by Various Aza-Peptide Esters

P4	P3	P2	P1	P1′	IC_{50} (μM) HLE[a]	IC_{50} (μM) PPE[b]
R[c]	Val	Pro	ANva	OPh	0.28	
R	Val	Pro	ANva	OBzl	0.88	
Boc-Gly	Val	Gly	Val	OBzl		400
Boc	Val	Gly	ANva	OBzl		8.9
Boc	Val	Gly	AAla	OBzl		66
R	Val	Gly	AAla	OBzl		3.3
R	Val	Gly	AAla	OPh		0.4
R	Val	Gly	AAla	ONp[d]		>400

[a] Conditions were pH 8.0, 0.05 M Tris, 0.05 M NaCl, 40 nM HLE, with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the substrate. [b] Conditions were pH 8.0, 0.2 M Tris, 7.5 μg PPE, with Suc-Ala-Ala-Ala-*p*-nitroanilide as the substrate. [c] R = MeOCO-CH(CH_2CHMe_2)-NH-CO-. [d] Np = nitrophenyl.

site but does not acylate the enzyme.[409] Elmore and Smyth thought an aryl ester might be reactive enough to acylate the enzyme, so they synthesized the nitrophenol analogue, Ac-APhe-ONp (HONp = *p*-nitrophenol), which stoichiometrically acylates chymotrypsin and more slowly trypsin.[405,410−412] As a result, Ac-APhe-ONp can be used as an active site titrant to determine the absolute molarity of active chymotrypsin in solution.[410,411,413] Orr and Elmore prepared a series of Ac-APhe-ONp derivatives for chymotrypsin with different para substituents on the aromatic ring of the leaving group.[414]

Gupton et al. studied the inhibition of the chymotrypsin-like enzymes (chymotrypsin, subtilisin BPN′, subtilisin Carlsberg, and human leukocyte cathepsin G) with aza-peptide esters.[405] Aza-peptide nitrophenyl esters containing aza-amino acids with long alkyl side chains (AVal, ANva, ALeu, and AIle) acylated chymotrypsin stoichiometrically with no measurable turnover.[405] The simple phenyl analogue of Ac-APhe-OEt, Ac-APhe-OPh, also rapidly inactivates chymotrypsin, and this activation is only slowly reversible (first-order rate constant for reactivation = 1.2×10^{-4} s^{-1}) but can be accelerated by hydroxylamine.[408] In effect, Ac-APhe-OPh is an active-site-directed irreversible inhibitor of chymotrypsin and can be used as a ligand for covalent affinity purification of chymotrypsin.

Cathepsin G. Because Ac-Ala-Ala-ANle-ONp behaves like an irreversible inhibitor by acylating cathepsin G stoichiometrically to produce an acyl enzyme with a very small turnover rate, Gupton et al. investigated the inhibition by varying the leaving groups in hopes of increasing specificity. The compounds Ac-Ala-Ala-ANle-OPh and Ac-Ala-Ala-ANle-OCH_2CF_3 irreversibly inhibit cathepsin G (see Table 43).[405] Inhibitors containing ANva at the P1 position and ethyl esters are poorer inhibitors. In summary, the order of reactivity of aza-peptide esters is ONp > OPh > OCH_2CF_3 > OEt.

Elastase. Aza-peptides with a P1 aza-amino acid residue react with elastases to form stable carbazyl enzyme intermediates.[401,415] Aza-peptide *p*-nitrophenyl esters acylate both porcine pancreatic elastase and human leukocyte elastase.[401] It was found that when a particular amino acid yields a more reactive substrate, the corresponding aza-amino acid compound forms a more stable acyl enzyme and has a slower deacylation rate (k_{cat}). However, the deacylation rates were considerably faster than those observed with chymotrypsin and cathepsin G. The less effective interactions at the shallower elastase S1 pocket, versus the deep pockets in cathepsin G and chymotrypsin, allow a compound to occasionally occupy a suitable conformation for deacylation with an increased deacylation rate. The tetrapeptide Cbz-Ala-Ala-Pro-AAla-ONp but not the dipeptide Ac-Ala-AAla-ONp inhibits elastase, and the resulting carbazyl enzyme deacylates slowly, indicating that an extended substrate structure is required for binding to elastase.[412,416] In addition to acting as inhibitors, the compounds Ac-Ala-Ala-AAA-ONp (where AAA = AAla, ANle, and ANva) are active site titrants for porcine pancreatic elastase and human leukocyte elastase. The ALeu and ANle analogues form the most stable carbazyl enzyme complexes (k_{cat} for reactivation < 0.00019 s^{-1}), so derivatives of these compounds with different leaving groups were analyzed as HLE inhibitors.[401] The order of reactivity was OPh > OCH_2CF_3 > OEt, which is the same for inhibition of cathepsin G (see Table 43). The NH of the P1 aza-amino acid is essential for acylation of elastase as the substitution with methyl (Ac-Ala-Ala-MeAAla-ONp) results in no reaction with HLE or PPE.[401] Some aza-peptides do not acylate elastase but instead act as competitive inhibitors such as Ac-Ala-Ala-Pro-NHN(CH_3)CO_2CH(CH_3)CO-NH-$CH_2C_6H_5$ (K_i = 2.2×10^{-5} M).[417]

Aza-peptides that contain different peptide sequences, leaving groups, and various aza-amino acid substitutions at P1 have been investigated as HLE and PPE inhibitors (see Table 44).[418,419] The phenyl ester at P1′ is more potent than the benzyl ester, and ANva at P1 is more effective than AAla.

The compound Ac-Ala-Ala-Pro-AAla-Lac-R (Lac is the alanine isostere lactic acid) inhibits both PPE and HLE, when Lac-R is an amide or hydrazide, but inhibits only PPE if Lac-R is an ester (Table 45).[417] If the P1 or P1′ residue is a simple alanine residue, then potency is reduced (Table 45). Because the structural integrity of the inhibitor is maintained, these compounds do not appear to function as acylating agents, but as noncompetitive inhibitors.

Dipeptidyl Peptidase IV. Unlike other aza-peptide-serine protease systems, aza-peptides are not as effective with dipeptidyl peptidase IV. However,

Table 45. Inhibition of Elastase by Aza-alanine Analogues

							ID$_{50}$ (μg/mL)	
P5	P4	P3	P2	P1	P1′	P2′	PPE[a]	HLE[a]
Ac	Ala	Ala	Pro	AAla	Lac[b]	OEt	1	NI
Ac	Ala	Ala	Pro	AAla	Ala	OEt	100	NI
Ac	Ala	Ala	Pro	Ala	Lac	OEt	167	NI
Ac	Ala	Ala	Pro	AAla	Lac	NH_2	0.4	8

[a] Conditions were pH 7.5, 0.05 M potassium phosphate buffer, 25 °C, with Ac-Ala-Ala-Pro-Ala-*p*-nitroanilide as the substrate. [b] Lac = -O-CH(CH_3)-CO-.

Table 46. Inhibition of Recombinant Human Prostate-Specific Antigen by Aza-peptides

inhibitor	k_{inact}/K_i[a] (M^{-1} s^{-1})
Ac-Phe-APhe-OPh	31
Ac-Phe-ATyr-OPh	410
Boc-Ser-Phe-APhe-OPh	2600
Boc-Ser-Phe-ATyr-OPh	32000

[a] Conditions were 1 M potassium phosphate buffer, pH 7.0, and 4.5 mM Suc-Ala-Ala-Pro-Phe-*p*NA (NA = nitroanilide) at 25 °C.

they have been developed by Neumann et al. and Borloo and De Meester.[420,421] Neumann et al. prepared and evaluated a series of phenyl ester substrates for DPP IV, which had aza-alanine or aza-proline at the P1 position.[420] These compounds formed fairly stable acyl enzyme complexes, but reactivation of the enzyme occurred after a few minutes. Aza-alanine compounds (Ala-AAla-OPh = 0.0038 s^{-1}) reactivated a little more slowly than aza-proline derivatives (Ala-APro-OPh = 0.0367 s^{-1}) in phosphate buffer. Compounds containing an aza-proline underwent rapid decay in solution, probably due to intramolecular cyclization. The aza-proline may not be able to distort the substrate conformation enough so that deacylation is effectively prevented. Rate constants for the inactivation of DPP IV by Gly-APro-OPh and Ala-AAla-OPh are 609 and 154 M^{-1} s^{-1}, respectively. Unfortunately, these compounds did not lead to potent inhibitors of dipeptidyl peptidase IV.

Trypsin. Gray and Parker expanded on earlier work with chymotrypsin and designed Bz-AOrn-OPh, which rapidly inhibits trypsin and more slowly chymotrypsin, but undergoes spontaneous deacylation (first-order rate constant = 2.1×10^{-4} s^{-1}).[422,423] Unfortunately, Bz-AOrn-OPh is an unstable inhibitor at neutral pH, probably due to cyclization to form the corresponding oxadiazolone with liberation of phenol. Gray et al. then discovered a more stable, effective inhibitor of trypsin, Et-O-CO-AOrn-OPh, which does not affect chymotrypsin or urokinase but strongly inhibited thrombin.[423] Ferraccioli et al. have also synthesized ALys and AOrn phenyl and *p*-nitrophenyl esters, which rapidly inactivate thrombin and trypsin by forming very stable acyl enzymes.[424]

Prostate-Specific Antigen (PSA). Aza-peptides were also shown to inhibit the recombinant human prostate-specific antigen (rh-PSA).[425] The lead compound was Ac-Phe-AGly-OPh ($k_{inact}/K_i = 31$ M^{-1} s^{-1}). Substitution of PSA's preferred amino acids into the P1−P3 positions increased inhibition of rh-PSA. The results show that PSA prefers Tyr over Phe in P1 by 12-fold (Table 46). Placing Ser in the P3 position increases the inhibitory potency by a factor of 80. The PSA aza-peptide inhibitors Boc-Ser-Phe-APhe-OPh ($k_{inact}/K_i = 2600$ M^{-1} s^{-1}, $t_{1/2} = 4.4$ h) and Boc-Ser-Phe-ATyr-OPh (Figure 82, $k_{inact}/K_i = 32{,}000$ M^{-1} s^{-1}, $t_{1/2} = 3.8$ h) both produce stable acyl enzyme intermediates that are hydrolyzed slowly.

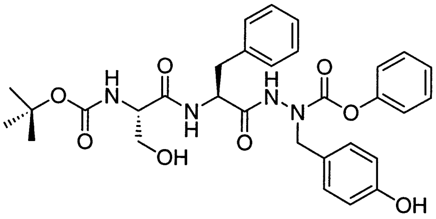


Figure 82. PSA inhibitor Boc-Ser-Phe-ATyr-OPh.

Hepatitis C Virus NS3 Serine Protease. Zhang et al. have performed SAR studies on aza-peptide-based inhibitors of the hepatitis C virus (HCV) NS3 serine protease.[426] The NS3 protease is a chymotrypsin-like serine protease responsible for the processing of the HCV polyprotein. For the most part, these inhibitors exhibited nonacylating, competitive inhibition, as <10% of the protease was trapped in an inactive, acylated state. Compounds with the peptide recognition element Ac-Asp-Thr-Glu-Asp-Val-Val-Pro, which contain ANva at P1 and either a thioester ($K_i = 160$ μM), ketone ($K_i > 100$ μM), α-ketoester ($K_i = >100$ μM), or sulfonyl group ($K_i = >100$ μM) on the prime side, were weak or inactive inhibitors. However, compounds that contain an ester with a good leaving group such as Ac-Asp-Thr-Glu-Asp-Val-Val-Pro-ANva-ONp were more potent inhibitors ($K_i = 2$ μM).[426]

Structure—Activity Relationships: Cysteine Proteases. Acyloxymethyl ketones selectively inactivate cysteine proteases because of the greater nucleophilicity of the cysteine thiol compared with the serine hydroxyl. Magrath and Abeles proposed that the nucleophilicity of the thiol might also lead to selective reaction with aza-peptides.[402] Some enzymes are specific for certain amino acids in the S1′ site, and aza-peptide alkyl esters could be designed with alkyl groups to satisfy these S′ binding requirements. Aza-peptide alkyl esters are unreactive with serine proteases, so they could be the most peptide-like selective cysteine protease inhibitors. Papain was inhibited by Ac-Phe-AGly-O*i*Bu ($k_2/K_i = 18$ M^{-1} s^{-1}) and Ac-Phe-AGly-OPh ($k_2/K_i > 11000$ M^{-1} s^{-1}) to form a stable acyl enzyme. The rate of inhibition was slowed in the presence of another inhibitor, no reactivation was observed after dialysis ($t_{1/2} > 118$ h), and valine methyl ester completely reactivated the inhibited enzyme within 2 h.[402,403] Aza-glycine esters ($k_2/K_i = 18$ M^{-1} s^{-1}) were more effective than aza-alanine esters ($k_2/K_i = 0.02$ M^{-1} s^{-1}) for inhibiting papain.[402,403] It was proposed that the reactive conformation of the aza-glycine residue, as compared to the aza-alanine residue, is such that the hydrogen atom is not directed into a binding pocket but is directed toward the surface of the enzyme where the bulkier methyl group of aza-alanine would not fit.[402] Another explanation is that papain has high selectivity for L- versus D-amino acid conformations at the

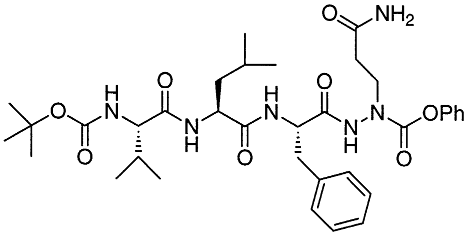


Figure 83. Structure of Boc-Val-Leu-Phe-AGln-OPh.

P1 position and aza-amino acid derivatives have a planar configuration.[403]

Baggio et al. have synthesized aza-peptide pseudosubstrates, which react with papain to form slowly hydrolyzing acyl enzymes.[427] The inhibitors contain an aza-peptide at P1 and incorporate amino acids at the prime side, which interact with the leaving group binding site (S′) and accelerate the rate of reaction of inhibitor with enzyme. One such inhibitor is Ac-Phe-AGly-OLeu-NMe$_2$ ($k_{on} = \sim 10^5$ M^{-1} s^{-1}) compared with Ac-Phe-AGly-OMe ($k_{on} = 13$ M^{-1} s^{-1}).[427]

Xing and Hanzlik confirmed and expanded on the work of Magrath and Abeles by evaluating ester and amide derivatives of aza-glycine, aza-alanine, and aza-phenylalanine as inhibitors of cathepsin B and papain.[403] Amide and thioamide derivatives are not substrates, nor do they irreversibly inactivate papain, but they are weak competitive inhibitors (K_i values between 0.2 and 4 mM). An aza-glycine analogue with a nitro-phenyl ester inactivates papain rapidly ($k_2/K_i > 70000$ M^{-1} s^{-1}) but also decomposes quickly in solution with release of nitrophenol. The rate of inactivation depends on the leaving group electronegativity and hydrophobicity and the substrate specificity. Compounds designed for cathepsin B and papain did not inactivate cathepsin C. Studies indicate that aza-peptide esters may be useful active site titrants of cysteine proteases because they are easy to synthesize and stable in solution.

Rhinovirus 3C Protease. Aza-peptides that resemble the natural substrate sequence for rhinovirus 3C protease and contain a reactive leaving group (−OAr) inhibit the rhinovirus 3C protease by carbamoylating the active site cysteine.[428] The compound Boc-Val-Leu-Phe-AGln-OPh (Figure 83) was a slow-turnover substrate that gave transient inhibition as it underwent hydrolysis. The compound Boc-Val-Leu-Phe-AGly-OPh was a very slow, but irreversible, inhibitor. These two results indicate that the glutamine side chain may be more important in deacylation than acylation when the pseudo-substrate contains a reactive leaving group.

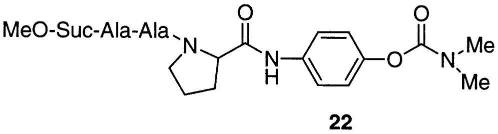
Aza-peptides that contain the leaving groups −OR and −NHR such as Boc-Val-Leu-Phe-AGln-OBn, Boc-Val-Leu-Phe-AGln-OCH$_2$CCl$_3$, and Boc-Val-Leu-Phe-AGln-Gly-Pro-NH*i*Bu do not inactivate and are not hydrolyzed by the enzyme. This can be attributed to the semicarbazide moiety being insufficiently reactive to carbamoylate the enzyme's active site thiol. Compounds lacking the P3 and P4 residues such as Boc-Phe-AGln-OPh and Boc-Phe-AGly-OPh show no inhibitory activity, which reflects the importance of these residues for substrate recognition by the 3C protease. It also shows the dependence of the inactivation process on the same intermolecular interactions, which contribute to catalysis with normal peptide substrates. Kati et al. also showed that the aza-peptide ester and amide, Boc-Phe-AGln-OCH$_3$ and Boc-Phe-AGln-NH-CH$_3$, did not significantly inhibit the rhinovirus 3C protease ($k_{inact}/K_{inact} < 1$ M^{-1} s^{-1} for both compounds).[103]

B. Carbamates

Peptidyl carbamate esters (RNHCOOR′) and thiocarbamates (RNHCOSR′) with appropriate substitutions have been designed that are stable to enzymatic hydrolysis and specifically inhibit PPE and HLE with no effect on trypsin or chymotrypsin.[429−433] Two inhibitors are MeO-Suc-Ala-Ala-Pro-CH$_2$-N(CH(CH$_3$)$_2$)-CO-ONp ($K_i = 4.24 \times 10^{-5}$ M) and MeO-Suc-Ala-Ala-Pro-CH$_2$-N(CH(CH$_3$)$_2$)-CO$_2$-Ph ($K_i = 3.00 \times 10^{-5}$ M, **23**, Figure 84). The inhibitors in Figure 84 are carbamate analogues that have the peptide portion placed on the nitrogen (**23**) or the peptide portion placed on the ester oxygen (**22**).[429] However, only the carbamate **23** and related compounds are inhibitors of PPE.

Structure—Activity Relationships. One thiocarbamate inhibitor (Figure 85, RXCONR′R″ with X = S) with a methyl thiotetrazole leaving group, which is less toxic than *p*-nitrophenol, was active against PPE ($K_i = 1.95 \times 10^{-5}$ M) and HLE ($K_i = 4 \times 10^{-6}$ M).[432] Altering the methyl thiotetrazole leaving group to a phenyl thiotetrazole and placing lysine or ornithine (desmosine-like residues) at the P3 or P4 positions (represented by Ala-Ala in Figure 85) created a new class of slow binding, selective HLE inhibitors (K_i values between 3.23×10^{-7} and 9.70×10^{-8} M).[431,434] Incorporation of a desmosine-like residue was intended to mimic the natural HLE substrate elastin. One of these compounds prevents corneal ulceration and vascularization after prolonged soft contact lens wear in the rabbit.[435]

Other low molecular weight thiocarbamate esters containing an electrophilic thiocarbamate functionality and a primary or secondary aliphatic or aromatic amine substitution were also designed as irreversible HLE inhibitors (see structure in Table 47).[436] Straight-



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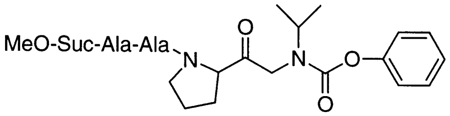

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Figure 84. Peptidyl carbamate inhibitors **22** and **23**.

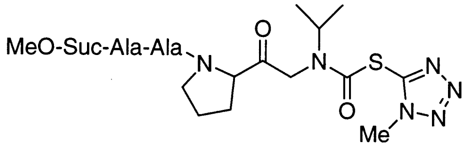
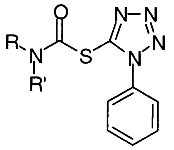


Figure 85. Carbamate inhibitor of PPE and HLE.

Table 47. Inhibition of HLE by Novel Thiocarbamates



R	R′	$k_{obs}/[I]^a$ (M^{-1} min^{-1})
CH_3-CH_2-CH_2-CH_2	H	2.1×10^5
$(CH_3)_2$-CH	H	3.3×10^3
Ph	H	1.9×10^2
CH_3-CH_2	CH_3-CH_2-CH_2-CH_2	1.8×10^2

[a] Conditions were 0.1 M Hepes buffer, pH 7.5, 0.05 M NaCl, and 3.7×10^{-5} M HLE in 0.05 M NaAc buffer at pH 5.5, with MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide as the substrate.

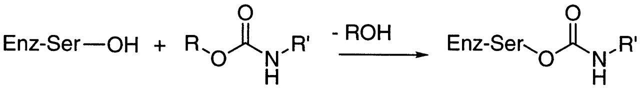


Figure 86. Mechanism of inhibition of serine proteases by carbamates.

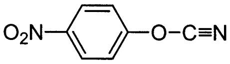


Figure 87. Structure of *p*-nitrophenyl cyanate.

chain nonpolar aliphatic substituents on the nitrogen of the thiocarbamate may be essential for high inhibitory activity ($k_{obs}/[I] = 2.1 \times 10^5$ M^{-1} min^{-1}, see Table 47).

Alkyl isocyanates inhibit chymotrypsin and pancreatic elastase specifically by reaction at the active site serine residue (Ser 195) to form *N*-alkyl carbamyl derivatives.[437,438] Chymotrypsin is inactivated stoichiometrically by octyl isocyanate and nearly stoichiometrically by butyl isocyanate, whereas elastase is inactivated only by butyl isocyanate. However, neither isocyanate inactivates trypsin. This selectivity corresponds with their known substrate specificities and can be explained on the basis of the structure of their substrate binding pockets observed in three-dimensional structures.

Baggio et al. has synthesized pseudo-substrates, which react with chymotrypsin to form slowly hydrolyzing acyl enzymes.[427] These carbonate esters contain leaving groups consisting of amino acids in order to interact with the leaving group binding site of chymotrypsin. Changing the leaving group R in benzyl-OCO-R from OMe ($k_{on} = 9$ M^{-1} s^{-1}) to OCH(Me)CO-Leu-Arg-OMe ($k_{on} = > 23000$ M^{-1} s^{-1}) had a significant effect on the rate of inactivation.[427]

Mechanism. The mechanism of inhibition of elastase by carbamates involves the transfer of an acylating moiety (HR′NCO) to the enzyme (Figure 86).

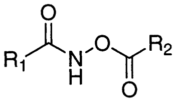
Crystal Structure. Aryl cyanates, the reactivity of which can be controlled by the nature of the substituents on the aromatic ring and the pH of the reaction medium, have been shown to carbamylate the active site residues of serine proteases. Using *p*-nitrophenyl cyanate (Figure 87), Robillard et al. prepared a stable, inhibited carbamoyl derivative of bovine chymotrypsin A with no nonspecific side reactions.[439] This compound reacts with both Ser 195 and Cys 1 in chymotrypsin Aπ and Aδ to give dicarbamylated products, but reacts with only the active site Ser 195 in chymotrypsin Aα. A hydrogen-bonding network consisting of the carbonyl oxygen of the carbamyl group, the backbone carbonyl of Phe 41, and the backbone amide of Gly 193 stabilizes the carbamyl group. This structure is a model for a stable acyl enzyme derivative in which the acyl carbonyl is not in the proper orientation for deacylation to occur.

Biological Activity. Despite their high inhibitory potency in vitro, therapeutic usage of small peptidyl carbamates has been limited due to short biological half-lives. As a result, macromolecular forms of peptidyl carbamate inhibitors of elastase have been designed for the prolongation of their half-lives in biological systems.[434,440,441] These derivatives consist of a low molecular weight peptidyl carbamate coupled to a linear hydrophilic polymer. This covalent linkage between the flexible linear polymer and the peptidyl carbamate inhibitor does not affect the in vitro inhibitory potency toward HLE (K_i values from 2 to 36 nM or 2×10^{-9} M). One such macromolecule was able to protect a hamster lung from HNE-induced emphysema ($t_{1/2}$ = 421 min) after intratracheal administration.[441]

C. Peptidyl Acyl Hydroxamates

Peptidyl acyl hydroxamates or *O*-acylhydroxylamines were originally designed in the early 1980s to inhibit serine proteases, in particular, dipeptidyl peptidase IV.[442] However, it was later shown that this class of inhibitors strongly inactivates cysteine proteases with second-order rate constants up to 10^6 M^{-1} s^{-1}.[443,444] Hydroxamates are irreversible inhibitors of both serine and cysteine proteases.[443-451] The mechanism of inactivation is not yet fully understood and depends on the nature of the enzyme and the reaction conditions. The general structure of peptidyl hydroxamates is shown in Figure 88. Potent and specific inhibitors for cysteine proteases are designed by varying the *N*-acyl and *O*-acyl groups of the hydroxylamine moiety. The electronic nature of the *O*-acyl leaving group can alter the reaction rate. Peptidyl hydroxamates were reviewed by Brömme and Demuth in 1994.[450]

Stability. Peptidyl hydroxamates exist as monoanions at neutral pH and are stable under acidic conditions, but are less stable toward strong alkali.[452] In aqueous solution, *N*-peptidyl-*O*-acyl hydroxamates are degraded spontaneously into the corresponding hydroxamic acid and a carboxylic acid. The decomposition rate is dependent on the nature of the leaving group. The nonenzymatic degradation occurs more quickly when the *O*-acyl residue has strong electron-withdrawing groups or is derived from acids with a lower pK_a value.[445] Half-lives of spontaneous decomposition of hydroxamates range from several



R_1-CO = an amino acid residue or peptidyl
R_2 = alkyl, aryl, or peptidyl

Figure 88. General structure of peptidyl hydroxamates.

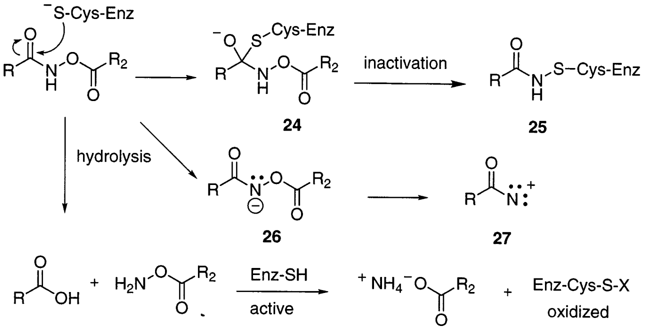


Figure 89. Proposed mechanism for inhibition of cysteine proteases by *N*,*O*-acyl hydroxylamines.

minutes to 15 h.[450] The P1 residue of the peptide affects the stability of the inhibitor. The *N*-peptidyl hydroxamates with a proline residue at P1 are up to 3 orders of magnitude more stable than inhibitors with other residues.[453] Compounds that have a Gly residue appear to be more stable than inhibitors having Phe, Ala, or Val in this P1 position.[449]

Mechanism. *N*,*O*-Diacyl hydroxylamines are irreversible inhibitors of serine and cysteine proteases. During the past decade, a variety of mechanistic studies have elucidated the mechanism of action of peptidyl acyl hydroxamates. Kinetic studies established the formation of an enzyme–inhibitor complex, which breaks down to form an irreversible enzyme adduct.[445,446] The heterolytic unimolecular fission of the inhibitor N–O bond, to generate an acyl nitrene and a carboxylic acid derivative, is the rate-determining step. This was determined by measuring the enthalpy of activation, the entropy of activation, and the solvent isotope effect, using an inhibitor with a ^{15}N label in the presence of ^{18}O-labeled water.[452] Evidence of covalent inactivation has been provided by ^{13}C and ^{15}N NMR, mass spectrometry, and X-ray crystallography.

The interaction of cysteine proteases with peptidyl *O*-acyl hydroxamates has been explored using papain and the peptidyl *O*-mesitoylhydroxamate Cbz-Phe-Gly-NHO-CO-(2,4,6-Me_3)Ph.[454,455] The inhibition process involves two competing reaction pathways, which provide different inactive enzyme products depending on conditions. In the presence of thiols, the formation of a highly reactive species (**24**) causes the inhibition reaction to form a sulfenamide adduct or a thiolhydroxylamine derivative (**25**, Figure 89). One proposed mechanism involves the formation of the tetrahedral intermediate (**24**) followed by the migration of the enzyme thiol group from the carbonyl at P1 residue to the nitrogen of the hydroxylamine. Another postulated mechanism involves binding of the deprotonated, negatively charged inhibitor (**26**) in the active site,[456] followed by formation of a carbonyl nitrene intermediate (**27**) by cleavage of the N–O bond and release of a mesitoic acid derivative. This reactive intermediate reacts with the nucleophilic cysteine to form the thiolhydroxylamine derivative (**25**), which has been identified by ^{13}C and ^{15}N NMR studies.[454] This mechanism is similar to the one observed with serine proteases at pH 5, and a similar hydroxylamine product is formed.

In the absence of a reducing thiol, overall hydrolysis and reduction of papain by the turnover product takes place.[454,455] At neutral pH, a number of *N*-peptidyl-*O*-benzoylhydroxylamines exist in solution as monoanions (pK_a = 5–6), where the N–O bond is not expected to be stable.[445,452] Hydrolysis of the inhibitor generates a free peptide acid and a hydroxamic acid. The activated enzyme is then oxidized by the hydroxamic acid. Robinson et al. showed that when papain is inactivated by Cbz-Phe-Gly-NHO-CO-(2,4,6-Me_3)Ph, in the absence of thiol, the end products are Cbz-Phe-Gly (identified by HPLC and ^{13}C NMR), NH_4^+ (identified by ^{15}N NMR), mesitoic acid (identified by HPLC), and inactive papain.[454] The thiol group of the enzyme active site is oxidized, possibly forming the sulfenic acid (Enz-SOH) or sulfinic acid (Enz-SO_2H).[455] A reducing thiol can reactivate the inactive papain.

Serine proteases are inactivated by *N*-peptidyl-*O*-hydroxamates by several competing pathways, which can lead to either irreversible inhibition (Figure 90) or inhibitor hydrolysis (Figure 91). The mechanism of inhibition of serine proteases by *N*-peptidyl-*O*-aroylhydroxylamines was explored by determining the structures of the final inactivated adduct by X-ray crystallography. Two products are formed upon inhibition of porcine pancreatic elastase by Boc-Ala-Ala-HNO-Nbz (Nbz = 4-nitrobenzoyl).[457] The structure of the enzyme–inhibitor complex depends on the pH at which the inactivation reaction occurs. A nitrene intermediate (**28**) can be generated due to the

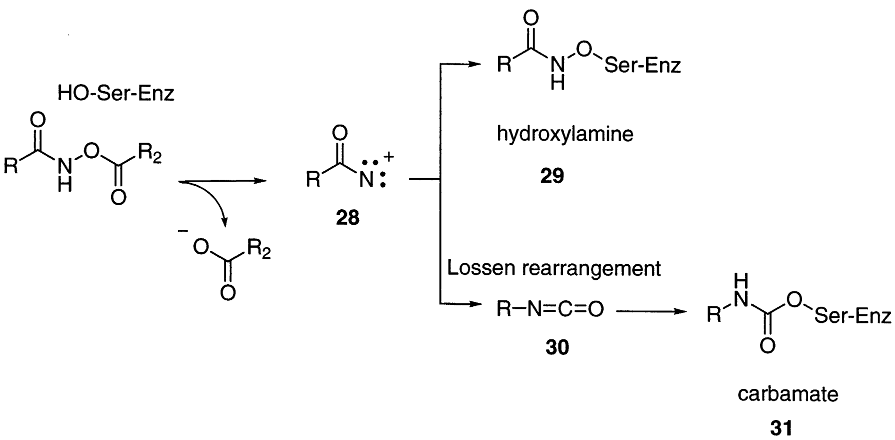


Figure 90. Mechanism of inactivation of serine proteases by *N*,*O*-acyl hydroxamates.

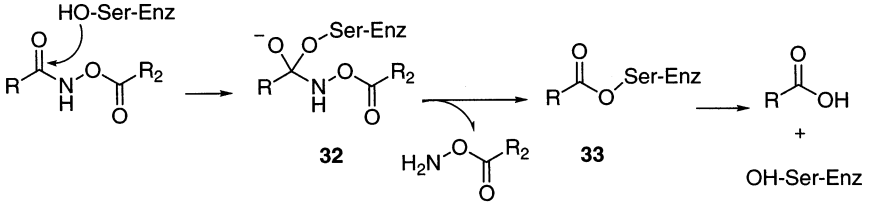
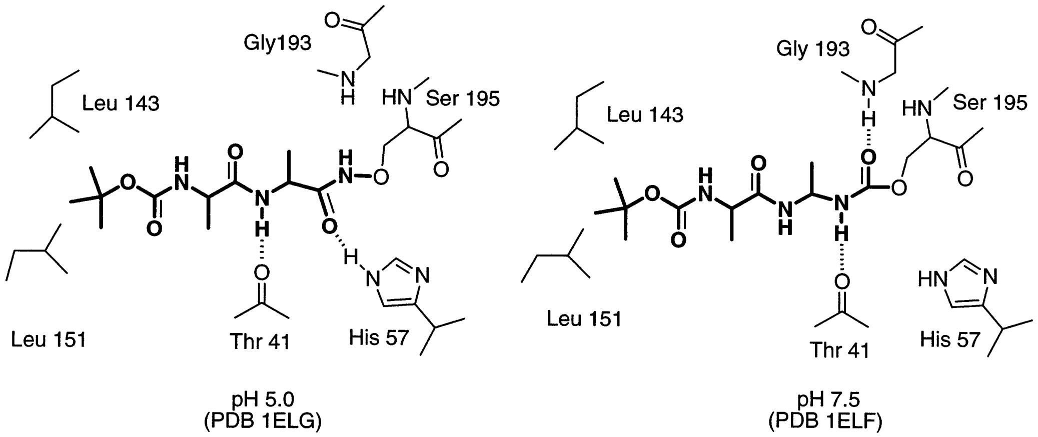


Figure 91. Serine proteases inactivation by enzyme-catalyzed hydrolysis by *N*,*O*-acylhydroxylamines.



pH 5.0
(PDB 1ELG)

pH 7.5
(PDB 1ELF)

Figure 92. Binding mode of Boc-Ala-Ala-NHO-Nbz to elastase at pH 5 and 7.5.

unimolecular N–O fission of the hydroxamate. At pH 5 the main reaction is the rapid formation of a covalent bond between the nitrene intermediate formed and the oxygen atom of Ser 195 (PDB code 1ELG). The resulting hydroxylamine derivative (**29**) is clearly seen in the X-ray structure. At pH 7, the predominant reaction is a Lossen-type rearrangement on the acyl nitrene intermediate, which takes place before the attack of the active site serine. The oxygen of Ser195 attacks the alkyl isocyanate (**30**) to form a carbamate derivative (**31**), which is present in the X-ray structures of the reaction products of PPE with Boc-Ala-Ala-HNO-Nbz (PDB code 1ELG) and subtilisin Carlsberg inhibited by Boc-Ala-Pro-Phe-NHO-Bz (PDB code 1SCN).[456]

The differences between the mechanism of inhibition of serine and cysteine proteases by peptidyl O-acyl hydroxamates can be attributed to the strength and reactivity of the nucleophilic residue in the enzyme active site (thiol versus hydroxyl group), to the stability of *O*-acylhydroxamate in conjunction with the pH at which the inhibition takes place, and to the binding mode of the inhibitor in the active site.

Serine proteases can also turnover *N*,*O*-diacyl hydroxamates (pseudo-substrates) as shown in Figure 91. Attack of the active site serine residue on the carboxyl carbon of the inhibitor results in a tetrahedral intermediate (**32**). The next step is the formation of the acyl enzyme (**33**) and the release of the acyl hydroxamic acid, which further decomposes to ammonia and a carboxylic acid. This mechanism was proposed for the reaction of hydroxamates with dipeptidyl peptidase IV, where enzyme-catalyzed turnover of the inhibitor is observed.[446]

Crystal Structure and Binding Mode. X-ray crystallography studies are available only for two serine proteases, porcine pancreatic elastase and subtilisin Carlsberg, complexed with *N*-peptidyl-*O*-aroylhydroxylamines. The structure of elastase inhibited by Boc-Ala-Ala-NHO-Nbz was studied at pH 5 and 7.5 (PDB codes 1ELG and 1ELF).[457] At pH 5, the complex formed between Ser 195 and Boc-Ala-Ala-NHO-Nb is a hydroxylamine derivative (Figure 92). The inhibitor binds in the S′ subsites, and the carbonyl oxygen of the alanine residue at P1 does not bind in the oxyanion hole to form hydrogen bonds with the backbone amides of Gly 193 and Ser 195. The hydroxylamine conformation is stabilized by two hydrogen bonds between the carbonyl oxygen of Ala residue at P1 and His 57 and the amide nitrogen of alanine and the carbonyl oxygen of Thr 41. The *tert*-butoxycarbonyl end of the inhibitor is in the hydrophobic pocket formed by Leu 149 and Leu 156. It is stabilized through a network of hydrogen bonds formed between two water molecules, the hydroxyl oxygen of Tyr 35, and the carbonyl oxygen of His 40.

At pH 7.5, the binding mode is comparable to that of the complex formed at pH 5, with the peptide part located in the S′ binding site of the enzyme. However, the final adduct formed by the covalent linkage between Ser 203 and the inhibitor is a carbamate.[457] Unlike the pH 5 complex the carbonyl oxygen of the Ala residue at P1 is in the oxyanion hole forming a hydrogen bond with the amide nitrogen of Gly 193 and an electrostatic interaction with the amide nitrogen of Ser 195 (3.3 Å). The same hydrophobic interactions stabilize the Boc group, but the water molecules and the hydrogen network are not present at pH 7.5.

A carbamate derivative is observed in the structure of subtilisin inactivated by Boc-Ala-Pro-Phe-NHO-Bz at pH 7.5.[456] The inhibitor binds in the S subsites, and the phenyl ring of the inhibitor is found in the S1 pocket. The oxygen of the carboxyl group of the carbamate protrudes into the oxyanion hole, where it forms hydrogen bonds with the side-chain nitrogen

Table 48. Inactivation of Cysteine Proteases by Peptidyl Hydroxamates

enzyme	inhibitor	k_2/K_i (M^{-1} s^{-1})	ref
cathepsin B	Cbz-Phe-Ala-NHO-Mes[a]	640000	443
	Cbz-Phe-Gly-NHO-Mes	580000	443
	Boc-Phe-Ala-NHO-Nbz[b]	14000	444
	Boc-Ala-Phe-Leu-NHO-Nbz	12000	444
	Cbz-Phe-Lys-NHO-Nbz	35000	448
	Cbz-Phe-Gly-NHO-CO-Leu[d]	1170	448
cathepsin H	H-Phe-HNO-Nbz	32000	447
	Boc-Phe-NHO-Nbz	1860	447
	Boc-Phe-Ala-NHO-Nbz	21	444
	Cbz-Phe-Lys-NHO-Nbz	760	448
cathepsin L	Cbz-Phe-Lys-NHO-Nbz	3540000	448
	Cbz-Phe-Phe-NHO-Ma[c]	1220000	444
	Cbz-Phe-Gly-NHO-Nbz	932000	449
	Boc-Gly-Phe-Phe-NHO-Nbz	800000	444
	Cbz-Phe-Val-Lys-NHO-Nbz	578000	448
	Cbz-Phe-Gly-NHO-CO-Phe[d]	68000	451
	Cbz-Phe-Gly-NHO-CO-Leu	19500	451
cathepsin S	Cbz-Val-Val-Lys-NHO-Nbz	606000	448
	Cbz-Phe-Lys-NHO-Nbz	471000	448
	Boc-Gly-Phe-Phe-NHO-Nbz	267000	444
	Boc-Ala-Phe-Leu-NHO-Nbz	229000	444
	Boc-Phe-Ala-NHO-Nbz	42000	444
	Cbz-Phe-Phe-NHO-Ma	21000	444
	Cbz-Phe-Gly-NHO-CO-Phe	8400	451
	Cbz-Phe-Gly-NHO-ClBz[e]	97900	449
papain	Cbz-Phe-Gly-NHO-CO-Phe	1080	451
	Cbz-Phe-Gly-NHO-CO-Leu	510	451
	Cbz-Phe-Gly-NHO-Nbz	5670	449
	Cbz-Phe-Gly-NHO-ClBz	2600	449

[a] Mes = mesitoyl. [b] Nbz = 4-nitrobenzoyl. [c] Ma = methacroyl ($CH_2{=}C(CH_3)$-CO-). [d] *O*-carbamoyl amino acid residue. [e] ClBz = 4-chlorobenzoyl.

Table 49. Inactivation of Serine Proteases by Peptidyl Hydroxamates

enzyme	inhibitor	k_2/K_i (M^{-1} s^{-1})	ref
DPP IV[a]	H-Ala-Pro-NHO-NBz[b]	1.9	445
chymotrypsin	Boc-Gly-Phe-NHO-NBz	0.4	442
PSE	Boc-Ala-Pro-NHO-NBz	2.5	446
elastase	Boc-Ala-Pro-NHO-NBz	12	446
	Boc-Ala-Pro-Val-NHO-NBz	15	446
trypsin	Cbz-Ala-Ala-Pro-Lys-NHO-NBz	930	448
	Cbz-Phe-Val-Lys-NHO-NBz	1960	448
thrombin	Cbz-Ala-Ala-Pro-Lys-NHO-NBz	48	448
	Cbz-Phe-Lys-NHO-NBz	69	448
thermitase	Boc-Ala-Ala-NHO-NBz	138	442
	Boc-Gly-Phe-NHO-Bz[c]	652	446
	Cbz-Gly-Phe-NHO-NBz	1170	449
	Cbz-Gly-Phe-NHO-ClBz[d]	428	449
subtilisin	Boc-Gly-Phe-NHO-Bz	1020	446
	Boc-Ala-Leu-Phe-NHO-Bz	36000	450
	Cbz-Gly-Phe-NHO-NBz	128	449
	Cbz-Gly-Phe-NHO-Bz	96	449
thiosubtilisin	Cbz-Gly-Phe-NHO-ClBz	3.8	449
	Cbz-Gly-Phe-NHO-Bz	2.5	449
t-PA	Cbz-Ala-Ala-Phe-Lys-NHO-NBz	1	448
plasmin	Cbz-Phe-Lys-NHO-NBz	69	448
	Cbz-Ala-Ala-Phe-Lys-NHO-NBz	48	448

[a] DPP IV = dipeptidyl-peptidase IV; PSE = proline-specific endopeptidase; *t*-PA = tissue plasminogen activator. [b] Nbz = 4-nitrobenzoyl. [c] Bz = benzoyl. [d] ClBz = 4-chlorobenzoyl.

of Asn 155 and the amide nitrogen of Ser 221. The inhibitor, which does not form an antiparallel β-sheet interaction with the enzyme, is stabilized by a network of water molecules and sticks out of the active site.

Crystal structures of cysteine proteases and diacyl hydroxylamine derivatives are not available in the Protein Data Bank.

Structure—Activity Relationships. *N*,*O*-Diacyl hydroxamates permit variations at the *N*-acyl and *O*-acyl residues and, therefore, can be tailored toward specific enzymes. The *N*-acyl residue usually contains peptidyl groups, whereas the *O*-acyl group can be any alkyl acid, aromatic acid, amino acid, or peptidyl residue. There is considerable difference in the second-order inhibition rate between serine and cysteine proteases (Tables 48 and 49). Dipeptidyl derivatives inactivate cysteine proteases, such as cathepsins, with a rate constant up to 10^6 M^{-1} s^{-1} (Table 48). However, inhibition of serine proteases is much slower, with rates in the range of 10−1000 M^{-1} s^{-1}. The results are consistent with the difference in k_2/K_i values, which differ by 4−5 orders of magnitude.[449]

The inhibitory specificity of peptidyl hydroxamates toward cysteine proteases is similar to the results obtained with peptide substrates (Table 48). A proline residue in P1 or P2 decreases the efficiency of inhibition, whereas lysine residues at P1 are well tolerated. Cathepsin L favors bulky aromatic residues at P1, such as leucine or phenylalanine, whereas cathepsin S prefers smaller residues in P2 and P3. Smith et al. designed the first hydroxylamine inhibitors for cathepsin B by using lipophilic *O*-acyl groups. The inhibitor Cbz-Phe-Ala-HNO-O-mesityl inhibited cathepsin B with a second-order inactivation rate of 640000 M^{-1} s^{-1}.[443] Cathepsin H inhibitors show significantly lower rates when compared with the other cathepsins, but H-Phe-HNO-Nbz is considered to be one of the most effective inhibitors of cathepsin H, with a second-order rate constant of inhibition of 31800 M^{-1} s^{-1}. The degree of selectivity of hydroxamates relative to individual cysteine proteases spans over 4−5 orders of magnitude (Table 48).

The rate of inhibition can be increased by using electron-withdrawing groups at the *O*-acyl moiety.[445] Substitution of a benzoyl group as the *O*-acyl residue with methacryl led to 5−20-fold more potent inhibitors.[444] Inhibition rates can vary up to 6-fold depending on the nature of the benzoyl substituent.[449] The introduction of a carbonyl group at the *O*-acyl moiety (peptidyl-NHO-CO-AA, where AA = amino acid) allows extension at the P′ site of the inhibitor with amino acids or peptides. The *N*-peptidyl-*O*-carbamoyl amino acid hydroxamates have at least 1 order of magnitude lower reactivity than *O*-nitrobenzoyl hydroxamates (Table 48) but are useful in probing the S′ site of cysteine proteases.[448,451]

Peptidyl hydroxamates are poorer inhibitors of serine proteases than cysteine proteases (Table 49).

Biological Studies. Several hydroxamates have been examined in vitro as dipeptidyl peptidase IV inhibitors. The Phe-Pro-HNO-Nbz and Ala-Pro-HNO-Nbz inhibit lymphocytes DPP IV with IC_{50} values of 20 and 30 μmol/mL, respectively.[458] DNA synthesis after stimulation of mononuclear cells by mitogenic lectins is impaired by the presence of the DPP IV inhibitor Ala-Pro-HNO-Nbz.[459] Therefore, the DPP IV peptidyl hydroxamate inhibitors suppress lymphocyte proliferation and may have therapeutic value in immune disorders.

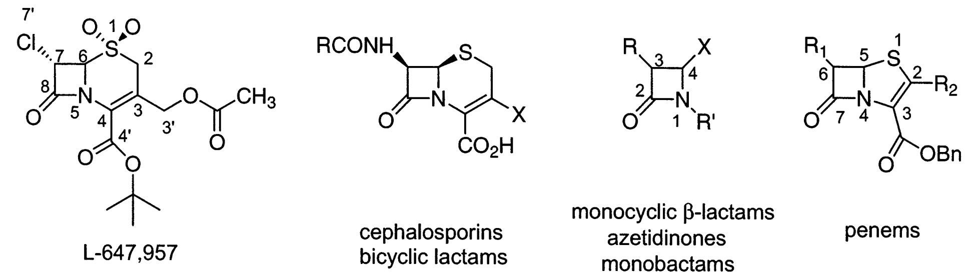


Figure 93. Structure and numbering of L-647,957, a 7-chloro-substituted cephalosporin derivative, and general structures of cephalosporins, penems, and monocyclic *β*-lactams.

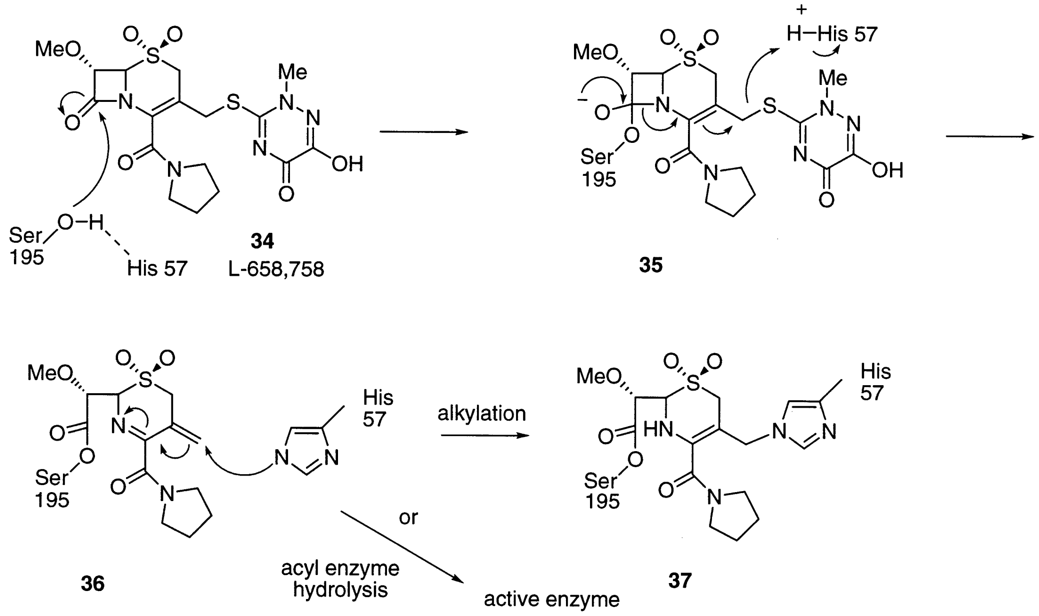


Figure 94. Proposed mechanism of inhibition of HLE by a 7-methoxy-substituted cephalosporin derivative (L-658,758).

D. *β*-Lactams and Related Inhibitors

β-Lactams were serendipitously discovered to inhibit serine proteases by Morris Zimmerman at Merck in 1986. While working on furyl saccharin and related derivatives as inhibitors for elastase (see saccharin section), Zimmerman was asked for some inhibitors by another Merck researcher who was studying *β*-lactamases. Furyl saccharin was an effective inhibitor of that *β*-lactamase, and Zimmerman decided to test *β*-lactamase inhibitors on elastase. Thus, he discovered that benzyl clavulanic acid was an elastase inhibitor ($IC_{50} = 5\ \mu M$), whereas clavulanic acid itself was inactive.[460] This led to a major effort at Merck to develop *β*-lactams as elastase inhibitors. That effort has still not borne fruit with the development of a commercial drug for the treatment of emphysema. In hindsight, it is quite logical to expect that *β*-lactamase inhibitors might inhibit serine proteases because both families of enzymes use an active site serine residue in their catalytic mechanism.

The Merck group pioneered the use of *β*-lactams for therapeutic uses besides their common use as antibacterials. The *β*-lactam inhibitors currently being synthesized as protease inhibitors consist of monocyclic *β*-lactams and bicyclic structures such as the cephalosporins or penems (Figure 93). Cephalosporin derivatives are also called bicyclic lactams, and monocyclic *β*-lactams have also been referred to as azetidinones or monobactams. The *β*-lactams inhibit the serine and cysteine proteases HLE, PPE, *E. coli* signal peptidase, PSA, cathepsin G, chymotrypsin, thrombin, trypsin, plasmin, chymase, HNE, human cytomegalovirus protease (hCMV), and poliovirus and human rhinovirus 3C proteases. The *β*-lactams *N*-(2-oxo-4-phenylazetidin-1-acetyl)-L-alanyl-L-valine benzyl ester and benzyl-(*S*)-2-(benzyloxycarbonyl)-azetidin-1-acetate have also been shown to be potent inhibitors of the cysteine protease papain.[461,462] The major research emphasis has been directed toward identifying inhibitors of human leukocyte elastase.

Mechanism. The mechanism of the inhibition of serine proteases by cephalosporin derivatives has been studied by numerous investigators.[463−467] Inhibition was generally believed to originate from the inability of the enzymes to undergo efficient deacylation once the inhibitors bound to the enzyme via acyl enzyme formation. Figures 94 and 96 show the proposed mechanisms for the inhibition of HLE by a 7-methoxy-substituted cephalosporin derivative[464] and of PPE by a 7-chloro-substituted cephalosporin derivative,[466] respectively. As seen in Figure 94, the first step is the attack on the *β*-lactam ring by the active site Ser 195 to form a tetrahedral intermediate (**35**). This intermediate collapses, opening the *β*-lac-

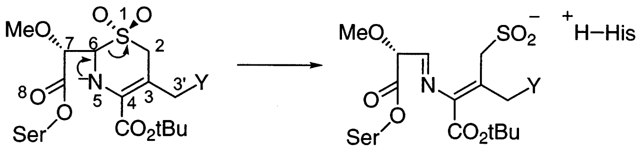


Figure 95. Mechanism for formation of sulfinic acid. In the structure, Y can be any group.

tam ring and eliminating the group from the cephalosporin 3′ position (for numbering, see Figure 93), to form the reactive acyl enzyme intermediate (**36**). The acyl enzyme intermediate (**36**) can either undergo hydrolysis to regenerate the active enzyme or undergo Michael addition of the active site His 57 to the 3′ position to form an inhibitor–enzyme complex (**37**) that is stable to nucleophiles such as hydroxylamine. In the crystal structure of HLE with L-658,758 the methoxy group at C-7 is present, which supports the mechanism shown in Figure 94.[464] If there is no leaving group on the 3′ position, it has been proposed that a double bond forms between N-5 and C-6, resulting in the opening of the dihydrothiazine ring to liberate a sulfinic acid, which could be stabilized by His 57 or by a conformational change (Figure 95).[463,468–470]

The mechanism with 7′-chlorocephalosporin derivatives involves a slightly different pathway (Figure 96). The acyl enzyme (**38**) can undergo elimination of HCl to yield the unsaturated acyl enzyme (**39**). This elimination of the acetoxy group at the cephalosporin 3′ position and of the chloride atom at C-7 to form the double bond has been confirmed by X-ray crystallography.[466] The acyl enzyme (**38**) can then undergo hydrolysis or reaction with the active site His 57 to give a second covalent bond as shown in Figure 96. The crystal structure demonstrates both acylation of the active site serine and alkylation of the histidine in a “double-hit” mechanism, which is also observed with saccharin and isocoumarin inhibitors. The dihydrothiazine ring of the inhibitor remains intact in the crystal structure.[466]

The inhibition of enzymes by monocyclic β-lactams proceeds by a mechanism similar to the mechanism shown in Figure 94 (see Figure 97).[467,471,472] However, the mechanism is complicated by the observation of multiple complexes and products, which indicate the presence of multiple pathways for inactivation and reactivation. All of the routes are initiated with the attack by the active site serine on the C-2 carbonyl of the β-lactam (**40**), which leads to the opening of the β-lactam ring between C-2 and N-1 and can result in the expulsion of the leaving group at C-4 (**41**) by forming a double bond between N-1 and C-4 (**42**). This double bond could isomerize into conjugation (between C-3 and C-4, enamine tautomer), permitting attack by the active site histidine at C-4 in a Michael addition to form a stable enzyme–inhibitor complex.[467,473] Alternatively, a water molecule could

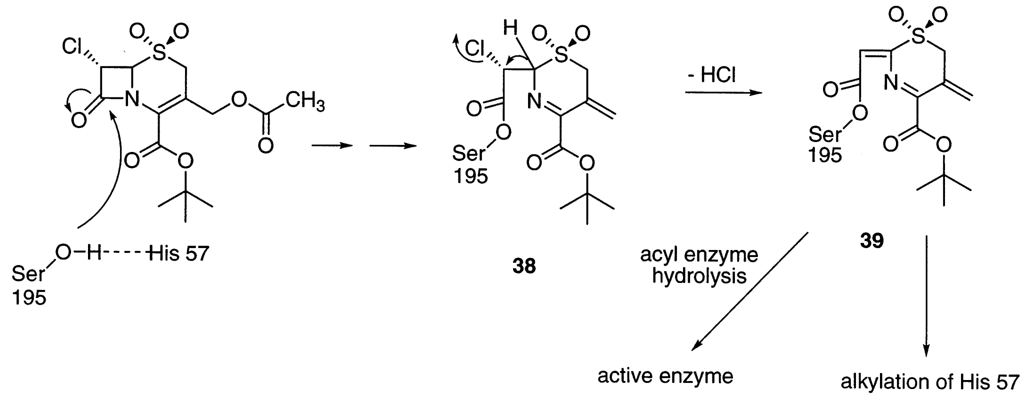


Figure 96. Proposed mechanism of inhibition of PPE by a 7-chloro-substituted cephalosporin derivative.

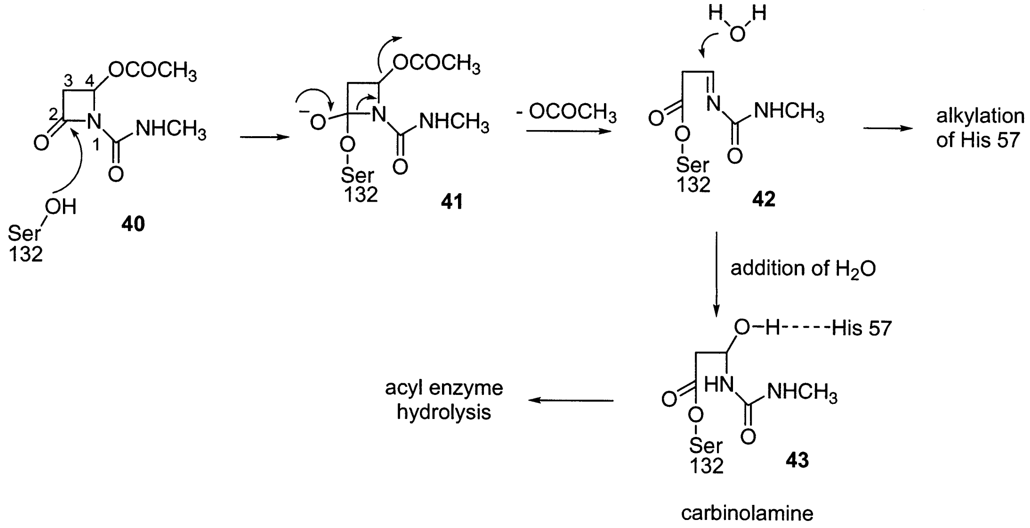


Figure 97. Proposed mechanism of inhibition of serine proteases (such as hCMV protease) by a monocyclic β-lactam.

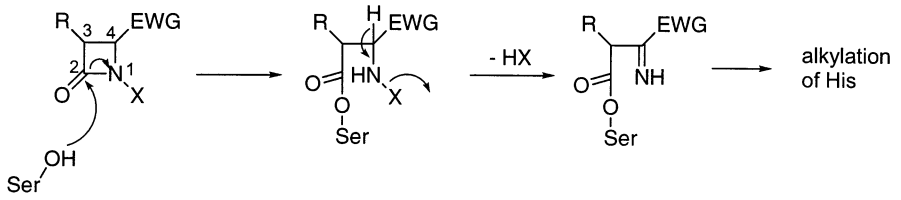


Figure 98. Mechanism of inhibition of serine proteases by β-lactams with the leaving group on the N-1 position (EWG = electron-withdrawing group).

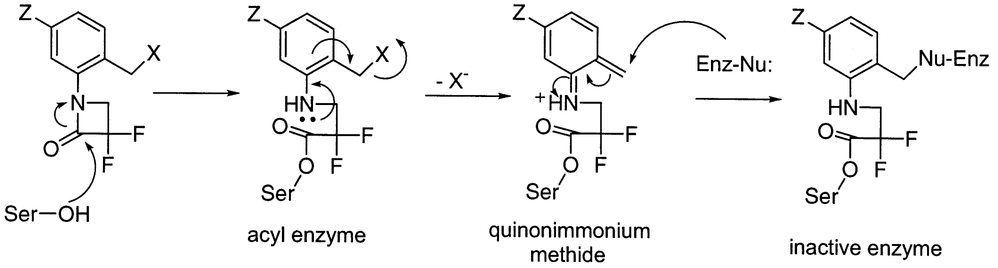


Figure 99. Mechanism of inhibition of HNE by *N*-aryl azetidinones.

attack the imine (**42**) to form a carbinolamine (**43**), the hydroxyl group of which could be stabilized by hydrogen bonding with a histidine residue in the enzyme. The carbinolamine (**43**) could also eliminate the substituted urea and produce an aldehyde.[474,475] The ability of monocyclic β-lactams to acylate the protease depends on the leaving group or substituent at C-4.[476] The stability of the acyl enzyme complex is insensitive to the nature of the C-4 leaving group, but does depend on the nature of the substituted urea.[477] In the case of hCMV, the histidine does not react with the imine (**42**) as the active enzyme is recovered over time. This recovery was confirmed using a fluorogenic β-lactam derivative.[472,476,478]

Monocyclic β-lactam HLE inhibitors have been designed that have the leaving group on the N-1 position instead of the C-4 position as in Figure 97.[467] This would cause the leaving group on the nitrogen to depart via β-elimination after the acylation step (Figure 98). To stimulate this elimination, an electron-withdrawing group must be present at C-4 to acidify the C-4 proton. The resulting Schiff base can serve as an electrophile for reaction with another active site nucleophile. Knight et al., using ESI-MS, have shown that HLE does not catalyze the β-elimination of *p*-nitrophenylsulfinate as Firestone et al. suggested. Knight et al. suggest that a double hit of both the active site serine and histidine is not required to form a stable acyl enzyme.[465]

Functionalized *N*-aryl azetidinones (functionalized monocyclic β-lactams), effective inhibitors of human neutrophil elastase, have a slightly different mechanism of action that involves formation of a powerful electrophilic quinonimmonium methide ion (Figure 99).[479] This can trap an enzyme nucleophile at yet another location on the inhibitor structure.

Two structurally related monocyclic β-lactams upon reaction with PPE form different covalent complexes.[477] These compounds contain an aryloxy substituent at C-4 but vary in the nature of the C-3 substituent. For both inhibitors, after attack by the catalytic serine and release of the C-4 substituent, a double bond (imine) is formed between C-4 and N-1 as discussed in Figure 98. The presence of a diethyl substituent at C-3 on the β-lactam ring (**45**, Figure 100) allows for the addition of water to the intermediate imine to form a carbinolamine as seen in Figure 97. However, a new mechanism arises when a hydroxyethyl moiety is present at C-3 (**44**, Figure 100). Elimination of the hydroxyethyl group will occur by retro-aldol reaction to form the acyl enzyme intermediate with concomitant release of acetaldehyde. The resulting double bond between C-3 and C-4 forms a stabilized, planar, conjugated system that is resistant to water hydrolysis.[477]

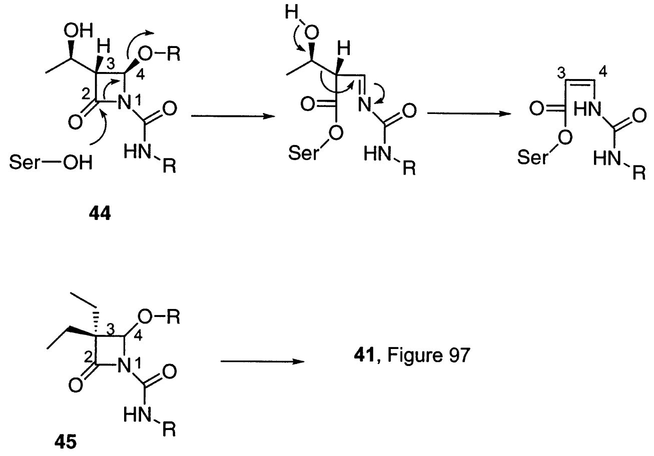
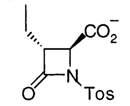
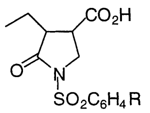
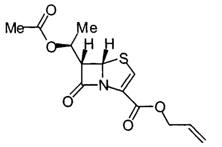
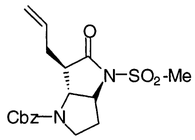


Figure 100. Mechanism of inhibition of PPE by a monocyclic β-lactam with a hydroxyethyl moiety at C-3 (**44**) versus a diethyl substituent at C-3 (**45**).

Crystal Structure. A number of crystal structures of serine proteases inhibited by β-lactams and analogues have been reported (Table 50).

Navia et al. describe the structure of the complex of PPE reacted with the time-dependent irreversible inhibitor L-647,957 (Figure 93), a 7-chloro-substituted cephalosporin derivative (Figure 101).[466] The active site Ser 195 is covalently bound to the inhibitor at C-8 by an ester bond. The carbonyl at C-8 is in hydrogen-bonding distance of the backbone amides of Gly 193 and Ser 195. One of the sulfonyl oxygens has a hydrogen bond with the side chain of Gln 192,

Table 50. Crystal Structures of Enzymes Complexed with β-Lactams and Analogues

enzyme	inhibitor	PDB Code	ref
PPE		1BTU	480
PPE	L-647,957		466
PPE		1E34 – 1E38	513
E. coli signal peptidase			481
PPE			517

and the other sulfonyl oxygen hydrogen bonds with the backbone amide of Val 216. The His 57, covalently attached to the 3′ position of the inhibitor, is now in the vicinity of Asp 60, which is in contact with the indole ring of Trp 94.

Wilmouth et al. describe the crystal structure of PPE and a monocyclic β-lactam (PDB code 1BTU).[480] The ring-opened β-lactam is in the active site, but the ester carbonyl is rotated by ~120° and is not located within the oxyanion hole. The hydrolytic water is displaced, thus disfavoring hydrolysis of the acyl enzyme complex (Figure 102). The ethyl side chain at C-3 is located in the S1 pocket as expected. One oxygen of the C-4 carboxylic acid forms hydrogen bonds with His 57 and a water molecule, whereas the other oxygen makes hydrogen bonds with two water molecules. The sulfonyl oxygens are in position to hydrogen bond with a water molecule and the side chain of Gln 192. The phenyl ring of the tosyl group sits deep in the S2 site near Trp 94 and Val 99 and parallel to His 57 (π–π interaction). The proposed mechanism involves acyl enzyme formation and then a conformational change, which involves the rotation of the ester carbonyl out of the oxyanion hole. This conformational change results in the breaking of two hydrogen bonds between the carbonyl oxygen and the backbone NH of Ser 195 and Gly 193.

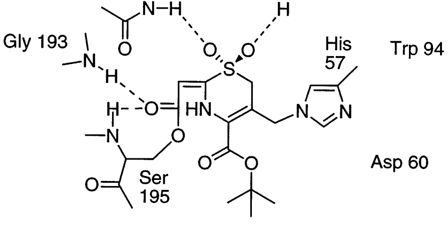


Figure 101. Schematic of PPE complexed with the cephalosporin derivative L-647,957.

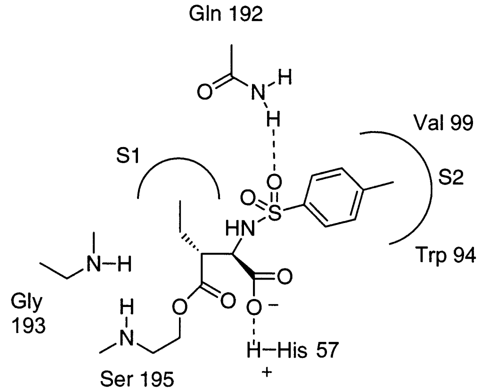


Figure 102. Schematic of PPE complexed with a monocyclic β-lactam inhibitor.

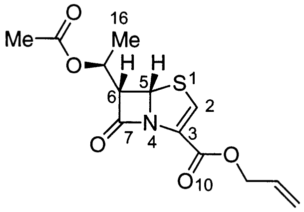
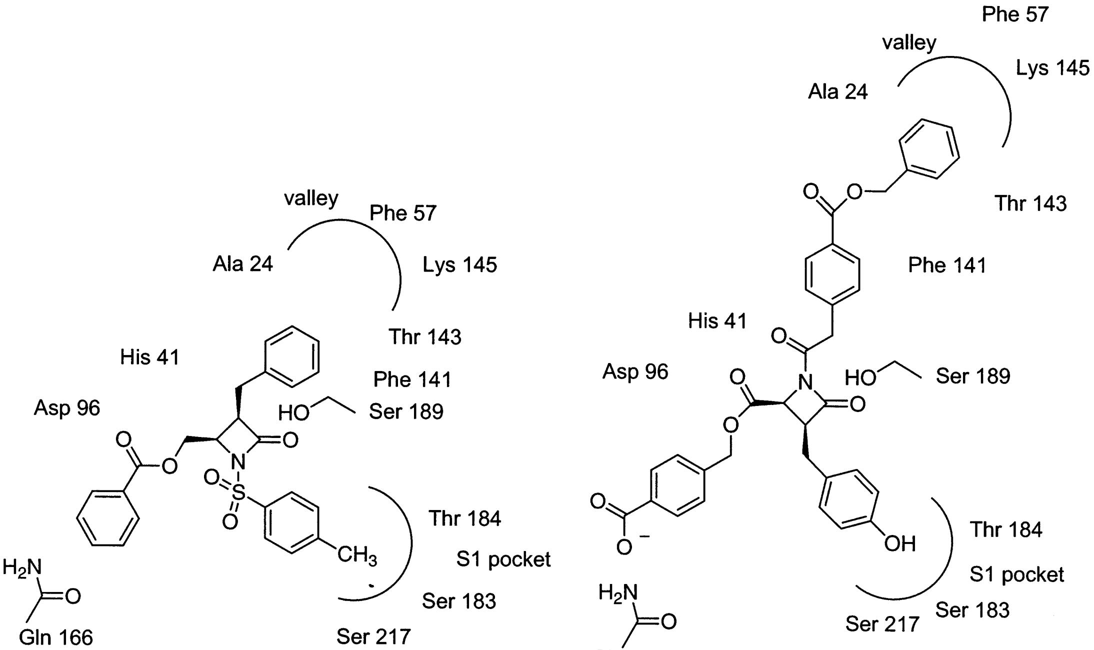


Figure 103. SPase inhibitor allyl (5*S*,6*S*)-6-[(*R*)-acetoxyethyl]-penem-3-carboxylate.

Signal peptidases (SPases) are essential for cell viability and function and release proteins that have been translocated into the inner membrane from the cell interior by cleaving off their signal peptides. Paetzel et al. have determined the crystal structure of a catalytically active soluble fragment of *E. coli* signal peptidase in complex with the β-lactam inhibitor, allyl (5*S*,6*S*)-6-[(*R*)-acetoxyethyl]-penem-3-carboxylate (Figure 103).[481] Bacterial SPases are not inhibited by standard protease inhibitors, but are inhibited by β-lactams with 5*S*-stereochemistry. The catalytic Ser 90 residue attacks the *si* face of the β-lactam, cleaving the lactam ring between C-7 and N-4 to form an acyl enzyme. The substrate-binding site and catalytic residues are contained in a large, exposed hydrophobic surface. The surface may be involved in the insertion of the enzyme into the membrane lipid bilayer. Because the carbonyl oxygen (O-10) of the inhibitor forms hydrogen bonds with Ser 278, Lys 145 is positioned to act as a general base in acylation and deacylation. The side-chain methyl group (C-16) is located in the S1 binding pocket and probably mimics the Ala side chain of the substrate.

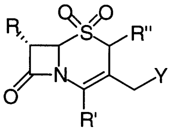
A homology model of PSA with 2-azetidinone inhibitors has been reported by Adlington et al.[482] In the normal esters (where the C-4 position contains the $-CO_2R$ group), the C-3 side chain of 2-azetidinone fills the S1 pocket, an interaction that has also been proposed in the inhibition of HLE and thrombin by 2-azetidinones. The N-1 side chain fits snugly into the valley region of the enzyme (**46**, Figure 104). An inverted mode of binding was proposed for a series of distinct reverse ester monocyclic 2-azetidinones (**47**, Figure 104). The reverse ester series have the



46 **47**

Figure 104. Molecular model of PSA containing a reverse ester inhibitor (**46**, $IC_{50} = 4.79\ \mu M$) and a normal ester (**47**, $IC_{50} = 3.5\ \mu M$).

Table 51. Inhibition of Elastase by Various Bicyclic *β*-Lactam Derivatives



				k_{obs}/[I] ($M^{-1}\ s^{-1}$)	
R	R′	R″	Y	HLE	ref
Cl	CH_2OCO-*p*-F-Ph	H	OAc	7600[a]	486
Cl	CO_2tBu	H	OAc	161000	463
OCOH	CO_2tBu	H	OAc	59000	463
OCH_3	CO_2tBu	H	OAc	19000	463
OCH_3	CO_2tBu	H	OCONHCH-(CH_2Ph)CO_2H	63900	526
OCH_3	CO_2tBu	H	OCOPh	32500	468
OCH_3	CO_2tBu	H	$=CH_2$	6095	468
OCH_3	CO_2CH_2Ph-3-CO_2H	H	OAc	62000	484
OCH_3	$CON(CH_2CH_2)_2O$	H	OAc	35200	526
OCH_3	CO_2tBu	α-OCH_3	OAc	125200	483
OCH_3	CO_2CH_2Ph-*p*-CO_2tBu	α-CH_3	OAc	118000	483

[a] Rate with PPE was 580 $M^{-1}\ s^{-1}$.

−O−CO−R group at the C-4 position instead of the $-CO_2R$ moiety. It was proposed that they bind by a rotation of 180° relative to the C-4 ester of the normal series.

Structure—Activity Relationships: Elastase. Doherty et al. discovered that neutral cephalosporins could be modified to become potent time-dependent inhibitors of HLE, PPE, and, to a lesser extent, α-chymotrypsin.[460] Kinetic data of various bicyclic *β*-lactam inhibitors of HLE can be found in Table 51. Most of the cephalosporins are inactive against trypsin and cathepsin G, but some have activity with plasmin and thrombin. The substituents at the 7′-α position (Figure 93) must be short and sterically small as it is proposed that they correspond to the side chain of L-amino acid substrates and fit in elastase's shallow S1 pocket.[460,463] Cephalosporins with 7′-*β* substituents are weak or inactive compared to the 7′-α analogues. Inhibitors with sulfones at S-1 have higher inhibitory potency compared to sulfoxides or sulfides, possibly by providing hydrogen-binding sites or by enhancing the chemical reactivity of the *β*-lactam ring.[463] Most substitutions at C-2 are tolerated and result in an increased inhibitory activity against HLE compared to the unsubstituted parent.[483] A crystal structure suggests that substituents at C-2 would not strongly interact with any particular structural feature of the enzyme.[466] Unlike other enzymes inhibited by *β*-lactams, HLE requires the carboxyl group of the *β*-lactam at C-4 to be esterified for effective inhibition.[460] In general, esters are more active than hydrophilic amides at the C-4 position. However, the C-4 substituent can be eliminated.[484]

Baici analyzed cephem sulfones, which contain thioesters or ketones at C-4 instead of esters and amides.[485] The C-4 ketones (−COPh, $t_{1/2} = 28$ h in human plasma in vitro) were the first cephem derivatives to possess sufficient chemical stability adequate for systemic administration. Balsamo et al. analyzed cephem derivatives, analogues of the previous C-4 esters, which contained an inverted ester at the C-4 position (−OCO instead of $-CO_2$, similar to the compounds in Figure 104).[486] These compounds were less potent against PPE but remained good inhibitors of HLE. Koteva et al. synthesized cephem derivatives that contain substituents, such as the tryptophanyl-9-fluorenylmethyl ester attached to the carbonyl at the C-4′ position, as novel HLE inhibitors.[487]

The 3′ substituent has a strong influence on the reactivity of the *β*-lactam ring and different substituents at the C-3′ position have been analyzed.[468] The

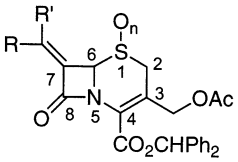


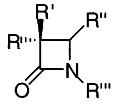
Figure 105. Structure of 7-alkylidenecephalosporins where the compound R = R′ = Br and $n = 0$ has an IC_{50} = 6.42 mM for PPE and 0.26 mM for HLE.

inhibitory potency of different β-lactams may vary depending upon the electron-withdrawing ability and leaving group ability of the 3′ side chain. The C-3 substituent is in an open area, so the enzyme can tolerate large groups, and acidic or basic moieties at this position (unlike C-4, see Figure 101). Time-dependent inhibitors exist, even if the C-3′ substituent is not a leaving group (i.e., CH_3, $k_{obs}/[I] = 1630$ M^{-1} s^{-1}), suggesting a different mechanism of action for these compounds.[468]

Buynak et al. have made some cephalosporin derivatives with variations at the C-7 position such as 7-alkylidenecephalosporins (Figure 105) and 7-vinylidenecephalosporins that are potent inhibitors of HLE.[488] In contrast to the earlier cephalosporin derivatives, the 7-haloalkylidene cephems have optimal inhibitory activity as sulfides rather than as sulfones. Modeling studies suggest that the alkylidene substituent readily docks in the S1 pocket. The proposed mechanism involves formation of the acyl enzyme followed by an isomerization of the alkylidene double bond to C-3 and C-4, generating a reactive allyl bromide, which is attacked by the histidine with the release of HBr. After isomerization, the compound could also undergo loss of the 3′ substituent and attack by the histidine at 3′ as proposed for cephalosporin derivatives.

The poor hydrolytic stability of bicyclic β-lactams (modified cephalosporins) precluded their use as orally active therapeutic agents. However, monocyclic β-lactams showed improved stability and specificity for elastases, showing only minor activity with other serine proteases.[489−493] Kinetic data of various monocyclic β-lactam inhibitors of HLE can be found in Table 52. Shah et al. discovered that monocyclic β-lactams, azetidinones, were the first orally active β-lactam inhibitors of HLE (Figure 106).[489,492] These compounds, containing a diethyl substituent at C-3, were evaluated for their activity in vitro against HLE and in vivo in a hamster lung hemorrhage model. Potent compounds in vitro and in vivo contained either a methyl ($k_{obs}/[I]$ = 4000 M^{-1} s^{-1}, ED_{50} = 10 mg/kg) or methoxy group ($k_{obs}/[I]$ = 5200 M^{-1} s^{-1}, 60% inhibition in lung hemorrhage model) in the para position of the phenyl ring of the N-1 benzyl urea. A compound similar to that in Figure 106, but with only one ethyl at C-3 and the *trans*-(3*R*, 4*R*)-configuration, was orally bioavailable in marmosets.[492]

Table 52. Inhibition of Elastase by Various Monocyclic β-Lactam Derivatives



R	R′	R″	R‴	HLE rate (M^{-1} s^{-1})	ref
OMe	H		CH_2OAc	19000[b]	498
Et	Et	OPh-4-CO_2H	$CONHCH_2Ph$	2200[a]	495
Me	Me	OPh-4-CO_2H	$CONHCH_2Ph$	280[b]	496
Et	*n*-Pr	(*S*)-OPh-4-CO_2H	$CONHCH_2Ph$	5200[b]	496
Et	Et	OPh-4-NO_2	$CONHCH_2Ph$	6800[b]	494
Et	Et	ON=C(CH_3)-Ph-4-F	$CONHCH_2Ph$	98300[a]	495
Et	Et	SO_2Ph	CH_2OCOPh	67.5[b]	498
Et	Et	OPh-4-CO_2H	$CONHCH_2$-2-naphth	21300[b]	489
Et	Et	(*S*)-4-hydroxyphenylacetic acid	CONHCH(*R*-propyl)-C_8H_5O	980000[a]	473
Et	Et	(*S*)-4-hydroxybenzoic acid	CONHCH(*R*-propyl)-$C_{10}H_{11}$	2930000[a]	473

[a] k_{inact}/K_i. [b] $k_{obs}/[I]$.

Modeling studies suggest that substituents at the C-4 position do not interact strongly with HLE. These C-4 substituents are released upon inhibition but have an important effect on the in vivo activity.[494] The best oral activity in the lung hemorrhage assay was obtained with C-4 aryl carboxylic acid ethers, such as the 4-hydroxybenzoate ($k_{obs}/[I]$ = 1500 M^{-1} s^{-1}, 68% inhibition in the lung hemorrhage model). 4-Hydroxybenzoic acid is found in the urine of healthy adults.[494] Compounds in which the leaving group at C-4 is a methyleneaminoxy moiety were effective at decreasing the lung hemorrhage induced by HLE.[495] Placing a second alkyl group at C-3 improved in vivo efficacy as it resulted in compounds that were stable in the blood and that had sufficient stability for oral absorption.[496] Of the various substituents tested at C-3, the 3,3-diethyl substitution was optimal ($k_{obs}/[I]$ = 1500 M^{-1} s^{-1}, $t_{1/2}$ = 7.7 h). Modeling with HLE suggests that the 3α-substituent occupies the S1 site. The *S*-configuration at C-4 allows the phenoxy moiety to lie in the S-2 to S-3 groove, and the benzyl of the urea moiety at N-1 lies along a hydrophilic surface in the S′ region. In the S′ region, substituents with the *R*-configuration at the methylene adjacent to the phenyl ring can project into a small hydrophobic pocket and enhance potency.[496]

An alkoxycarbonyl function (−CO−OR) on N-1 of monocyclic β-lactams[497] is proposed to increase the β-lactam carbonyl reactivity. The alkoxycarbonyl group would provide a potential leaving group (alkoxy moiety), which could generate an intermediate isocyanate function that could react with the histidine (Figure 107). However, the hydrolysis products contain the urethane functional group, so alkoxide expulsion must be too slow to generate the isocyanate function.

The *N*-acyloxymethyl- and *N*-aminocarbonyloxymethyl-2-azetidinones with different substituents at the β-lactam C-3 and C-4 positions were found to be

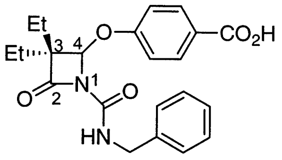


Figure 106. First orally active β-lactam inhibitor of HLE with $k_{obs}/[I]$ = 1500 M^{-1} s^{-1} and 56% inhibition lung hemorrhage model.

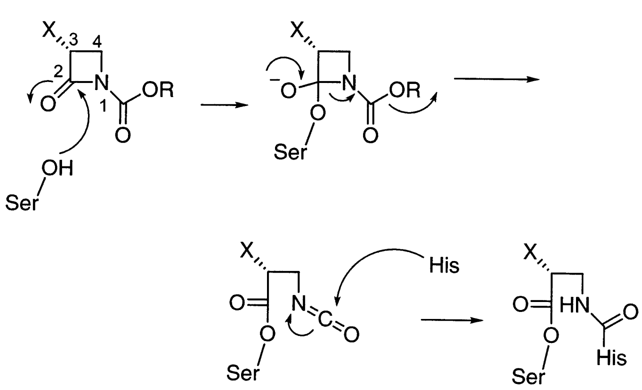
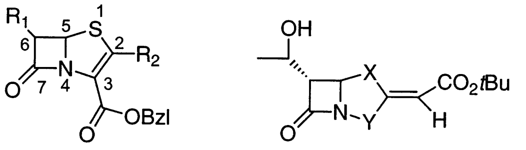


Figure 107. Mechanism of inhibition of serine proteases by monocyclic *β*-lactams, which contain an alkoxycarbonyl function (−CO−OR) on the N-1 position.



48 **49**

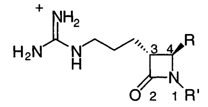
Figure 108. Structures of penem benzyl esters (**48**) and bicyclic *β*-lactam sulfenamides (**49**) as HLE inhibitors.

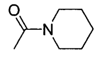
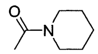
potent, selective, mechanism-based inhibitors for HLE.[498] Clemente et al. proposed that the carboxylate RCO_2^- is lost from the N-1 position, leaving an $N{=}CH_2$ that is susceptible to attack by the active site histidine. Penem benzyl esters substituted at the 6-position with small alkyl groups and at the 3-position with a variety of carbon and heteroatom groups (Figure 108, **48**) were tested as inhibitors of HLE[499] as were novel 3,2,0 bicyclic *β*-lactam sulfenamides and selected carbon isosteres (Figure 108, **49**).[500]

Thrombin. Han et al. have designed a series of 3-(3-guanidinopropyl)-azetidin-2-one derivatives as inhibitors of thrombin and trypsin, some of which have specificity against plasmin (see Table 53).[501] The presence of an azetidin-2-one nitrogen activating group increases potency. The C-4 substituent was essential for good inhibition, and polar substituents enhanced selectivity for thrombin compared to plasmin. A *trans* relationship between the C-3 and C-4 substituents was shown to be superior to the *cis*-conformation, which differs from inhibitors of the PSA. This study represents the first description of *β*-lactam derivatives as rationally designed inhibitors of thrombin.

Chymase. Aoyama et al. developed a series of 1-oxacephem-based (**50**, Figure 109) inhibitors of human chymase.[502−504] A docking model of **50** with human chymase shows the benzamide at the 7*β* position enclosed in the S1 pocket near Phe 191. The benzyl ester at the 4-position occupies the hydrophobic S1′ site, whereas its terminal carboxylic acid, deep in the S1′ pocket, electrostatically interacts with the side chain of Lys 40. On the basis of inhibition data for the 1-oxacephem **50** and the insights from some elastase inhibitors, Aoyama et al. then designed a novel class of 3-benzylazetidine-2-one derivatives (**51**, Figure 109).[471] The *β*-lactams **50** and **51** had high activity against human chymase and were selective over elastase, but had insufficient stability in human plasma. Structure−activity studies on compound **51** resulted in compound **52** (Figure 109), which has high potency against human chymase (IC_{50} = 3.1 nM), selectivity against α-chymotrypsin (IC_{50} = 15.3 nM), cathepsin G (IC_{50} = 35.4 nM), trypsin (IC_{50} = 26600 nM), thrombin (IC_{50} > 10000 nM), elastase (IC_{50} > 10000 nM), and plasmin (IC_{50} > 10000 nM), and also enhanced stability in human plasma ($t_{1/2}$ = 6 h). In a docking model of compound **52** with human chymase, the benzyl substituent at C-3 is nestled in the S1 pocket and capped with the side chain of Phe 191. The phenoxy substituent at C-4 occupies the S2 site, whereas its carboxyl group extends into solution. The urea moiety at N-1 is located in the prime site region of the enzyme with each benzene ring in a hydrophobic pocket (S1′ and S2′).

Table 53. Inhibition of Thrombin and Related Proteases by Azetidin-2-one Derivatives



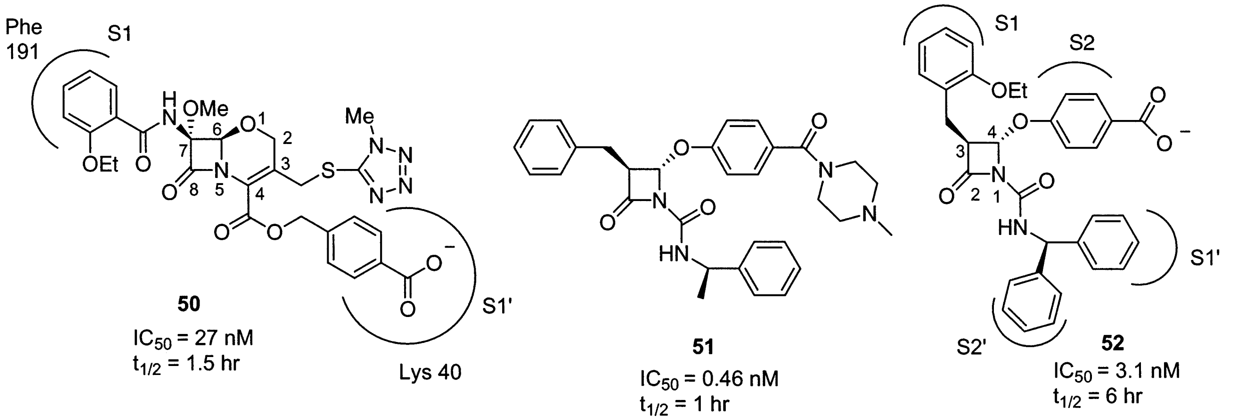
R	R′	k_2/K_i ($M^{-1}min^{-1}$)	IC_{50} (μM)		
		thrombin[d]	thrombin[e]	trypsin[f]	plasmin
CH_2CH_2Ph	H	1760	25[a]	> 100	72
CH_2CH_2Ph	$COCH_3$	1500000	0.21	0.47	0.0042
CO_2CH_3	$COCH_3$	19000000	0.002	0.04	3
	$COCH_3$	420000[b]	0.09	0.009	3.13
	$COCH_3$	43000[c]			
H	$COCH_3$	250000	0.1		

[a] K_i value. [b] *Trans* isomer. [c] *Cis* isomer. [d] Assay buffer was 145 mM NaCl, 5 mM KCl, 30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 1 mg/mL polyethylene glycol, pH 7.4. [e] Versus cleavage of substrate S-2231. [f] Assay buffer was 2 mM $CaCl_2$, 50 mM Tris/Cl, pH 8.0, with substrate Cbz-Val-Gly-Arg-pNA.

Aoyama et al. demonstrated that compounds containing the 1,3-diazetidine-2,4-diones (Figure 110) are effective inhibitors of cathepsin G, α-chymotrypsin, human chymase, and HNE with IC_{50} values of 0.39, 0.69, 0.85, and 58 nM, respectively.[504]

Rhinovirus 3C Protease. Because serine and cysteine proteases have similar hydrolytic mechanisms, Skiles and McNeil thought *β*-lactams might inhibit the viral 3C proteases. The spiro-*β*-lactam inhibitor (Figure 111) was a good inhibitor of both poliovirus and human rhinovirus 3C proteases (IC_{50} = 20 *μ*g/mL) and inhibited HLE (IC_{50} = 0.4 *μ*g/mL) and cathepsin G (IC_{50} = 4 *μ*g/mL).[505]

Human Cytomegalovirus Protease. Monocyclic *β*-lactams are mechanism-based inhibitors of hCMV protease.[472] The inhibitor **53** (Figure 112) is a good hCMV inhibitor (IC_{50} = 17 *μ*M, K_i = 5.7 *μ*M), is selective against elastase (IC_{50} > 100 *μ*M), chymotrypsin (IC_{50} > 100 *μ*M), and acetylcholine esterase (IC_{50} > 100 *μ*M), and possesses good aqueous stability



50
$IC_{50} = 27$ nM
$t_{1/2} = 1.5$ hr

51
$IC_{50} = 0.46$ nM
$t_{1/2} = 1$ hr

52
$IC_{50} = 3.1$ nM
$t_{1/2} = 6$ hr

Figure 109. Structure and schematic of β-lactam inhibitors interacting with chymase.

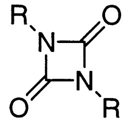


Figure 110. Structure of 1,3-diazetidine-2,4-diones.

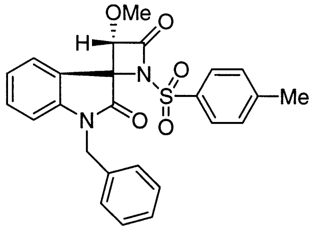
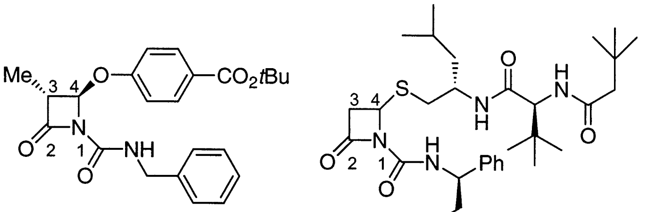


Figure 111. Spiro-β-lactam inhibitor of rhinovirus and poliovirus 3C proteases.

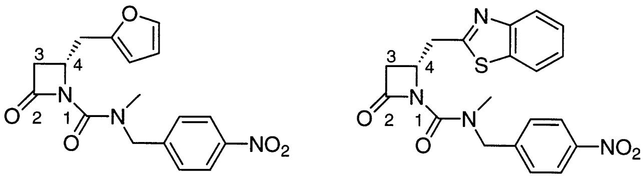


53 **54**

Figure 112. Structures of human cytomegalovirus protease inhibitors.

($t_{1/2} = 22.8$ h). The order of potency of inhibitors with various substituents at the C-3 position was Me ~ Et ~ *i*Pr < *n*Bu < CH_2Ph. As with thrombin, the *trans*-isomer was more potent.

During the study of substrate-based activated carbonyl inhibitors of hCMV protease, a peptidyl β-lactam was discovered that inhibited hCMV ($IC_{50} = 33$ μM) and was specific against PPE ($IC_{50} > 300$ μM), α-chymotrypsin ($IC_{50} > 75$ μM), cathepsin B ($IC_{50} > 300$ μM), and HLE ($IC_{50} > 300$ μM).[506] Deziel and Malenfant expanded on this work and developed both a peptidyl and nonpeptidic series (4-thioaryl derivatives) of more potent monocyclic β-lactams for hCMV.[507] Compound **54** (Figure 112, $IC_{50} = 0.07$ μM), one of the most active inhibitors of hCMV reported to date, was discovered and found to be selective against HLE, PPE, α-chymotrypsin, and cathepsin B ($IC_{50} > 75$ μM for all four enzymes). The peptidic series exhibited good in vitro potency and selectivity against a number of serine proteases, whereas some compounds in the nonpeptidic series showed activity in the viral replication assay.



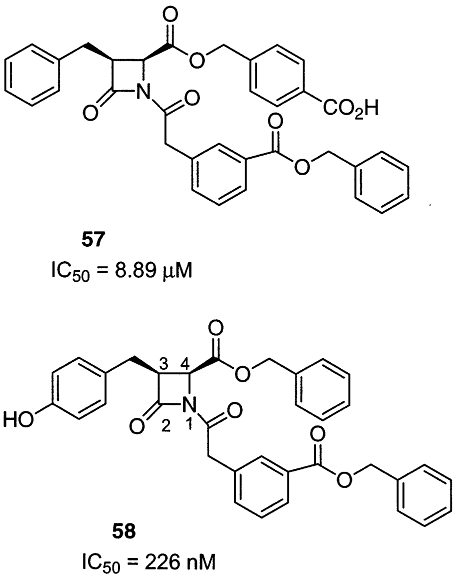
55
$IC_{50} = 3.1$ μM

56
$IC_{50} = 0.7$ μM

Figure 113. Structures of hCMV inhibitors that contain a heterocycle linked by a methylene group at the C-4 position.

It was proposed that the peptidic nature and large molecular weight of some inhibitors could result in poorer activity, so Yoakim et al. designed monobactam inhibitors that incorporate a benzyl side chain at the C-4 position and a urea moiety at N-1.[508] Compounds without substitutions at C-3 were more selective against PPE, HLE, and chymotrypsin. It was found that by introducing heterocycles linked by methylthio substituents at the C-4 position, one could improve both enzymatic and cell culture activity, but only to a certain level.[509] To make further improvements, Ogilvie et al. synthesized compounds that contain a heterocycle linked by a methylene group at the C-4 position.[510] Compounds with 2-furyl, 2-thiophenyl, 4-methyl-2-tetrazole, and 2-benzothiazole as the heterocycle were active in the plaque reduction assay, having potencies between 0.7 and 7.1 μM and EC_{50} values below 150 μM, and showed selectivity toward HLE, PPE, and cathepsin B (Figure 113, **55** and **56**).

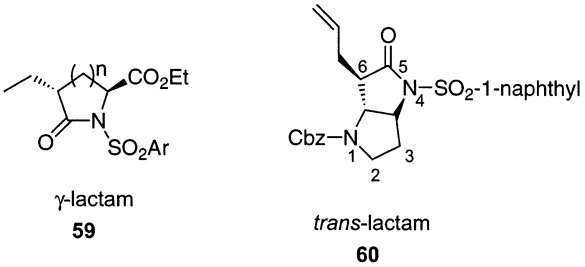
Prostate Specific Antigen. The design of monocyclic β-lactam inhibitors for PSA began with a lead compound (Figure 114, **57**, $IC_{50} = 8.98$ μM) that is a slow binding, time-dependent inhibitor which forms a stable acyl enzyme complex with catalytic Ser 189.[511] Adlington et al. then created a homology-derived molecular model of PSA to develop a variety of novel 2-azetidinone inhibitors for PSA[482] and analyzed the binding importance of all three side chains appended to the 2-azetidinone. PSA prefers an amino methylene group ($-CO_2CH_2Ph$-*p*-CH_2NH_2-TFA, $IC_{50} = 1.34$ μM) or a nonpolar functionality ($-CO_2CH_2Ph$, $IC_{50} = 1.43$ μM) at C-4. Amino or carboxyl functional groups cannot be chemically



57
IC_{50} = 8.89 μM

58
IC_{50} = 226 nM

Figure 114. Structures of *β*-lactam inhibitors of prostate-specific antigen.



59

60

Figure 115. Lactam inhibitors of serine proteases.

protected in potent inhibitors, which differs from HLE. Incorporation of a hydroxyl moiety into the C-3 side chain provided the greatest increase in PSA inhibition with a single modification ($IC_{50} = 348$ nM). An extended side chain at N-1 with two aryl rings is essential for PSA inhibition.[482] By using molecular modeling for the SAR studies, the most potent inhibitor of PSA known to date was discovered [Figure 114, **58** (3*S*,4*S*), $IC_{50} = 226$ nM].

Other Lactams and Lactones. *γ*-Lactams can also inhibit serine proteases even though the lactam carbonyl has reduced reactivity toward nucleophilic attack. Westwood et al. demonstrated that *γ*-lactams (for example, *γ*-lactam **59** in Figure 115) can acylate elastases (and potentially other serine proteases) in a manner similar to that of *β*-lactams.[512] With *β*-lactams, the acylation of PPE is effectively irreversible as the acyl enzyme intermediate is sufficiently stable to hydrolysis. With *γ*-lactams, the acylation step is a reversible process as the acyl enzyme readily undergoes deacylation. The reaction of a monocyclic *γ*-lactam with elastase occurs via reversible formation of a hydrolytically labile acyl enzyme complex.[512]

Wright et al. studied structures from crystals of PPE complexed with *γ*-lactams (similar to the structure of *γ*-lactam **59**, Figure 115) that were subjected to pH jumps (PDB codes 1E34, 1E35, 1E36, 1E37, and 1E38).[513] The results indicate that the conformation of the acyl enzyme species in the active site is dependent on pH. The side chain of His 57 can "flip" between two conformations, depending on pH. Other interactions are similar to Figure 93 such as Gln 192/Val 216 and carboxylate of inhibitor, ethyl group of inhibitor in S1, and the *γ*-lactam carbonyl and Ser 195/Gly 193. At pH 5, the *γ*-lactam−PPE complex is relatively stable, as the His 57 is rotated 90° from its normal position, thereby hindering deacylation. It is possible that the *p*-toluenesulfonyl group at the N-1 position displaces the hydrolytic water from the active site.

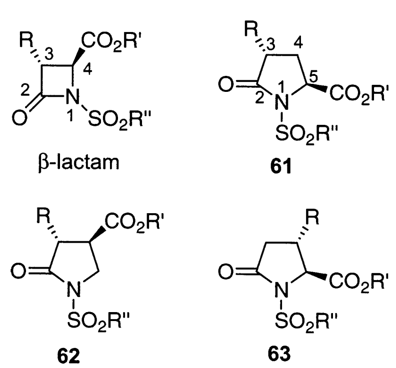
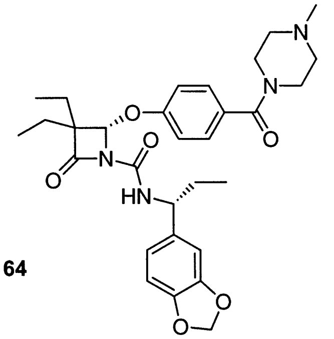


Figure 116. Structures of the three different types of *γ*-lactam inhibitors.

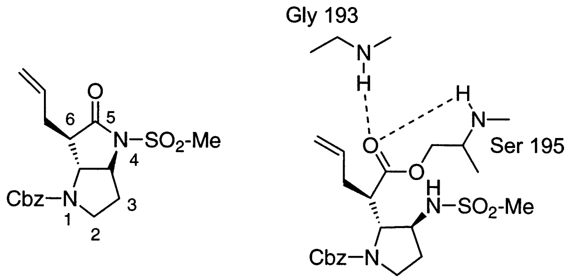


64

Figure 117. Structure of the *trans*-lactam DMP-777 (**64**), which is in clinical trials.

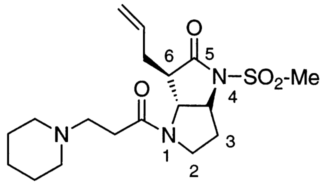
Wilmouth et al. studied the acyl enzyme complexes formed from different *γ*-lactams with PPE.[513,514] The lactams **61** (Figure 116) with a methylene inserted between C-3 and C-4 of the *β*-lactam template were weak inhibitors of PPE. The second series (**62**, Figure 116) with the methylene between C-4 and N-1 of the *β*-lactam template were good inhibitors and formed stable acyl enzyme complexes. This is probably a result of the carboxylic acid at C-4 forming hydrogen bonds with Gln 192 and Val 216. The third series (**63**, Figure 116) with the methylene between C-2 and C-3 of the *β*-lactam template were neither hydrolyzed nor inhibitors. The group at C-4 probably cannot productively orient the lactam carbonyl in the active site for nucleophilic attack by the serine.

Novel pyrrolidine *trans*-lactams and *trans*-lactones have been designed to be low molecular weight nonpeptidic inhibitors of HNE.[515] These are highly strained compounds, such as the *trans*-lactam **60** (Figure 115). These strained *γ*-lactams have similar inhibitory potency as L-694,458, a potent *β*-lactam elastase inhibitor. They have good stability in human plasma ($t_{1/2} = 2$ h) and blood ($t_{1/2} = 4.5$ h). An orally active pyrrolidine *trans*-lactam, DMP-777 (**64**, Figure 117), has entered phase II clinical trials for inflammatory-related disorders.[515]



65

Figure 118. Schematic of PPE complexed with the *trans*-lactam inhibitor **65**.



66

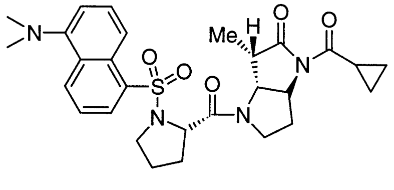
Figure 119. Structure of *trans*-lactam inhibitor **66**.

It was previously believed that the bicyclic *trans*-fused γ-lactones and γ-lactams, which inhibit HNE, were powerful acylating agents as a result of the inherent strain energy in the bicyclic structure, which was released upon ring opening. However, Sykes et al. showed that these bicyclic compounds are no more reactive than simple analogous γ-lactams and γ-lactones.[516] They created a reactivity index k_{OH} as a guide for determining the usefulness of an inhibitor as an acylating agent.

A schematic of the crystal structure of compound **65** ($IC_{50} = 0.047\ \mu M$ with HNE) with PPE is shown in Figure 118.[517] As with the other β-lactam crystal structures with PPE, one sees hydrogen bonds between the backbone amides of Gly 193 and Ser 195 (oxyanion hole) and the carbonyl oxygen of the inhibitor. The allyl group is in the S1 binding pocket.

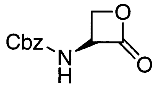
A *trans*-lactam inhibitor (**66**, Figure 119) with a methanesulfonyl group as the activating group at N-4, a propyl group at C-6, and a piperidine salt at N-1 has been developed as an intracellular neutrophil elastase inhibitor ($IC_{50} = 3.0\ \mu M$ in human whole blood, a measure of intracellular activity), which is stable in hamster liver microsomes.[517]

The γ-lactam design has also been applied to inhibitors for the hCMV.[518] Borthwick et al. designed compounds, similar to the *trans*-lactam (**60**, Figure 115), that bind covalently and reversibly in a time-dependent manner to the viral enzyme. The best compound ($IC_{50} = 13\ \mu M$ with hCMV; $IC_{50} > 100\ \mu M$ with thrombin; $IC_{50} = 9.08\ \mu M$ with elastase) has an α-methyl substituent at C-6 and $-COCH_2OCOMe$ at the N-4 position. Activity decreases when the α-methyl is eliminated (desmethyl) and changed to β-methyl, which is opposite to the preference of thrombin and elastase. Modifications of the parts of the *trans*-lactam that interact with S1′ and S3 of hCMV yielded inhibitors with low nanomolar potency against hCMV.[519] The preferred chirality for the α-Me substituent adjacent to the lactam carbonyl, which accesses the S1 site, is *S*.



67

Figure 120. Structure of hCMV protease inhibitor.



L-*N*-Cbz-serine β-lactone

Figure 121. Structure of the HAV 3C protease inhibitor L-*N*-Cbz-serine β-lactone.

One of the best inhibitors for the hCMV protease is **67** (Figure 120) ($K_i = 20$ nM, $IC_{50} = 0.34\ \mu M$), which has a dansyl-(*S*)-proline group on the α-methyl-5,5-*trans*-lactam template and is selective (elastase $IC_{50} > 10\ \mu M$; thrombin $IC_{50} > 200\ \mu M$; acetylcholine esterase $IC_{50} > 100\ \mu M$). Using modeling, Borthwick et al. showed that the (*S*)-proline occupies the S3 pocket, with the dansyl ring making hydrophobic interactions with the enzyme, and the cyclopropylcarbonyl moiety extends into the S′ site. In the tetrahedral transition state, both sulfonamide oxygens can hydrogen bond with Ser 135.[519]

β-Lactones are a new class of cysteine protease inhibitors for the hepatitis A virus (HAV) 3C protease developed by Lall et al.[520,521] These N-substituted serine and threonine β-lactones are potent irreversible inhibitors of the HAV 3C protease and the human rhinovirus 3C protease serotype 14. One such inhibitor, L-*N*-Cbz-serine β-lactone, shown in Figure 121, irreversibly inhibits the HAV 3C protease with $k_{inact}/K_i = 63\ M^{-1}\ s^{-1}$, whereas its enantiomer displays competitive reversible inhibition.[520,521] Nucleophilic attack of the cysteine thiol (Cys 172) at the β-position of the oxetanone ring results in inactivation of the enzyme. The β-lactone ring is important for binding as other related analogues with an *N*-Cbz side chain, such as the γ-lactones, four-membered ring β-lactam, 2-methylene oxetane, cyclobutanone, and 3-azetidinone, do not inhibit the HAV 3C protease.

Biological Studies. Some of the biological data have been discussed in the SAR section. The biological data obtained with β-lactam serine protease inhibitors have been reviewed by Hamilton-Miller.[522] The biological properties of cephalosporin-based inhibitors of PMN elastase have been reviewed by Davies et al.[523] Cephalosporin-based β-lactams that contain a C-7 methoxy group and a C-4 ester or amide are potent inhibitors of human polymorphonuclear leukocyte elastase (PMN elastase). The β-lactam L-659,286 (Figure 122, $k_{inact}/K_i = 12{,}800\ M^{-1}\ s^{-1}$), is a selective inhibitor of PMN elastase in vitro and in vivo (IC_{50} to inhibit elastinolysis by HLE is 1−2 μg/mL).[524] When administered locally, L-659,286 inhibits the lung damage in hamsters that results when they are treated intratracheally with human PMN elastase.[525] This elastase inhibitor is suitable

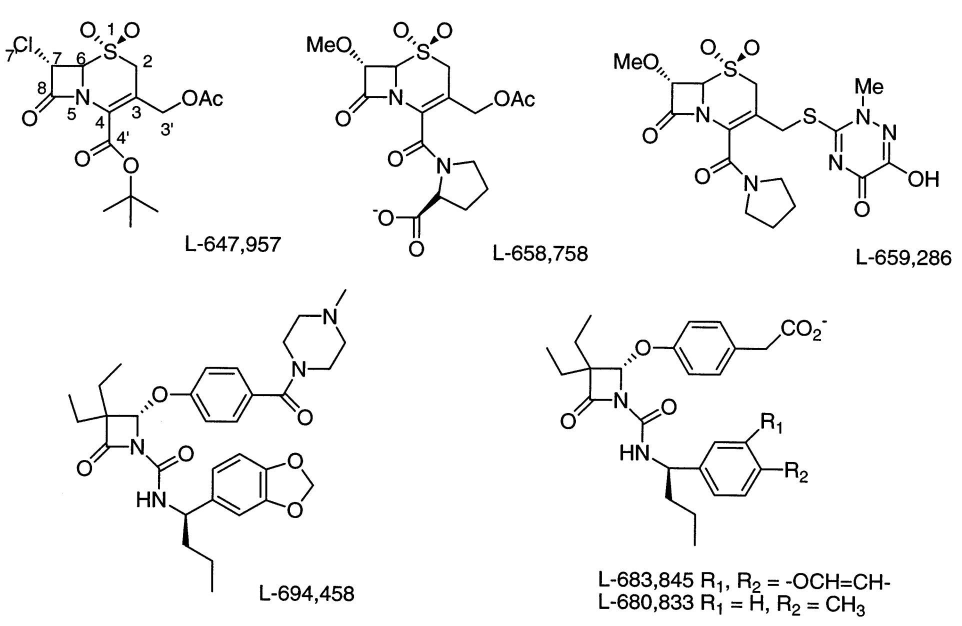


Figure 122. Structures of various *β*-lactam inhibitors.

for aerosol administration and protects terminal airways of the lung from PMN elastase damage and development of emphysema.

The selective, potent, time-dependent cephalosporin-based HLE inhibitor, L-658,758 (Figure 122, in vitro $k_{obs}/[I] = 3800\ M^{-1}\ s^{-1}$; in vivo $ED_{50} = 5\ \mu g$/animal) developed by Finke et al., could also be used clinically as a topical aerosol drug.[526] The C-4 esters were more potent in vitro, but the less active, more polar, and hydrolytically stable C-4 amides were more effective in vivo. These C-2 amides have potent, topical HLE inhibitory activity in the intratracheal hamster lung hemorrhage assays. These compounds (Figure 122, L-658,758, $t_{1/2} = 9$ h; L-659,286, $t_{1/2} = 6.5$ h) are functionally irreversible inhibitors of HLE as recovery of activity after inactivation and formation of enzyme−inhibitor complex are quite slow.[464] The *β*-lactam L-658,758 effectively inhibits elastinolysis by HNE, proteinase-3, and cystic fibrosis sputum elastase, but not metalloelastases, human and mouse macrophage metalloelastase, or *Pseudomonas* elastase.[527]

Potential HLE inhibitors, after being screened in vitro, are frequently assessed for oral activity in hamsters and mice that have HLE instilled in the lung. Pharmacokinetic studies are then used to determine bioavailability after oral administration to rats and monkeys. The monocyclic *β*-lactam inhibitors L-694,458 and L-683,845 (Figure 122) both have good bioavailability.[528,529] The metabolic pathway for L-694,458 involves an unusual cleavage of the piperazine ring. The potent, selective, time-dependent inhibitor of polymorphonuclear leukocyte elastase L-680,833 (Figure 122, $k_{inact}/K_i = 622000\ M^{-1}\ s^{-1}$) exhibits good oral bioavailability in rats and rhesus monkeys.[530] Another *β*-lactam, *N*-(2-chloromethylphenyl) 3,3-difluoroazetidin-2-one, is efficient in preventing the elastase-induced degradation of lung elastin fibers ($IC_{50} = 0.8\ \mu g$/mL) and has low cellular toxicity.[531] Some monocyclic *β*-lactams, which showed anti-inflammatory activity, also demonstrated very light analgesic activity.[532]

***β*-Lactone Inhibitors of the Proteasome.** Lactacystin **68** (Figure 123) is a *Streptomyces* metabolite that inhibits cell cycle progression and induces differentiation in a murine neuroblastoma cell line.[533] The cellular targets of lactacystin are the multiple *β*-subunits of the 20 S proteasome.[534] Lactacystin loses *N*-acetylcysteine in aqueous conditions to form an active intermediate, *clasto*-lactacystin *β*-lactone (**69**, Figure 123).[535] The *β*-lactone penetrates cell membranes and reacts with the proteasome. The *β*-lactone ring is attacked by the *β*-hydroxy group of the amino-terminal threonine residues of *β*-type proteasome subunits to form a stable ester between the inhibitor and enzyme (**70**, Figure 123).[535,536] When the active *β*-lactone intermediate is added to mammalian cells in culture, it can react with the sulfhydryl of glutathione to form a thioester adduct (lactathione, **71**), which is analogous to lactacystin. It is suggested that the formation of this lactathione concentrates the inhibitor inside the cells.[536]

Lactacystin is an irreversible, covalent inhibitor of the chymotrypsin-like ($k_{obs}/[I] = 675\ M^{-1}\ s^{-1}$) and trypsin-like ($k_{obs}/[I] = 29.9\ M^{-1}\ s^{-1}$) activities of the proteasome, but is a weak inhibitor of the peptidylglutamyl peptidase activity (PGPA, $k_{obs}/[I] = 3.71\ M^{-1}\ s^{-1}$) of the proteasome.[322,533−535] Despite initial reports that lactacystin was specific (not affecting serine or cysteine proteases such as chymotrypsin, trypsin, and papain),[533,534] there have been reports that it inhibits other cellular proteases such as cathepsin A and tripeptidyl peptidase II.[537] Unlike peptide aldehyde inhibitors, lactacystin does not inhibit lysosomal degradation of an endocytosed protein but does inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation.[534]

E. Heterocyclic Inhibitors

Heterocyclic compounds are acylating agents or mechanism-based inhibitors that inactivate mostly serine proteases. In many cases, the acyl enzymes

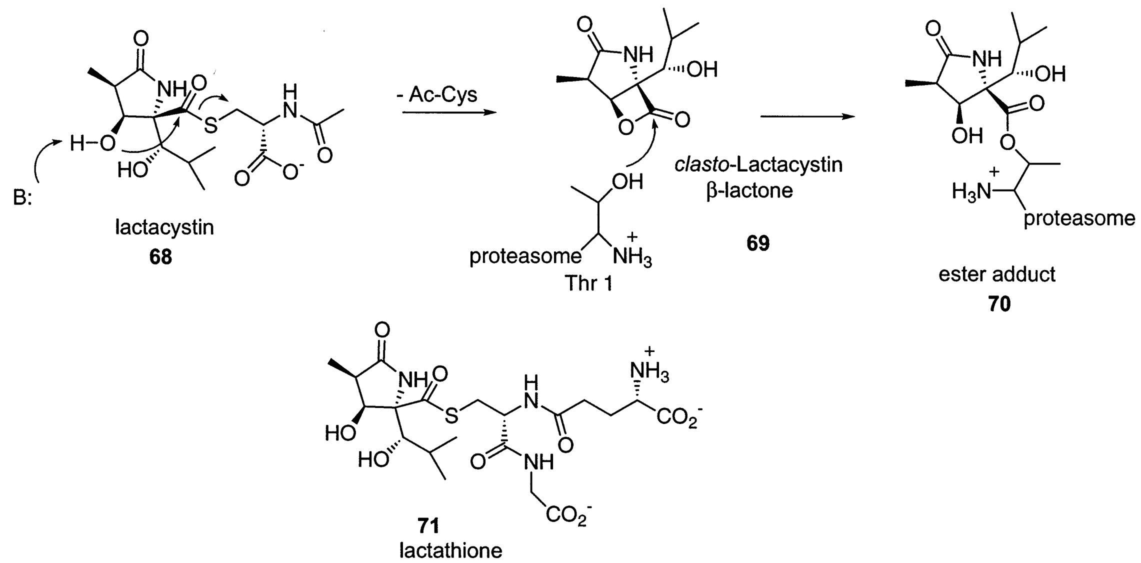
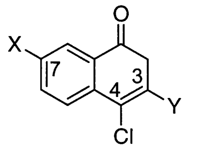


Figure 123. Inhibition of the proteasome by lactacystin's active intermediate *clasto*-lactacystin *β*-lactone.



DCI (3,4-dichloroisocoumarin)	X = H	Y = Cl
3-alkoxy-7-amino-4-chloroisocoumarin	X = NH_2	Y = OR
3-alkoxy-4-chloro-7-guanidinoisocoumarin	X = $NHC(=NH_2^+)NH_2$	Y = OR
NH_2-CiTPrOIC (ACITIC)	X = NH_2	Y = $O(CH_2)_3$-$SC(=NH_2^+)NH_2$

Figure 124. General structure of isocoumarins. The isothiureidoisocoumarins are abbreviated 7-amino-4-chloroisothiureidopropoxyisocoumarin (NH_2-CiTPrOIC).

formed are unstable and rapidly deacylate. However, geometric or electronic effects can often induce greater stability to specific acylated enzymes. Most of the time, heterocycles are considered to be irreversible inhibitors. Many classes of heterocyclic inhibitors have been developed, such as N-substituted saccharins,[538-540] benzoxazinones,[541-544] isocoumarins,[545] isotoic anhydrides,[546-548] oxazine-2,6-diones,[549] halomethylcoumarins,[550-553] nitroso amines,[554] haloenol lactones,[555-560] ynenol lactones,[561,562] isobenzofuranones,[563] chloropyrones,[564,565] *N*-hydroxysuccinimides,[566-569] and thiazolidinones.[570-573] The mechanisms of action for many of these inhibitors have not yet been demonstrated, and there are no representative X-ray crystal structures available in the Protein Data Bank for every class of heterocyclic inhibitors.

1. Isocoumarins

Isocoumarins are potent, irreversible heterocyclic inhibitors of serine proteases.[545,574,575] Many isocoumarins are mechanism-based inhibitors, and the inactivation occurs by opening of the isocoumarin ring by the active site serine residue to form an acyl enzyme derivative, which is quite often stable.[575,576] Isocoumarins can also be “suicide” inhibitors when a new reactive structure is unmasked during the acylation reaction, and this reactive species can further react with a nucleophile, such as solvent or His 57. The various mechanisms have been confirmed by X-ray structures of complexes of serine proteases with different isocoumarins.[577-581] This class of inhibitors reacts with all serine proteases tested so far, the proteasome, several esterases, and some cysteine proteases.

Isocoumarins are a class of heterocyclic structures that are rich in possible masked functional groups. Many derivatives have been synthesized and evaluated for inhibitory activity. Isocoumarin derivatives mostly contain substitutions at the 3- and 7-positions of the heterocyclic ring (Figure 124). The 3-alkoxy-4-chloro-7-substituted derivatives are more specific inhibitors of serine proteases, including HLE,[545,575,581,582] PPE,[545,575,583] proteinase 3,[584] cathepsin G,[545,575] chymotrypsin rat mast cell protease I and II,[545,575] tryptases,[585] granzymes,[586] trypsin-like enzymes that are involved in blood coagulation,[587-589] and the complement cascade.[590] The 3-alkoxy group provides selectivity for certain serine proteases,[545] whereas derivatives containing basic substituents such as a guanidine group are potent inhibitors of trypsin-like enzymes.[579,586,588,591,592] The nomenclature and general structure of isocoumarin inhibitors are shown in Figure 124.

The most widely used isocoumarin derivative, 3,4-dichloroisocoumarin (DCI), is a general serine protease inhibitor[575] that inhibits all of the serine proteases with the exception of C2a and Bb from the

Table 54. Inhibition of Enzymes by 3,4-Dichloroisocoumarin

enzyme	[I] (μM)	k_{obs}/[I] (M^{-1} s^{-1})	ref
serine proteases			
human leukocyte elastase	1.1	8920	575
porcine pancreatic elastase	8.1	2500	575
human proteinase 3	3.6	2600	584
		6340	593
bovine chymotrypsin A_α	13	570	575
human leukocyte cathepsin G	49	28	575
rat mast cell protease I	38	260	575
rat mast cell protease II	11	580	575
human skin chymase	92	27	575
S. griseus protease A	136	310	575
subtilisin		substrate	575
bovine trypsin	127	198	575
human thrombin	340	10	575
bovine thrombin	127	25	575
bovine factor Xa	422	0.2	575
bovine factor XIa	239	27	575
human factor VIIa	44	31	591
human factor XIIa	135	64	575
porcine pancreatic kallikrein	127	27	575
human factor D	109	192	575
human C2a	330	NI[a]	590
human Bb	330	NI	590
human C1s	44	170	590
human C1r	470	42	590
murine granzyme A	45	50	586
murine granzyme B	4.2	4200	586
human granzyme H			594
S. aureus protease V-8	18	2770	575
sheep lymph capillary CIP	460	39	599
protease La	82	30	600
dipeptidyl peptidase IV	50	18%	600
proteasome			
chymotrypsin-like activity	4	147	592
glutamyl-hydrolyzing activity	12	32.9	592
trypsin-like activity	40	11.6	592
cysteine proteases			
papain	422	turnover	575
leucine aminopeptidase	422	NI	575
calpain			601
caspase-1 (ICE)			595
caspase-3			595
caspase-6			595
caspase-7			595
caspase-9			595
other enzymes			
acetylcholinesterase	157	<0.6	600
influenza C virus esterase	6.3	410	596
glycogen phosphorylase *b*	100	3.4	597
β-lactamase	385	NI	575
phospholipase A2			598

[a] NI = no inhibition.

complement cascade. Subtilisin turns over DCI.[575,590] The rates of inhibition of serine proteases by DCI vary from $k_{obs}/[I] = 8900$ M^{-1} s^{-1} for HLE to 0.2−200 M^{-1} s^{-1} for most trypsin-like enzymes (Table 54). DCI is most effective against elastases, granzyme B, and proteinase 3 and inactivates factor X and thrombin more slowly.[586,593,594] It also inhibits the three enzymatic activities of the proteasome.[592] DCI is fairly specific for serine proteases, as it does not inhibit metalloproteases or aspartic proteases but does inhibit some cysteine proteases such as calpain and recombinant caspase-3, -6, -7, and -9.[595−601] Papain is turned over by DCI.[595−599]

Most serine proteases are inhibited with half-lives in the range of 0.3−5 min, given a DCI concentration ranging between 50 and 400 μM. The acyl enzyme formed as result of inactivation has varying stabilities, with some half-lives for reactivation of >24 h when excess inhibitor is not removed. The rate constants for deacylation are usually in the order of 20 s^{-1}, with a half-life between 6 and 10 h.[575] DCI is a useful alternate to the more toxic diisopropylfluorophosphate (DFP) and is generally much more reactive toward serine proteases than phenylmethanesulfonyl fluoride (PMSF).[575]

Dichloroisocoumarin has been very useful for characterization of the role of serine proteases in biological processes, such as lymphocyte granule-mediated cytolysis, serine proteases-dependent DNA fragmentation, apoptosis, and caspase activation.[595,602−604] DCI labeled with ^{14}C was used to understand the catalytic functions of the subunits of the proteasome.[605] Biotinylated isocoumarins are used for detection, localization, and isolation of serine proteases.[606] Isocoumarins have been reviewed several times.[59,60,600]

Stability. Isocoumarins have limited stability in biological buffers and plasma, and they are hydrolytically destroyed by physiological nucleophiles such as glutathione. Dichloroisocoumarin is reasonably stable in buffer and has half-lives of 18 and 48 min for its hydrolysis in pH 7.5 HEPES and phosphate buffer, respectively. In the presence of 0.2 mM glutathione in HEPES buffer, its half-life decreases to 1 min. In general, 3-alkoxy-7-amino-4-choroisocoumarins are more stable than DCI.[575,585] For example, 7-amino-4-chloro-3-methoxyisocoumarin has $t_{1/2}$ values for hydrolysis of 200 and 815 min in HEPES and phosphate buffer, respectively.[545] Isothioureidoalkoxy derivatives have a half-life between 18 and 168 min in pH 7.5 HEPES buffer.[589] Isocoumarin inhibitors that contain the guanidine group at the 7-position are unstable in plasma ($t_{1/2} = 4-8$ min).[588]

Mechanism. The general mechanism of inhibition of serine proteases involves opening of the isocoumarin ring by the active site serine residue to form an acyl enzyme. Isocoumarins have several potential advantages in the inhibition process due to the possible formation of either an acyl enzyme derivative or an alkylated enzyme derivative. The acyl enzyme can be reactivated by hydrolysis or treatment with a nucleophile, whereas the alkylated enzyme is stable and unreactive. The mechanism of inhibition is supported by X-ray crystal structures of serine proteases, such as elastase and trypsin, with a variety of isocoumarins.[577−581,607]

The inhibition mechanism of serine proteases by 3-alkoxyisocoumarin derivatives involves the reaction with the active site Ser 195 to form an ester functional group in the acyl enzyme intermediate that can further deacylate to form the active enzyme (Figure 125). Isocoumarin ring opening catalyzed by the enzyme occurs simultaneously with the enzyme inactivation reaction. The mechanism of inhibition of DCI (**72**), which has a 3-chloro substituent, involves the formation of an acid chloride (or ketene) functional group (**73**) in the active site of the enzyme.[575] This then forms an acyl enzyme, which is stabilized by a salt link between the carboxylate of

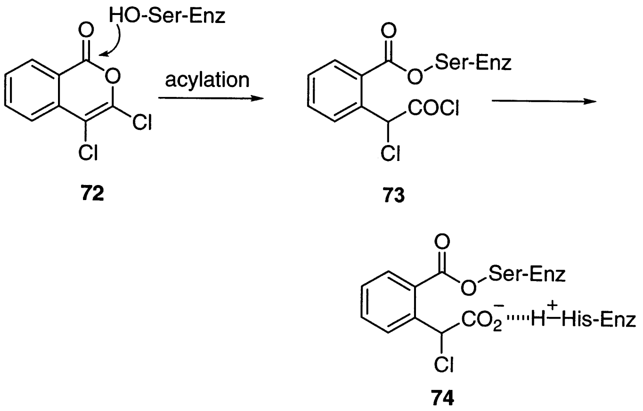


Figure 125. Mechanism of inactivation of serine proteases by 3,4-dichloroisocoumarin.

Table 55. PDB Codes for X-ray Crystal Structures of Serine Proteases and Isocoumarin (IC)-Based Inhibitors

enzyme	inhibitor	PDB code	ref
PPE[a]	7-amino-4-chloro-3-methoxyIC[b]	1JIM	577
trypsin	4-chloro-7-guanidino-3-ethoxyIC	NA	578
PPE	4-chloro-7-guanidino-3-ethoxyIC	8EST	579
PPE	7-amino-4-chloro-3-(2-bromoethoxy)IC	9EST	580
PPE	7-(Tos-Phe-amino)-4-chloro-3-methoxyIC	NA	581
factor D	3,4-dichloroIC	1DIC	607

[a] PPE = porcine pancreatic elastase. [b] IC = isocoumarin.

the inhibitor and the protonated His 57 (**74**). This intermediate was proposed as a result of proton release studies and has been detected by electrospray mass spectroscopy.[81] Treatment with hydroxylamine reactivates the initial acyl enzyme derivative.

The mechanism of inhibition by 7-substituted isocoumarins is shown in Figure 126. The acyl enzyme intermediate is formed similarly to the derivatives that lack substitution at the 7-position. However, the acyl enzyme (**75**) can eliminate the chlorine to generate a quinone-imine methide intermediate (**76**). This intermediate can further react with a nearby enzyme nucleophile such as His 57 to give an alkylated acyl enzyme derivative (**77**), which is doubly covalently bound to the enzyme, or with a solvent molecule to give a simple acyl enzyme derivative (**78**). All three binding modes have been observed in the crystal structures. The X-ray structures of PPE with 4-chloro-3-ethoxy-7-guanidinoisocoumarin and 7-(Tos-Phe-amino)-4-chloro-3-methoxyisocoumarin show that the chlorine is still present in the acyl enzyme.[579,581] In the complex formed by PPE with 7-amino-3-(2-bromoethoxy) isocoumarin the inhibitor forms an alkylated acyl enzyme, with covalent bonds to both Ser 195 and His 57, as was seen with β-lactams.[580] Only 30% of the enzyme could be reactivated from the enzyme–inhibitor complex, suggesting that at least 70% of the enzyme is double-linked. The acyl enzyme derivative in which the chlorine group has been replaced by an acetoxy group has been reported with 7-amino-4-chloro-3-methoxyisocoumarin–PPE complex (**78**, with Nu = $OCOCH_3$).[577] The alkylated acyl enzyme is stable and cannot be reactivated by hydroxylamine, whereas the acyl enzymes can be reactivated. In general, the inhibition of PPE, HLE, and chymotrypsin by 3-alkoxy-7-amino-4-chloroisocoumarins can be reactivated partially with NH_2OH. The majority of the inhibited enzyme is in the form of a stable alkylated acyl enzyme derivative, and only 15–43% of the enzyme activities are regained. The proposed mechanism of inhibition of serine proteases by isocoumarins (Figure 126) is consistent with the enzyme–inhibitor complexes described by the X-ray crystal structures.

Crystal Structures and Binding Modes. The binding mode of isocoumarins in the active site of serine proteases depends on the type of acyl enzyme that is formed. The available X-ray structures of serine proteases inhibited by isocoumarins are listed in Table 55.

In the complex of PPE with 7-amino-4-chloro-3-methoxyisocoumarin (PDB code 1JIM),[577] an acyl enzyme is formed at Ser 195 and an acetate from the solvent has displaced the chlorine and occupies the S1 subsite (Figure 127). The benzoyl carbonyl oxygen is partially in the oxyanion hole due to a hydrogen bond between the carboxyl group and the amide group of Gly 193. The His 57 residue is rotated into

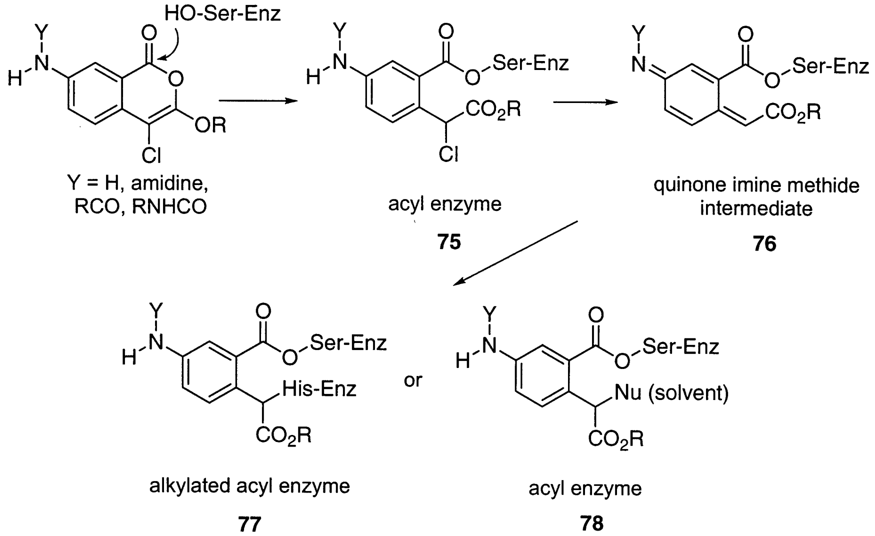


Figure 126. Mechanism of inhibition of serine proteases by 7-substituted isocoumarin derivatives.

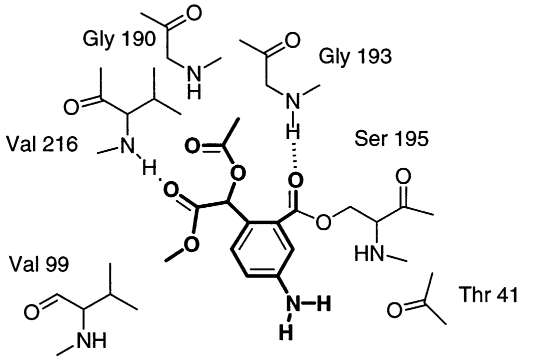


Figure 127. PPE−7-amino-4-chloro-3-methoxyisocoumarin complex (PDB code 1JIM).

the "out" position in common with benzoxazinones (see section of benzoxazinones)[608] and is not able to interact with the inhibitor. The carbonyl of the methoxy group makes a hydrogen bond with the backbone amide of Val 216. In this complex, the aromatic ring is displaced and the amino group is far away from Thr 41 and is not able to form a hydrogen bond.

In the complex of PPE with 7-guanidino-substituted isocoumarin (PDB code 8EST), the chlorine is present in the acyl enzyme.[579] The acyl carbonyl group is twisted out of the oxyanion hole and the 3-ethoxy group occupies the S1 pocket. The imidazole ring is rotated into the "out" position, as seen in the structure of the 7-aminoisocoumarin. There are hydrogen bonds between the guanidinium group, Thr 41 (both carbonyl group and hydroxyl), and water. The aromatic ring is bent further away from the serine residue in this complex than in the 7-aminoisocoumarin complexes, and the planes of the two rings form an angle of 38.4°. The difference in the acyl enzyme is most probably due to the buffer conditions used for crystallization at pH 5 (0.1 M acetate for the 7-amino structure, and 0.1 M phosphate buffer for the 7-guanidino structure). The structure of this guanidine isocoumarin complexed with trypsin was determined.[578] With trypsin, both the chloroacyl enzyme derivative at Ser 195 and the double-covalent adduct with His 57 exist in the crystal. The 7-guanidinium group occupies the S1 pocket, where it interacts with Asp 189 and a water molecule facilitating the hydrogen bonding. The two structures (PPE and trypsin) are different, with orientation of the isocoumarin ring twisted almost 180° between the two structures. Clearly, these examples show how the same inhibitor can have dissimilar binding mode interactions with two distinct enzymes, PPE and trypsin.

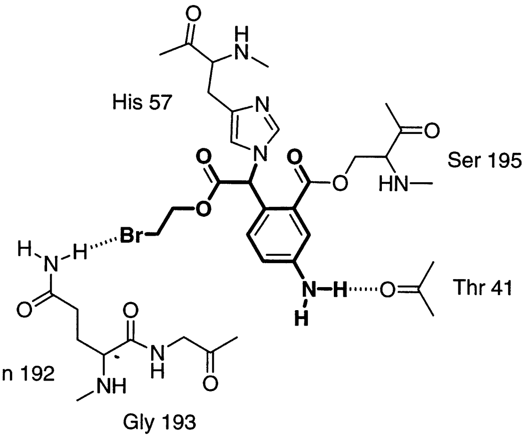


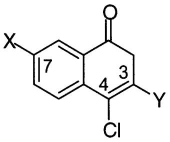
Figure 128. PPE−7-amino-3-(2-bromoethoxy)-4-chloroisocoumarin complex (PDB code 9EST).

An alkyl acyl enzyme that is double covalently bound to the enzyme is observed in the complex of PPE with 7-amino-3-bromoethoxyisocoumarin (PDB code 9EST).[580] The histidine residue is in the "in" position and forms a covalent bond with the inhibitor (Figure 128). The benzoyl carbonyl oxygen is far removed from the oxyanion hole. The bromoethoxy chain is displaced toward the S3 pocket, formed by Gln 192, Val 216, and Ser 217, and the bromine atom forms a weak hydrogen bond with the side chain of Gln 192. The 7-amino substituent points toward the S2′ subsite where it makes a hydrogen bond with the carbonyl group of Thr 41. Comparison of the double-covalent complex in elastase and trypsin reveals that the aromatic ring of the inhibitor is flipped by 180°, going from the S1 to the S2′ subsite. The chirality of the covalent adduct at the carbon attached to His 57 is *S*) in the elastase complex, whereas it is *R* in both doubly covalent complexes and in the chloroacyl enzyme derivative of trypsin. The retention of configuration *S* is consistent with the postulated mechanism involving the formation of the quinone imine methide intermediate.

Structure—Activity Relationships. To enhance selectivity of isocoumarins for specific subclasses of serine proteases, a large number of inhibitors have been prepared. There are two main classes of isocoumarin inhibitors: 3-alkoxy-4-chloroisocoumarin and 7-subsituted-3 alkoxy-4-chloroisocoumarins.

Generally, simple 3-alkoxy-4-chloroisocoumarins are potent acylating agents for elastases and chymotrypsin-like enzymes (Table 56). The most reactive 3-alkoxy-4-chloroisocoumarins have $k_{obs}/[I]$ values as high as 650000 M^{-1} s^{-1}. Derivatives with small alkoxy groups, such as methoxy or ethoxy at the 3-position, are potent inhibitors of HLE and moderate inhibitors of PPE. Introduction of more bulky alkoxy groups such as benzyloxy or phenylethoxy results in good inhibitors of chymotrypsin and moderate inhibitors of cathepsin G (Table 56).[575,582] Similarly, rat mast cell protease I, a chymotrypsin-like enzyme, reacts most rapidly with 4-chloro-3-(4-fluorobenzyl)-isocoumarin ($k_{obs}/[I] = 46000$ M^{-1} s^{-1}).[545] Trypsin and other coagulation serine proteases are slowly inhibited by 4-chloro-3-ethoxyisocoumarin and 7-amino-4-chloro-3-ethoxyisocoumarin with $k_{obs}/[I]$ values of 0.6−370 M^{-1} s^{-1}. Substitution with a 3-aminopropoxy group results in a 54-fold increase in reactivity with trypsin and slow inhibition of thrombin.[588] Introduction of another basic group, such as isothiureidoalkoxy, increases the potency toward trypsin-like proteases 2−200-fold compared to the 3-(aminopropoxy)-substituted isocoumarins. This group resembles an arginine residue and can make additional hydrogen bonds in the S1 pocket. The isothioureidoalkoxy derivatives are good inhibitors of tryptases with $k_{obs}/[I]$ values up to 10^5 M^{-1} s^{-1}. However, most of these isocoumarins do not form stable enzyme−

Table 56. Inhibition Rates of Elastase and Chymotrypsin-like Enzymes by Isocoumarin



	inhibitor			
enzyme	X	Y	$k_{obs}/[I]$ ($M^{-1}s^{-1}$)	ref
HLE[a]	H	OEt	43000	575
	Tos-Phe-NH	OMe	190000	575
	$PhCH_2CH_2CONH$	OMe	130000	581
	NH_2	OCH_2CH_2Br	200000	582
	PhNHCONH	OCH_2CH_2Br	1200000	582
	$C_{10}H_7$-NHCONH	OCH_2CH_2Br	390000	582
	(*S*)-Ph($C_{10}H_7$)CHNHCONH	OCH_2CH_2Br	310000	582
	$PhCH_2NHCSNH$	OPr	143000	582
PPE[b]	$PhCH_2NHCSNH$	OEt	12000	579
	Tos-Phe-NH	OMe	6500	545
	(*o*-CH_3OOC)PhCONH	OMe	7100	581
	PhNHCONH	OMe	7500	581
	*t*Bu-NHCONH	OCH_2CH_2Br	6600	582
	$PhCH_2CONH$	OCH_2CH_2Br	4950	582
	Boc-Trp-NH	OMe	5800	582
cathepsin G	H	OCH_2Ph	1140	545
	NO_2	OPh	>2600	545
	NO_2	$OCH_2CHBrCH_3$	1660	582
chymotrypsin	H	OC_6H_4-4F	32000	545
	H	OPh	16000	545
	NO_2	OCH_2CH_2Br	98000	582
	$PhCH_2CONH$	OCH_2CH_2Br	82000	582
	$PhCH_2CONH$	$OCH_2CHBrCH_3$	140000	582
	NO_2	$OCH_2CHBrCH_3$	200000	582
	p(CH_3)C_6H_4CO-L-Phe-NH	OCH_2CH_2Br	49000	582
RMCP I[c]	H	OPh	29560	545
	H	OC_6H_4-4F	46000	545
RMCP II	NO_2	OC_6H_4-4F	>10500	545
	H	OPh	9600	545
	NO_2	OEt	>7000	545
HSC[d]	H	OPh	19500	545
	H	OC_6H_4-4F	11500	545
proteinase 3	NH_2	OCH_2CH_2Br	4700	584
	CH_3CONH	OPr	2200	584

[a] HLE = human leukocyte elastase. [b] PPE = porcine pancreatic elastase. [c] RMCP = rat mast cell protease. [d] HSC = human skin chymase.

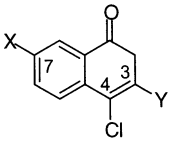
inhibitor complexes, and the inhibited enzymes regained 70−100% activity upon standing for periods >15 min.[585]

The reactivity of 3-alkoxy-substituted isocoumarins usually agrees with the substrate preferences at the P1 site of the various serine proteases.[609,610] The structure of the 3-alkoxy group determines the reactivity toward a particular serine protease. Results of the SAR studies imply that the 3-alkoxy group is interacting with the S1 subsite of the enzyme, which is proven by X-ray structures.

A variety of substituents at the 7-position of the heterocyclic ring have been introduced and yielded a new class of isocoumarins, 7-substituted-3-alkoxy-4-chloroisocoumarins. Addition of a 7-amino group to the 3-alkoxy-4-chloroisocoumarin ring resulted in 3-alkoxy-7-amino-4-chloroisocoumarins, which are potent inactivators of elastase and chymase (Table 56).[545] The effect of the 7-amino substituent in 7-amino-3-methoxy-4-chloroisocoumarins was evaluated in a series of acyl, urea, and carbamate derivatives.[581] Most of the *N*-acyl derivatives were 3−7 times more reactive toward HLE than the nonacylated isocoumarins. Hydrophobicity of the substituent is directly related to inhibition of HLE. The N-Tos-Phe derivative is the best inactivator of HLE from this series ($k_{obs}/[I] = 200000\ M^{-1}\ s^{-1}$), whereas the *N*-phenylurea derivative was the best inhibitor of PPE ($k_{obs}/[I] = 7500\ M^{-1}\ s^{-1}$). To obtain potency and selectivity for HLE, another SAR study evaluated inhibitors in which both the 7-amino and 3-alkoxy groups were varied. In the 2-bromoethoxy series, the compound with the 7-NHCONHPh substituent was very selective and potent for HLE with a $k_{obs}/[I]$ value of $1.2 \times 10^6\ M^{-1}\ s^{-1}$. Compounds with a 7-phenylacetylamino substituent were good inhibitors of chymotrypsin, whereas the tosyl-L-Phe derivative is more selective toward chymotrypsin. Elaboration on the 7-position substituent combined with a better fitting 3-alkoxy substituent led to highly potent and selective inhibitors for HLE. However, potency and selectivity was reduced when the side chain of the 7-substituent in the 3-(3-bromoethoxy) series was more rigid and extended.

Other SAR studies were aimed at finding better isocoumarin inhibitors for trypsin and blood coagula-

Table 57. Inhibition Rates of Trypsin-like Enzymes by Substituted Isocoumarins



enzyme	inhibitor		$k_{obs}/[I]$ (M^{-1} s^{-1})	ref
	X	Y		
bovine trypsin	NH_2	$OCH_2CH_2CH_2iT$[a]	410000	588
	guanidino	OMe	310000	588
	$PhCH_2CONH$	$OCH_2CH_2CH_2iT$	165000	585
	Boc-Phe-NH	$OCH_2CH_2CH_2iT$	300000	585
rat skin tryptase	H	$OCH_2CH_2CH_2iT$	650000	585
	Boc-D-Phe-NH	OCH_2CH_2iT	360000	585
human lung tryptase	H	$OCH_2CH_2CH_2iT$	260000	585
	$PhCH_2NHCONH$	OCH_2CH_2iT	280000	585
	NH_2	OCH_2CH_2iT	28800	585
human skin tryptase	D-Phe-NH	OCH_2CH_2iT	62000	585
	H	$OCH_2CH_2CH_2iT$	83000	585
bovine thrombin	guanidino	OMe	290000	588
	guanidino	OEt	55000	588
	NH_2	$OCH_2CH_2CH_2iT$	630	588
	(*S*)-$Ph(CH_3)CHNHCONH$	OCH_2CH_2iT	41000	589
	(*R*)-$Ph(CH_3)CHNHCONH$	OCH_2CH_2iT	29500	589
human thrombin	PhNHCONH	OCH_2CH_2iT	22400	589
	$PhCH_2NHCONH$	OCH_2CH_2iT	11680	589
	(*S*)-$Ph(CH_3)CHNHCONH$	OCH_2CH_2iT	21000	589
	(*R*)-$Ph(CH_3)CHNHCONH$	OCH_2CH_2iT	12000	589
PP[b] kallikrein	guanidino	OEt	200000	588
	NH_2	$OCH_2CH_2CH_2iT$	110000	588
HP[c] kallikrein	guanidino	OEt	500000	588
	guanidino	OCH_2CH_2Ph	270000	588
human factor VIIa	Phe-NH	OCH_2CH_2iT	4010	589
	PhNHCONH	OCH_2CH_2iT	3140	589
	PhNHCONH	$OCH_2CH_2CH_2iT$	720	589
human factor IXa	$PhCH_2CONH$	OCH_2CH_2iT	2620	589
	$PhCH_2CONH$	$OCH_2CH_2CH_2iT$	610	589
	(*S*)-$Ph(CH_3)CHNHCONH$	OCH_2CH_2iT	3920	589
	guanidino	OEt	2850	589
bovine factor Xa	guanidino	OMe	3100	588
	guanidino	OEt	27000	588
	guanidino	OCH_2CH_2Ph	96000	588
human factor Xa	guanidino	OEt	11000	589
	PhNHCONH	OCH_2CH_2iT	4740	589
human factor XIa	guanidino	OEt	60000	588
	H	$OCH_2CH_2CH_2iT$	47000	588
	PhNHCONH	OCH_2CH_2iT	104000	589
	$PhCH_2NHCONH$	OCH_2CH_2iT	105000	589
human factor XIIa	H	OCH_2CH_2iT	39000	588
	H	$OCH_2CH_2CH_2iT$	27000	588
	Phe-NH	OCH_2CH_2iT	107000	589
	D-Phe-NH	OCH_2CH_2iT	82000	589
human factor D	guanidino	OMe	250	590
	guanidino	OMe	190	590
C1s	H	$OCH_2CH_2CH_2iT$	130000	590
	NH_2	$OCH_2CH_2CH_2iT$	23000	590
C1r	H	$OCH_2CH_2CH_2iT$	6600	590
	NH_2	$OCH_2CH_2CH_2iT$	1300	590
murine granzyme A	guanidino	OEt	26000	586
	H	$OCH_2CH_2CH_2iT$	18000	586
human granzyme A	H	$OCH_2CH_2CH_2iT$	18000	586
	NO_2	OEt	7200	586

[a] iT = isothiureido. [b] PP = porcine pancreatic. [c] HP = human plasma.

tion serine proteases. Isocoumarin derivatives that contain basic functional groups, such as 3-aminoalkoxy, 7-guanidino, and 3-isothiureidoalkoxy, are good inhibitors of trypsin, porcine pancreatic kallikrein, and several blood coagulation enzymes.[588] The most potent inhibitors for the coagulation enzymes were 3-alkoxy-4-chloro-7-guanidinoisocoumarins, with $k_{obs}/[I]$ values in the range of 10^3−10^5 M^{-1} s^{-1} (Table 57). These inhibitors are 2−5 orders of magnitude more reactive than 7-amino-4-chloro-3-ethoxyisocoumarin, which does not contain a basic substituent. The various 3-alkoxy groups, methoxy, ethoxy, and 2-phenylethoxy, often had a significant effect on reactivity. For example, the values of inhibition for bovine factor Xa increased 31-fold upon going from methoxy to ethoxy to 2-phenylethoxy,

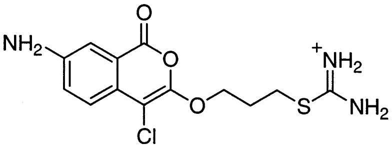


Figure 129. Structure of the isocoumarin derivative 7-amino-4-chloro-(3-isothiureidoalkoxy)isocoumarin (ACITIC).

whereas with bovine thrombin the values increased almost 10-fold going in the opposite direction. The inhibitor 4-chloro-7-guanidino-3-methoxyisocoumarin is 94-fold more selective toward bovine thrombin than factor Xa. In contrast, the nature of the 3-alkoxy group has little effect on bovine trypsin, human plasma kallikrein, or human factor Xa–XIIa. The complexes formed from the inactivation of trypsin or thrombin by the guanidino derivatives are very stable.

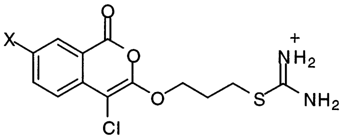
Various substituents at the 7- and 3-positions of 7-amino-4-chloro-3-(3-isothiureidoalkoxy)isocoumarin were evaluated for their efficiency to inhibit coagulation enzymes.[589] Substituted isocoumarins with an isothiureidoethoxy group at the 3-position and a large hydrophobic group at the 7-position are potent inhibitors for thrombin, factor VIIa, factor IXa, factor Xa, factor XIa, and factor XIIa. A few derivatives inhibit human thrombin quite potently and have $k_{obs}/[I]$ values up to 4×10^4 M^{-1} s^{-1}. The anticoagulant activity of the most potent inhibitors of thrombin was tested in vitro.

Biological Studies. In addition to kinetic studies with the purified coagulation serine proteases, the more potent isocoumarin inhibitors were tested for their effect on the prothrombin time (PT) and activated partial thromboplastin time (APTT) in human, rabbit, or pig plasma. The PT assay measures the coagulant activity in the extrinsic pathway, where factor VIIa, factor Xa, and thrombin are involved. The APTT measures the coagulant activity in the intrinsic pathway, where factor XIIa, factor XIa, factor IXa, factor VIIIa, factor Xa, and thrombin are involved. The isocoumarin derivatives ACITIC (Figure 129) and 4-chloro-3-ethoxy-7-guanidinoisocoumarin showed anticoagulant activity in vitro.[588,591]

ACITIC and 4-chloro-3-ethoxy-7-guanidinoisocoumarin (20–30 μM) were effective anticoagulants using the PT and APTT assays (2–5-fold increase in clotting time). Interestingly, ACITIC, which has the longest lifetime in plasma, is more effective in the APTT assay than the 4-chloro-3-ethoxy-7-guanidinoisocoumarin. This may be a reflection of the poor inhibitory potency of ACITIC toward thrombin, whereas the 7-guanidinoisocoumarin is a good inhibitor for most of the coagulation serine proteases. Alternatively, ACITIC appears to be a slightly better inhibitor of factor VIIa than the 4-chloro-3-ethoxy-7-guanidinoisocoumarin ($IC_{50} = 0.70$ μM) when assayed in the presence of thromboplastin.[589] The general serine protease inhibitor 3,4-dichloroisocoumarin (330 μM) prolonged the prothrombin time to 2 min, but had no effect after incubation for 3 min in plasma.[589]

Derivatives of ACITIC also showed potent anticoagulant activity in pig plasma, and the results are shown in Table 58. The PT is prolonged by 1.4–3-fold at an inhibitor concentration of 32 μM. All four isocoumarins were very effective as anticoagulants in the APTT assay (>6.5-fold increase in clotting time). These four compounds are 7-carbamoylamino derivatives of ACITIC, are as stable as ACITIC, and are more stable than 4-chloro-3-ethoxy-7-guanidinoisocoumarin in plasma.

Table 58. Effect of 7-Substituted 4-Chloro-3-(2-isothiureidoethoxy)isocoumarins on Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) in Pig Plasma



inhibitor X	[I] (μM)	PT (s)	APTT (s)
control	0	18.6	17.7
PhNHCONH	16	28.5	>120
	32	58.3	>120
$PhCH_2NHCONH$	32	31.1	>120
(*S*)-$Ph(CH_3)CHNHCONH$	32	30.0	>120
(*R*)-$Ph(CH_3)CHNHCONH$	32	26.2	>120

ACITIC has excellent anticoagulant activity in human and rabbit plasma as measured using the PT and APTT assays. This drug prolongs PT ~1–2-fold and APTT 3.6–4.5-fold in human or rabbit plasma at concentrations of 20–30 μM. ACITIC was also evaluated in 14 New Zealand white rabbits (2.5–3.5 kg) using continuous infusion (1 mL/min) of the drug dissolved in saline into the jugular vein.[611] At a dose of 0.4 mg/mL/min, ACITIC prolonged APTT 2-fold over the control value, whereas at the highest dose of 1.2 mg/mL/min, APTT was increased 3–4-fold. The half-life of ACITIC in rabbits is effectively 1–2 min, and the APTT returned to normal a few minutes after cessation of the infusion. By 15 min postinfusion, there was essentially no demonstrable effect of ACITIC on the APTT.

ACITIC was also evaluated in a rabbit thrombosis model, which involved introduction of a constriction into the jugular vein, which is then followed by the formation of a clot and cessation of bleeding. ACITIC at 1 mg/mL completely prevented thrombus formation in 10 veins, whereas complete thrombosis and no bleeding were observed in 9 of the 10 control veins.[611] The inhibitor, 4-chloro-3-ethoxy-7-guanidinoisocoumarin, had activity in human and rabbit plasma when tested with the PT and APTT assays. However, no effect was observed when it was administered to rabbits due to its instability in the rabbit plasma.

Rabbit models have been used extensively in evaluating other potential anticoagulants, and the above results show that ACITIC is a potent, short-acting in vivo anticoagulant in rabbits. The compound exhibits a linear dose–response curve, and its effects are highly reproducible. These results are also consistent in in vitro experiments that demonstrate the anticoagulant action of substituted isocoumarins.[588]

More recently, ACITIC was shown to inactivate chondrocyte-mediated cartilage proteoglycan breakdown occurring in response to proinflammatory cytokines.[612] This compound is also a rapid inactivator of urokinase-type plasminogen activator. The functional half-life of the inactivator in culture medium was 95 min, and its concentration in cartilage was 2.5-fold higher than in the surrounding medium.

Isocoumarin derivatives and DCI have been used as molecular probes to characterize the biological role of lymphocyte granule proteases in cytotoxic lymphocytes (CTL) cells.[586,594,603,613] DCI inhibits all five granzymes and inactivates cytolysis.[602,603] The lysis is restored when the inhibited granzymes are reactivated by hydroxylamine, which indicates that serine proteases are essential for hydrolysis. Biotinylated isocoumarins were successfully used in the detection of granzymes in CTLs.[606,614,615]

DCI and other isocoumarin derivatives have been proven to be effective in identifying DCI-resistant components of the proteasome,[616,617] whereas ^{14}C-labeled DCI was used to understand the catalytic functions of subunits of the proteasome.[605] The radiolabeled incorporation is found in the β-type subunits, LMP2 and X, which is consistent with the rapid inactivation of the chymotrypsin-like activity. DCI is used frequently to understand the function of the proteasome and the biological processes in which it is involved.[618−622]

In a recent study, DCI inhibited caspase activation under cell-free conditions in vitro and purified recombinant human caspase-3, -6, -7, and -9.[595] DCI inhibited caspase activity by 50% at concentrations ranging from 500 μM to 1 mM. Caspase-9 inactivation by DCI was proven in experiments with anti-neoepitope antiserum. DCI has helped to elucidate a variety of biological processes, such as serine-protease-dependent DNA fragmentation,[623] programmed cell death in various blood lines,[604,624−626] processing and release of tumor necrosis alfa,[627] activation of the heat shock factor 1 (HSF1),[628] oxidative damage of apolipoprotein A-I,[629] post-traumatic brain injury, neuronal apoptosis,[630] and many more.

2. Benzoxazinones

Substituted benzoxazin-4-ones have been well characterized as heterocyclic acylating agents of serine proteases. Teshima et al. first reported this class of alternate substrate inhibitors in 1982 and showed that they were potent irreversible inhibitors of HLE, PPE, cathepsin G, and chymotrypsin.[541] Since then the inhibitory effects of benzoxazinones have been studied extensively on HLE[543,544,631] and extended to cathepsin G,[632] C1r serine protease of the complement system,[633,634] human chymase,[635] thrombin,[547] factor X,[636] and even viral proteases, such as herpes simplex virus type 1 (HSV-1) protease[637] and hCMV protease.[638] Benzoxazinones inhibit serine proteases according to a mechanism that involves an acyl enzyme intermediate.[542,639] Potency is achieved by rapid acylation rates combined with a slow deacylation rate.[543,640] This class of heterocyclic non-peptide inhibitors has a core structure of two fused aromatic rings, which offers the possibility of broad chemical variation and optimization for a particular target protease (Figure 130).

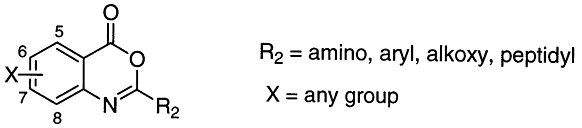


Figure 130. General structure of benzoxazin-4-ones.

Mechanism. The mechanism of inhibition of serine proteases by benzoxazinones involves the nucleophilic reaction with Ser 195 to form a stable acyl enzyme intermediate.[542,639] Attack of the active site serine occurs with the C-4 carbonyl of the benzoxazinone as shown in Figure 131. As a result, the heterocyclic ring opens with the formation of an ortho-substituted benzoyl acyl enzyme intermediate. The formation of the acyl intermediate is demonstrated by spectral changes accompanying benzoxazinone ring opening and methanol trapping of the acyl enzyme.[542]

The initial kinetic studies, the isolation of the acyl enzyme intermediate, and deacylation products were later supported by X-ray crystallography of the enzyme−benzoxazinone complexes that contained the acyl enzyme structure.[608] Benzoxazinones have been identified as mechanism-based inhibitors of the HSV-1 protease, and the formation of an acyl enzyme adduct was demonstrated by MALDI-TOF mass spectroscopy.[637]

The intermediates are stable due to the electron-donating properties of the ortho substituents. The acyl enzyme is a substituted derivative and should be considered to be a vinylogous carbamate rather than an ester, the normal acyl enzyme structure formed in peptide bond hydrolysis. The anthranyl derivative of chymotrypsin was first made as a fluorescent spectral probe for the active site of chymotrypsin. At that time it was discovered to be stable toward deacylation. The acyl enzyme derivative can undergo hydrolysis to form an *N*-acylanthranilic acid regenerating the active enzyme. In the case of 2-amino-substituted benzoxazinones, the ortho substituent is a urea. This urea substituent can attack the benzoyl carbonyl in the acyl enzyme derivative and accelerate deacylation.

Crystal Structures and Binding Modes. The structures of the acyl enzyme formed upon reaction of the benzoxazinones with serine proteases have been investigated by X-ray crystallography.[608] Coordinates for the complex of PPE inhibited by two valine-derived benzoxazinone inhibitors are available (PDB code 1INC). The slow binding inhibitor, (1-(5-chloro-4-oxo-4*H*-3,1-benzoxazin-2-yl)-2-methylpropyl) carbamic acid (1,1-dimethylethyl) ester, forms an acyl enzyme intermediate through a covalent bond with Ser 195 (Figure 132). It is important to note that the ester carbonyl oxygen atom of the inhibitor is not in the oxyanion hole formed by the backbone NH's of Ser 195, His 57, and Asp 102. Instead, the valyl carbonyl group, which is formed as a result of benzoxazinone ring opening, is located in the oxyanion hole. Surprisingly, the imidazole ring of His 57 is displaced and rotated out into solution, probably due to a large conformational change after the formation of the acyl enzyme. A water molecule bridges the Asp 102 carboxylate with the benzoyl carbonyl group of the heterocycle. An electrostatic

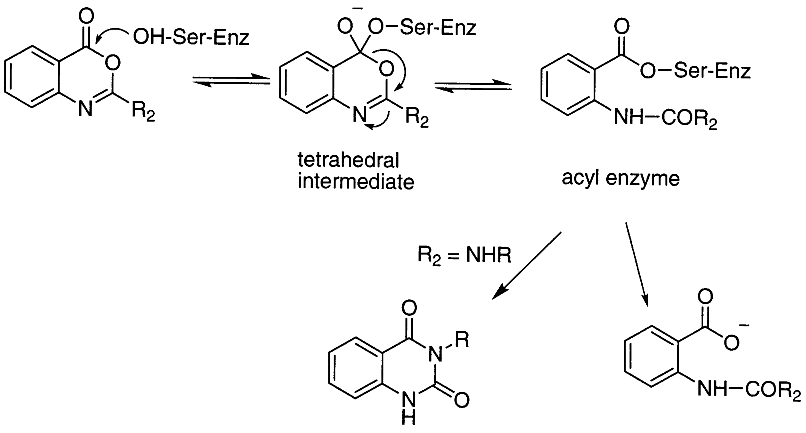


Figure 131. Mechanism of inactivation of serine proteases by benzoxazinones.

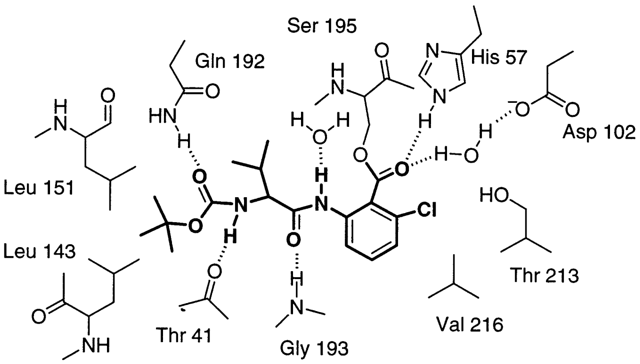


Figure 132. Schematic drawing of a PPE–benzoxazinone complex.

interaction (3.91 Å) between the imidazole ring and the carboxylate group of the heterocycle also occurs (Figure 132). The displaced conformation of the imidazole ring of His 57 is observed in all PPE complexes with heterocyclic inhibitors.[608,641]

The enzyme–inhibitor complex is stabilized by a variety of hydrogen-bonding and hydrophobic interactions. The inhibitor hydrogen bonds with the backbone amide group of Gly 193, the backbone carbonyl of Thr 41, the side-chain NH of Gln 192, and two water molecules. Hydrophobic interactions are mainly between the S1 pocket formed by Thr 213 and Val 216 and the 5-chlorobenzoyl. The *tert*-butyl group lies in a hydrophobic pocket formed by the S2′ and S1′ subsites consisting of Leu 143, Leu 151, and Gln 192 (Figure 132).

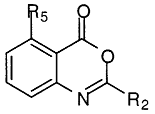
The slow deacylation step of the acyl enzyme complex[639] is explained by the geometry of the inhibitor in the active site. In order for hydrolysis to take place, a nucleophilic water molecule must attack the ester carbonyl. However, on the basis of X-ray structure there is no solvent accessible to the ester carbonyl group. The *re* face of the ester is blocked by the valine amide group of the inhibitor, whereas the *si* face is oriented toward the S1 and is protected by the 5-chloro substituent.

Structure—Activity Relationships. A large variety of benzoxazinones have been designed and synthesized due to the multiple variations that can be achieved on this heterocyclic ring structure. Acylation rates, deacylation rates, and the hydrolytic stability of the bezoxazin-4-ones can be manipulated by changing the nature of the substituents on both rings.[543,640] In search of selective and potent HLE benzoxazinones inhibitors, Krantz and co-workers have synthesized hundreds of compounds by multiple substitutions at the R_2 position (3-position substituent of the benzoxazinones) and positions around the phenyl ring (5-, 6-, 7-, 8-substituents). These studies mapped the design criteria for producing potent and selective benzoxazinone inhibitors for serine proteases. Ideal inhibitors have substituents that enhance the acylation rate and limit the deacylation step.

One of the first SAR studies examined the hydrolysis rates, k_{on}, k_{off}, and K_i, and the relationship of the substitutions on the heterocyclic ring to the potency of benzoxazinones inhibitors for human leukocyte elastase.[543] Alkaline hydrolysis rates and k_{on} varied by 6 orders of magnitude, and k_{off} rates span 5 orders of magnitude. Regardless of their common benzoxazinone core structure, size, and hydrophobicity, K_i values for the compounds span 8 orders of magnitude.[543] Substitutions at 7- and 8-positions with bulky groups do not affect hydrophobicity, whereas substitution at the 6-position is unfavorable. The alkyl groups at the 5-position and electron-withdrawing groups at R_2 inhibit enzyme-catalyzed deacylation. The deacylation rates can be decreased by substituting R_2 with small alkyl groups linked via heteroatoms. The most potent inhibitor for human leukocyte elastase, 2-ethoxy-5-ethylbenzoxazinone, has a K_i = 0.042 nM (Table 59).[543] Electron-withdrawing substituents on both rings increase the acylation and deacylation rates, whereas electron-donating groups increase stability and bulky substituents often slow the deacylation rates.

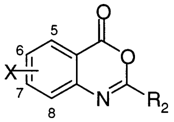
After targeting elastase, the search continued for benzoxazinones inhibitors of C1r (a serine protease of the complement system), human chymase, and cathepsin G. Some examples of benzoxazinones inhibitors and their IC_{50} values are listed in Table 60. The most studied class of benzoxazinone inhibitors contain 2-aryl or 2-amino substitutions at the R_2 position. Derivatives such as 3,1-benzothiazin-4-ones show little activity against chymotrypsin,[642] whereas the isosteric benzene–thiophene replacement in 4*H*-thieno[2,3-*d*][1,3]oxazin-4-ones enhances the stability of the HLE inhibitors (Figure 133).[643,644]

Table 59. Inhibition of Serine Proteases by Benzoxazinones



enzyme	inhibitor R_2	 R_5	K_{on} (10^3 $M^{-1}s^{-1}$)	K_{off} (10^{-4} s^{-1})	K_i (nM)	ref
chymotrypsin	CH_3	H			3700	541
	$CF_2CF_2CF_3$	H			10	541
	$NHCH_2Ph$	H	810	270	33	635
	$N(CH_3)Ph$	H	82	9	11	635
	$N(CH_3)CH_2Ph$	H	810	270	23	635
	OCH_2Ph	H	1650	2	0.12	643
	OCH_2Ph	CH_3	1340	2.6	0.19	632
chymase	$NHCH_2Ph$	H	623	31	5	635
	$N(CH_3)Ph$	H	79	11	14	635
	$N(CH_3)CH_2Ph$	H	19	2.2	11	635
cathepsin G	$CF_2CF_2CF_3$	H			250	541
	OCH_2Ph	H	7	2.4	30	632
	OCH_2Ph	CH_3	70	11.6	20	632
	$NHCH_2Ph$	H	6.5	0.9	10	632
	$N(CH_3)CH_2Ph$	H	1.3	0.2	10	632
HLE	$CF_2CF_2CF_3$	H			9	541
	CH(*i*Pr)NH-Boc	H	4.1	19	280	639
	CH(*i*Pr)NH-Boc	CH_3	770	11	1.3	639
	NH-*i*Pr	CH_2CH_3	70	0.66	0.94	631
	OCH_2CH_3	CH_2CH_3	340	0.14	0.04	543
	OCH_2CH_3	CH_2Br	1120	0.76	0.07	543
	OCH_2CH_3	*i*-Pr	780	0.55	0.07	543
	Pro-Leu-Gly-NH_2	H	11	2.30	21	543
thrombin	$CF_2CF_2CF_3$	H	1		90	640
	OCH_2CH_3	H	53	13	1000	547
proteinase 3	OCH_2Ph	H	6.3	2.3	37.2	643
	OCH_2Ph	CH_3	111	2.0	1.8	643

Table 60. IC$_{50}$ Values for the Inhibition of Serine Proteases by Benzoxazinones



enzyme	inhibitor X	 R_2	IC$_{50}$ μM)	ref
C1r	H	Ph-2-I	1.37	633
	7-CF_3	NH(2-I-C_6H_4)	0.40	634
	H	$N(CH_3)$(2-I-C_6H_4)	1.40	634
C1s	H	Ph-2-I	1.25	633
trypsin	H	Ph-2-I	15.3	633
	H	$N(CH_3)$(2-I-C_6H_4)	4.50	634
plasmin	H	Ph-2-I	6.68	633
kallikrein	H	Ph-2-I	9.89	633
thrombin	H	Ph-2-I	0.51	633
	6-CH_3	C_6H_4-2,6-F_2	8.00	636
	5-NO_2	3-pyridyl-2-Cl	9.70	636
FXa	5-NO_2	Ph-2-OCH_3	5.80	636
FX activation[a]	5-Cl, 8-Cl	C_6H_4-2-F	1.20	636
	7-NO_2	C_6H_4-2,6-F_2	0.82	636
	7-CF_3	C_6H_4-2,6-F_2	1.40	636
HSV-1 protease[b]	5-Cl, 8-Cl	OCH_2CH_3	1.50	637
	H	O-C_4H_6-4-OCH_3	2.50	637
	7-(Cbz-Ala-NH)	NH-*i*-Pr	5.00	637
hCMV protease[c]	5-CH_3	NH-(1*R*)-$CH(CH_3)$-C_6H_5	0.92	638
	5-CH_3, 6-(pyrrolidine-CH_2CONH)	NH-(1*R*)-$CH(CH_3)$-C_6H_5	2.40	638

[a] TF/FVIIa-catalyzed activation of FX. [b] Herpes simplex virus type 1 protease. [c] Human cytomegalovirus protease.

In search of a selective inhibitor for complement serine protease C1r, Gilmore et al. found that 2-(2-iodophenyl)-4*H*-3,1-benzoxazin-4-one acts as a good inhibitor with an IC$_{50}$ = 1.37 μM.[633,634] However, it is not selective toward other serine proteases such as thrombin (Table 60).[633] Other 2-aryl-substituted 4*H*-3,1-benzoxazin-4-ones have been reported as specific inhibitors of the tissue factor (TF)/factor VIIa-

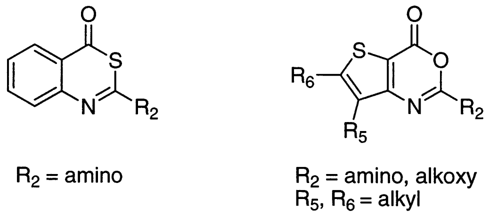


Figure 133. Sulfur-containing benzoxazinone derivatives.

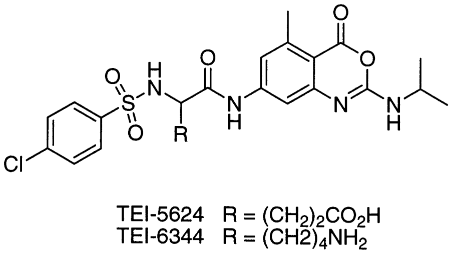


Figure 134. Chemical structures of TEI-5624 and TEI-6344, two potent inhibitors of HLE.

induced pathway of coagulation.[636] The goal was to identify small molecules as potential drugs for oral antithrombotic treatment without side effects. Inhibitors that contain two or more electronegative substituents, such as F, Cl, or NO_2, in the 2-aryl moiety had IC_{50} values in the range of 0.17−40 μM and showed a 50-fold selectivity toward factor X versus thrombin.[636]

Another major class of substituted benzoxazinones are 2-amino-4*H*-3,1-benzoxazin-4-ones, which have been exploited to obtain selective and stable inhibitors for serine proteases. HLE inhibition by this class of compounds has been studied extensively.[543,631,645] The 2-amino-5-methyl-substituted benzoxazinones are potent HLE inhibitors and are stable in an aqueous buffer.[543] Derivatives such as 7-substituted 5-methyl-2-isopropylamino-4*H*-3,1-benzoxazin-4-ones inhibit elastase selectively and only weakly inhibit cathepsin G, chymotrypsin, plasmin, trypsin, and thrombin (300−45000-fold less).[645] The potent arylsulfonylamino acid derivatives, TEI-5624 and TEI-6344, as shown in Figure 134, inhibit HLE with K_i values of 6.91 and 16.3 nM and IC_{50} values of 2.9 and 30 nM, respectively.[645]

In another study, selectivity for chymase over HLE was achieved by incorporation of aromatic structures into the 2-substituent of the benzoxazinone ring.[635] However, none of the chymase inhibitors exhibited specificity for chymase over chymotrypsin, which is difficult to achieve due to the structural similarities of these two serine proteases. Benzoxazinones have also been used effectively to selectively inhibit the hCMV protease[638] and the HSV-1 protease type 1.[637] Inhibition data are shown in Table 60.

Biological Studies. The stability of a number of benzoxazinones has been analyzed in aqueous solution, by measuring their half-lives at different pH values. For example, the arylsulfonyl amino acid derivatives (TEI-5642 and TEI-6344) have long survival times in human plasma at pH ranging from 6.0 to 7.0.[544] The half-life in human plasma is >3 h and is at least 1 h in hamster plasma. In vitro, TEI-5624 and TEI-6344 also prevent degradation of insoluble elastin by stimulated polymorphonuclear leukocytes.[544] Another inhibitor, 2-(6′-methoxy-3′4′-dihydro-1′-naphthyl)-4*H*-3,1-benzoxazin-4-one, inhibits elastase in vitro and is active in vitro in a hemorrhagic assay.[646]

Newly synthesized benzoxazinone derivatives against viral proteases, such as HSV-1 and hCMV, were tested for ability to inhibit the target enzyme. Several benzoxazinone inhibitors of HSV-1 were analyzed for their stability in aqueous solution by monitoring the half-lives at pH 7.5.[637] The inhibitors, which had IC_{50} < 50 μM, had a wide range of half-lives (1−171 h). There was no correlation found between the potency and reactivity of the inhibitors. A series of six substituted 2-amino analogues that inhibit hCMV protease were tested for their ability to prevent viral replication in rapid cell culture assay. When tested for in vitro human plasma activity, the inhibitors displayed half-lives ranging from 0.5 to 2 h.[638]

The therapeutic efficacy of benzoxazinone inhibitors with human elastase has been evaluated in in vivo studies using hamsters in lung injury models.[544] Intratracheal administration of TEI-5624 or TEI-6344 to hamsters inhibits HLE-induced lung injury, preventing both the acute and chronic phases of lung injury. Intratracheal administration was preferable to intravenous administration. The compound TEI-6344 was delivered to the lung at a 300-fold higher concentration than TEI-5624 and was eliminated from the lung with a half-life of 4 h. These biological studies suggest that TEI-6344 and TEI-5624 could be useful therapeutic agents for the treatment of HLE-mediated diseases such as emphysema, respiratory distress syndrome, cystic fibrosis, and septic shock.

3. Saccharins

Saccharins are irreversible acylating agents of serine proteases, first discovered in the early 1980s by Zimmerman et al. as inhibitors for HLE and cathepsin G.[538,539] Potent and selective saccharin inhibitors have been designed and tested in vitro and in vivo for their activity against HLE[540] and human mast cell tryptase.[647] Saccharins are also known as 1,2-benzisothiazol-3-one 1,1-dioxide. Initially there were two types of saccharins, *N*-acylsaccharins and *N*-arylsaccharins. The most potent *N*-acylsaccharins inhibit HLE, pancreatic elastase, cathepsin G, and trypsin, with IC_{50} values in the order of 10^{-6} M. The most widely known *N*-acylsaccharin is *N*-furoylsaccharin (Figure 135), which has been evaluated for its ability to prevent the development of emphysematous lesions in animal models.[648,649] *N*-Arylsaccharins are potent acylating agents of HLE and chymotrypsin but

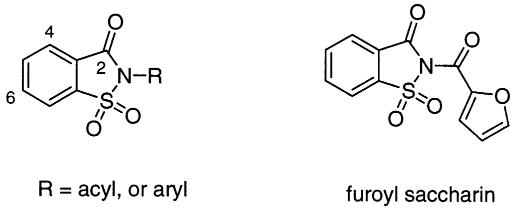


Figure 135. General structures of saccharin and furoyl saccharin.

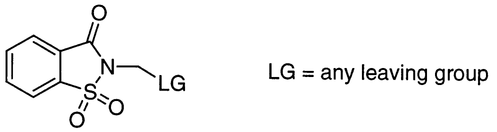


Figure 136. General structure of *N*-methylene-substituted saccharins.

do not inhibit pancreatic elastase, cathepsin G, or trypsin. This class of saccharin derivatives behaves as alternate substrate inhibitors.[539] The general structure of these heterocyclic compounds is shown in Figure 135.

The next generation of saccharin inhibitors were suicide inhibitors and contained the general structure shown in Figure 136, where LG is a good leaving group.[650] Saccharins of this class can easily be derivatized by changing the leaving group and introducing substitutions on the phenyl ring of the heterocyclic core structure. Thus, researchers at Sterling Winthrop-Kodak were able to develop orally bioavailable benzisothiazolone inhibitors of HLE.[540] Saccharins as serine protease inhibitors have been previously reviewed by Martyn et al.[651]

Mechanism. The probable mechanism of action of *N*-acyl- and *N*-arylsaccharins involves attack on the amide bond of the heterocyclic ring by the active site serine to form an acyl enzyme intermediate. The active site serine protease could potentially attack either the carbonyl group at C-2 or the sulfonamide sulfur of the saccharin to give either a stable acyl derivative or a sulfonyl derivative. Labeling studies using ^{35}S-labeled *N*-furoylsaccharin as inhibitor for HLE and PPE demonstrated that the label becomes covalently and stoichiometrically bound to serine proteases upon acylation.[538] This experiment eliminated the possibility that the furoyl carbonyl was attacked. The enzyme acyl intermediates are stable for days at neutral pH, because deacylation is slow.

The *N*-acyl group of saccharins must be electronegative in order to activate the C−N bond for acylation. Simple *N*-aryl group saccharins were not able to inhibit HLE even when they contained electron-withdrawing substituents.[539] Substitution of the *N*-arylsaccharin with electron-withdrawing groups led to potent inhibitors. Alkaline hydrolysis of furoylsaccharin cleaves the N−C bond, and not the S−N bond, as no sulfonic derivative is observed. The proposed mechanism (Figure 137) suggests that the carbonyl group in the heterocyclic ring is the probable site of enzyme attack.

Following the discovery of acyl- and arylsaccharin protease inhibitors, a group of mechanism-based or suicide inhibitors was discovered. The saccharins contain a *N*-methylene group with an attached leaving group (Figure 136). This class of saccharins has the potential to generate a reactive intermediate that could covalently link to a second active site nucleophile such as His 57. The proposed mechanism is shown in Figure 138. Acylation of Ser 195 by the saccharin gives a tetrahedral intermediate (**79**), which can eliminate the leaving group, giving the reactive acyl enzyme (**80**). Slow deacylation leads to the formation of active enzyme and *o*-carboxybenzenesulfonamide (**82**).[538,650] Another pathway involves Michael addition of His 57 imine with formation of a doubly covalently attached complex to the enzyme (**82**). This doubly covalent acyl enzyme derivative has been observed in X-ray structures of serine proteases with isocoumarin inhibitors.[578,580]

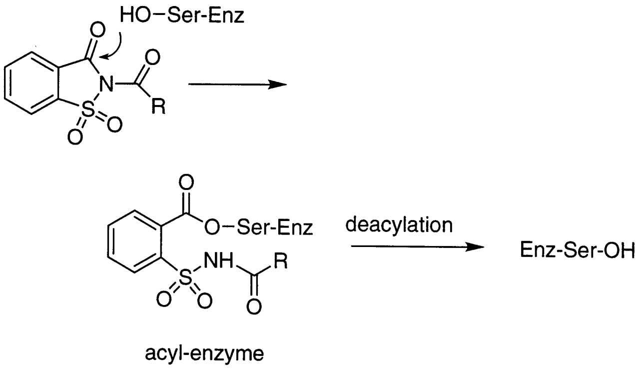


Figure 137. Mechanism of inhibition by *N*-acyl saccharins.

The mechanism of inhibition by saccharin derivatives that contained a leaving group has been explored by high-field ^{13}C NMR, HPLC, product analysis, comparative SAR, and molecular modeling studies.[650,652,653] There is no X-ray structure available in the Protein Data Bank for protease−saccharin complexes. An initial NMR study has identified *o*-carboxybenzenesulfonamide (**82**) as the product[650] when a saccharin derivative was reacted with sodium methoxide. The mechanism of action of *N*-fluoromethylsaccharin with α-chymotrypsin was investigated using high-field ^{13}C NMR, HPLC, and spectroscopy assays.[653] The major product formed by the inactivation of α-chymotrypsin with *N*-fluoromethylsaccharin was found to be saccharin (**83**), whereas *o*-carboxybenzenesulfonamide was not detected. Inhibition of chymotrypsin by ^{13}C-radiolabeled *N*-phenylsulfoxymethylsaccharin resulted in identification of formaldehyde hydrate, saccharin, and phenylsulfinic acid. This indicates that the deacylation reaction probably involves a cyclization to saccharin (**83**). Due to the limited aqueous stability of the inhibitor, ^{13}C NMR was not able to detect the formation of the acyl enzyme intermediate or any other enzyme−inhibitor complexes.

The properties of the leaving group have been correlated with the differences in acylation rates. According to the mechanism of inhibition shown in Figure 138 the rates of deacylation should be independent of the identity of the leaving group. An initial study of HLE inhibitors showed that inactivated elastase regained 80−90% of its activity after 24 h.[650] The half-lives of reactivations ranged between 12 and 15 h with inhibitors containing different leaving groups, which is consistent with a common intermediate.

Structure−Activity Relationships. Many saccharin derivatives have been designed and synthesized in a search for more selective, potent, and bioavailable inhibitors. SAR studies have focused on the nature of the leaving group and substitution around the phenyl ring of the heterocycle.[650,652−654] Hlasta and co-workers discovered several orally bioavailable benzisothiazolone inhibitors of

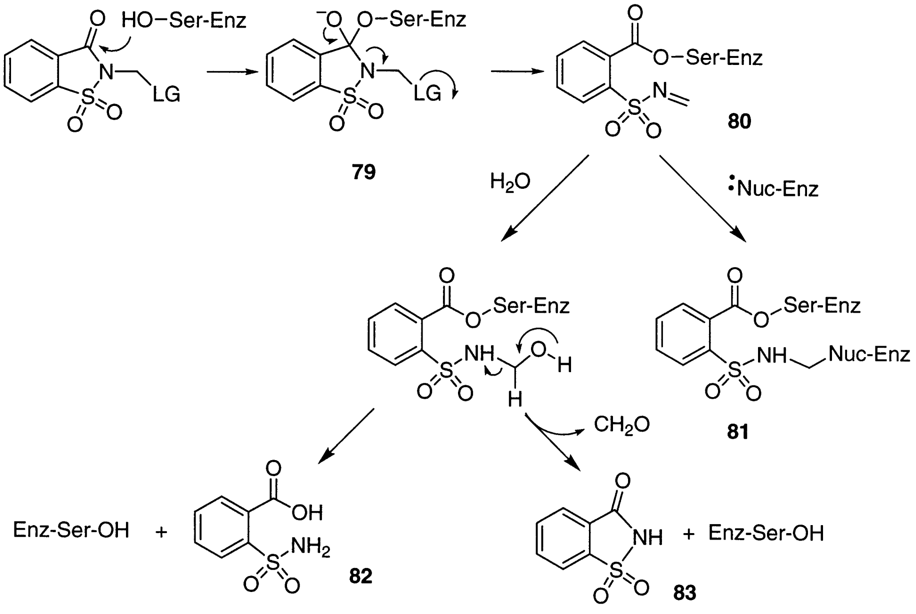


Figure 138. Mechanism of inhibition of serine proteases by saccharins.

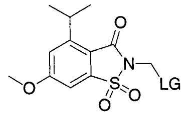
HLE.[540,652,654−660] The potency and stability of the HLE inhibitors are dependent on the C-4 and C-6 substitution and the nature of the leaving group (see Figure 135 for numbering). Some of the most potent and stable inhibitors are derivatives of 4-isopropyl-6-methoxybenzisothiazolone (Table 61). The isopropyl substituent at C-4 improved not only the inhibitory potency but also the stability in human blood.[656] The electron-donating methoxy group at C-6 of saccharins with 2,6-dichlorobenzoate as the leaving group also improves the blood stability, in some examples, as much as 5-fold.[540,654] However, there is no clear effect of the leaving group on HLE inhibition and blood stability. Many leaving groups such as phenylmercaptotetrazole,[654,656] 2,6-dichlorobenzoates,[659,660] phosphonates and phosphinates,[657] and β-dicarbonyl systems,[655] including 5,5-dimethyltetronates and 2-hydroxypyrido[1,2-*a*]pyrimidin-4-one, were tested. The 2,6-dichlorobenzoate leaving group was the most potent and retained stability in human blood.[654,656] However, due to poor aqueous stability, a series of compounds with aqueous solubilizing substituents such as amines and carboxylic acids were also evaluated. The best inhibitors of these series, WIN 64733 and WIN 63759 (Table 61), are specific inhibitors of HLE and do not inhibit cathepsin G, thrombin, plasmin, chymotrypsin, fibroblast collagenase, or stromelysin.[540]

Saccharin or 1,2-benzisothiazol-3-one 1,1-dioxide derivatives have been designed and tested as selective and potent inhibitors of human mast cell tryptase. Commercially available carboxylic acids were used to prepare a library of 300 *N*-(acyloxy) benzisothiazolone derivatives.[647] The lead compound contained a benzyloxy carbamate moiety linked by a methylene spacer to the saccharin nitrogen (Table 62). From the SAR studies it was concluded that the optimal distance between the benzisothiazolone 1,1-dioxide ring and the benzyloxy carbamate ring was four carbons. Inhibitory activity was decreased when an additional amide group was inserted. The SAR studies suggest that the interaction between the inhibitor and the S′ region of tryptase are important for inhibitor recognition. The inhibitors are specific for tryptase, as they inhibit elastase and thrombin 40- and 100-fold less potently, respectively, and do not inhibit thrombin, plasmin, *t*-PA, urokinase, or factor Xa. Another library of compounds, with similar structures and potency, were synthesized using solid phase chemistry (Table 62).[661]

Biological Studies. Furoylsaccharin was the first saccharin derivative to show antielastase activity in vivo.[648,649] This acylating agent was tested for its ability to prevent elastase-induced emphysema in two acute animal models using hamsters and rabbits. Intratracheal administration in hamsters resulted in a partial inhibition, whereas in rabbits furoylsaccharin prevented the changes due to elastase-induced emphysema in a dose-related manner.[648]

Benzisothiazolone-based inhibitors, designed by Hlasta et al., show good potency, specificity, and blood stability when tested with HLE. The best inhibitor from the series, WIN 634759, has the best pharmacokinetic properties in dogs, where it is orally bioavailable (21% absolute bioavailability).[540] In vivo, the inhibitor produces a dose-related inhibition of HNE-induced pulmonary hemorrhage when administered intravenously ($ED_{50} = 3$ mg/kg; 4.5 mol/kg) or subcutaneously ($ED_{50} = 19$ mg/kg; 28.5 mol/kg) in hamsters.[662] However, WIN 634759 is not orally active in hamsters, rats, or monkeys; upon oral administration it is rapidly degraded in the intestines and liver.[540,662] Other studies showed that the compound is stable in dog blood and jejunum and liver S9 homogenates.[540] Bronchoalveolar lavage studies in dogs following oral administration show that WIN 63759 reaches the lung at concentrations 3−5-fold higher than those found in plasma.[662] Its oral bioavailability in humans is predicted using the in vitro metabolism in dogs. Many mechanism-based inhibitors of HLE have poor hydrolytic and metabolic stability and as a result have poor in vivo activity by parenteral or oral administration.[663] Saccharins, such as WIN 63759 and its derivatives, show relatively

Table 61. Inhibition of HLE by 4-Isopropyl-6-methoxybenzisothiazolone Derivatives



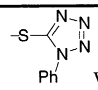
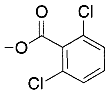
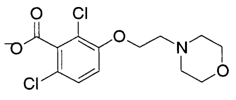
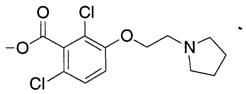
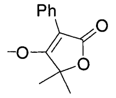
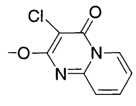
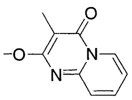
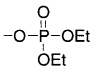
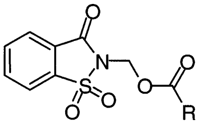
inhibitor LG	K_i (nM)	human blood stability $t_{1/2}$(hr)	ref
 WIN 63395	0.270	4.3	654
 WIN 63394	0.023	2.3	654
 WIN 63759	0.013	0.5	540
 WIN 64733	0.014	1.8	540
	0.021	2.6	655
 WIN 65936	0.066	3.0	655
	0.250	5.8	655
 $-O-P(=O)(OEt)-OEt$	0.035		657

Table 62. IC_{50} Values for Inhibition of Human Mast Cell Tryptase



inhibitor R	IC_{50} (μM)	ref
$(CH_2)_2NH$-Cbz	0.85	647
$(CH_2)_4NH$-Cbz	0.11	647
C_6H_4-4-(Cbz-NH)	0.06	647
C_6H_4-$CH_2NHCONHPh$	0.16	647
$(CH_2)_3CONHCH_2$-(3,4-Cl_2-C_6H_3)	0.23	661
$(CH_2)_3CONHCH_2$-(4-OCH_3-C_6H_4)	0.43	661

good potency, specificity, and blood stability when tested with HLE.

A potent and selective inhibitor of mast cell tryptase, (1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3H)-yl)methyl *N*-[(phenylmethoxy)carbonyl]-β-alaninate (Table 62), was tested in a delay-type hypersensitivity (DTH) mouse model of skin inflammation.[647] Applications of a 5% solution of inhibitor in acetone stopped the increase in ear weight due to edema by 69%. The myeloperoxidase content, an enzyme marker of polymorphonuclear leukocyte infiltration, was decreased by 96%. Thus, this particular saccharin derivative is both a potent and selective tryptase inhibitor in vitro and is effective in an animal model of inflammatory skin disease.

4. Miscellaneous Heterocyclic Inhibitors

1,2,5-Thiadiazolidin-3-one 1,1-Dioxide Derivatives. This class of heterocyclic compounds are based on the 1,2,5-thiadiazolidin-3-one 1,1-dioxide scaffold and are potent and selective inhibitors of serine proteases such as elastase (HLE), cathepsin G, proteinase 3, and chymase.[664,665] The mechanism of inhibition of serine proteases is postulated to be similar to that of saccharins (see Figure 138). Numerous compounds were designed by changing the nature of the substituents at the C-2 position and the leaving group (see Table 63). The nature of the leaving group varies from phosphonates[665] to functionalized sulfonamides[666] to carboxylates[667] and amino acid residues.[571] Structure−activity studies resulted in inhibitors for elastase with second-rate constants up to 10^6 M^{-1} s^{-1} (see Table 63). These heterocyclic inhibitors bind to both S and S′ subsites of the enzyme and are selective between neutral, basic, and acidic serine proteases.

1,2,4-Thiadiazoles. 1,2,4-Thiadiazoles are a new class of heterocyclic inhibitors that react only with cysteine proteases.[58] Depending on the substituents on the heterocyclic ring, the inhibition can be reversible or irreversible. Substituents at the C-3 position control the reactivity, whereas substituents at the C-5 position act as a recognition arm for enzyme selectivity. The general structure is shown in Figure 139.

Mechanism. The inhibition mechanism involves nucleophilic attack of the cysteine residue on the sulfur atom of the heterocyclic ring and formation of a disulfide bond (Figure 139) at the same time ring opening takes place. The mechanism of action has been proven by X-ray crystallography of a papain−inhibitor complex, which clearly shows the formation of the disulfide bond between Cys 25 and the sulfur atom of the thiadiazole moiety.[58]

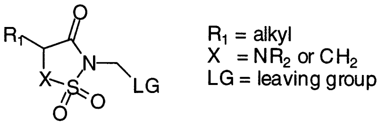
Structure—Activity Relationships. 1,2,4-Thiadiazoles are potent inhibitors of cathepsin B. The potency is dependent on the substituents at C-3 (R_1) and C-5 (R_2). The best inhibitor has R_1 = OMe and R_2 = Leu-Pro-OH and a second-order rate of 5630 M^{-1} s^{-1}. However, when R_1 is replaced with a methyl group, the inhibitory potency decreases 100-fold.

V. Phosphonylation Agents

A. Peptide Phosphonates

Peptide phosphonate serine protease inhibitors were developed on the basis of diisopropylfluorophosphate (DFP). DFP irreversibly inhibits many serine

Table 63. Inhibition of Serine Protases by 1,2,5-Thiadiazolidin-3-one 1,1-Dioxide Derivatives



inhibitor[a]			k_{obs}/[I] ($M^{-1}s^{-1}$)			
R_1	X	LG	elastase	cathepsin G	proteinase 3	ref
$(CH_3)_2CH_2CH$	NCH_2Ph	$O(PO)(OCH_2Ph)_2$	6000000	89120	120	665
$(CH_3)_2CH_2CH$	NCH_3	$OCO\text{-}C_6H_4\text{-}2,6\text{-}Cl_2$	4928300	60	33400	667
$(CH_3)_2CH_2CH$	NCH_2Ph	$OCO\text{-}C_6H_4\text{-}2,6\text{-}Cl_2$	2381000	30	14400	667
$(CH_3)_2CH_2CH$	CH_2	$OCO\text{-}C_6H_4\text{-}2,6\text{-}Cl_2$	469900	2200		667
$(CH_3)_2CH_2CH$	NCH_3	$N(SO_2CH_3)(COCH_2NHCbz)$	229400	60	27400	571
$(CH_3)_2CH_2CH$-(*S*)	NCH_3	O←L-Phe-Cbz	637900	140	126200	571
$(CH_3)_2CH_2CH$-(*S*)	NCH_3	O←D-Phe-Cbz	1056800	50	157300	571
$(CH_3)_2CH_2CH$	CH_2	O←D-Phe-Cbz	170500	80		571

[a] LG = leaving group; ← peptide chain runs in reverse direction.

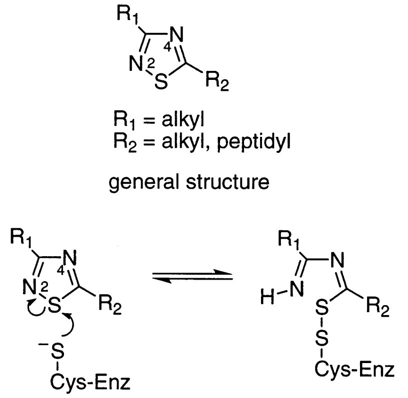


Figure 139. General structure and mechanism of inhibition of 1,2,4-thiadiazoles.

proteases and is one of the first irreversible inhibitors described for serine proteases (see phosphonyl fluoride section). The reactivity and chemical stability of organophosphorus inhibitors as irreversible inhibitors of serine proteases is determined by the electrophilicity of the phosphorus atom. In the case of phosphonyl fluoride inhibitors, the phosphorus atom is adjacent to a fluorine atom, resulting in high reactivity and low chemical stability. Some phosphonyl fluorides are very potent inhibitors, but they also undergo rapid nonenzymatic hydrolysis. Phosphonyl fluorides also react with acetylcholinesterase, which makes them extremely toxic. Thus, these inhibitors are considered to be compounds that lack therapeutic utility.

Oleksyszyn et al. developed a new class of organophosphorus inhibitors called peptidyl α-aminoalkylphosphonates.[668] Peptidyl α-aminoalkylphosphonates are irreversible inhibitors of serine proteases (Figure 140). The design of the inhibitor structure was based on a compromise between the reactivity and the chemical stability. Compared to the fluorine atom, the adjacent phenoxy group is less electronegative. This makes the phosphorus atom sufficiently electrophilic to undergo a nucleophilic displacement reaction by the active site serine hydroxyl group. Peptidyl phosphonate esters are hydrolytically stable and do not react with acetylcholinesterase. Considerable specificity is obtained among different serine proteases, where the peptide sequence of the inhibitor is altered to match the specificity of the target enzyme.

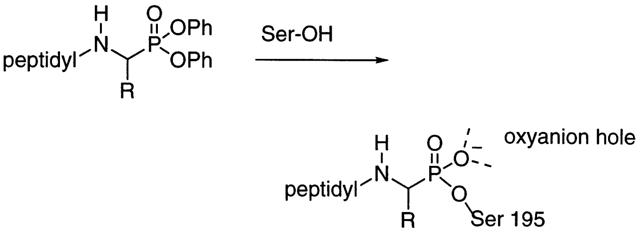


Figure 140. Inactivation of serine proteases by peptide phosphonates.

Nomenclature. Peptidyl α-aminoalkylphosphonates $R'CO{-}NHCH(R_1)P({=}O)(OR)_2$ are abbreviated $R'CO\text{-}AA^P(OR)_2$, where AA is the amino acid residue corresponding to the α-aminoalkylphosphonate residue, P is the phosphorus atom, and R is an alkyl or aryl group.

Crystal Structure and Inhibition Mechanism. The mechanism of inhibition of serine proteases by peptidyl phosphonates has been established by NMR and X-ray crystallographic studies. Oleksyszyn et al. first determined via ^{31}P NMR that the geometry around the phosphorus atom in the inhibitor–enzyme complex is tetrahedral.[669] The inhibitor Suc-Val-Pro-$Phe^P(OPh)_2$ showed two signals at 19.59 and 18.75 ppm in the ^{31}P NMR, signals corresponding to the two diastereomers. When bound to chymotrypsin, one broad signal at 25.98 ppm corresponding to the Ser 195 phosphonate ester is obtained. The ^{31}P was shifted more than 6 ppm, indicating a tetrahedral geometry at the phosphorus atom. Oleksyszyn et al. proposed the mechanism of inhibition of serine proteases by peptidyl phosphonate esters as an addition elimination mechanism as shown in Figure 141.[670]

The active site Ser 195 hydroxyl group attacks the phosphorus atom, forming a pentacoordinate intermediate (**85**). In the next step, one of the phenoxy groups leaves and a stable tetravalent phosphonylated derivative (**86**) is formed. A slow aging process then occurs, and the tetravalent phosphonylated derivative loses the second phenoxy group. A stable serine phosphomonoester is formed where one of the phosphonate oxygens is extended into the oxyanion hole. The geometry around the phosphorus atom was determined to be tetrahedral by ^{31}P NMR.

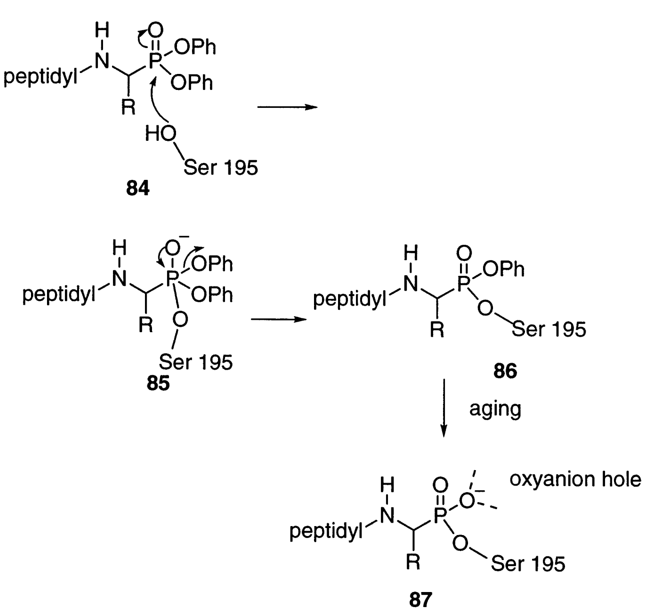


Figure 141. Proposed mechanism of inhibition of serine proteases by peptide phosphonate esters.

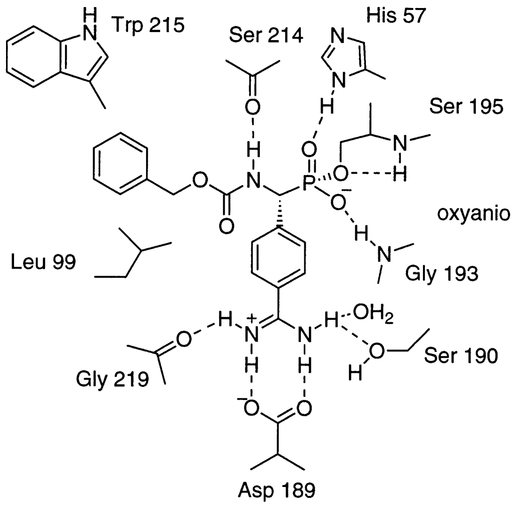


Figure 142. Structure of trypsin complexed with Cbz-(4-AmPhGly)^P(OPh)$_2$.

Crystal structural data are consistent with this mechanism. The crystal structure of Cbz-(4-AmPhGly)^P(OPh)$_2$ bound to bovine trypsin shows a tetrahedral phosphorus atom that is covalently bound to the active site Ser 195 Oγ (Figure 142). The covalent bond formation is accompanied by the loss of both phenoxy groups.[671]

Two structures were observed in complexes of human α-thrombin inhibited by Cbz-D-Dpa-Pro-Mpg^P-(OPh)$_2$ (Dpa = diphenylalanine, Mpg = α-amino-δ-methoxypropyl phosphonate).[672] Crystal structures of α-thrombin with the phosphonate inhibitor were determined independently using crystals of different ages (Figure 143). The first complex, determined from 7-day-old crystals, is a reversible pentacoordinate phosphorus intermediate. The second complex, determined from 12-week-old crystals, is a tetracoordinated phosphorus atom covalently linked to the active site Ser oxygen. In complex I, the typical antiparallel β-sheet interaction with the active site residues 214–216 is not seen with the inhibitor. The inhibitor adopts an approximately cyclic conformation and makes hydrogen bonds to the enzyme via water molecules. The P2 Pro occupies the hydrophobic pocket consisting of Tyr 60A and Trp 60D. The β,β-diphenylalanine (Dpa) at the P3 position makes hydrophobic interactions with Leu 99, Ile 174, and Trp 215. Only the P3 amino group makes hydrogen bonds to the active site Gly 216.

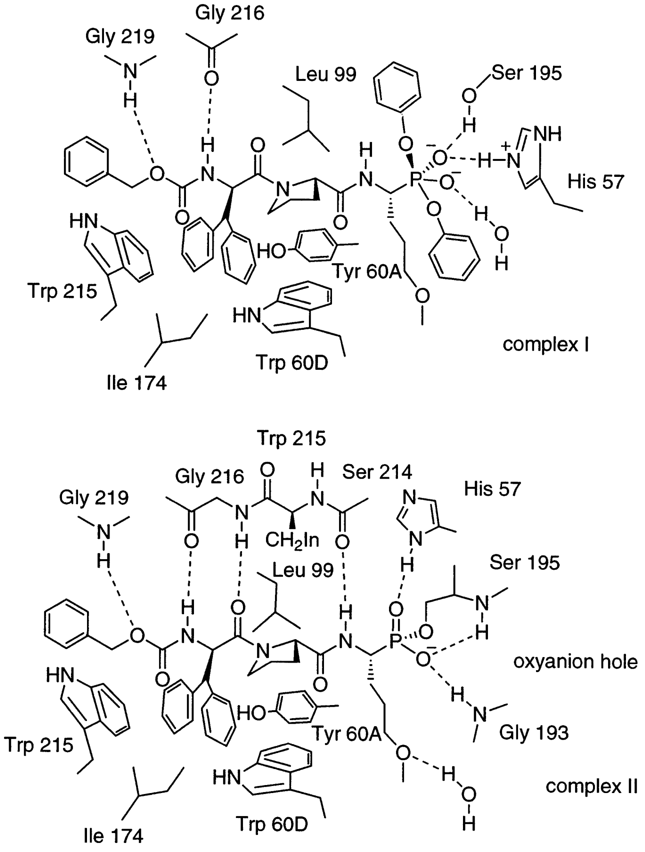


Figure 143. Structures of thrombin complexed with Cbz-D-Dpa-Pro-Mpg^P(OPh)$_2$ (complexes I and II).

In the second complex (Figure 143), the inhibitor makes antiparallel β-sheet interactions in the enzyme's active site residues, Ser 214–Gly 216, as seen with typical thrombin–inhibitor complexes. The P1 methoxypropyl side chain makes a hydrogen bond to a water molecule in the S1 pocket. The P2 and P3 interactions remain the same as in complex I.

In complex I, the phosphorus atom is attacked by a water molecule to give the pentacoordinate complex. The active site Ser 195 O and His 57 N make hydrogen bonds to the oxygen from the water molecule, which is attached to the phosphorus atom. The serine is not covalently linked to the inhibitor. The other phosphorus oxygen atom hydrogen bonds to a nearby water molecule, and the two phenoxy groups are still covalently attached to the phosphorus atom. Complex I ultimately decays into complex II by attack of Ser 195 and sequential elimination of the phenoxy groups. It is not possible to predict which one would be lost first, but most probably the one that is more exposed to the solvent leaves first. The loss of the second phenoxy group takes place by a slow aging process. The oxyanion can then interact with the oxyanion hole, making hydrogen bonds to Ser 195 and Gly 193.

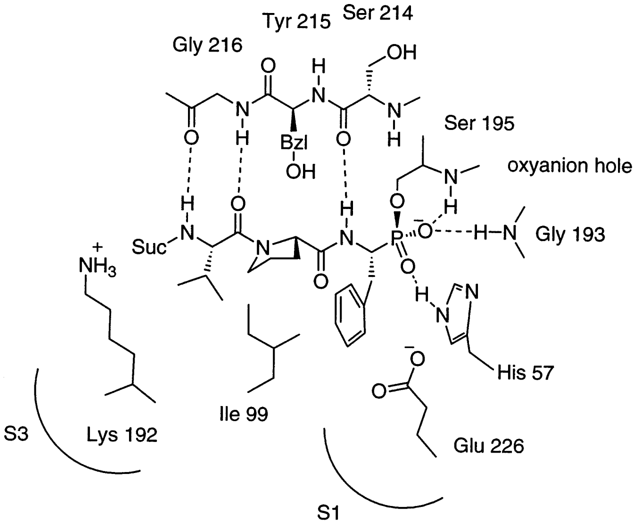


Figure 144. Structure of cathepsin G complexed with Suc-Val-Pro-Phe^P(OPh)$_2$.

The crystal structure of human neutrophil cathepsin G with the peptidyl phosphonate inhibitor Suc-Val-Pro-Phe^P(OPh)$_2$ indicates covalent bond formation of the inhibitor to the enzyme's active site Ser 195 Oγ as seen in Figure 144.[673] The geometry around the phosphorus atom is tetrahedral. One of the phosphonate oxygens extends into the oxyanion hole, making hydrogen bonds to Gly 193 and Ser 195. Both phenyl groups are lost in the inhibitor–enzyme complex during or after the reaction with Ser 195. The inhibitor backbone makes antiparallel β-sheet interactions with the residues Ser 214–Lys 218 in a twisted manner. The phenyl ring in the S1 pocket resides partially between Tyr 215–Gly 216 and Ala 190–Lys 192. The bottom part of the S1 pocket is divided in half by the Glu 226. This is a unique feature of cathepsin G compared to other serine proteases. Thus, cathepsin G has a double-headed (Janus-like) substrate specificity for both basic and aromatic residues. The Pro residue at the P2 position allows the inhibitor to form a kinked conformation to fit into the active site. The Pro ring makes hydrophobic interactions with Ile 99. The isopropyl side chain of the Val residue extends into the S3 pocket and makes hydrophobic interactions with the Lys 192 side chain.

The crystal structure of α-lytic protease with the extended phosphonate inhibitor Boc-Ala-Ala-Pro-Val^P(OPh)-OCHMeCO-Ala-OMe has been determined (Figure 145).[674] The inhibitor is covalently attached to the enzyme's active site Ser 195 Oγ. The phosphorus atom is tetracoordinated, and the inhibitor backbone makes hydrogen-bonding interactions with the residues Ser 214–Gly 216 in an antiparallel β-sheet form as seen in other trypsin-family serine proteases. The P1 phosphonate residue forms hydrogen bonds with the amide protons of Ser 195 and Gly 193. The active site His 57 does not interact with the phosphonate. Its side chain extends into the solution because there are no negatively charged oxygen atoms nearby. The S2 subsite is a hydrophobic pocket consisting of Tyr 171, Phe 94, and His 57 that surrounds the Pro residue at the P2 position. The P3 Ala makes hydrogen bonds to the Gly 216. Extension of the peptide sequence to the P5 position does not provide additional interactions with the active site. Incorporation of two residues in the P1′ and P2′ positions allows the formation of two additional hydrogen bonds. However, there is little adjustment in the structure of the enzyme in this region, suggesting that these interactions do not participate in the enzymes' specificity.

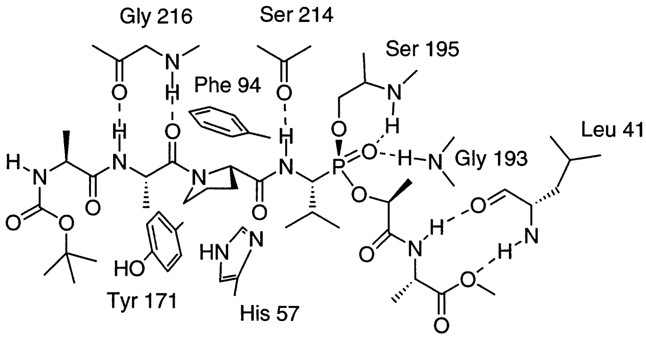


Figure 145. Structure of α-lytic protease complexed with Boc-Ala-Ala-Pro-Val^P(OPh)-OCHMeCO-Ala-OMe.

Table 64. Inhibiton of Chymotrypsin by Peptide Phosphonates[668,669]

	k_{obs}/[I] (M^{-1} s^{-1})		
inhibitor	chymotrypsin	HNE	PPE
MeO-Suc-Ala-Ala-Ala-Phe^P(OPh)$_2$	12000	NI[a]	NI
MeO-Suc-Ala-Ala-Pro-Val^P(OPh)$_2$	21	7100	7100
MeO-Suc-Ala-Ala-Pro-Phe^P(OPh)$_2$	11000	NI	NI
Cbz-Phe-Pro-Phe^P(OPh)$_2$	17000	NI	1.5
Cbz-Val-Pro-Val^P(OPh)$_2$	560	7000	480
Suc-Val-Pro-Phe^P(OPh)$_2$	44000	NI	NI
Cbz-Ala^P(OPh)$_2$	NI	NI	NI
Cbz-Phe^P(OPh)$_2$	1200	NI	NI

[a] NI = no inhibition.

Structure—Activity Relationships. Peptidyl phosphonate structures incorporating the tetrahedral phosphorus moiety in the place of the scissile carbonyl group of a peptide substrate were first tested for inactivation of chymotrypsin. The second-order rate constants were only 12–27 M^{-1} s^{-1}.[675] Peptidyl phosphonates, incorporating the phosphorus moiety at the C terminus with a diphenyl ester and appropriate peptide sequences, were tested for inhibitory activity against bovine chymotrypsin, PPE, and HLE.[668] Peptidyl phosphonates inhibit those enzymes specifically and irreversibly with k_{obs}/[I] values in the range of 10^3–10^4 M^{-1} s^{-1} (Table 64).

The monopeptide derivatives were in general weaker inhibitors. Addition of amino acid residues to the peptide chain improved the inhibitory potency significantly. The inhibitor MeO-Suc-Ala-Ala-Pro-Phe^P-(OPh)$_2$ is quite potent and specific for chymotrypsin with a k_{obs}/[I] value of 11000 M^{-1} s^{-1}. Peptidyl phosphonates inhibit elastases very slowly. All of the inhibitors have a Phe residue at the P1 position in accordance with the P1 preference for chymotrypsin. The inhibitor Suc-Val-Pro-Phe^P(OPh)$_2$ is the best inhibitor for chymotrypsin with a rate constant of 44000 M^{-1} s^{-1}. PPE and HNE are not inhibited by Suc-Val-Pro-Phe^P(OPh)$_2$. However, cathepsin G and RMCP II are inhibited by this inhibitor with rate constants of 36000 and 15000 M^{-1} s^{-1}, respectively.

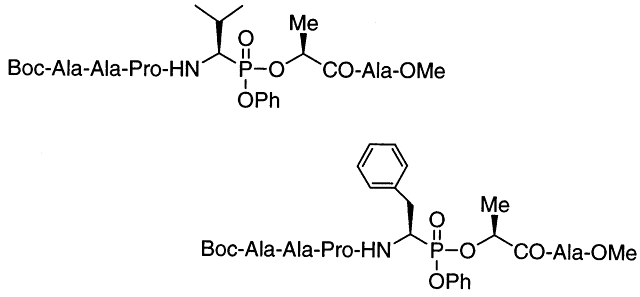


Figure 146. Hexapeptide phosphonate analogues.

The potent chymotrypsin inhibitor Cbz-Phe-Pro-Phe^P-$(OPh)_2$ inhibited cathepsin G preferably over RMCP II with a rate constant of 5100 compared to 32 M^{-1} s^{-1}. When the residue Val occupied the P1 position of the peptide sequence, potent and selective inhibitors for elastases PPE and HNE were obtained. The inhibitor MeO-Suc-Ala-Ala-Pro-Val$^P(OPh)_2$ was quite specific for both PPE and HNE ($k_{obs}/[I] = 7100$ M^{-1} s^{-1}) and inhibited chymotrypsin with a rate constant of only 21 M^{-1} s^{-1}. Boc-Val-Pro-Val$^P(OPh)_2$ is the best elastase inhibitor with inhibition rate constants 11000 and 27000 M^{-1} s^{-1} for PPE and HNE, respectively, and no inhibition was observed with chymotrypsin.[668,669]

To investigate the relative contribution of each region of the active site of HNE, hexapeptide phosphonate esters with Phe^P and Val^P incorporated at the P1 position and -OCHMeCO-Ala-OMe in P1′–P2′ positions have been developed (Figure 146).[676] The hexapeptide phosphonate analogues irreversibly inhibit HNE, α-lytic protease (ALP), and subtilisin with second-order rate constants in the range of 10^{-3}–10^{1} M^{-1} s^{-1}. These results imply that the interactions at P′ subsite do not play an important role in binding to HNE.

In accordance with the subsite specificity of trypsin-like serine proteases, arginine and lysine analogues, the unusual side-chain derivatives with (α-amino-*δ*-methoxypropyl)phosphonyl (Mpg^P) or (α-amino-*n*-hexyl)phosphonyl (Pgl^P) residues at the P1 position, and peptidyl phosphonates containing *β*,*β*-diphenylalanine (Dpa) at the P2 position have been developed (Figure 147).[677–679] The arginine and lysine analogues, Ac-D-Phe-Pro-Arg$^P(OPh)_2$ and Ac-D-Phe-Pro-Lys$^P(OPh)_2$, were slow binding inhibitors of thrombin with IC_{50} values in micromolar range (1–125 μM). The peptidyl phosphonate inhibitors, D-Phe-Pro-Mpg$^P(OPh)_2$ and D-Dpa-Pro-Mpg$^P(OPh)_2$, inhibited thrombin selectively with IC_{50} values of 1.70 and 1.50 μM, respectively.

Oleksyszyn et al. developed arginine analogues of peptidyl phosphonates for the trypsin-like serine proteases thrombin, trypsin, factor XIIa, and kallikrein. The results are shown in Table 65.[670] The parent compound Cbz-(4-AmPhGly)$^P(OPh)_2$ is a potent inhibitor of human plasma kallikrein with a second-order rate constant of 18000 M^{-1} s^{-1}. The AmPhGly$^P(OPh)_2$ or 4-amidinophenylglycine residue is an arginine analogue (see Figure 142). The inhibitor Boc-D-Phe-Pro-(4-AmPhGly)$^P(OPh)_2$ inactivates thrombin at least 5-fold more selectively than the other enzymes tested. The Boc derivative is a more potent inhibitor, suggesting that there might be a different binding mode for the AmPhGly peptide derivatives complexed with thrombin compared to the other peptidyl phosphonate inhibitors.

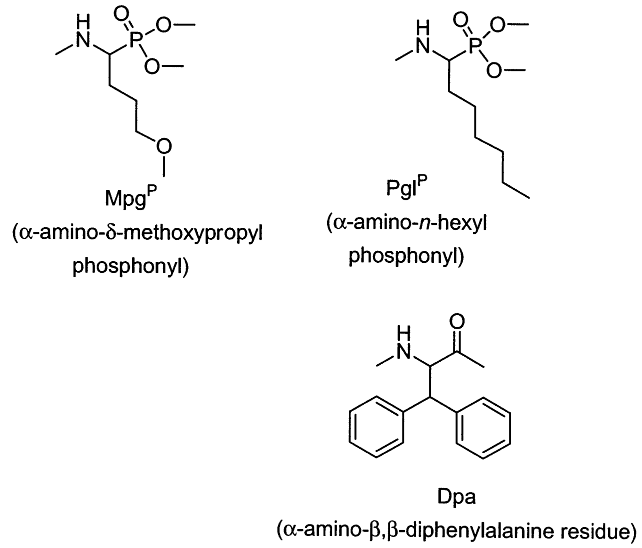


Figure 147. Examples of α-aminoalkyl phosphonates with unusual side chains and structure of the P2 diphenylalanine residue.

To obtain more potent inhibitors of serine proteases, halophenyl ester phosphonates have been developed.[680] It has been suggested that the increased electrophilic character of phosphonates should also increase the inhibitory potency. In general, the peptide halophenyl phosphonate esters are potent inhibitors of HNE and PPE, but compared to the phenyl esters, halophenyl ester phosphonates are equally or less potent inhibitors of HNE and PPE. The inhibitors, Val-Pro-Val^P(OPh-*p*Cl)$_2$ and Val-Pro-Val^P(OPh-*m*Cl)$_2$, inhibit HNE potently with second-order rate constants of 13000 and 9700 M^{-1} s^{-1}, respectively (Table 66). Inhibitors with a free N terminus are better inhibitors than those with Boc or Cbz groups in the same position. In contrast to HNE, PPE is more potently inhibited by inhibitors with a Boc or Cbz group at the N terminus. The inhibitors Boc-Val-Pro-Val^P(OPh-*p*Cl)$_2$ and Boc-Val-Pro-Val^P(OPh-*m*Cl)$_2$ inhibit PPE with second-order rate constants 4400 and 4100 M^{-1} s^{-1}, respectively. The inhibitors with one of the phenoxy groups replaced by a phenyl group (phenylphosphinate esters) showed little to no inhibition toward both elastases. Only Cbz-Val-Pro-Val$^P(Ph)(OPh)_2$ inhibited HNE at a moderate rate (670 M^{-1} s^{-1}) and PPE very slowly (50 M^{-1} s^{-1}).[680] It is hypothesized that chlorine may interfere with the binding of the inhibitor in the active site of two elastases. In the transition state, the chlorine atom may affect the location of the phenoxy group on the phosphorus atom.

In contrast to elastases, the halophenyl phosphonate esters are more reactive toward chymotrypsin and dipeptidyl peptidase IV (DPP IV). Apparently, the active sites of chymotrypsin and DPP IV can accommodate the chlorophenoxy group more effectively. The single diastereomer dipeptide inhibitor Ala-Pip^P(OPh-*p*Cl)$_2$ inhibits DPP IV with a second-order rate constant of 1300 M^{-1} s^{-1} (Figure 148).[681]

Table 65. Inactivation of Trypsin-like Serine Proteases by Peptide Phosphonate Derivatives[670]

	k_2/K_i (M^{-1} s^{-1})				
inhibitor[a]	human thrombin	bovine thrombin	human factor XIIa	human plasma kallikrein	bovine trypsin
Cbz-(4-AmPhGly)^{P}(OPh)$_2$	80	170	20	18000	2000
Boc-D-Phe-Pro-(4-AmPhGly)^{P}(OPh)$_2$	11000	12000	52	160	2200
D-Phe-Pro-(4-AmPhGly)^{P}(OPh)$_2$	700	730	2.8	250	110
Ac-D-Phe-Pro-Arg^{P}(OPh)$_2$[679]	1200				

[a] 4-AmPhGly^{P} = 4-amidinophenylglycine phosphate residue.

Table 66. Inactivation of Elastase by Peptide Phosphonates and Phenylphosphinates[680]

	k_2/K_i (M^{-1} s^{-1})		
inhibitor	chymotrypsin	HNE	PPE
Boc-Val-Pro-Val^{P}(OPh)$_2$		25000	11000
Cbz-Val-Pro-Val^{P}(OPh-*p*Cl)$_2$	560	7000	480
Boc-Val-Pro-Val^{P}(OPh-*p*Cl)$_2$	50	1400	4400
Val-Pro-Val^{P}(OPh-*p*Cl)$_2$	125	13000	500
Cbz-Val-Pro-Val^{P}(OPh-*m*Cl)$_2$	830	5700	490
Boc-Val-Pro-Val^{P}(OPh-*m*Cl)$_2$	100	1100	4100
Val-Pro-Val^{P}(OPh-*m*Cl)$_2$	60	9700	590
Cbz-Val-Pro-Val^{P}(Ph)(OPh)		670	50
Val-Pro-Val^{P}(Ph)(OPh)		NI[a]	NI

[a] NI = no inhibition.

Table 67. Inactivation of Trypsin-like Serine Proteases by 4-Amidinophenylglycine Phosphonate Diphenyl Ester Derivatives[683]

	k_2/K_i (M^{-1} s^{-1})		
inhibitor[a]	granzyme A	granzyme K	trypsin
Cbz-(4-AmPhGly)^{P}(OPh)$_2$	1180	3.8	2000
Cbz-Ala-(4-AmPhGly)^{P}(OPh)$_2$	1670	NI[b]	1850
Cbz-Thr-(4-AmPhGly)^{P}(OPh)$_2$	2220	3	97
Cbz-Leu-(4-AmPhGly)^{P}(OPh)$_2$	6.3	NI	50
Cbz-Pro-(4-AmPhGly)^{P}(OPh)$_2$	790	34	910
Cbz-Phe-Ala-(4-AmPhGly)^{P}(OPh)$_2$	1770	4.2	3010
$PhCH_2SO_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$	3650	87	37060
3,3-diphenylpropanoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$	250	1830	2660
3-phenylpropanoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$	1510	50	1520

[a] 4-AmPhGly^{P} = 4-amidinophenylglycine phosphate residue. [b] NI = no inhibition.

Alternatively, the reason for decreased potency with elastases may be an electronic effect.

Diphenyl phosphonate analogues of aspartic and glutamic acids have been tested for inhibitory activity against *S. aureus* V8 protease and granzyme B.[682] *S. aureus* V8 protease prefers both Asp and Glu, and granzyme B prefers Asp at the P1 position. Two inhibitors, Ac-Asp^{P}(OPh)$_2$ and Ac-Glu^{P}(OPh)$_2$, inhibit *S. aureus* V8 protease with second-order rate constants of 5000 and 5300 M^{-1} s^{-1}, respectively. No activity against granzyme B was observed.

Diphenyl phosphonate esters have also been evaluated with the trypsin-like enzymes granzymes A and K and mast cell tryptase.[683] Generally, granzyme A is inhibited more effectively than granzyme K, and most of the inhibitors were ineffective with tryptase (Table 67). The dipeptide derivative Cbz-Thr-(4-AmPhGly)^{P}(OPh)$_2$ is quite selective for the two granzymes. Cbz-Thr-(4-AmPhGly)^{P}(OPh)$_2$ inhibits granzyme A potently (2220 M^{-1} s^{-1}), whereas it is a weak inhibitor of granzyme K (3.0 M^{-1} s^{-1}). The dipeptide derivative 3,3-diphenylpropanoyl-Pro-(4-AmPhGly)^{P}(OPh)$_2$ is the best granzyme K inhibitor (1830 M^{-1} s^{-1}), and it is moderately reactive with granzyme A (250 M^{-1} s^{-1}). The tripeptide derivative $PhCH_2SO_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$ is the best granzyme A inhibitor within this series with a second-order rate constant 3650 M^{-1} s^{-1}. $PhCH_2SO_2$-Gly-Pro-(4-AmPhGly)^{P}(OPh)$_2$ has little activity toward granzyme K (87 M^{-1} s^{-1}), but it potently inactivates trypsin (37060 M^{-1} s^{-1}). However, selectivity can be obtained among granzyme A and trypsin in vivo. The granzymes are active in granules and more susceptible to inhibition than trypsin. In contrast, trypsin is stored in the pancreas in its inactive zymogen form. By the time trypsin is activated, the concentration of the inhibitors would be too low to show significant inhibitory activity against trypsin.

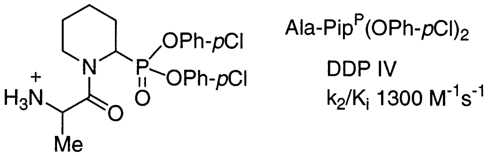


Figure 148. DPP IV inhibitor Ala-Pip^{P}(OPh-*p*Cl)$_2$.

Stability. Peptidyl phosphonate esters are stable in buffer solutions and in human plasma. No change was observed in the UV spectrum of the inhibitor MeO-Suc-Ala-Ala-Pro-Leu^{P}(OPh)$_2$ in 0.1 M HEPES and 0.5 M NaCl, pH 7.5, at 25 °C after 7 days. Addition of glutathione also had no effect on the spectrum. The stability of the inhibitor Suc-Val-Pro-Phe^{P}(OPh)$_2$ in human plasma was tested by its ability to inactivate chymotrypsin. The half-life of inhibition was not changed for the first 8 h. After 24 h of incubation, an increase in half-life was observed, indicating partial destruction of the inhibitor.[669] Peptidyl phosphonates form very stable derivatives with serine proteases. The half-life for reactivation of the elastases HNE and PPE after inhibition with MeO-Suc-Ala-Ala-Pro-Val^{P}(OPh)$_2$ was >48 h. Chymotrypsin regained activity slowly with $t_{1/2} = 10$ h with the same inhibitor. With trypsin, some AmPhGly derivatives showed no loss of inhibitory potency after incubation in neutral pH buffers for one month, and the inhibited enzyme recovered no significant activity during the same time period.

Biological Studies. The stability of peptidyl phosphonate esters toward hydrolysis makes them suitable for use in vivo. Several fluorescent derivatives of phosphonate esters have been used for cellular localization of serine proteases.[684] Fluorescent derivatives with the appropriate peptide recog-

Table 68. Inactivation by Fluorescent Peptide Phosphonate Derivatives[684]

inhibitor[a]	k_2/K_i (M^{-1} s^{-1}) chymotrypsin	HNE	PPE
FTC-Aca-Ala-Ala-Met^P(OPh)$_2$	190	22	13
FTC-Aca-Phe-Leu-Phe^P(OPh)$_2$	9500	252	16
Boc-Ala-Ala-Met^P(OPh)$_2$	3	2	3
Cbz-Phe-Leu-Phe^P(OPh)$_2$	110	27	NI[b]

[a] FTC = 5-fluoresceinyl(thiocarbamoyl); Aca = 6-aminocaproyl. [b] NI = no inhibition.

nition sequence are potent inhibitors of chymotrypsin, HNE, and PPE as shown in Table 68. The specificity of these derivatives was not affected by the fluorescent label. The inhibitor FTC-Aca-Ala-Ala-Met^P(OPh)$_2$ irreversibly labeled discrete granule-like regions of the NK cell line, RNK-16 [FTC = 5-fluoresceinyl(thiocarbamoyl), Aca = 6-aminocaproyl].

The serine protease inhibitor biotinyl-Aca-Aca-Phe-Leu-Phe^P(OPh)$_2$ was also shown to inactivate a granule chymase and block natural killer (NK)-mediated cytotoxicity in rat RNK-16 granule extracts.[685] Perforin-dependent lysis mediated by cytotoxic lymphocyte granules was inactivated by using 1 mM concentration of the serine protease inhibitors Cbz-Ala-Ala-Ala^P(OPh)$_2$, Cbz-Met^P(OPh-*p*Cl)$_2$, Cbz-Leu-Phe^P(OPh)$_2$, and biotinyl-Aca-Aca-Phe-Leu-Phe^P-(OPh)$_2$.[686]

DPP IV is a membrane-bound serine protease. To further investigate the biological function of DPP IV, the DPP IV inhibitor prodipine [Pro-Pro^P(OPh)$_2$] has been utilized in vivo.[687] Male rabbits (3−4 kg) injected with 10 mg of prodipine had decreased DPP IV activity after 1 h, which remained unchanged for 24 h. Systematic treatment with prodipine inhibited plasma DPP IV activity and tissue DPP IV activity in circulating mononuclear cells, the kidney cortex, thymus, spleen, lung, and liver.

The inhibitor Cbz-(4-AmPhGly)^P(OPh)$_2$ has been evaluated for antitrypanosomal activity against the bloodstream form of *Trypanosomai brucei brucei*.[688] The target enzyme is a serine oligopeptidase called oligopeptidase B. The compound Cbz-(4-AmPhGly)^P-(OPh)$_2$ was curative at 5 mg/kg/day in an in vivo mouse model of infection, but it was toxic at higher doses. There was no significant correlation between the inhibitory potency and the in vitro antitrypanosomal activity, suggesting that there might be multiple targets within the parasite for peptide phosphonates.

B. Phosphonyl Fluorides

Phosphonyl fluorides have been known to be very potent inhibitors for serine proteases and esterases for over 80 years. Classical phosphonyl fluoride inhibitors such as diisopropyl phosphonofluoridate (DFP), isopropylmethyl phosphonofluoridate (Sarin), and 1,2,2-trimethylpropylmethyl phosphonofluoridate (Soman) do not resemble a peptide substrate and hence are nonselective toward a particular serine protease. These phosphonylating agents are also extremely toxic due to their reactivity with acetylcholinesterase. However, they have been useful tools for the identification and classification of new serine proteases.

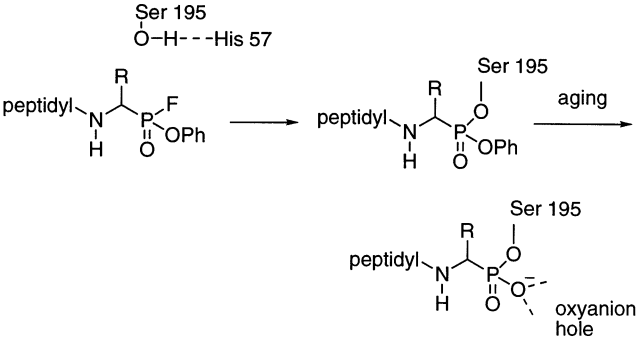
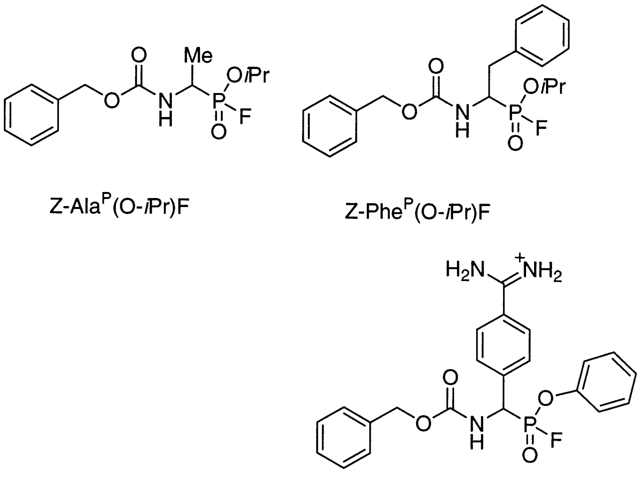


Figure 149. Inactivation of serine proteases by peptide phosphonyl fluorides.



Z-Ala^P(O-*i*Pr)F

Z-Phe^P(O-*i*Pr)F

Z-(4-AmPhGly)^P(OPh)F

Figure 150. Examples of phosphonyl fluoride inhibitors.

Relatively few peptide phosphonyl fluoride derivatives have been reported. The general structure is RCONHCH(R)-PO(OPh)F. They are usually synthesized from the corresponding amino alkyl phosphonate diphenyl ester by partial hydrolysis followed by formation of the phosphonyl fluoride.

Mechanism. The mechanism of inactivation of serine proteases by phosphonyl fluorides probably involves nucleophilic replacement of the fluoride group on the phosphorus atom by the active site serine hydroxyl group, resulting in a stable enzyme−inhibitor complex (Figure 149).

Structure−Activity Relationships. The structures of several phosphonyl fluoride inhibitors, which resemble the peptide substrates for serine proteases, are shown in Figure 150. These peptide phosphonyl fluorides exhibited relatively good selectivity toward their target serine protease. The inhibitors Cbz-Ala^P-(O-*i*Pr)F and Cbz-Phe^P(O-*i*Pr)F inactivated elastase and chymotrypsin with second-order rate constants of 2000 and 180000 M^{-1} s^{-1}, respectively. The inhibitor Cbz-Phe^P(O-*i*Pr)F appeared to be quite selective for chymotrypsin over elastase and had an inhibition rate constant of only 160 M^{-1} s^{-1} for elastase.[689] Table 69 has some representative inhibition data.

Phosphonyl fluorides with a benzamidine group at the P1 position have been developed as inhibitors for trypsin-like serine proteases.[690] The positively charged side chain of these inhibitors is suitable for the

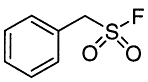
Table 69. Inhibition of Serine Proteases by Phosphonyl Fluorides[675]

	k_2/K_i (M^{-1} s^{-1})	
inhibitor	elastase	chymotrypsin
Cbz-Ala^P(OMe)F	2000	
Cbz-Ala^P(O*i*Pr)F	1280	8830
Cbz-Ala^P(NH*i*Pr)F	160	
Cbz-Phe^P(NH*i*Pr)F	160	180000
DFP		15000
Sarin		23000
Soman		200000

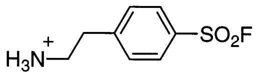
Table 70. Inactivation of Trypsin-like Serine Proteases by Cbz-(4-AmPhGly)^P(OPh)F[690]

enzyme	k_2/K_i (M^{-1} s^{-1})
bovine trypsin	260000
human thrombin	100000
mast cell tryptase	40000
plasmin	59000
kallikrein	90000
α-chymotrypsin	29000
human neutrophil elastase	2700
porcine pancreatic elastase	NI[a]
acetylcholinesterase	NI

[a] NI = no inhibition.



PMSF
88



AEBSF
89

Figure 151. Structures of PMSF (**88**) and AEBSF (**89**).

negatively charged S1 pocket of trypsin-like serine proteases. The inhibitor Cbz-(4-AmPhGly)^P(OPh)F (4-AmPhGly = 4-amidinophenylglycine) was found to be a potent inhibitor of trypsin, thrombin, mast cell tryptase, and plasmin with second-order rate constants over a range of 10^5 M^{-1} s^{-1} (Table 70). Chymotrypsin and human neutrophil elastase are inhibited much more slowly by this phosphonyl fluoride than trypsin-like serine proteases. PPE and acetylcholinesterase are not inhibited at all.

Stability. Although phosphonyl fluorides are effective serine protease inhibitors, they are often unstable in aqueous conditions. A DMSO solution of the inhibitor Cbz-(4-AmPheGly)^P(OPh)F lost 80% of its inhibitory activity after 1 h at room temperature. To prevent the hydrolysis, the inhibitor was dissolved in dry DMF and stored at 0° C. It was stable for 1 h, during which kinetic measurements were made.[690]

VI. Sulfonylating Agents

A. Sulfonyl Fluorides

Sulfonyl fluorides have been widely studied as inhibitors of serine proteases since their initial discovery by Fahrney and Gold in 1963. Sulfonyl fluorides inhibit most serine proteases such as chymotrypsin,[691−693] trypsin,[691,694] elastase,[695] and complement, coagulation, and fibrinolytic serine proteases.[696] Two well-known sulfonyl fluorides are PMSF (**88**, Figure 151) and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, **89**, Figure 151). Sulfonyl fluorides are not very specific, as PMSF is a general serine protease inhibitor and has also been shown to inhibit the cysteine protease papain at pH 7.[697] The benzenesulfonyl fluoride derivative AEBSF is more stable in physiological medium than PMSF, is commercially available, and is widely used as a broad-spectrum serine protease inhibitor.

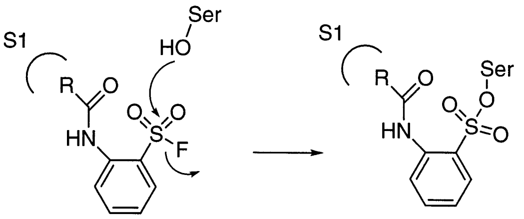


Figure 152. Mechanism of inhibition of serine proteases by sulfonyl fluorides.

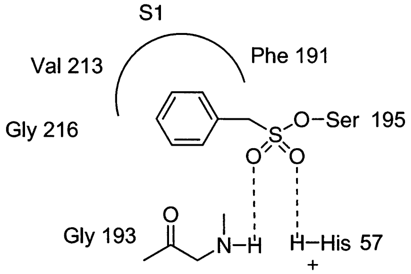


Figure 153. Structure of human chymase inhibited by PMSF (PDB code 1KLT).

Mechanism. Sulfonyl fluorides inhibit serine proteases such as α-chymotrypsin by reacting with the hydroxyl of the active site serine residue Ser 195 (sulfonylation) to form a sulfonyl enzyme derivative (Figure 152), which is stable for long periods of time, except at high pH.[698,699] Benzenesulfonyl fluoride with additional substituents can interact with the S1 subsite, and this increases the specificity and potency of the inhibitor. For example, inhibitors with a fluoroacyl group can interact with the S1 binding site of elastase to increase the inhibitor's specificity.

Crystal Structure. Several sulfonyl enzyme derivatives, such as tosyl chymotrypsin,[700,701] tosyl elastase,[702] and *p*-chloromercuribenzenesulfonyl−chymotrypsin,[703] have been studied by X-ray crystallographic methods. The compounds *p*-toluenesulfonyl fluoride (tosyl fluoride) and *p*-iodobenzenesulfonyl fluoride (pipsyl fluoride) were invaluable aids in the early crystal structure determination of α-chymotrypsin, γ-chymotrypsin, elastase, and subtilisin BPN′.[704−707]

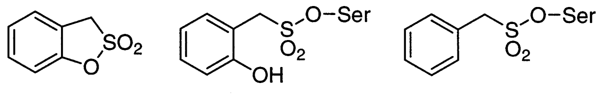
A crystal structure of human chymase treated with PMSF has been determined and shows sulfonylation of the active site Ser 195 with phenylmethanesulfonic acid (Figure 153, PDB code 1KLT).[708] The phenylmethane moiety of the inhibitor is positioned at the mouth of the S1 pocket, which allows the phenyl ring to have some contacts with Phe 191, Val 213, and Gly 216 and the methyl of PMSF to be 3.6 Å from Ser 214. The sulfur is covalently linked to the hydroxyl of Ser 195, and the sulfonyl oxygens interact with the oxyanion hole (backbone amide of Gly 193) and His 57, which is in the *trans*-conformation. The Lys 40 near the S1′ pocket explains the preference of chymase for Asp and Glu in P1′.

Structure–Activity Studies: Serine Proteases. In general, sulfonyl fluorides inhibit HLE and chymotrypsin more rapidly than PPE and cathepsin G, respectively, whereas trypsin is the least reactive of the enzymes. The order of reactivity of simple sulfonyl fluorides for chymotrypsin and trypsin is phenylmethane > 2-phenylethane > benzene > 2-methylpropane > methanesulfonyl fluoride, whereas only the methane and benzene derivatives react with acetylcholinesterase.[691] The specificity of sulfonyl fluoride inhibitors toward elastase, cathepsin G, and chymotrypsin can be improved by structural modifications to the inhibitor.[695] The reactions of various sulfonyl fluorides with trypsin and chymotrypsin have been studied in detail.[692–694,709–712] The compounds *p*-[*p*-(*p*-fluorosulfonylbenzamido)phenoxypropoxy]benzamidine and α-(2-carboxy-4-chlorophenoxy)-*N*-[*m*-(*m*-fluorosulfonylphenylureido)benzyl]acetamide are excellent active-site-directed irreversible inhibitors of trypsin and chymotrypsin, respectively.

Benzenesulfonyl fluorides (arylsulfonyl fluorides) that contain 2-fluoroacyl groups are potent and selective elastase inhibitors.[713] They have also been shown to be chymotrypsin inhibitors.[714] The best HLE inhibitor, 2-(CF_3CF_2CONH)$C_6H_4SO_2F$ ($k_{obs}/[I] = 1700$ M^{-1} s^{-1}, hydrolysis rate $= 1.8 \times 10^3$ s^{-1}), was slightly better than the chloromethyl ketone MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl and selective against cathepsin G (130-fold slower) and chymotrypsin (57-fold slower). The compound 2-(CF_3CONH)$C_6H_4SO_2F$ ($k_{obs}/[I] = 2300$ M^{-1} s^{-1}, hydrolysis rate $= 2.3 \times 10^3$ s^{-1}) was the best PPE inhibitor. The most reactive substituted benzenesulfonyl fluoride for chymotrypsin A_α ($k_{obs}/[I] = 3300$ M^{-1} s^{-1}), rat mast cell proteases I and II (RMCP I, $k_{obs}/[I] = 2500$ M^{-1} s^{-1}, RMCP II $k_{obs}/[I] = 270$ M^{-1} s^{-1}), and human skin chymase ($k_{obs}/[I] = 1800$ M^{-1} s^{-1}) was 2-(Cbz-Gly-NH)-$C_6H_4SO_2F$ (hydrolysis rate $= 3.5 \times 10^3$ s^{-1}).[96,713] Hydrolysis rates and inhibition data were obtained at 30 °C in a pH 7.5 buffer containing 0.1 M Hepes, 0.5 M NaCl, and 10% DMSO.

Unfortunately, arylsulfonyl and arylalkylsulfonyl fluorides, like those discussed in the preceding paragraph, are unstable because they are susceptible to hydrolysis in aqueous media such as buffers.[713,715,716] Approximate half-lives of PMSF in buffer solutions (10 mM buffer and 150 mM NaCl) at 25 °C are 110, 55, and 35 min at pH 7.0 (sodium phosphate), pH 7.5 (Hepes), and pH 8.0 (Tris-HCl), respectively. However, stock solutions of PMSF in 2-propanol are fully stable at room temperature for months.[715]

The compound (*p*-amidinophenyl)methanesulfonyl fluoride (*p*-APMSF) is a specific irreversible inhibitor of plasma serine proteases such as trypsin and thrombin (K_i values between 1 and 2 μM), which prefer positively charged side chains (lysine or arginine), but *p*-APMSF does not inactivate chymotrypsin.[696] The metal-containing derivatives *p*-antimonybenzenesulfonyl fluoride and *p*-mercuribenzenesulfonyl fluoride are as active as PMSF at irreversibly inhibiting chymotrypsin, trypsin, and a chromosomal protease.[717] The compound *N*-(β-pyridylmethyl)-3,4-dichlorophenoxyacetamide-*p*-fluorosulfonylacetanilide bromide (PDFA) is a potent inhibitor of chymotrypsin (second-order rate constant $= 5.41 \times 10^4$ M^{-1} min^{-1}) and a weak irreversible inhibitor of trypsin, pancreatic kallikrein, elastase, and thrombin.[718]



sultone **90** **91** **92**

Figure 154. Structures of a sultone and sulfonyl enzyme complexes.

Several spin label derivatives of benzenesulfonyl fluorides have been used to probe and compare the active site conformations of chymotrypsin and related serine proteases.[719,720] Radiolabeled sulfonyl fluorides have been useful in structural studies of serine proteases such as α-chymotrypsin.[721] Fluorescent labeling of proteins such as chymotrypsin, subtilisin Carlsberg, and trypsin at the active site can be accomplished by reacting them with 5-dimethylaminonaphthalene-1-sulfonyl fluoride (dansyl-fluoride).[722] The active enzyme concentration of subtilisin BPN′ from *Bacillus amyloliquefaciens* has been determined by titration with PMSF.[723]

Biological Studies. PMSF delays the recovery from the crush of peripheral nerves in hens.[724] Systemic PMSF can protect central endorphins from enzymatic destruction. Intraperitoneal injection of PMSF in Sprague–Dawley rats produced dose-dependent analgesia.[725]

Other Sulfonylating Compounds. Nitrophenyl esters of benzenesulfonic acid and phenylmethanesulfonic acid containing various positively charged groups on the benzene ring have been studied as inactivators of trypsin-like proteases.[726] For example, *p*-nitrophenyl *p*-amidinothiomethylbenzenesulfonate inactivates thrombin with a k_2 of 580 M^{-1} min^{-1}.

Sultones, five-membered cyclic sulfonates, are highly reactive toward chymotrypsin.[727] The sultone **90**, *o*-hydroxy-α-toluenesulfonic acid sultone (Figure 154, $K_i = 1.4 \times 10^{-3}$ M), and 2-hydroxy-3,5-dinitro-α-toluenesulfonic acid sultone react with α-chymotrypsin in a rapid, stoichiometric reaction to form catalytically inactive sulfonyl enzymes that decompose slowly over time.[727–729] The 2-hydroxyl group is a structural requirement for the desulfonylation reaction, because a sulfonyl enzyme formed from a compound with a 3-nitro group does not desulfonylate at neutral pH value. The sultones sulfonylate the active site serine hydroxyl (Ser 195) in a similar fashion as do the sulfonyl fluorides.[727,730] The only difference between the structures of the sulfonyl enzymes resulting from the reaction with the sultone (**91**, Figure 154) or PMSF (**92**, Figure 154) is the presence of the 2-hydroxyl group.

VII. Miscellaneous Inhibitors

Miscellaneous Acylating Agents. Sulfonate salts of amino acids are irreversible inhibitors of serine proteases.[731] For example, the sulfonate salt of norvaline (Figure 155, *N*-(sulfocarbonyl)-L-norvaline 1-methyl ester, inhibits HLE with a k_2/K_i of 860 M^{-1} s^{-1}.

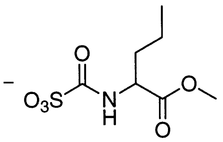


Figure 155. Sulfonate salt of the methyl ester of L-norvaline.

VIII. Summary and Perspectives

Very effective and specific irreversible inhibitors are now available for many serine, cysteine, and threonine proteases. Some of the irreversible inhibition rates are close to diffusion controlled. Particularly noteworthy examples of highly reactive inhibitors are shown in Table 71. It is clear that high inhibition rates can be obtained with a variety of warhead groups.

Inhibitor Design. A wide variety of approaches has been utilized in the design of potent inhibitors for serine, cysteine, and threonine proteases. The most common approach is the attachment of a warhead (alkylating, acylating, phosphonylating, or sulfonylating functional group) to the appropriate peptide sequence or other binding/recognition structure. Occasionally, library screening is used to discover new inhibitors, although this approach is less common with irreversible inhibitors. Once an inhibitor is developed for a protease, extensive modifications are often made to the inhibitor structure to improve the specificity and potency of the inactivator. Very frequently, a large number of crystal structures of enzyme−inhibitor complexes are determined, and molecular modeling is used to guide the design work. Clearly, most of the important warhead functional groups have already been described in the literature. However, there are still new classes of inhibitor structures that remain to be discovered. In addition, systematic modification of current inhibitor structures offers many opportunities for improving inhibitor potency and selectivity. One current trend is the replacement of the peptide backbone of peptide inhibitors with peptide mimics. These so-called peptidomimetics often have improved bioavailability and stability in biological systems.

One particularly noteworthy group of inhibitors are the double-hit inhibitors. These inhibitors are mechanism-based or suicide inhibitors that react with two active site nucleophiles. In the case of serine proteases, double-hit inhibitors usually react both with the active site serine and with histidine. Examples of double-hit inhibitors include isocoumarins, β-lactams, and saccharins as heterocyclic inhibitors for serine proteases, and epoxomicin, a natural product inhibitor of the proteasome that covalently links both the amino group and the hydroxyl group of Thr 1. It is clear that there are many opportunities for the design of new double-hit inhibitors containing a variety of reactive groups concealed in heterocyclic structures.

Structural and kinetic studies have frequently led to interesting insights into inhibition mechanisms. For example, it is clear that many heterocyclic acylating agents of serine proteases undergo conformational changes during the inhibition reaction to produce stable acyl enzyme derivatives, which are improperly oriented for a rapid deacylation. Usually, the active site histidine is pushed into an out conformation and the acyl carbonyl group is twisted out of the oxyanion hole. As a result, the acyl enzyme is in an incorrect orientation for deacylation. Several examples have been observed with β-lactams (Figure 94) and isocoumarin inhibitors of serine proteases. The same inhibitor structure can also give different products with different proteases. For example, the heterocyclic inhibitor 4-chloro-3-ethoxy-7-guanidinoisocoumarin gives orthogonal binding modes with trypsin and porcine pancreatic elastase.

Many irreversible inhibitor designs utilize the S′ subsite to obtain additional potency or specificity. Examples of inhibitors that use the S′ subsites are epoxysuccinates, vinyl sulfones, β-lactams, and acyloxymethyl ketones. For example, in the case of acyloxymethyl ketones the pyrazolyloxy leaving group at P1′ in the inhibitor structure appears to give the acyloxymethyl ketone inhibitors specificity for caspase-1 versus other cysteine proteases such as cathepsin B and calpain. A few of the newer inhibitors such as extended epoxysuccinate inhibitors make extensive use of both S and S′ subsites. In the future, we would expect to see increasing use of S′ subsites and distant S subsites in inhibitor design. Related proteases often have considerable differences in these regions.

Protease Clan and Family Differences. There are significant differences in the active site structures of serine, cysteine, and threonine proteases that are useful in designing specific inhibitors. In particular, there are differences in the oxyanion hole between serine and cysteine proteases that affect inhibitor reactivity. For example, phosphonates require a rigid oxyanion hole and are moderate inhibitors of serine proteases but do not inhibit cysteine or threonine

Table 71. Potent Inhibitors of Serine, Cysteine, and Threonine Proteases

enzyme	inhibitor	rate (M^{-1} s^{-1})
thrombin	D-Phe-Pro-Arg-CH_2Cl	9600000
cathepsin B	Cbz-Phe-Ala-CH_2OCO-2,6-$(CF_3)_2$-Ph	1600000
cathepsin L	Cbz-Phe-Cys(Bzl)-CH_2OCO-2,6-$(CF_3)_2$-Ph	10700000
caspase-1	$PhCH_2CH_2CO$-Val-Ala-Asp-CH_2OCO-2,6-$(Me_2)_2$-Ph	1200000
cathepsin L	HO-(2*S*,3*S*)-Eps-Phe-NHBzl	27800000
cathepsin B	MeO-Gly←Gly←Leu←(2*S*,3*S*)-Eps-Leu-Pro-OH	1520000
rhinovirus 3C protease	Michael acceptor AG7088	1470000
cruzain	Cbz-Phe-Hph-VS-ONp	6500000
cruzain	Cbz-Phe-Hph-VS-OPh	16800000
cathepsin S	Mu-Np2-Hph-VS-2Np	56000000
cathepsin L	Cbz-Phe-Lys-NHO-Nbz	3540000

proteases. The active site histidine acts as a general base in serine proteases with the serine uncharged. In cysteine proteases the histidine is usually protonated and interacts with the negative thiolate of the active site cysteine. This difference in the protonation state of the active site histidine explains why vinyl sulfones are specific for cysteine proteases and do not react with serine proteases. The vinyl sulfones require protonation by the histidine in the inhibition mechanism. Another example of a class of inhibitors with different reactivity toward serine and cysteine proteases is peptide *N*,*O*-diacylhydroxamates. The poorly nucleophilic serine in serine proteases reacts to form a carbamate via a Lossen rearrangement. The more nucleophilic cysteine of cysteine protease yields products formed by a direct nucleophilic displacement. Unlike serine and cysteine proteases, the proteasome has a totally different active site structure involving Thr 1 and reacts with only a few of the many irreversible inhibitors described in the literature.

In general, all peptide inhibitors bind to serine proteases, cysteine proteases, and the proteasome in an extended conformation. However the protease structure that recognizes this extended peptide can be quite different. For example, serine proteases and caspases form an antiparallel β-sheet structure with the inhibitor, whereas the peptide binding site is a deep canyon in clan CA proteases (papain family). Individual proteases will have significant structural variation in the extended substrate binding sites, especially in subsites away from the active site residues. Some proteases can accommodate quite unusual side chains in their binding pockets. For example, thrombin accommodates unusual non-peptide-like side chains in S1, whereas other coagulation enzymes are much less tolerant. Frequently, different binding modes are observed with different inhibitors bound to the same enzyme. For example, subtilisin BPN′ prefers aromatic residues at P1 but will accommodate a Lys side chain at P1 by interacting with the methylene side chain of Lys in S1 and the amino group of Lys on the surface of the enzyme.

One of the major challenges for the future is the design of inhibitor structures and warhead groups to utilize the unusual combinations of catalytic groups and new protease folds that are being discovered in recently described proteases. Along with the proteasome, there are a number of new protease folds with differing active site residues that offer a multitude of opportunities for inhibitor design and mechanistic studies. It appears that serine proteases can use a Ser-Lys diad, a Ser-His-Asn triad, a Ser-His-Glu triad, and a Ser-His-His triad in addition to the classic Ser-His-Asp triad. In contrast, the proteasome uses a Thr hydroxyl-α-amino group diad. Another interesting class of enzymes is the serine carboxyl peptidase group, which may be a mechanistic cross between serine proteases and aspartate proteases. With such a variety of unique active site residues, it is likely that future researchers will be able to use considerable creativity in developing interesting and novel mechanism-based inhibitor structures for these new classes of proteases.

Protease Biological Function and Functional Proteonomics. Protease inhibitors are used by many scientists to elucidate the role of various proteases in models of biological function. Table 72 lists synthetic protease inhibitors that are widely available. Frequently, the inhibitors used in biological studies do not have the expected specificity or selectivity, which has led to many unwarranted conclusions in the literature. The specificity of many new inhibitors is usually not examined with many other proteases, and yet the inhibitor is often claimed to be “specific”. For example, the fluoromethyl ketone Asp-Glu-Val-Asp-CH_2F, frequently used as a caspase-specific inhibitor in apoptosis models, inhibits calpains I and II more effectively than most caspases. This fluoromethyl ketone is also an excellent inhibitor of many cathepsins including cathepsin B. Thus, it would be wise to further test the specificity of new inhibitors or widely used inhibitors with a greater library of potential protease targets before using them in biological studies.

One advantage of irreversible inhibitors is their irreversibility. Once the target enzyme is killed, it cannot usually be reactivated and the organism must resynthesize the enzyme. The inhibitor will usually stay covalently bound to the inhibited enzyme until the enzyme is degraded into its constituent pieces. If the protease inhibitor has an attached fluorophore or biotin molecule, the inhibitor can be used to localize various proteases in cells and tissues. Bogyo and co-workers have made several particularly elegant uses of radiolabeled and fluorescently labeled irreversible cysteine protease inhibitors in the functional proteomic profiling of cysteine proteases in cancer, malaria, and cataract formation.[732] Irreversible inhibitors clearly have a distinct advantage in proteomic profiling and, in the future, it is likely that libraries of related reagents will be developed for other classes of proteases.

Therapeutic Potential of Irreversible Inhibitors. Although there is great potential for the use of irreversible protease inhibitors for therapy, no such compounds have reached the market as drugs. Several reversible protease inhibitors have reached the market, most notably angiotensin converting enzyme inhibitors for the treatment of hypertension and HIV protease inhibitors for the treatment of AIDS. A strong bias against irreversible inhibitors exists in the pharmaceutical industry, and it is an uphill battle to get irreversible inhibitors considered as potential clinical candidates. Even though widely used drugs, such as aspirin and β-lactam antibiotics, are irreversible enzyme inhibitors, the lore in the pharmaceutical industry suggests that irreversible inhibitors will cause immune disorders upon long-term use. Many reversible protease inhibitors have also failed in clinical trials due to poor bioavailability or various toxicities. Notable examples are trifluoromethyl ketone elastase inhibitors for the treatment of emphysema and benzamidine-based inhibitors of the mast cell tryptase for the treatment of asthma. Thus, each new protease inhibitor should be considered on its individual merits (potency, selectivity, bioavailability, etc.) as a potential drug, rather than be

Table 72. Commercially Available Synthetic and Low Molecular Weight Irreversible Protease Inhibitors

inhibitor (source)[a]
chloromethyl ketones with an acidic P1 residue
Ac-Tyr-Val-Ala-Asp-CH_2Cl (ms), Boc-Asp(OBzl)-CH_2Cl (b), Cbz-Ala-Ala-Asp-CH_2Cl (a), Cbz-Ala-Ala-Asp(OMe)-CH_2Cl (e), Cbz-Tyr-Val-Ala-Asp-CH_2Cl (b), Ac-Leu-Glu(OMe)-His-Asp-CH_2Cl (b), Cbz-Asp-Glu-Val-Asp-CH_2Cl (b), biotinyl-Tyr-Val-Ala-Asp-CH_2Cl (a, b), biotinyl-Ala-Ala-Asp(OMe)-CH_2Cl (e)
chloromethyl ketones with a basic P1 residue
Tos-Lys-CH_2Cl (TLCK, ms), Gly-Arg-CH_2Cl (e), D-Val-Phe-Lys-CH_2Cl (c, e), Glu-Gly-Arg-CH_2Cl (b, c, e), Cbz-Gly-Gly-Phe-CH_2Cl (b), 1,5-dansyl-Glu-Gly-Arg-CH_2Cl (c), Phe-Pro-Arg-CH_2Cl (e), D-Tyr-Pro-Arg-CH_2Cl (b), D-Phe-Phe-Arg-CH_2Cl (b), D-Phe-Pro-Arg-CH_2Cl (PPACK, a, b, c), decanoyl-Arg-Val-Lys-Arg-CH_2Cl (a, b), biotinyl-PPACK (b, c), biotinyl-Glu-Gly-Arg-CH_2Cl (c), D-Val-Leu-Lys-CH_2Cl (b)
chloromethyl ketones with a neutral P1 residue
Tos-Phe-CH_2Cl (TPCK, ms), Leu-CH_2Cl (b, e), Phe-CH_2Cl (b, e), Cbz-Leu-CH_2Cl (b), Cbz-Phe-CH_2Cl (b), Cbz-Phe-Ala-CH_2Cl (b, e), Cbz-Phe-Phe-CH_2Cl (e), Cbz-Leu-Tyr-CH_2Cl (b), Ala-Ala-Phe-CH_2Cl (b, c, e), Cbz-Ala-Pro-Phe-CH_2Cl (c), Cbz-Gly-Leu-Phe-CH_2Cl (e), Ala-Ala-Pro-Val-CH_2Cl (e), MeO-Suc-Ala-Ala-Pro-Ala-CH_2Cl (b, c, e), MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl (b, c), MeO-Suc-Ala-Pro-Pro-Val-CH_2Cl (c), MeO-Suc-Phe-Gly-Ala-Leu-CH_2Cl (e), Suc-Ala-Ala-Pro-Phe-CH_2Cl (e), Mu-Ala-Ala-Pro-Val-CH_2Cl (e), Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Gly-CH_2Cl (b), biotinyl-Phe-CH_2Cl (e), biotinyl-Ala-Ala-Phe-CH_2Cl (e), biotinyl-Ala-Ala-Pro-Phe-CH_2Cl (e)
fluoromethyl ketones with an acidic P1 residue
Cbz-Val-Ala-Asp(OMe)-CH_2F (a, b, bm, c, e), Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (b, c, e), Boc-Asp(OMe)-CH_2F (c, e), Cbz-Tyr-Val-Ala-Asp-CH_2F (b), Cbz-Tyr-Val-Ala-Asp(OMe)-CH_2F (c, e), Cbz-Trp-Glu(OMe)-His-Asp(OMe)-CH_2F (a, c, e), Cbz-Val-Glu(OMe)-Ile-Asp(OMe)-CH_2F (c, e), Cbz-Ile-Glu(OMe)-Thr-Asp(OMe)-CH_2F (c, e), Cbz-Leu-Glu(OMe)-His-Asp(OMe)-CH_2F (c, e), Cbz-Leu-Glu(OMe)-Thr-Asp(OMe)-CH_2F (a, e), Cbz-Asp(OMe)-Gln-Met-Asp(OMe)-CH_2F (a, e), Cbz-Leu-Glu(OMe)-Val-Asp(OMe)-CH_2F (a, e), Cbz-Leu-Glu(OMe)-Glu(OMe)-Asp(OMe)-CH_2F (a, e), Cbz-Ala-Ser-Thr-Asp(OMe)-CH_2F (e), Cbz-Ala-Glu(OMe)-Val-Asp(OMe)-CH_2F (a, c, e), Cbz-Val-Asp(OMe)-Val-Ala-Asp(OMe)-CH_2F (e), biotinyl-Val-Ala-Asp(OMe)-CH_2F (e), Cbz-Val-Lys(biotinyl)Asp(OMe)-CH_2F (e), biotinyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-CH_2F (e)
fluoromethyl ketones with a neutral P1 residue
Cbz-Phe-Ala-CH_2F (c, e), Mu-Val-Hph-CH_2F (c, e), Mu-Phe-Hph-CH_2F (e), Cbz-Phe-Phe-CH_2F (c, e), Cbz-Leu-Leu-Tyr-CH_2F (c, e), Cbz-Leu-Leu-Leu-CH_2F (e), biotinyl-Phe-Ala-CH_2F (e)
bromomethyl ketones
Cbz-Asp(OtBu)-CH_2Br (b)
diazomethyl ketones
Gly-Phe-CHN_2 (e), Fmoc-Tyr-Ala-CHN_2 (b), Cbz-Phe-Tyr(*t*Bu)-CHN_2 (b, c), Cbz-Phe-Phe-CHN_2 (b, e), Cbz-Phe-Ala-CHN_2 (e), Cbz-Phe-Pro-CHN_2 (e), Cbz-Tyr-Ala-CHN_2 (e), Cbz-Leu-Leu-Tyr-CHN_2 (e), Cbz-Val-Val-Nle-CHN_2 (b)
acyloxymethyl ketones
Cbz-Asp-CH_2−2,6-Cl_2−Ph (a, b, bm, pi), Ac-Tyr-Val-Ala-Asp-CH_2-2,6-Me_2-Ph (b), Ac-Tyr-Val-Lys(biotinyl)-Asp-CH_2-2,6-Me_2-Ph (b), Cbz-Glu-Lys(biotinyl)-Asp-CH_2-2,6-Me_2-Ph (pi), Cbz-Phe-Lys-2,4,6-Me_3-Ph(b)
epoxides
E-64 (ms), E-64c (b, bm, i, p), E-64d (b), CA-074 (ms), CA-074Me (ms), epoxomicin (ms)
vinyl sulfones
4-hydroxy-5-iodo-3-nitrophenylacetyl-Leu-Leu-Leu-VS (NLVS, c), 4-hydroxy-3-nitrophenylacetyl-Leu-Leu-Leu-VS (NP-L_3VS, c)
acyl hydroxamates
Boc-Ala-Ala-NHO-Bz (c), Boc-Gly-Phe-NHO-Bz (c), Boc-Val-Phe-NHO-Bz-*p*Cl (c), Cbz-Phe-Gly-NHO-Bz (c), Cbz-Phe-Gly-NHO-Bz-*p*Me (c), Cbz-Phe-Gly-NHO-Bz-*p*OMe (c), Boc-Pro-Phe-NHO-*p*Cl (c), Boc-Ala-Ala-Ala-NHO-Bz (c), Boc-Ala-Pro-Phe-NHO-Bz (c)
β-lactams
lactacystin (ms)
isocoumarins
3,4-dichloroisocoumarin (DCI, bm, i)
sulfonyl fluorides
phenylmethanesulfonyl fluoride (PMSF, ms), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, ms)

[a] Sources: a, Alexis Corp. (Carlsbad, CA); b, Bachem (King of Prussia, PA; Torrance, CA); bm, Biomolecular Research Laboratory Inc. (Plymouth Meeting, PA); bo, Boston Biochemistry (Cambridge, MA); c, Calbiochem-Novabiochem AG (Laufelfingen, Switzerland); cn, CN Biosciences, Inc. (Darmstadt, Germany); e, Enzyme Systems Products (Livermore, CA); i, ICN Biomedicals, Inc. (Costa Mesa, CA); p, Peptides International, Inc. (Louisville, KY); pi, Peptide Institute (Osaka, Japan); ms, many sources.

summarily excluded due to its mechanism of inhibition.

Notable examples of irreversible inhibitors that have been tested for possible therapeutic use include β-lactams and saccharins as inhibitors of neutrophil elastase for the treatment of emphysema and the epoxysuccinate E-64 for the treatment of muscular dystrophy. The epoxysuccinate E-64c is active in animal models of muscular dystrophy, but was not orally active, whereas the corresponding ethyl ester E-64d has oral activity in humans. A number of other bioavailable or orally active inhibitors are now available including benzoxazinones and saccharins, such as WIN 63759 as inhibitors for elastase and viral proteases and vinyl sulfones. Several inhibitors are undergoing clinical trials, including a γ-lactam inhibitor that is being tested in trials for inflammatory disorders. For therapeutic use, some inhibitors may be more useful than others. Fluoromethyl ketones, for example, may exhibit toxicity due to their degradation to fluoroacetate, whereas other classes may not have similar problems. In one case, a peptide fluoroketone was toxic after 3 days (calcipain) in vivo but the corresponding vinyl sulfone was not.

Use of irreversible inhibitors for short-term therapeutic administration might raise fewer red flags. Thus, bacterial, viral, and parasitic diseases are one area in which irreversible protease inhibitors could have great potential.[733] In diseases such as malaria, Chagas' disease, sleeping sickness, malaria, leishmaniasis, and rhinovirus infections, cysteine proteases such as cruzain, rhodesain, falcipain, cpL and cpB, and the rhinovirus 3C protease are essential. In addition, investigators are beginning to examine the possibility of treating periodontal disease with inhibitors of gingipain and related bacterial enzymes. With these diseases, it may be necessary to treat with the drug for only a short period of time to cure the disease. If treatment with an irreversible inhibitor is limited to a few days or a few weeks, then there would be little concern about immunological problems. In the case of long-term treatment with irreversible inhibitors in diseases such as arthritis, then immunological problems could be a real concern in some patients. Currently, vinyl sulfone inhibitors of cruzain for the treatment of Chagas' disease and vinyl sulfone inhibitors of the rhinovirus for the treatment of the common cold are undergoing clinical trials. The 3C protease inhibitors, such as AG7088, have good oral bioavailability. It is likely that irreversible protease inhibitors will be used in the future for the treatment of bacterial, viral, and parasitic diseases.

If the goal is the development of potential drugs, high enzyme inhibition rates are not sufficient. It is frequently the case that potent inhibitor structures have very poor bioavailability. For example, SAR studies with vinyl sulfones produced very potent inhibitors for the rhinovirus protease, but many compounds had very poor bioactivity. In general, it is necessary to minimize the number of hydrogen bond donors and acceptors and charges to obtain good bioavailability.[734] In inhibitor design, there is always a delicate balance between reaching for distant subsites and trying to keep the size of the inhibitor within a reasonable molecular weight range. Navia showed that orally bioavailable drugs have a roughly Gaussian distribution of molecular weight between 150 and 550 Da, with oral bioavailability quite low in compounds beyond 550 Da.[735] Thus, the challenge of the medicinal chemist is always to keep the molecule small while maintaining potency and specificity. It is likely that increasing use of biological assays, along with enzyme assays, will be made in the early states of a drug development program to assay for bioavailability.

Finally, a novel use of irreversible inhibitors for the serine protease thrombin has led to an interesting use of an enzyme−inhibitor complex. Thrombin can be acylated by *trans*-cinnamoyl active esters to give stable acyl enzyme derivatives.[736] If the cinnamoyl group has a *o*-hydroxy group, then it can easily be deacylated photochemically regenerating active thrombin. This led to the concept of "surgical glue", which is being developed by Porter and co-workers. The acyl thrombin derivative can be applied to any desired surgical site and then activated with a laser. Thrombin then generates fibrin, which "glues" the tissue together. Surgical glue is currently being developed by an Atlanta biotechnology company.

In the future, we expect to see many novel irreversible inhibitor structures developed for serine, cysteine, and threonine proteases, along with many new inhibitor applications. In addition, there are hopeful signs that irreversible inhibitors will soon be used for the treatment of some disease states.

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X. Note Added in Proof

Since the acceptance of this review, further pyrrolidine *trans*-lactams have been discovered as inhibitors of human neutrophil elastase.[765]

The structure of human cathepsin F with the irreversible vinyl sulfone inhibitor, 4-morpholin-4-yl-piperidine-CO-Phe-Nva-VS-Ph, was determined by Somoza et al.[766] New aza-peptide inhibitors for papain, cathepsin B, and cathepsin K were designed on the basis of the Z-Arg-Leu-Val-AGly sequence.[767] Aza-peptide epoxides, a new class of irreversible inhibitors, are potent and selective for clan CD of cysteine proteases such as caspases and legumain.[768]

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