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Review Article

RECENT DEVELOPMENTS IN INHIBITING CYSTEINE AND SERINE PROTEASES

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Some 20 years ago, affinity labelling was introduced to help gather information on active-site catalytic groups and the mechanisms of proteolytic enzymes. Now this knowledge is used to produce specific and selective inhibitors for these enzymes. The concept of "biospecific drug design" has stimulated progress in turning the inhibitors into therapeutically applicable agents. For instance, sales of antihypertensive drugs based on inhibitors of the angiotensin converting enzyme are expected to be over 2 billion US-$ in 1992. This partly illustrates the efforts made by many researchers to introduce strategies in potease inhibition for medicinal purposes. This review discusses some of the concepts.

KEY WORDS: protease, proteinase, peptidase, inhibitor, affinity label, transition-state analogue, acyl-enzyme inhibitor, enzyme-activated inhibitor.

INTRODUCTION

Pharmacological research relies more and more on information provided by molecular biology and biochemistry. This development was induced by clinical medicine requesting therapeutic agents with well-defined features and also by the requirements of today's society to reduce chemical pollution of both the individual and the environment. This general orientation resulted in increasing costs of pharmaceutical production but conversely stimulated the introduction of new methods of quantitative structure-activity analysis.[1] Today, often detailed biochemical knowledge is the prerequisite in the development a medicine.[2–4] New methodology in genetics and protein chemistry, as well as theoretical methods such as computer-aided structure design are widely used to reduce the cost of R. & D. The predicition of the possible activity of anticipated structures abolishes time-consuming and costly synthetic work.[5–8] One example of this "biospecific drug design" is the development of mechanism-based inhibitors of proteolytic enzymes.[3,9–12] The growing realization of their significance in numerous pathogenic processes has led to increased activity in the field of inhibitor synthesis for proteolytic enzymes.[18–20] Specific protease inhibitors are already used as medicines or are under development with the following therapeutic goals:

(a) the treatment of emphysema, arthritis, pancreatitis and thrombosis, (b) the treatment of skin disorders, different types of cancer and muscular dystrophy, (c) the treatment of high blood pressure, (d) use of antiviral drugs and immunosuppressives.

A new challenge for drug design is the HIV-1-aspartate protease, a key enzyme in

virus maturation. An appropriate inhibitor of that proteinase could be a valuable tool in AIDS-treatment.[21-23]

For further reading on proteolytic enzymes, their physiological and pathophysiological significance recent monographs and papers[4,11-18,24-32] are available. This review will focus especially on recent developments in cysteine and serine protease inhibition using low molecular weight inhibitors.

2. PROTEASES

2.1. General Aspects

Proteolytic enzymes are often named and distinguished by criteria derived from their occurrence and special properties: (i) localization in species, tissues and compartments (pancreatic elastase, human neutrophil elastase, HIV-protease), (ii) substrate specificity and selectivity (collagenase, elastase, enkephalinase, angiotensin-converting-enzyme), (iii) substrate size and/or cleavage-site (proteinases and peptidases, amino- and carboxypeptidases).

Depending on their physiological task classification as signal peptidases, digestive proteases, regulatory proteases are quite common. For recent discussions on nomenclature see references.[32,33] Besides differences in specificity, localization and function proteases differ mainly in their way of promoting peptide bond hydrolysis. At present, there are four reaction mechanisms established leading to the following hydrolase subclasses: metallo proteases, aspartic proteases, serine proteases, cysteine proteases.

All these enzymes catalyze the cleavage of the amide bonde linking two amino acids by nucleophilic attack on the scissile carbonyl carbonatom. While the nucleophile is an integral part of the enzyme's structure in serine and cysteine proteases, metallo and aspartic enzymes use an activated water molecule located in the active site so that the tetrahedral intermediate (TI in Figure 1) is held *noncovalently* within the active site during catalysis.[18,34-38] Consequently, strategies leading to useful inhibitors have evolved very differently in recent years. Successful inhibition of the non-covalent catalyzing enzymes is often achieved with chemically unreactive substances mimicking the scissile peptide bond, the tetrahedral intermediate or functional groups of the

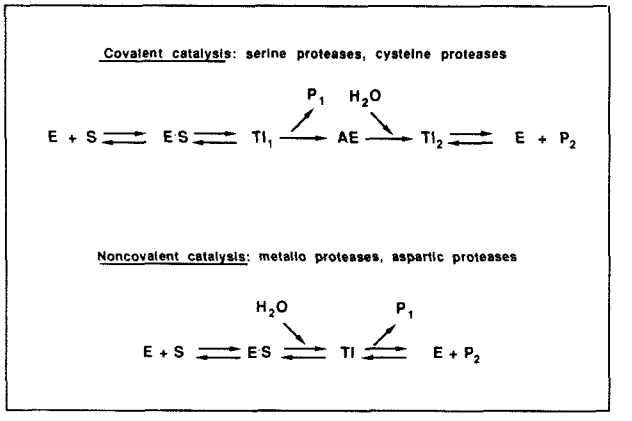

FIGURE 1 Hydrolytic mechanisms by proteolytic enzymes

products.[37,39] Among those structures, peptide bond isosteres and statine derivatives are the most powerful inhibitors of aspartic proteases.[35–41]

Compounds which easily complex the catalytic metal ion have been shown to be useful inhibitors of metallo proteases. Among them, phosphorus-containing compounds, hydroxylamine- and thiol group-containing structures have led to powerful inhibitors (see recent reviews and papers[12,18,41–45]). The aim of the following text is to examine the design of inhibitors for the *covalent* catalyzing serine and cysteine proteases. Reviews dealing with particular enzymes, strategies and concepts leading to special inhibitor types have recently appeared and are referred to.[3,4,9–11,46–53] A collection of enzyme inhibition data has also been published.[54]

2.2. Mechanistic Aspects

Despite numerous therapeutic goals and the considerable interest engendered on serine protease catalysis, clinic applications of their low molecular weight inhibitors have failed to materialize. Thus, in the last decade numerous attempts based on the enzyme's mechanism have been made to overcome the undesirable features of high unspecific reactivity of some active-site directed inhibitors.[55] During the catalytic cycle (Figure 1) several covalent adducts are formed between enzyme and substrate. The following elements of the catalytic machinery, particulary of nucleophilic catalytic enzymes are mainly involved in the formation and breakdown of the transition-states and reaction intermediates and have been considered for inhibitor design (compare Figure 1): (i) binding: noncovalent interactions occurring between elements of the protein and the substrate, promoting more or less the productive transition to a reaction intermediate, (ii) nucleophilicity of the attacking nucleophile, (iii) catalysis of the nucleophilic attack of enzyme groups (TI_1-formation and breakdown to the acyl enzyme (AE), i.e. proton abstraction from the nucleophile and its transfer to the leaving group by the catalytic triad), (iv) transition-state stabilisation by the protein matrix (electrostatic stabilisation of the oxyanion), (v) catalysis of the nucleophilic attack by the water molecule at the acyl enzymes (TI_2-formation and breakdown to free enzyme and products).

The extent to which a particular enzyme can use the potential of its catalytic apparatus to reduce the energy barriers between reaction intermediates during hydrolysis, depends upon a variety of factors such as the substrate structure and the electronic nature of the leaving group. Most important seems to be the optimal fitting of the substrate structure to the active site, thus recruiting its catalytic machinery and even changing the rate limiting steps.[56–58] Much of our knowledge in this respect has come from NMR and crystallographic studies of enzyme-substrate/inhibitor complexes (for recent papers see[56–67,92]). Knowledge about the velocity of the individual steps within a catalytic event have come from kinetic studies, including isotope effects on enzymatic reactions.[68,71,85,93] The advent of protein engineering has added detailed informations, about the degree to which the above elements of the catalytic machinery are involved in the catalytic event.[72–79] Comparing the catalytic features of serine and cysteine proteases the following main differences are important for inhibitor design: (i) in serine protease catalysis the P_1-aminoacid is of primary importance for binding and specificity-in cysteine proteases the P_2-aminoacid has a greater significance for the specificity (Figure 2),[80–82] (ii) in serine proteases the catalytic machinery, especially the nucleophile becomes activated by a complex response of the protein upon substrate binding, involving the deprotonation of the serine nucleophile by the catalytic triad-

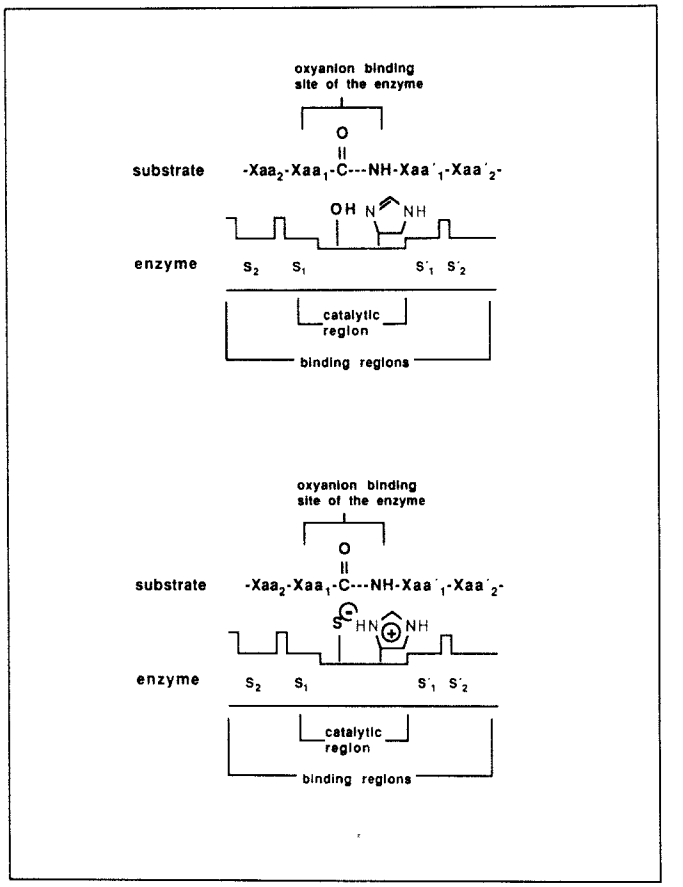

FIGURE 2 Catalytic important structural elements of serine and cysteine proteases

cysteine proteases are activated (deprotonated nucleophilic thiol) in the gound state of the enzyme (Figure 2),[80,83] (iii) in serine proteases the negatively charged tetrahedral intermediate formed by nucleophilic attack is stabilized by a complex hydrogen bonding system - in cysteine proteinases electrostatic oxyanion stabilisation seems not to be so crucial for catalysis.[68,84–94]

These features, especially the greater nucleophilicity of the cysteine residue, are responsible for some of the differences in inhibitor efficacy described below and are to be considered in future inhibitor design.

2.3. Inhibitors

Enzyme inhibitors form either noncovalent or covalent complexes with their target enzymes. Depending on which catalytic step they exhibit their action they have been classified as substrate or product analogues, affinity labels, transition-state analogues, acyl enzyme inhibitors and enzyme-activated inhibitors. A pre-requisite for all the above inhibitor types is their recognition and binding by the target enzyme (see also[4,94–103]). The typical characteristics of each class are compiled in Table I, and a

TABLE 1
Selected structural classes for small molecule protease inhibitors (for references see text)

Type	Structural Classes	Characteristics and Comments
affinity labels	halomethyl ketones, diazomethyl ketones, haloethyl ketones, diazoethyl ketones, acyloxy methyl ketones, sulphonium methyl ketones, epoxy derivatives.	-covalent, irreversible. -alkylation of active site histidine (serine proteases) and of cysteine (cysteine proteinases) – a result of intruding chemical reactivity into the active site by affinity labelling. -target step: Michaelis-complex (E·S). -parameter: K_i(binding), k_{inact} (inactivation rate), k_{inact}/K_i (specificity).
transition-state analogues	peptidyl aldehydes, peptidyl methyl ketones, peptidyl phosphonic acids, peptidyl boronic acids.	-noncovalent, reversible. -simulation of transition-states or reaction intermediates. -target step: tetrahedral intermediate (TI). -parameter: K_i(also k_{on} and k_{off}, rate constants in tight binding inhibition).
acyl enzyme inhibitors	azapeptides, carbaminic acids, "inverse" esters and amides, heterocyclic esters and amides.	-covalent, reversible. -during hydrolysis by the target enzyme formation of stable, slowly hydrolysing acylenzymes. -target step: acyl enzyme (AE). -parameter: K_i, k_{ac} (rate constant of acyl enzyme formation), k_{deac} (deacylation rate constant).
enzyme-activated inhibitors	heterocyclic or aliphatic ester and amides bearing latent reactive nitrogen and carbon intermediates.	-covalent, irreversible. -during catalysis, release of hidden chemical reactivity leading to modification of several active site residues. -target step: all intermediates and transition-states of the catalytic process. -parameter: K_i, k_{inact} (inactivation rate constant), k_{cat} (rate constasnt of simultaneously occurring catalysis), r = partition ratio (k_{cat}/k_{inact}).

schematic representation of inhibitor types is shown in Figure 3. Different kinetic approaches have been developed to estimate the characteristic kinetic parameters. For recent papers and reviews dealing with these procedures in detail see references[4] and [104–112]. To simplify comparison in the following text, where applicable, the apparent second order rate constant (k_{obs}/I in $M^{-1} \cdot s^{-1}$), the second order rate constant ($k_{inact}K_i$ in $M^{-1} \cdot s^{-1}$) or the binding constants (K_i in M) have been used to illustrate specificity. Sometimes the rates or the half-lives of the inactivation or reactivation processes are referred to.

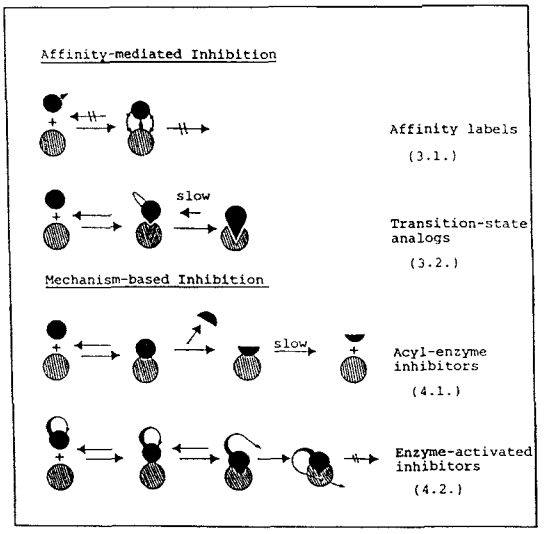

FIGURE 3 Inhibitor Classes (schematic)

3. BINDING-MEDIATED INHIBITION

3.1. Affinity Labelling

The discovery that enzymes are inhibited by the products of proteolysis has stimulated the development of affinity labelling.[94] A chemically reactive structure may diffuse into the active site of a target enzyme due to the affinity of an attached product or substrate analogue fragment (Figure 3). There it can react with the essential catalytic groups of the enzyme and thus block the enzyme activity.[95] The most studied protease inhibitors among affinity labelling agents are the peptide halomethyl ketones. The work of E. Shaw and his coworkers on the primary and secondary specificity of plasma proteinases using peptidyl arginyl chloromethyl ketones has greatly enhanced the knowledge of secondary enzyme-inhibitor-interactions.[113-117] In contrast to former postulates, recent kinetic, NMR and X-ray studies support the formation of intermediate hemiketales during the inactivation of serine proteases.[118-121]

While halomethyl ketone derivatives specifically modify the active-site histidine of serine proteases, in cysteine proteases the sulphur of the cysteine becomes alkylated (Figure 4).[95] Typcially specifity constants of the inactivation of proteases are shown in Table II.

To overcome the undesirable high electrophilic reactivity of the peptidyl chloromethyl ketones, several investigators have developed synthetic routes to the more stable peptidyl fluoromethyl ketones.[128-130] It had been expected that the flourine atom might be too inert for nucleophilic desplacement and that irreversible inactivation might not be achievable with this type of derivative in contrast to the chloromethyl ketones. However, with serine and cysteine proteases, alkylation of the enzymes occurs. Using model compounds there is a decrease in the degradation rate of the fluoro compounds by about 3 orders of magnitude compared to the corresponding chloromethyl ketones. While the reactivity as cysteine proteases inhibitors

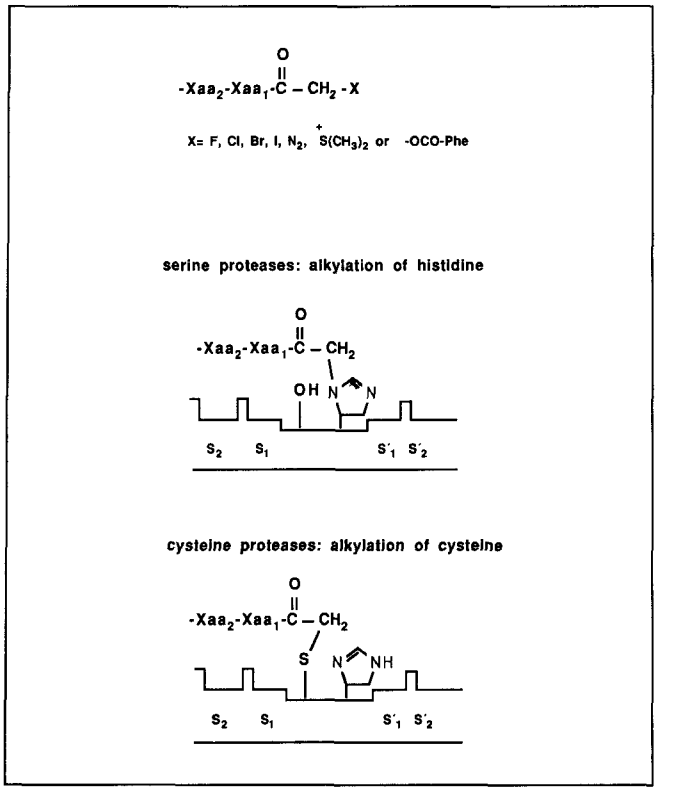

FIGURE 4 Affinity labelling of serine and cysteine proteases with methyl ketone derivatives

drops only slightly, a significant loss of inactivating power of peptidyl fluoromethyl ketones towards serine proteases has been found (Table III).

Even more specific cysteine protease inhibitors are the diazomethyl ketones[131–135] where a high degree of selectivity between calpain II and the cathepsins B and L was also demonstrated. A second order rate constant of $1.5 \times 10^6 M^{-1} \cdot s^{-1}$ was estimated for the inactiviation of cathepsin L using Z-Leu-Leu-Tyr-CHN_2.[133]

In contrast to the stressed high selectivity of peptidyl diazomethyl ketones for cysteine proteases the serine enzyme, prolyl endopeptidase is inhibited at comparable rates by the substrate analogue chloromethyl ketone ($150 M^{-1} \cdot s^{-1}$) and a peptidyl diazomethyl ketone ($1300 M^{-1} \cdot s^{-1}$).[132,134]

Several new approaches to the making of affinity labels more suitable for pharmaceutical purposes have provided alternatives to the diazo- and halomethyl ketones i.e. peptidyl diazoethyl ketones,[136] peptidyl chlorethyl ketones,[136] peptidyl sulphonium methyl ketones,[137–141] peptidyl acyloxymethyl ketones.[142] Reagents have been tested with an additional methyl side-chain near the reactive grouping with the goal of diminishing side reactions in biological fluids. As expected the reactivity of the chloroethylketones and diazoethyl ketones towards model nucleophiles dropping by about 60%. However, the compounds exhibit remarkably less reactivity towards several cysteine proteases while the reactivity against serine proteases does not change so dramatically.[136]

TABLE II
Inactivation of proteases with peptidyl chloromethyl ketones

Inhibitor	Specificity (k_{inact}/K_i in $M^{-1}s^{-1}$)	Enzyme (type)		References
Z-Ala-Ala-Phe-CH_2Cl	7 900	chymotrypsin	(serine)	122
	4 760	subtilisin BPN'	(Serine)	122
Z-Phe-CH_2Cl	152	chymotrypsin	(serine)	122
	17	subtilisin BPN'	(serine)	122
MeOSuc-Ala-Ala-Pro-Val-CH_2Cl	1 560	porcine pancreatic elastase	(serine)	123
	3 500	human leukocyte elastase	(serine)	124
D-Phe-Pro-Arg-CH_2Cl	10 080 000	thrombin	(serine)	125
Z-Phe-Phe-CH_2Cl	91 300	cathepsin B	(cysteine)	126
Z-Phe-Ala-CH_2Cl	4 520	cathepsin B	(cysteine)	127

TABLE III
Inactivation of proteases with peptidyl fluoromethyl ketones

Inhibitor	Specificity (k_{inact}/K_i in $M^{-1} \cdot s^{-1}$)	Enzyme (type)		References
D-Phe-Pro-Arg-CH_2Cl	10 080 000	thrombin	(serine)	125
D-Phe-Pro-Arg--CH_2F	60 000	thrombin	(serine)	129
Z-Phe-Phe-CH_2Cl	910 300	cathepsin B	(cysteine)	126
Z-Phe-Phe-CH_2F	392 860	cathepsin B	(cysteine)	126

The differences described for the inhibitory potency of halo- and diazoalkyl ketones reflect the potential opportunities for applying a combination of steric and electronic factors in determining the efficiency of a desired inhibitor. However, to produce inhibitors for pharmacological applications with suitable physical properties i.e. solubility, permeability etc. the above compounds are somewhat limited with regard to the structure of the substrate (product) analogue recognition part of the molecule.

In 1988 E. Shaw invented peptidyl sulphoniummethyl ketones[137] so providing the opportunity for structural modifications at the P′-site of the inhibitor molecule. Z-Phe-Ala-$CH_2^+S(CH_3)_2$ inactivates papain with an apparent second order rate constant of $2.8 \times 10^5\ M^{-1} \cdot s^{-1}$. These inhibitors are extremely specific for cysteine endo- and exopeptidases such as the cathepsins B, C and H. Some sulphonium salts derived from lysine were examined as inhibitors for the cysteine protease clostripain and a number of trypsin-related serine proteases.[138–139] A strategy breaking with the tradition of the highly reactive methylketone derivatives has been followed by Smith and coworkers. On the basis of the methyl ketone concept, the developed inhibitors with difficult to displace leaving groups whose reactivity could be controlled by substituents.[142] The peptidyl acyloxymethyl ketones offer the potential of affinity labels of low chemical reactivity taking advantage of the high explicit reactivity of cysteine proteases and have been proven to be potent inactivators of the cathepsin B. Depending on the electronegativity of the leaving acyl residue the specificity changes between $80\ M^{-1} \cdot s^{-1}$ (Z-Phe-Ala-CH_2-OCO-(3,5-CH_3)Ph) and $1.6 \times 10^6\ M^{-1} \cdot s^{-1}$ (Z-Phe-Ala-CH_2-OCO-(2,6-CF_3)$_2$)Ph) in inactivation of cathepsin B.

Peptidyl methyl ketone derivatives have been used practically as spectroscopic probes for serine protease,[143,144] to discriminate between zymogens and active proteases in the blood coagulation system,[145] to identify active cysteine proteases in virus-transformed mouse fibroblasts[146] or to visualize cysteine proteinases in human tumours[147] and they have been tested as potential antiviral agents.[148]

A completely different affinity labelling approach is based on the naturally-occurring epoxide inhibitor E-64.[149] E-64 and synthetic analogues are specific inactivators for cysteine proteinases and only weak competitive inhibitors of serine proteases.[149–155] Depending on the substrate analogue carrier peptide, selectivity may be achieved (Table IV). The initially proposed reversed-mode binding for epoxide inhibitors has been superceded by NMR and X-ray crystal structure analysis of the papain-inhibitor complex.[92,156] Furthermore, NMR-studies suggest that nucleophilic attack by the cysteine thiol group of papain on the C-3 carbon of the epoxide is the inactivating mechanism and that the role of the active-site histidine is more likely involved in binding than in catalysing the inactivation process.[157] Possibly, other similar vinyl aminoacid esters and peptides which have been introduced recently by Hanzlik and coworkers,[158,159] might act as cysteine protease inhibitors.

TABLE IV
Inactivation of cysteine proteases by epoxide inhibitors

Inhibitor	Specificity (k_{inact}/K_i in $M^{-1} s^{-1}$)	Enzyme (type)		References
E-64	89 400	cathepsin B	(Cys.)	151
	4 000	cathepsin H	(Cys.)	151
	96 250	cathepsin L	(Cys.)	151
	23 340	calpain	(Cys.)	154
	638 000	papain	(Cys.)	152
EP.475	298 000	cathepsin B	(Cys.)	151
	2 018	cathepsin H	(Cys.)	151
	206 000	cathepsin L	(Cys.)	151
	7 450	calpain	(Cys.)	154
	357 000	papain	(Cys.)	152

3.2. Transition-State Analogue Inhibitors

The idea of L. Pauling,[160] that enzymes are designed by nature to bind the reaction intermediates tighter than the substrates or products (compare Figure 3), stimulated in the seventies, the development of transition-state analogue inhibitors for proteolytic enzymes[161,162] (for recent reviews see[103,163]). These compounds lack the scissile carbonamide linkage but have groups, without cleavable residues, prone to each addition of nucleophiles. Stable, tetrahedral intermediate- resembling structures have been proved to be formed by proteases with peptide aldehydes, peptide methylketone derivatives, peptide boronic acids and peptide phosphonic acids (Figure 5).[164–169]

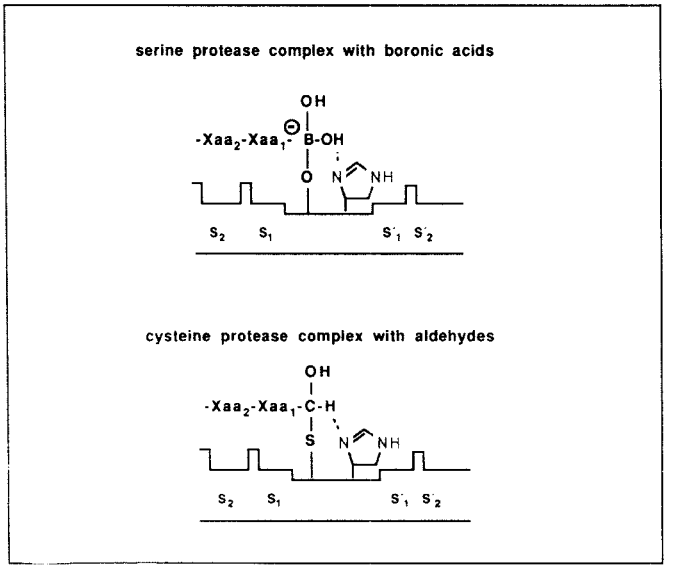

FIGURE 5 Transition-state analogue inhibitors

The compounds bind three to six orders of magnitude tighter to their targets than the corresponding carbonyl substrates. Crystallographic studies of the complexes formed between alpha-lytic protease or porcine pancreatic elastase and peptide boronic acids[62,170] demonstrate the multiplicity of enzymic groups involved in tetrahedral intermediate stabilisation.[63,64] Additionally, NMR-studies of the complex between Ac-Leu-Phe-CF_3 and chymotrypsin[171] support the existence of tetrahedral intermediates and their stabilisation by the oxyanion-binding site.[172–179,191] In many cases, time-dependent complex formation between enzyme and inhibitor has been observed (using conventional detection techniques, Table V). The kinetics of this "slow-binding" inhibition have been worked out by Morrison *et al.*[105,180,181]

The elastase inhibitor MeOSuc-Ala-Ala-Pro-(L)boroVal-OH was shown to suppress elastase-induced emphysema in an animal model.[182]

Peptides containing proline residues have been shown to be very resistant to proteolytic cleavage at these linkages.[183] Many regulatory peptides contain proline residues which determines the peptide chain conformation and biological activity.[184] To regulate the activity of such peptides, evolution created an exclusive set of proline specific peptidases.[185] Among them, prolyl endopeptidase (PE) and dipeptidyl peptidase IV (DP IV), are believed to be involved in peptide hormone processing and regulation.[183–186] Recently, a series of transition-state analogue inhibitors of PE were described.[187] Z-thioproline-thioprolinal was the most potent structure with a K_i-value of 10 pM. We used a similar approach to devise tight-binding inhibitors for DP IV and found Ile-thiazolid (K_i = 130 nM) and Val-thiazolidid (K_i = 270 nM) to be very effective. The compounds have also been shown to supress T-cell proliferation *in situ*.[188] Oral administration of PE-inhibitors in concentrations as little as 1 μg per animal prevents experimental amnesia in mice.[187]

Another important application of transition-state analogue compounds is in the development of catalytic antibodies.[75,78,79,189]

4. MECHANISM-BASED INHIBITORS

Mechanism-based inhibitors are a relatively new development in inhibitor research. Although the analysis of stable acyl enzymes for theoretical studies was described in the sixties[200] no enzyme inhibitor applications were noted in a review on protease inhibition in 1980.[100] Since then, progress has been impressive. In contrast to affinity labels and transition-state analogues, mechanism-based inhibitors are activated by the catalytic machinery of the target enzyme and chemically transformed into inhibitors. In principle their are two possibilities: (i) catalytic attack causes the formation of an acyl enzyme which deacylates more slowly than the natural enzyme-product-complexes. The inhibitor-enzyme complex is formed covalently with the active site nucleophile, but without damaging the target protein (acyl enzyme inhibitors, Figure 3, 4.1.), (ii) catalytic attack causes the formation of latent (hidden) chemcially-reactive intermediates, which form stable covalent bonds with different functional groups within or near to the active site, thus leading to an irreversibly modified protein (enzyme-activated inhibitors, Figure 3, 4.2.).

Both principles lead to the clinical advantage of having relatively chemically inert compounds circulating in biological fluids that are only activated by the target they are designed to inactivate.

TABLE V
Selected transition-state analogue inhibitors of serine and cysteine proteases

Inhibitor	Specificity (K_i in nM)	Enzyme (type)		Bindingtype	Ref.
Ac-Leu-Leu-Arg-H	5.0	cathepsin B	(Cys.)	slow binding	190,191
Z-Phe-Ala-CH_2F	500.0	cathepsin B	(Cys.)	slow binding	192
Z-Phe-Ala-H	21.0	cathepsin B	(Cys.)	slow binding	192
Z-Phe-Ala-CH_3	31 000.0	cathepsin B	(Cys.)	slow binding	192
Z-Ala-Ala-Phe-Phe-CH_3	17 000.0	papain	(Cys.)		193
	16 000.0	cathepsin B	(Cys.)		193
	1 200.0	cathepsin L	(Cys.)		193
Ac-Phe-$CH_2-C\equiv N$	6 800.0	papain	(Cys.)		177
	730.0	papain	(Cys.)		194
Bz-Phe-H	36 000.0	chymotrypsin	(ser.)		174
Z-Lys(Z)-Val-Pro-Val-CF_3	< 0.1	human leukocyte elastase	(Ser.)	slow binding	195
MeOSuc-Lys(Z)-Val-Pro-Val-CF_3	< 0.3	human leukocyte elastase	(Ser.)	slow binding	195
Z-Ala-Ala-Pro-Val-CF_3	1.0	human leukocyte elastase	(Ser.)	slow binding	196
MeOSuc-Ala_2-Pro-(L)boroPhe-OH					
	57.9	chymase	(Ser.)	slow binding	197
	-	trypsin	(Ser.)		197
BOC-Ala-Pro-(L)boroVal-OH	0.35	alpha-lytic protease	(Ser.)	slow binding	199
MeOSuc-Ala_2-Pro(L)Val-OH					
	6.4	alpha-lytic protease	(Ser.)	slow binding	199
	0.25	porcine pancreatic elastase	(Ser.)	slow binding	179
	0.57	human leukocyte elastase	(Ser.)	slow binding	198
MeOSuc-Ala_2-Pro-Val-H	78.0	porcine pancreatic elastase	(Ser.)		179

4.1. Acyl Enzyme Inhibitors

Acyl enzymes were found to be reaction intermediates during catalysis relatively early in protease research.[201–204] Depending on the substrate structure and the electronic properties of the leaving groups, the ratio between the acylation rate and deacylation rate may be altered to make the deacylation step rate determining for the whole process (compare Figure 1). Thus an acyl enzyme accumulates whose hydrolysis depends only on the electronic and steric properties of the remaining acyl residue.[205,206] This knowledge was used to prepare reagents for protease concentrations determinations[207] and to develop compounds as potential prodrugs for trypsin-like serine proteases.[53] Some representative examples are listed in Table VI and selected structures are shown in Figure 6:

Benzoic acid derivatives, inverse substrates Systematic work on the electronic effects of ring substituents in the acylation and deacylation of serine proteases with benzoyl derivatives was performed by Caplow & Jencks[205] and by Wang & Shaw.[206] In general, electron donating substituents stabilize the acyl enzymes while electron withdrawing groups destabilize them. Besides the electronic effects, steric factors play a role in stabilizing acyl enzymes. The high stability of p-guanidinobenzoyl trypsin induced the development of active site titrants for proteolytic enzymes.[207] Other useful inhibitor structures were derived from benzene sulphonic and phosphonic acids.[252] Recently, the inactivation of chymotrypsin and elastase using alpha-aminoalkylphosphonate diphenyl esters was reported. Depending on the peptide recognition part of the structures, the specificity constants were between $0.4\ M^{-1} \cdot s^{-1}$ and $1.7 \times 10^4\ M^{-1} \cdot s^{-1}$ (k_{obs}/I) were obtained. The reactivation half-lives were longer than 10 hours.[250]

The same acylating principle was used in designing "inverse" substrates. Here, the ability of enzymes to accommodate small changes in substrate structure stimulated development. Markwardt *et al.* and Tanizawa *et al.*[210–212] synthesized compounds with simply inverted ester linkages, thereby attaching the alcohol's hydroxyl group to the recognition part of the substrate and the carbonyl group to the original leaving group. Thus, after acylation, the former leaving group remains and stabilizes the acylenzyme. Acyl residues containing double bonds which are conjugated to aromatic structures

TABLE VI
Selected acyl enzyme inhibitors

Class	Deacylation (half-live, h)	Enzyme	References
benzoyl derivatives	> 30	trypsin, chymotrypsin, etc.	206–209
inverse substrates	> 15	plasmin, trypsin, etc.	53, 210–220
isatoic acid anhydrides	> 60	porcine pancreatic elastase	221–224
benzoxazinones (and oxazindiones	> 15	chymotrypsin	225–228
N-acylsaccharines	> 2000	elastases	229, 230
6-chloro-2-pyranones	23	chymotrypsin	231–236
arylenollactones	1–2	chymotrypsin	237
azapeptides	1–2	chymotrypsin, elastases	238–246
carbaminic acids	> 100	chymotrypsin, trypsin	247–249
aminoalkylphosphonates	> 10	chymotrypsin, elastases	250
alkynyl carboxylates	3	chymotrypsin, trypsin, elastase	251

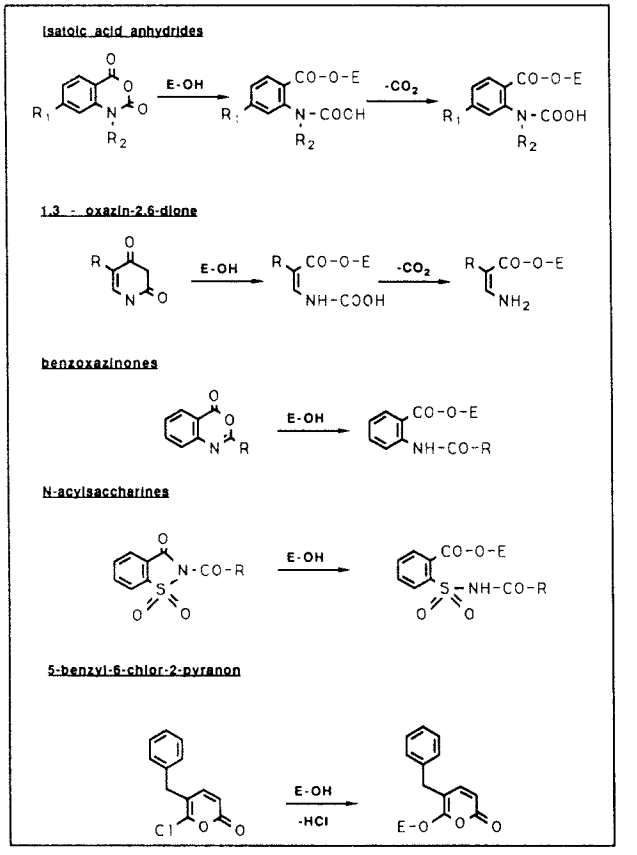

FIGURE 6 Acylenzyme inhibitors (selected structures)

as in indolylacrylic, furoylacrylic and cinnamic esters[204,217,208,209] have been demonstrated to be useful.

Of special importance among these structures is *o*-hydroxy-alpha-methyl-*p*-amidinophenyl cinnamate as a light-sensitive thrombin inhibitor.[217,219] The stable cis-cinnamic acyl enzyme may be reactivated by photolysis inducing isomerisation of the *o*-hydroxyl function which catalyzes the deacylation intramolecularly.[253,254] For further reviews on acyl enzyme inhibitors see references.[4,10,11,53,103,218]

Isatoic anhydrides, oxazindiones, benzoxazinones, acylsaccharines Unfortunately, benzoic acid esters have the same electronic effect on both deacylation and acylation.This disadvantage was circumvented with compounds where potential electron donating substituents have no effect during acylation. Upon nucleophilic attack they become unmasked and diminish the deacylation rate. Isatoic anhydrides introduced by Abeles group,[221–223] therefore behave in a certain sense as "enzyme-activated". The compounds also inhibit the elastases, alpha-lytic protease, trypsin, thrombin, and plasmin.[222] 1,3-Oxazin-2, 6-diones are similar, yielding intermediate carbaminic acids.[223] Benzoxazinones, which acylate by lactone ring opening,[224–228] provide additional possibilities for the introduction of electron donating substituents. Human

leukocyte elastase, for instance, is inactivated depending on the electronic nature of the substituents[228] in the range of (k_{inact}/K_i) 15 $M^{-1} \cdot s^{-1}$ to 7.3 $\times$ 10^5 $M^{-1} \cdot s^{-1}$.

Related to the acyl enzymes formed with benzoxaxinones are the products of serine protease reaction with N-acylsaccharines. During lactam ring cleavage stable acyl enzymes are formed.[229,230]

6-Chloro-2-pyranones, arylenol lactones Pyranone- and aryllactones were designed, with enzyme-induced alkylation of the enzyme active site in mind. But analysis of the reaction products has shown that acylation is responsible for the inhibition.[231-236] The position of the benzyl substituent in 6-chloro-2-pyranones determines the direction of the nucleophilic attack of the serine hydroxyl group of chymotrypsin. If the substituent is located in position 5, the formation of a pseudo-acylenzyme without ring opening takes place. If the pyranone is in the 3-position, benzyl substitued nucleophilic attack at the carbonyl carbon occurs followed by lactone ring opening. Arylenollactones were investigated parallel to haloenol lactone by Katzenellenbogen.[237] However, compared to this inhibitor class, the lactone ring opening results in stable hydroxymethyl ketones which do not alkylate the active site of chymotrypsin.

Azapeptides and peptide carbaminic acid derivatives All of the above acylating heterocyclic inhibitors exhibit a fairly low selectivity among several serine proteases. Only in a few cases is sufficient differentiation between trypsin- and chymotrypsin-like enzymes possible. In contrast, substrate analogue structures such as azapeptides and carbaminic acids provide higher selectivity. Structural modification of the P_1-aminoacid, similar to the inverse substrates, resulted in compounds with drastically diminished deacylation reaction rates. The nitrogen incorporated into the peptide chain, instead of the alpha-carbon of the P_1-aminoacid, is responsible for resonance stabilization with the scissile carbonyl group. To accomplish acylation, readily leaving

TABLE VII
Deacylation life-lives of azapeptidyl proteases (Date from references 238–242)

Compound	Deacylation (half-live, min)	Enzyme (type)	
Ac-azaPhe-ONp	> 62	chymotrypsin	(Ser.)
Ac-Ala-Ala-azaAla-ONp	no acylation		
Ac-aza-Leu-ONp	> 62		
Ac-azaPhe-ONp	20	subtilisin BPN′	(Ser.)
Ac-Ala-Ala-azaAla-ONp	0.12		
Ac-azaLeu-ONp	4.5		
Ac-azaPhe-ONp	15.5	cathepsin G	(Ser.)
Ac-Ala-Ala-azaAla-ONp	> 62		
Ac-azaLeu-ONp	no acylation		
Ac-Ala-Ala-azaAla-ONp	3.6	porcine pancreatic	
Ac-Ala-Ala-azaNle-ONp	> 62	elastase	(Ser.)
Ac-Ala-Ala-azaVal-ONp	0.1		
Ac-Ala-Ala-azaAla-ONp	3.5	human leukocyte	
Ac-Ala-Ala-azaNle-ONp	> 62	elastase	(Ser.)
Ac-Ala-Ala-azaVal-ONp	0.032		
Ala-azaPro-OPhe	0.3	dipeptidyl peptidase IV	
Ala-azaAla-OPhe	2.94		(Ser.)
Gly-azaPro-OPhe	0.28		

alcohols, such as nitrophenol or phenol must be used.[238-241] The resulting acylenzymes deacylate depending on the nature of the substrate analogue fragment (Table VII), i.e. Ac-azaPhe-chymotrypsin is around 6 orders of magnitude more slowly hydrolyzed than Ac-Phe-chymotrypsin (see Table VI).[202] The compounds have been used successfully as active site titrants. Peptide carbaminic acids follow the same general principle.[247-249] Besides chymotrypsin and the elastases, azapeptide ester inhibitors of trypsin-like proteases and proline-specific peptidases have been synthesized.[242-247] However, all these compounds have in common instability in water under physiological conditions caused by reactivity of the p-nitrophenyl ester.[240,242]

Enzyme-Activated Inhibitors

As seen in the last section, most of the acylating compounds are shown to be relatively unselective since proteases with different specificities are inhibited by the same compounds. Other substrate analogous structures such as azapeptides are on the other hand relatively unstable in aqueous media. Additionally, acylenzymes may be reactivated by nucleophiles and in some applications this might not be very desirable.

Bloch[255] in attempting to overcome these undesirable features introduced an inactivation principle which is summarized as follows: (a) the inhibitors are *a priori* chemically stable, e.g. they do not react with biomolecules other than their target enzymes, (b) they bind in an analogous manner to substrate in the active site of their target enzymes, (c) only the action of the anticipated target enzyme's catalytic apparatus releases the latent chemical reactivity of the inhibitor, which irreversibly modifies the active site.

Since 1977, a variety of chemical groups have been investigated as modifying agents. In general, all of them are activated or released by amidolytic or esterolytic reactions.

Since the reactive intermediates are often connected to the usual leaving part of the molecule their presence within the active site must be prolonged. This is in many cases realized with heterocyclic structures by bridging the acyl part of the substrate and the potential leaving group. However, often unproductive release of products is observed. Although this has been accepted as a typical criterium of enzyme-activated inhibition, diminishing this unproductive side reaction is often a problem. Reviews on enzyme-activated inhibitors for many different enzymes have appeared.[3,4,9,10,103,263-265] Below we shall characterize some of the developments in enzyme-activated inhibitors of serine and cysteine proteases from a more mechanistic viewpoint (Table VIII, structural examples in Figures 7-7c.

TABLE VIII
Enzyme-activated inhibitors, structural classes

Class	Reaction	References
haloenol lactones, ynenol lactones, *N*-nitrosamides, coumarines, cephalosporins.	alkylation and acylation of the active site of target enzymes by latent halomethyl ketones, carbonium intermediates, carbonyl chlorides.	266–272 273–275 276–280 281–289 290–294
N-imidazole carbozamides, diacyl hydroxylamines, *N*-(alkylsulphonyloxy) succinimides.	carbamylation and/or amidation by reactive nitrogen intermediates.	299–304 305–315 316–318

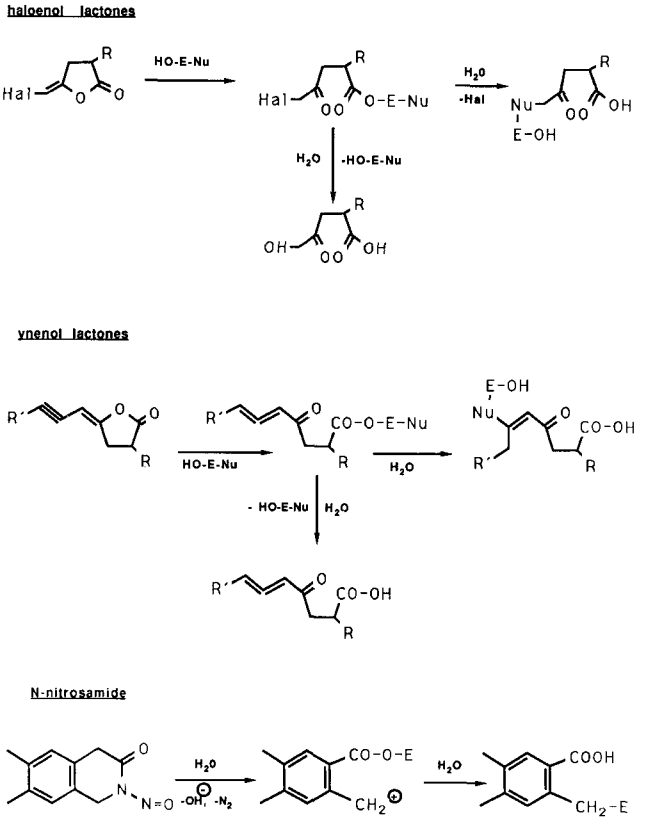

FIGURE 7 Enzyme-activated inhibitors (selected structures)

Haloenol and ynenol lactones R. Rando[255] suggested the use of haloenol lactones as potential enzyme-activated serine protease inhibitors in 1974. During hydrolyis of the lactone ring a latent halomethyl group could be formed which by analogy to the affinity labels of halogenomethyl ketones might alkylate the active-site histidine. This strategy has been followed by Katzenellenbogen's group, who have since invented numerous powerful serine protease inhibitors.[266–269] Systematic variation of the structural elements has shown that the size of the lactone ring is more important for affinity than the attached aryl substituent. Pyranones bind one order of magnitude tighter (K_i = 300 nM) to chymotrypsin than furanones. The inactivation also occurs faster using pyranones (half-lives in the range of minutes or seconds) while side reactions such as spontaneous hydrolysis and enzyme-catalyzed turnover are diminished.[268,269]

Powers and coworkers could demonstrate a reasonable selectivity between elastases and chymotrypsins using isobenzfuranones and benzpyranones.[270] Molecular mechanics and computer graphic analysis show that the aryl residue attached to the haloenol lactones binds in the hydrophobic pocket of chymotrypsin. Probably, His-57 becomes alkylated.[271,272] Treatment of the inactivated enzyme using hydroxylamine does not restore the activity.

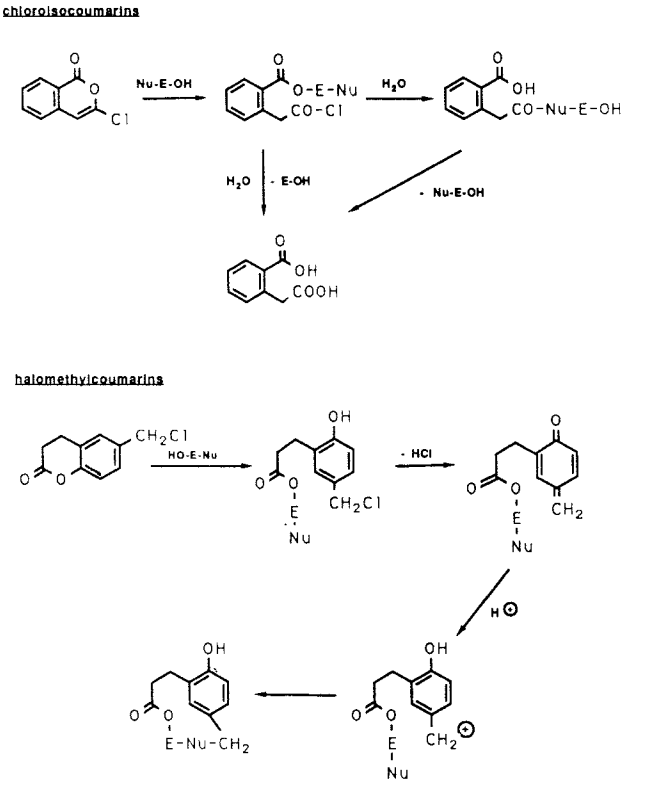

FIGURE 7a Enzyme-activated inhibitors (selected structures)

A similar concept was developed by Krantz and coworkers, who invented ynenol lactones as inhibitors for elastolytic enzymes.[273-275] The compounds carry a masked allenone which is released by enzymatic lactone ring cleavage from a conjugated enol-acetylenic-intermediate. Depending on the chemical nature of attached side chains, either unproductive deacylation occurs or the irreversible addition of the nucleophilic residue to the allenone takes place. Also, by variation of this side chains the selectivity between serine proteases exhibiting different substrate specificities is increased. With specificity constants (k_{inact}/K_i in $M^{-1} \cdot s^{-1}$) between 30 and 3×10^4 and binding constants K_i in the μM-range ynenol lactones are among the most effective inhibitors of human leukocyte elastase.

N-Nitrosamides Enzyme-mediated release of electron deficient intermediates during enzyme-inhibitor interactions should be very effective in inhibitor design. Such reactive structures could lead to fast and efficient enzyme inactivation. Accordingly, White and coworkers inactivated chymotrypsin using N-nitrosoadmide derivatives. During enzymatic action a diazonium intermediate releases nitrogen yielding an alkylating carboniumion derivative. By introducing cyclic nitroso lactames, unproductive diffusion of the reactive intermediates from the active side could be prevented.[276,277] Product analysis and molecular modelling emphasize that the carbonium inter-

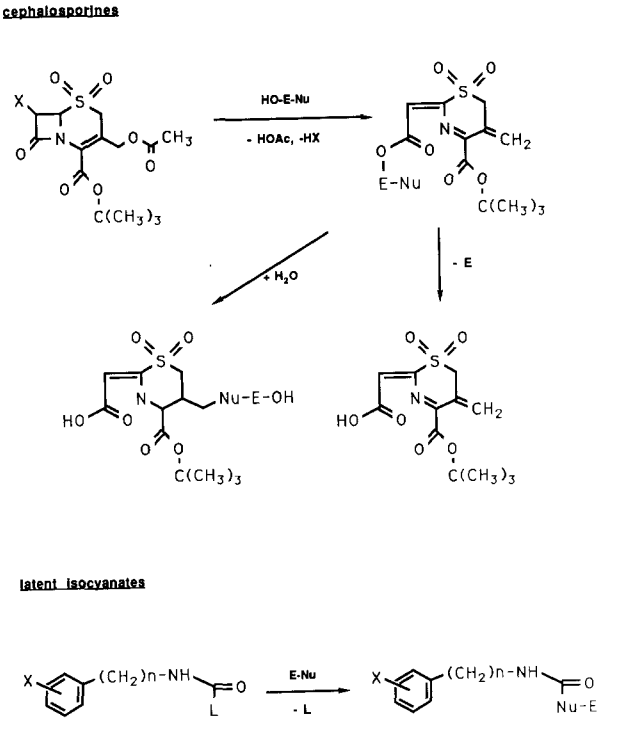

FIGURE 7b Enzyme-activated inhibitors (selected structures)

mediates react with different nucleophilic groups in the chymotrypsin active site.[278] The relatively low reactivity of the substrate analogue N-nitroso amides may be greatly enhanced by photolysis.[279] Because of the carcinogenicity of the nitroso group the compounds will probably not have therapeutic use.[280]

Coumarins By analogy to the reactions of haloenol lactones coumarins are activated by lactone ring opening induced by the nucleophilic attack of the target enzyme. But compared to haloenol lactones, some coumarins e.g. chloro isocoumarins, carry a second potential acylating function. Depending on the position of the ring substituent however, the subsequent chemical reactions by the emerging reactive groups proceed differently. Enzymatic attack on halomethyl coumarins and alkoxychloroamino coumarins releases reactive methylene groups or carbonium ions leading to stable modification of the target enzymes.[281–289]

Binding constants for the inhibitors are in the nM-range and the half-lives of the inactivation reactions are between 1 and 10 min. The selectivity of the inhibitors between proteases with different substrate specificities is surprising (Table IX).

Human leukocyte elastase is inhibited by 3-methoxy-4-chloro-7-(N-toluenesulphonyl-phenylalanyl)-amino isocoumarin with a second order rate constant of $k_{inact}/K_i = 2 \times 10^5\ M^{-1} \cdot s^{-1}$. Recently, selective compounds for the trypsin-like coagulation enzymes and kininases were described, showing that using heterocyclic structures very specific inhibitors may be designed (e.g. inactivation of human plasma kallikrein

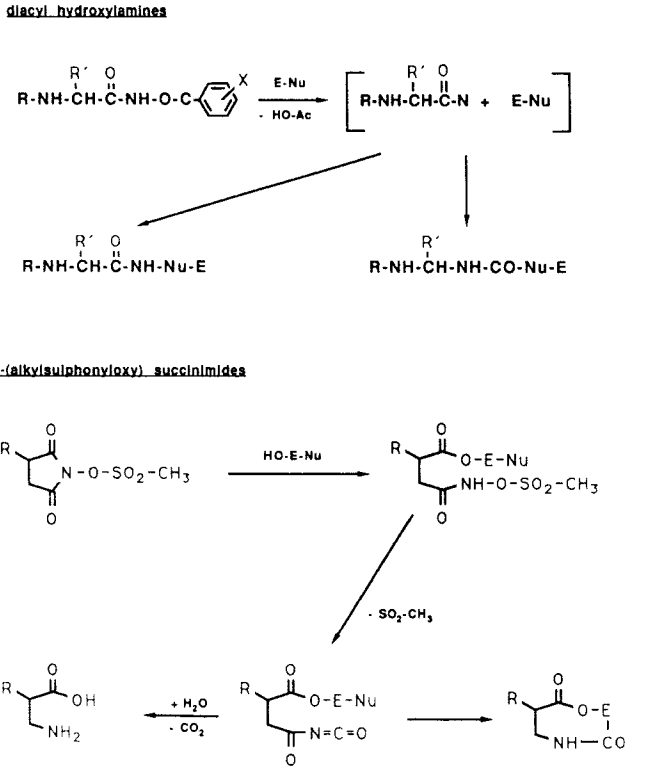

FIGURE 7c Enzyme-activated inhibitors (selected structures)

TABLE IX
Inactivation of serine proteases by 3-alkoxy-4-chloro-7-amino isocoumarin (data from 265)

Inhibitor	Specificity	Enzyme
(3-alkoxy substituent)	(k_{obs}/I in $M^{-1} \cdot s^{-1}$)	
methoxy	10000	human leukocyte elastase
	1000	porcine pancreatic elastase
	17	cathepsin G
	110	chymotrypsin
ethoxy	9400	human leukocyte elastase
	700	porcine pancreatic elastase
	200	cathepsin G
	270	chymotrypsin

by 3-ethoxy-4chloro-7-guanidino isocoumarin: $k_{inact}/K_i > 5 \times 10^5$ $M^{-1} \cdot s^{-1}$).[283] In Table X the most important structrual representatives are listed. For a recent review see reference.[265]

Cephalosporins Cephalosporins are well-known enzyme-activated inhibitors of serine transpeptidases involved in cell wall synthesis of bacteria.[290] Lactam ring cleavage by the active site serine of these D-Ala-D-Ala-peptidases results in an acyl enzyme which is anchored within the active site of the target enzyme by numerous

TABLE X
Coumarins as protease inhibitors - structural and mechanical examples

Compound	Reaction	Ref.
Chloro isocoumarins	lactone ring opening, acylation of a nucleophile by intermediate carbonyl chloride	281–283
3-alkoxy-4-chloro-7-amino coumarin	lactone ring opening, formation of a 4-quinone imine, alkylation of a nucleophile.	284–286
halomethyl coumarins	lactone ring opening, formation of a quinomethide, protonation, alkylation of a nucleophile.	287–289

interactions. The deacylation half-lives are longer than 500 hours making the compounds important antibiotics in clinical practice.

Looking for potent inhibitors of human leukocyte elastase, a group at Merck found cephalosporins with anti-elastolytic activity. Structural modification of the compounds resulted in inhibitors exhibiting binding constants in the micromolar range and inactivation half-lives of some seconds.[291,293]

According to x-ray analysis and molecular modelling of the complex between a beta-lactam antibiotic and porcine pancreatic elastase, the inactivation is initiated by lactam ring cleavage. Subsequently, bonds in the heterocyclic system undergo rearrangement and eliminates an acyl residue, forcing the formation of a reactive methylene which probably alkylates the active-site histidine. Work by Hachim and Smith[294] has previously suggested the presence of this intermediate which leads to oxazolinones in the inhibition of α-chymotrypsin by clinically used cephalothin and other cephalosporins with good 3-substituted leaving groups. However, recent pharmacological studies with one of the new compounds demonstrate reactivation within 3 days. With a specificity constant of $k_{inact}/K_i = 1.28 \times 10^4\ M^{-1} \cdot s^{-1}$ and no effect on thrombin, trypsin, plasmin, papain, chymotrypsin and cathepsin G, the substituted beta-lactam L-659, 286 specifically inhibits humam polymorphonuclear leukocyte elastase in an animal model.[295]

Reactive nitrogen intermediates: latent isocyanates and nitrenes Several organic reactions proceed via formation of reactive nitrogen intermediates. Nitrenes and nitrenium ions are, like carbenes and carbonium ions electron deficient, reactive intermediates of alpha-elimination reactions. Typically, the subsequent or concerted migration of the neighbouring aryl and alkyl residues yields the electrophilic isocyanates.[296,297] Brown and Wold used such alkyl isocyanates to inactivated serine proteases.[298,299]

To circumvent the high toxicity and spontaneous hydrolysis of isocyanates, Groutas and coworkers designed compounds in which masked isocyanates are enzymatically activated.

N-Imidazolyl carbonyl amines and *N*-imidazolyl aminoacid methyl esters form intermediate isocyanates after a base-catalyzed deprotonation of the carbaminic acid expelling the leaving imidazole.[300–302]

By variation of the ring substituents selectivity between elastase, trypsin and plasmin could be demonstrated. Human leukocyte elastase was inactivated with an apparent second order rate constant of $k_{obs}/I = 2.07 \times 10^3\ M^{-1} \cdot s^{-1}$.

Carbaminic acid sulphonate salts and carbaminic acid *N*-hydroxy succinimide esters developed especially as inhibitors for human leukocyte elastase, react in a similar fashion.[303–304]

In a different approach, reactive nitrogen intermediates were anticipated to be

generated in enzyme-induced Curtius- or Lossen rearrangements. The classic reactions are nucleophilic migrations from a carbon to a nitrogen atom. Losing a nucleofuge, the uncharged nitrogen atom with six electrons in its outer shell invites the migration of a proximale group carrying an electron pair. The migration and the nucleofuge elimination occur either by a concerted process yielding isocyanates directly or via a highly-reactive nitrene intermediate.[296,297]

Since migration of a peptide analogue residue R held within an enzyme's active site might be hindered, nucleophilic attack on the scissile carbonyl carbonamide linkage could promote alpha-elimination resulting in short lived highly electrophilic nitrenes.

In fact, the incubation of serine proteases with active-site directed carboyl azides resulted in the inactivation of chymotrypsin and dipeptidyl peptidase IV.[306] In contrast to hydrolytically labile carbonyl azides, the substrate analogue *N*-peptidyl-O-acyl hydroxylmaines were proved to be stable and selective inhibitors for serine and cysteine proteases (Table XI).[307,310–312] Using dipeptidyl peptidase IV as a model and analysing the mechanistic and kinetic properties of this new inhibitor class they could be characterized as mechanism-based inhibitors.[308,309] The structure of the compounds ideally permits the tailoring of their selectivity simply by exchange of acyl residues at the hydroxylamine linkage.[308,311] Since, depending on purpose and target enzyme, the stability and reactivity of the compounds may be predetermined the diacyl hydroxylamines may thus become suitable reagents for clinical purposes. The inhibitors have been used in biological investigations to evaluate the participation of depeptidyl peptidase IV in human T lymphocyte activation.[314–316] So far, the mechanism of protease inactivation by diacyl hydroxylamines has not been established. However, recent studies on the spontaneous degradation of *N*-peptidyl-*O*-(4-nitrobenzoyl) hydroxylamines in aqueous solution indicate heterolytic N-O- bond fission of the hydroxylamine linkage as the rate-determining step of this reaction in a lossen-reaction.[313]

TABLE XI
Inactivation of proteases with N-peptidyl-O-acyl hydroxylamines

Inhibitor	Specificity (k_{inact}/K_i in $M^{-1} \cdot s^{-1}$)	Enzyme (type)		Reference
H-Ala-Pro-NHO-Nb	1.9	DP IV	(serine)	308
BOC-Ala-Pro-NHO-Nb	2.5	PE	(serine)	310
BOC-Ala-Ala-NHO-Nb	12	porc. panc. elastase	(serine)	310
	166	thermitase	(serine)	310
	226	subtilisin	(serine)	310
BOC-Gly-Phe-NHO-Bz	652	thermitase	(serine)	310
	1 020	subtilisin	(serine)	310
Z-Phe-Gly-NHO-$(CH_3)_3$Bz	640 000	cathepsin B	(cysteine)	311
Z-Phe-Gly-NHO-Ac	980	cathepsin B	(cysteine)	311
Z-Phe-Phe-NHO-Ma	2 800	cathepsin B	(cysteine)	312
	1 222 000	cathepsin L	(cysteine)	312
	21 000	cathepsin S	(cysteine)	312
	19	cathepsin H	(cysteine)	312
BOC-Ala-Phe-Leu-NHO-Nb	12 000	cathepsin B	(cysteine)	312
	606 000	cathepsin L	(cysteine)	312
	229 000	cathepsin S	(cysteine)	312
	32	cathepsin H	(cysteine)	312

TABLE XII
Inactivation of serine proteases by N-(sulphonyloxy succinimides (data from 317–319)

Inhibitor (3-alkyl substituent)	(sulphonic acid)	Specificity (k_{obs}/I in $M^{-1} \cdot s^{-1}$)	Enzyme
benzyl	methane	9 000	chymotrypsin
		1 100	human leukocyte elastase
isobutyl	methane	50 000	human leukocyte elastase
isobutyl	trans-styrene	> 100 000	human leukocyte elastase
H	trans-styrene	inactive	human leukocyte elastase

Exploiting the above principle, masked heterocylic nitrene or isocyanate generating structures might be used to perform enzyme-activated protease inhibition. Groutas and coworkers recently studied N-hydroxy succinimide derivates as inhibitors of chymotrypsin and leukocyte elastase (Table XII).[317,318] Using 3-benzyl-*N*-(methanesulphonyloxy) succinimide the inactivation mechanism of chymotrypsin via a latent isocyanate was supported[319] by NMR studies.

CONCLUSION

A variety of structural and mechanistic ideas for protease inhibitor design have been developed and tested in the past decade. Among the above described synthetic inhibitors of serine and cysteine proteases are potential candidates far further pharmacological studies and perhaps may find use as clinical agents for the treatment of a variety of diseases. Increasing knowledge of the molecular basis for pathogenic processes will stimulate further development of low molecular weight protease inhibitors. The mechanism-based inhibitors especially, with their potential for tailoring to a particualr target enzyme are already being considered for use as drugs.

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