Chapter 27. The Inhibition of Proteolytic Enzymes

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This review will cover critically but not exhaustively the principal methods for efficient inhibition of proteolytic enzymes. Although the main emphasis is on serine proteases,[1-4] other proteases and other types of enzymes will be discussed to illustrate specific points. Much of the work over the years on enzyme inhibition has been purely theoretical, but the use of organophosphorus compounds (anticholinesterases) in insect control is well known, and the potential for specific enzyme inhibitors in medicine is becoming increasingly apparent.[5,6]

Concepts of Specific Enzyme Inhibition - An enzyme is specifically inhibited when its active site is blocked physically and/or chemically without significant alteration of the rest of the molecule. For the most part the kinetic complexities of inhibition[7,8] will not be dealt with. The emphasis is on various types of molecular interactions with active sites. Inhibitors may be categorized by the way they interact with active sites, but such categories may overlap and may not be assignable without considerable investigation. A broad division may be made between inhibitors that react chemically and those that interact only physically with an active site. Both groups include inhibitors that are for practical purposes either reversible or irreversible. It is a major thesis of this review that the efficiency of inhibition is directly related to the degree and correctness of binding for both reversible and irreversible inhibitors. As in the past, new types and examples of inhibitors will doubtless be discovered by accident, but as chemists learn more about the stereochemistry and reactivity of active site constituents, they will be increasingly successful in designing potent and selective inhibitors. It is really a question of exploiting the organic and physical chemistry of enzymes. The position has recently been taken that transition state inhibitors "are not likely to succeed in clinical utility" because they would "bind to the active site of the enzyme, i.e., where the enzymes from different cellular species show the greatest similarity or even identity."[9] Clinical utility is a very complex matter, but as will be seen, considerable selectivity in the inhibition of similar enzymes has already been achieved, and more can be expected from basic knowledge of the stereochemistry and mechanisms of action of active sites.

Substrates and Reversible Inhibitors - In order to design efficient and specific inhibitors of any type, it is necessary to know as much as possible about the mechanism of action and specificity of the enzyme and about the secondary binding sites, if any, near its active site. A classical method involves testing various substances as substrates, exemplified by the work of Bergmann's school on the specificity of proteolytic enzymes using small peptide substrates.[10] In a refinement of this method, oligopeptide substrates, sometimes containing D-amino acids, have been used to map the specificity and subsites of papain,[11,12] carboxypeptidase A,[13] and elastase.[14,15] Differences in the binding sites of

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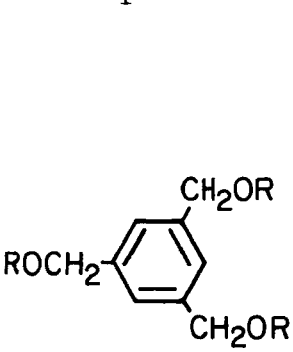
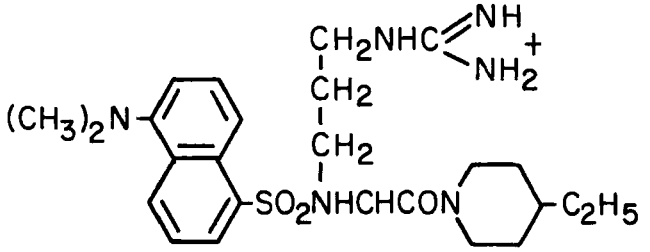
chymotrypsin, subtilisin Novo, and subtilisin Carlsberg were delineated by use of oligopeptide inhibitors.[16] Recently some oligopeptide inhibitors were developed for elastase in a search for medicinally useful compounds.[6] One of the most effective inhibitors was acetyl-Ala-Ala-Pro-Mec-Lac-$NHCH_3$ (Mec = 2-methylcarbazic acid; Lac = lactic acid), which has a K_i of 0.65 μM with pancreatic elastase.[6]

Much work has been done using simple molecules as reversible inhibitors to characterize binding sites of proteases. Some outstanding examples are the binding of amidines and guanidines to trypsin[17] and the inhibition (K_i's in the micromolar range) of trypsin, thrombin, and plasmin by aromatic amidines with ether functions[18] and by bis-amidines,[19] and tris-amidines for trypsinlike enzymes.[20] In the latter study, α,α',α''-tris(4-amidino-2-bromophenoxy)mesitylene (1) was found to inhibit pancreatic kallikrein with a K_i of 0.0243 μM, while α,α',α''-tris(3-amidinophenoxy)mesitylene was the best thrombin inhibitor with a K_i of 0.651 μM, and was a strong anticoagulant. Derivatives of dansylarginine are very good reversible thrombin inhibitors. One of the best is N^{α}-dansyl-L-arginine-4-ethylpiperidide (2; OM 205), which inhibits thrombin with an I_{50} of 0.03 μM but has little effect on plasmin (I_{50} 1 mM).[21] A well known reversible inhibitor that behaves almost irreversibly is methotrexate, which has a K_i of about 10^{-11} M in the inhibition of dihydrofolate reductase.[22]



1: R = 4-amidino-2-bromophenyl



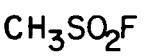
2 (OM 205)

X-ray crystallographic studies of proteases have yielded fascinating results on the mode of binding of polypeptides, substrates, and inhibitors to the active site regions of chymotrypsin, trypsin, elastase, and subtilisin.[1-4,23-25] Since it is now relatively easy to construct a useful model (compatible with the widely used CPK models) of the active site of chymotrypsin,[26] and since models of the active sites of any enzymes for which x-ray data are available can be similarly constructed, the interactions of substrates and inhibitors with enzymes can be readily visualized. Study of these models should stimulate ideas for the design of effective inhibitors.

Affinity Labeling - In 1961 the idea of affinity labeling arose independently in four laboratories, where it was applied to glutamic dehydrogenase,[27] a hapten-antibody system,[28] and chymotrypsin.[29,30] This concept has proved very useful, and a vast literature on it has

evolved.[31-36] The basic principle is that with knowledge of the specificity of the active site of an enzyme (or any other biological binding site) it is possible to design reagents which have both a group that binds specifically to the active site and a group that can react chemically with a constituent of (or group near) this site. Such reagents have been used frequently to identify groups in or near active sites and can be used to inactivate specifically one enzyme in a mixture.[37] In the design of affinity labeling agents a knowledge of techniques for chemical modification of proteins is essential.[38-40]

There are other reagents, besides affinity labeling agents, that react very specifically with an active site constituent on the basis of chemical reactivity. With the latter the reaction rate is often greatly accelerated by the concentration effect of the binding step, which may be evaluated by a Kitz-Wilson plot[41] from which a maximum rate constant (k_{max}) and a binding constant (K_m) can be obtained. Thus methanesulfonyl fluoride (3) irreversibly inactivates acetylcholinesterase without binding physically to the enzyme, while (3-hydroxyphenyl)trimethyl-ammonium iodide methanesulfonate (4) binds to the site before chemically reacting with it because of its quaternary ammonium group.[41] Compound 4



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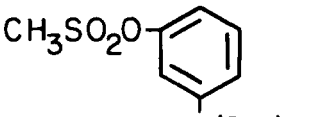

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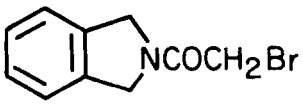
is therefore an affinity labeling agent while 3 is not. It is not commonly appreciated that correct binding of an affinity labeling agent may be of paramount importance for rapid reaction with an active site constituent. Table 1 shows kinetic constants for the inactivation of chymotrypsin by three α-bromoamides at pH 5. N-Bromoacetylisoindoline (5) and N-bromoacetylbenzylamine (6) are both affinity labeling agents that



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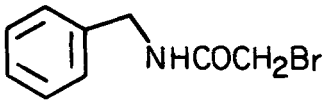

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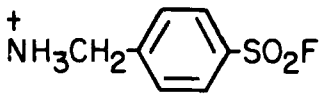
Table 1

Compound	10^4 k_{max}	10^3 K_m	10^4 k_2	$V_{\frac{1}{2}}/V_2$
5	5880	4.0	-	13×10^3
6	12.5	6.7	-	15
7	-	-	57.5	-

alkylate Met 192 near the active site but at vastly different rates, which depend upon neither the reactivity of the bromine nor the binding constant (K_m). In the last column of the table, their half-maximal

reaction rates are very roughly compared with the second order rate of inactivation by α-bromoacetamide (7), which does not show binding behavior. The difference in rate between 5 and 6 is due to the fact that the amide group is coplanar with the ring system in 5 and not in 6. Such conformational differences in these and other[42] site-specific α-bromoamides have been correlated[43] with the stereochemistry of the active site of chymotrypsin by use of the model mentioned above.[26] With the benzene ring of the coplanar α-bromoamide 5 in the binding site, the sulfur atom of Met 192 is in the right position for an attack on the back side of the CH_2Br group. Compound 6 does not fit correctly for an easy attack by Met 192.

Although α-haloamides[44,45] and α-haloketones[32] are very useful for studying active site constituents in vitro, they would probably not be of much use in vivo because they react rapidly with sulfhydryl groups. But the sulfonyl fluorides may be promising in vivo,[46,47] and they are much less toxic than organophosphates. For example, the oral LD_{50} in mice of the irreversible thrombin inhibitor, 4-aminomethylbenzenesulfonyl fluoride (8), is 1070 mg/kg, while that of diethyl 4-nitrophenyl phosphate is 8.0 mg/kg.[47] Diazomethyl ketones have recently been reinvestigated with cathepsin B_1 as affinity labeling agents that do not react



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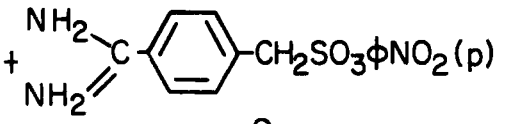

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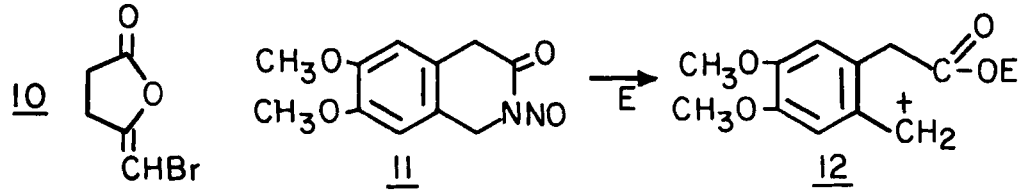
with sulfhydryl groups.[48] Although p-nitrophenyl esters might also not be desirable in vivo, p-nitrophenyl p'-amidinophenylmethanesulfonate (9) is worth consideration because of its high selectivity. It binds well to thrombin, trypsin, plasmin, chymotrypsin, and plasma kallikrein, but of these it irreversibly inactivates only thrombin.[49] The meta amidino analog is only a competitive inhibitor of these enzymes.[49] Further, the ortho and meta nitro analogs of 9 show much less selectivity: they irreversibly inactivate trypsin and kallikrein in addition to thrombin but do not affect plasmin.[50]

Occasionally there are reports that an enzyme is not inactivated by an inhibitor that might be expected to be effective. In such cases it is best to vary the reaction conditions and try other inhibitors of the same type. Urokinase is inhibited by DFP but not by tosyl-L-lysine chloromethyl ketone (TLCK),[51] which would be unexpected for a trypsin-like serine protease. The authors wisely did not, as some might, ascribe the lack of reactivity of urokinase toward TLCK to lack of a histidine residue at its active site. Rather they pointed to a difference between the active site of urokinase and that of other trypsinlike enzymes. It was later found that the peptide chloromethyl ketones acetyl-Gly-Lys-CH_2Cl and norleucyl-Gly-Lys-CH_2Cl inactivate urokinase by labeling a histidine residue at the active site.[52] In another case it was erroneously concluded that thrombin has no essential histidine at its active site from the fact that it was not activated by tosyl-L-phenylalanine chloromethyl

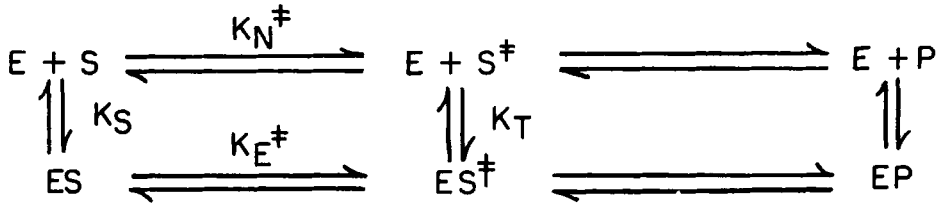
ketone (TPCK) and from the belief that TPCK is a reagent for histidine residues.[53] Actually TPCK is not specific for histidine, but it is specific for chymotrypsinlike enzymes. The same group later found that TLCK inhibits bovine thrombin,[54] a result consistent with the known specificity of thrombin.

Suicide Inhibitors - A fascinating new group of irreversible enzyme inhibitors developed in recent years are called k_{cat} or suicide inhibitors.[35,55,56] Such an inhibitor is basically an affinity labeling agent with a masked active group that can be activated by the target enzyme's active site mechanism. There is considerable promise in this type of inhibitor because the potentially reactive group may be rather innocuous in a mixture of enzymes or in vivo until the inhibitor reaches and is activated by the target enzyme.

Practically all of the work done with suicide inhibitors has been on nonproteolytic enzymes, especially pyridoxal- and flavin-dependent enzymes, and much of the recent work is reviewed in the preceding chapter of this volume by Jung. While it has been suggested that a bromolactone of the general type 10 could be a suicide inhibitor of chymotrypsin because the acyl enzyme would rearrange to an α-bromoketone,[55] no experimental work on this has appeared. Chymotrypsin has been irreversibly inactivated by the nitrosoamide 11. The acyl enzyme from 11 would be expected to convert to the carbonium ion 12, which can alkylate one or more groups in or around the active site, but the place(s) of attack has not been established.[57]



Transition State Inhibitors - These inhibitors have been developed mainly in the last few years.[58-60] Here the term transition state inhibitor is used to describe the inhibitor itself and transition state analog to describe the enzyme-inhibitor complex. Such inhibitors were proposed in 1948 by Pauling,[61] and were discussed prior to their current popularity by Jencks.[62] The rationale is based on the theory that the transition state of a substance undergoing enzymatic reaction should be more tightly bound to an enzyme than the initially bound substrate. While the formation of a normal transition state accelerates an enzymic reaction, the formation of an abnormal one should lead to inhibition. The following scheme applies,[58] where the upper line shows the nonenzymatic route and



the lower line the enzymatic route. The binding constants for these inhibitors can theoretically be much better than 10^{-10} M.[60] It has been stated that "it appears meaningless to distinguish between 'binding sites' and 'catalytic sites' since catalysis requires an enhanced degree of binding,"[59] but examples will be given to show that the primary and secondary specificity of binding (K_S) is related to the final degree of binding (K_T). Sometimes this selectivity shows up in the reactivity (k_{cat}) of a substrate rather than in K_S,[60] but whatever the exact kinetic situation, high specificity is necessary for the design of good inhibitors for particular enzymes. The lack of emphasis on K_S sometimes seen in the literature may be due to misinterpretation of the importance of increased binding in the transition state ($K_E\dagger$). The strain that is induced in a substrate in going from the Michaelis complex ES to the transition state ES† reduces the activation energy of the ensuing reaction but also results in an observed K_S less favorable than would be possible for an optimum fit.[62] In no way do these considerations reduce the importance of primary binding. In the above scheme K_S represents the initial binding of substrate that is enhanced in the second step ($K_E\dagger$); K_T represents the idealized binding of the substrate's transition state to a complementary conformation of the enzyme's active site, and it should be a much better binding constant than K_S. To the extent that a transition state analog approaches the true transition state, the binding constant should approach K_T.

The enhancement in binding of some transition state inhibitors has been less than predicted by theory, and this has been attributed to the imperfect nature of the inhibitors.[63,64] Changes in entropy have also been invoked to account for such binding.[64] It seems likely, however, that the less-than-expected binding "is probably largely due to the imperfect nature of the analogs rather than to the incorrectness of the theory."[63] This view not only seems reasonable but also provides a stimulus to design more nearly perfect inhibitors.

Very tight binding is characteristic of transition state inhibitors and must be measured with care.[8] But such binding in itself may be due to factors other than the formation of a transition state analog, which has to be determined in individual cases.[60] For example, the tight binding of methotrexate to dihydrofolate reductase[22] does not appear to be caused by approximation of the complex to a transition state.[65]

Some peptide aldehydes are very efficient transition state inhibitors of proteolytic enzymes. The rationale is that an aldehyde exists in hydroxylic media largely in a tetrahedral state as either a hydrate or an acetal. The aldehyde group should be able to react with the hydroxyl group of serine or the sulfhydryl group of cysteine at the active site of a proteolytic enzyme to form a tetrahedral intermediate (hemiacetal or hemithioacetal) that should be quite stable because it derives from an aldehyde and also because it mimics the normal tetrahedral transition state. This transition state analog is abnormal, however, since it lacks the leaving group of a typical substrate (amide or ester). Acetyl-L-phenylalanylaminoacetaldehyde is a very good inhibitor

(K_i 0.046 μM) of papain.[66] The importance of the detailed structure of this inhibitor and therefore of primary binding is borne out by the facts that benzoylaminoacetaldehyde had a K_i of 25 μM and propionaldehyde of about 1 M.[66] Recently, physical evidence was obtained to support the view that aldehydes form a hemithioacetal with the reactive sulfhydryl group of papain.[67,68] The serine protease elastase has been inhibited by synthetic peptide aldehydes, the most potent of which was acetyl-Pro-Ala-Pro-alaninal (K_i 0.8 μM). The corresponding alcohol was a much poorer inhibitor (K_i 600 μM), and the primary structure of the aldehyde was quite important: acetyl-Ala-Pro-alaninal had a K_i of 62 μM.[69]

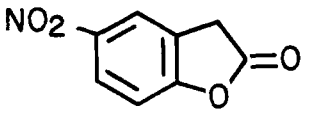
Peptide aldehydes of microbial origin had been discovered to be efficient inhibitors of proteolytic enzymes before the recent interest in transition state analogs, and much of this work has been reviewed.[70-72] Leupeptins, which terminate in argininal, inhibit trypsinlike enzymes[73] while synthetic analogs ending in aromatic amino acid aldehydes inhibit chymotrypsin.[74] Elastinal, an unusual tetrapeptide terminating in -Gln-alaninal,[75] is a good inhibitor of elastase but a very poor one for a number of other proteolytic enzymes.[76]

Boronic acids have been investigated as transition state inhibitors of proteases, but the best of these do not have outstanding binding constants. More potent inhibitors of this type might be obtained by synthesizing compounds analogous in structure to the better aldehyde inhibitors. There is, however, good evidence that boronic acids can form tetrahedral intermediates at the active sites of proteases.[77]

The much better inhibition of chymotrypsin by phenylethaneboronic acid (K_i 0.05 mM) and styreneboronic acid (K_i 0.026 mM) than by hydrocinnamaldehyde (K_i 0.38 mM) and cinnamaldehyde (K_i 2.6 mM) was attributed to transition state binding.[78,79] Phenylethaneboronic acid (K_i 2.6 mM) and benzeneboronic acid (K_i 0.8 mM) are far better inhibitors of subtilisin Carlsberg than methaneboronic acid (K_i 250 mM).[80] The most convincing evidence for a tetrahedral complex in these inhibitions was obtained by x-ray diffraction with adducts of benzene- and phenylethaneboronic acids with subtilisin BPN' (Novo): The boron atom was found to be coordinated tetrahedrally, with one of the two additional boronic acid oxygen atoms lying in the "oxyanion hole" and the other at the leaving group site. Moreover, the structure of the tetrahedral intermediate for substrate hydrolysis is isosteric with these boronic acid adducts. Thus the adducts can be considered good models for the transition state complex.[77]

Stable acyl enzymes can be formed by reaction with low molecular weight compounds, mostly active esters, which may be called titrants, because several have been used to titrate the concentration of proteolytic enzyme solutions.[81-83] In the case of crystalline indoleacryloylchymotrypsin, a correct transition state is difficult to achieve as the carbonyl group attached to Ser 195 is not properly oriented for deacylation.[24] Some natural protease inhibitors form stable acyl enzymes. The chemistry[84] and structure[85] of the complex between soybean trypsin inhibitor and trypsin have been especially well investigated.

A number of cyclic esters inhibit chymotrypsin.[83] For example, 13 and 14 react with it to form relatively stable acyl enzymes, but the products behave differently. While the acyl enzyme from 14 hydrolyzes



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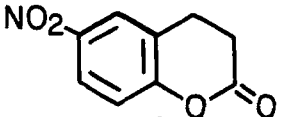

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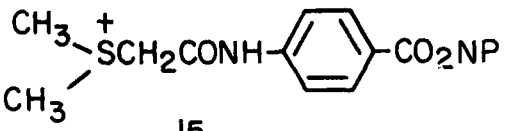
to give the free acid, that from 13 releases 13 itself. There is an equilibrium between free chymotrypsin, the acyl enzyme, and 13, which slowly hydrolyzes nonenzymatically to the corresponding acid.[86] To the extent that cyclic titrants can be regenerated readily, their effect is the same as that of reversible inhibitors, though the mechanism is different.

Most titrants studied are acyclic ones that lose the acyl substituent upon reaction with an enzyme. The majority of these are p-nitrophenyl (NP) esters, which are convenient for measuring reaction rates. Other esters can be used for enzyme inhibition,[87] and the particular ester chosen can affect specificity.[50] Perhaps the best known of titrants is p-nitrophenyl-p'-guanidinobenzoate (NPGB), which is used to determine the concentration of various trypsinlike enzymes. The acyl enzyme from trypsin itself is particularly stable.[81,87] In a study of deacylation rates of para-substituted benzoyl trypsins and chymotrypsins, compound 15 was found useful for titrating chymotrypsin in the presence of trypsin.[88]



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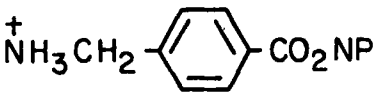

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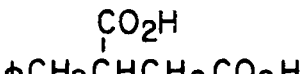
With compound 16 it is possible to titrate plasmin contaminated by thrombin (up to about 50%). The kinetics of the reactions of NPGB and 16 with trypsin, thrombin, and plasmin were compared and considered to support strongly the idea that acylation is the most significant parameter for specificity in these enzymes.[89] This is probably true, but specificity in acylation is intimately connected with specificity in binding. Further, the specificity of deacylation determines the efficiency of a titrant as an inhibitor. Although the principle of microscopic reversibility dictates that deacylation is the reverse of acylation,[90] it cannot be applied strictly to an acyclic titrant, since the latter loses its acyl substituent to the medium upon acylation. Because of this there is considerable scope in the design of titrants as inhibitors.

By-Product Analogs - This new type of inhibitor is so constructed that it resembles the collected substrates for the reverse of an enzymatic reaction all combined in one molecule. Consequently such an inhibitor binds with an affinity resembling the combined affinity of the products of the reaction. In the first good example of this sort of inhibitor, L-benzylsuccinate (17), which may be considered as part of the combined



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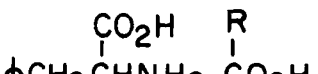

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structures (18; R = $\phi CONHCH_2$-) of hippuric acid and L-phenylalanine, had a K_i of 0.45 μM in the inhibition of carboxypeptidase A.[91] Recently S-(guanidinoethyl)mercaptosuccinic acid was found to be the most effective (K_i 4 μM) of a series of by-product analogs of carboxypeptidase B.[92] Perhaps the most fascinating example of this type of inhibitor is D-3-mercapto-2-methylpropanoyl-L-proline (SQ 14,225), which inhibits angiotensin converting enzyme with a K_i of 0.0017 μM[5] (cf Chapter 9 of this volume). In this case the second carboxyl group of the previously reported inhibitors of this class is replaced by a sulfhydryl group that can coordinate with the zinc atom at the active site of the enzyme. Several principles discussed and illustrated in this review were applied in the evolution of SQ 14,225: 1) The concept of inhibition was based upon the probable mechanism of action of the target enzyme. 2) The binding specificity of the enzyme was very important, and in this case had to be worked out in detail by testing a large number of compounds. 3) Chemical ingenuity led to the selection of an excellent substituent (sulfhydryl group) for the design of the best inhibitor.

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