Cysteine Proteases and Their Inhibitors

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Contents

1. Introduction

The large family of peptide-bond-cleaving hydrolases, the peptidases (= proteases, EC 3.4), can be categorized as endopeptidases (= proteinases, EC 3.4.21−99) and exopeptidases (EC 3.4.11−19), according to the point at which they break the peptide chain. These enzymes can be ordered further, according to the reactive groups at the active site involved in catalysis, into serine (EC 3.4.21), cysteine (EC 3.4.22), and aspartic endopeptidases (EC 3.4.23) and metalloendopeptidases (EC 3.4.24). Cysteine carboxypeptidases, i.e. exopeptidases of the cysteine class which cleave polypeptide chains at the C terminal, are found in the group EC 3.4.18. Enzymes whose reaction mechanism has not been completely elucidated are classed in the subgroup EC 3.4.99.[1] In terms of numbers, the serine peptidases have been most extensively investigated and are the best characterized subgroup. Cysteine proteases, which are mostly referred to as thiol proteases in older literature, have been found in viruses, bacteria, protozoa, plants and mammals.[2] Recently, cysteine proteases were also discovered in fungi.[3−5]

2. Cysteine Proteases: Occurrence and Structure

2.1. Viral Cysteine Proteases

Certain viruses such as the picornaviruses, which include rhinoviruses, poliomyelitis, and hepatitis A viruses (HAV), code their proteins in the form of a single precursor which is cleaved at specific points by a peptidase that also originates from this precursor protein.[6,7] These enzymes are cysteine proteases. The recently published X-ray structures of the 3C proteinase (picornain) of the HA virus[8a] and of the rhinovirus[8b] confirm the theory[9] that the three-dimensional structure and catalytic mechanism of these enzymes are similar to the serine proteases of the chymotrypsin family and that viral 3C proteases and serine proteases of the trypsin/chymotrypsin family originated from a common ancestor by divergent evolution.[8c]

2.2. Bacteria

Staphylococcal cysteine protease (EC 3.4.22.13; MW 13 000[13a]), isolated from the culture filtrate of

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Tanja Schirmeister, born in 1963, studied pharmacy from 1982 to 1987 at the Albert-Ludwigs-University in Freiburg. She passed her final examinations in 1987 and one year later got her license as a pharmacist. In 1993 she received her Ph.D. for a work on enzymatic hydrolyses of esters under the guidance of Professor Dr. H.-H. Otto. For this dissertation she was awarded with the Carl-Wilhelm-Scheele prize of the German Pharmaceutical Society DPhG. Since 1993 she has been working on the development of new cysteine protease inhibitors.

Staphylococcus aureus, is characterized by a very high substrate tolerance: it cleaves proteins on the carboxy and amino sites of most amino acids.[13b] The ability of the enzyme to degrade elastin may have a pathological role.[14] Streptococcal cysteine protease (streptopain; EC 3.4.22.10)[10] is unusual for a bacterial protease in that it is formed from an inactive zymogen by proteolysis and reduction.[11] Both the sequences of the zymogen[11] (337 amino acids) and of the active enzyme[10] (253 amino acids) are known. The specificity of this protease is similar to that of the papain family[12] (section 2.5). The identity of streptopain as SPE B (streptococcal pyrogenic exotoxin B) has been shown.[414] (See also section 2.3; see section 7.2.7 for use of cysteine protease inhibitors in the treatment of streptococcal infections *in vivo*). Clostripain[15] (EC 3.4.22.8), isolated from *Clostridium histolyticum*, is a dimeric peptidase[15] which can be activated by calcium.[16] The sequence of the shorter polypeptide is known (MW 15 398).[17] This enzyme is highly substrate specific: clostripain preferentially cleaves Arg−Xaa bonds and is similar in this respect to the serine protease trypsin.[18] Its specificity for proline at the P1′ position is relatively unusual for a protease.[207] Clostripain is considered to be a prototype protease which functions according to the general mechanism of the cysteine proteases but differs from the enzymes of the papain family in its specificity and sequence (see also Table 17a). Arg-gingipain (RGP) and Lys-gingipain (KGP) are cysteine proteases of multiple forms in *Porphyromonas gingivalis*, a rod which plays a major role in the pathogenesis of chronic periodontitis. These proteases have "trypsin-like" activity and act as virulence factors in periodontal diseases.[410,411,414] (See also section 2.3 for bacterial PepC proteases).

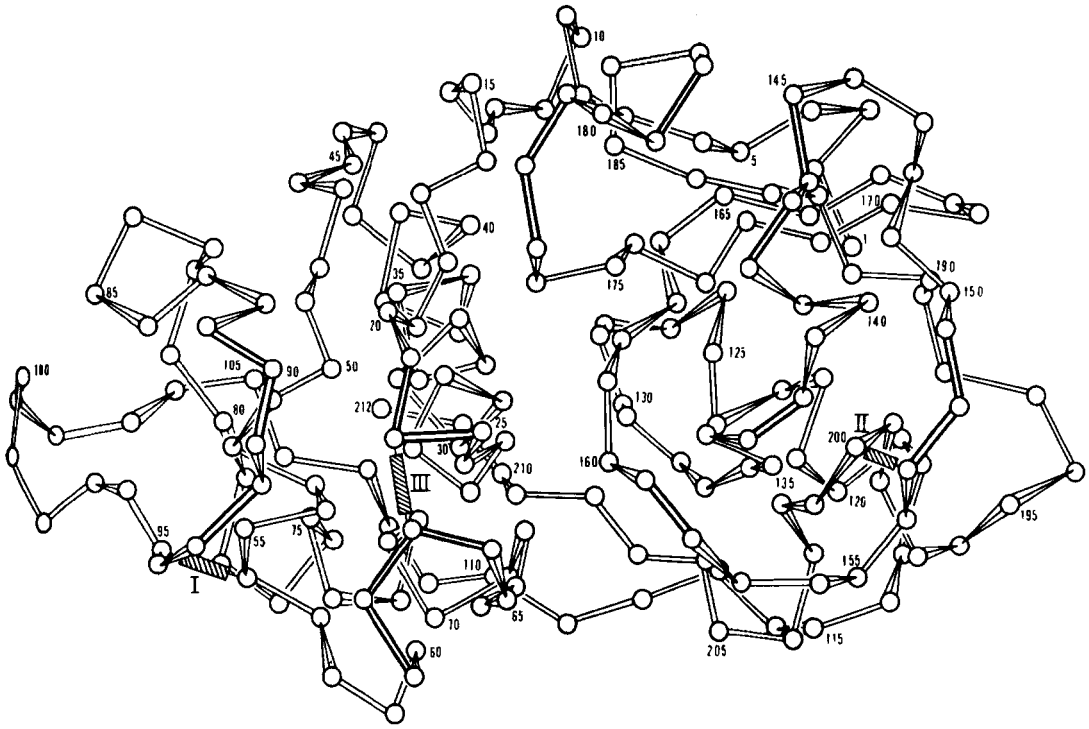
2.3. Fungi

There are very few reports of thiol-dependent enzymes in fungi. In 1989, cathepsin B (section 2.6.1) was found in a cell homogenate of *Aspergillus flavus*.[3] A calcium-dependent, membrane-bound cysteine endopeptidase has been isolated from yeast (proteinase yscF).[4,19] Since the gene sequence has significant homology in one domain to subtilisin, characterization of the enzyme as a cysteine protease is still questionable.[20] In 1993, a cytoplasmic enzyme was isolated from yeast[5] which is equivalent to mammalian bleomycin hydrolase[21] (BLH, responsible for bleomycin tumor resistance). The enzyme is a cysteine aminopeptidase,[5] and the sequence of the active site corresponds to that of the cysteine proteases of the papain family.[5] The crystal structure of Gal6, the homolog of BLH in yeast, is now known.[473] The enzyme has a *hexa*meric structure with a prominent central channel. The papain-like active sites are situated within this channel. Cysteine aminopeptidases (*PepC)* from *Streptococcus thermophilus* and *Lactococcus lactis* are also similar to the eucaryotic BLH[208,412,413] and may constitute a new family of thiol aminopeptidases.

2.4. Protozoa and Worms

Although parasitic protozoa are a very diverse group of organisms, most of the proteinases isolated from them belong to the cysteine proteases.[22] Some of these enzymes play a vital role in parasite nutrition and invasion of the host cells.[23] Many new publications (e.g. refs 428−431, 470, and 499) are concerned with these enzymes since these proteases are promising targets for the treatment of parasitic infections. Only a selection of the many cysteine proteases are described here. Cruzipain[24,25] (cruzain) is the common name for the enzyme with the highest proteolytic activity in *Trypanosoma cruzi*, the organism transferred to humans by various parasitic bugs which causes American trypanosomiasis (Chagas' disease[26]). The known fragment sequences of the 60 kDa glycoprotein, its specificity of proteolysis, and inhibition by E-64 (see section 7.2.10) all indicated a relationship to the proteases of the papain family[24] (Figure 13, $k_{2nd} = 20\ 800\ M^{-1}\ s^{-1}$) which was proven

Chart 1. Tertiary Structure of Papain: Position of the α-C Atoms and Disulfide Bridges. (Reprinted from ref 43. Copyright 1976 Elsevier.)

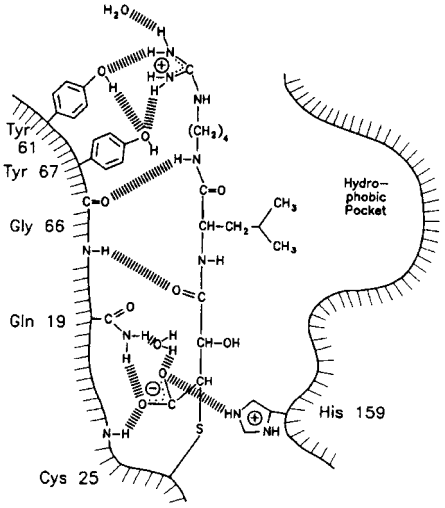


by the recently elucidated structure of this enzyme.[404] Possibly the enzyme is involved in protection of the parasite from the host immune system by degradation of IgG. Trypanopains[427] are cysteine proteases isolated from african trypanosomes. It is certain that invasion of the magna form (tissue form) of *Entamoeba histolytica*, which causes amoebiasis,[26] into the intestinal wall and lysis of the liver parenchyma cells are a result of cysteine protease activity.[426] Similarly, there seems to be a connection between virulence of an entamoeba strain and expression of these proteases.[27,424] The names amoebapain[28] and histolysin[29] have been suggested for two of the enzymes involved in these processes, although the proteases may possibly be identical. According to recent results an extracellular cysteine protease of entamoeba trophozoites may be involved in cleavage of complement components C3 and C5 and thus circumvent normal host immunity.[425] Twenty-three cysteine proteases have been found in *Trichomonas vaginalis*,[30] a trichomonades species which is pathogenic in humans. These differ from the well-characterized cysteine proteases of plants and animals in that they have an unusually high molecular weight (up to 96 kDa).[31] Successful tests with protease inhibitors have been performed *in vivo* with *Trichomonas vaginalis*.[32] Proteases also play a role in malaria, one of the most significant infectious diseases in humans. Malaria is caused by four different types of the species *Plasmodium*[26] and proteases are involved during the asexual phase which takes place in human.[33] The proteolytic activity is evenly distributed between serine, cysteine, and aspartic endoproteases.[34] Ten different enzymes are known at present which are involved in attack of the merozoites on erythrocytes, degradation of human hemoglobin as a source of nutrition for the schizontes and destruction of the erythrocytes.[33,34] New results indicate that a trophozoite cysteine protease is required for initial cleavage of globin by intact parasites.[423] The cysteine proteases which have been isolated from *Plasmodium* have a high degree of similarity with the enzymes of the papain family; on the other hand, there are sequences which are only conserved within the *Plasmodium* enzymes and are probably responsible for specific hydrolysis of the globin ("hemoglobinase" activity).[205] One of these enzymes was named falcipain.[422] Initial experiments with cysteine protease inhibitors have been performed with promising results[33−35] (see section 2.6.2 for the role of calpains; see also Table 17b). Erythrocyte hemoglobin is also an important source of nutrition for the adult form of the trematode *Schistosoma mansoni*, responsible for intestinal bilharzia. Probably several cysteine proteases are involved in degradation of hemoglobin by this organism.[36] Two are cathepsin-like enzymes (cathepsin B, refs 399 and 486; cathepsin L, refs 432 and 497) and another shows no homology to papain. This enzyme has sequences in common with the enzymes from *Plasmodium*.[205] It has been shown that legumain (see section 2.5) an atypical cysteine protease from legume seeds is a homolog of this hemoglobinase (Sm32).[399,432]

2.5. Plant Cysteine Proteases

The most extensively investigated plant cysteine protease is papain (EC 3.4.22.2) from the latex of *Carica papaya* (melon tree). Papain is a monomeric polypeptide with a molecular weight of 23 406, consisting of a chain of 212 amino acid residues with three disulfide bridges (Cys22-Cys63, Cys56-Cys95, Cys153-Cys200). The sequence[37] and three-dimensional structure[38,43] are known (Chart 1, from ref 43). X-ray structural analysis has also been performed on papain−inhibitor complexes with the inhibitors BPACK[39] and E-64[40] (Chart 2, from ref 40). The

Chart 2. Schematic Diagram of the Enzyme−Inhibitor Complex of E-64 and Papain. (Reprinted from ref 40. Copyright 1989 American Chemical Society.)



active site is formed by amino acid residues Cys25 and His159 (section 3). Figure 1 shows the selectivity

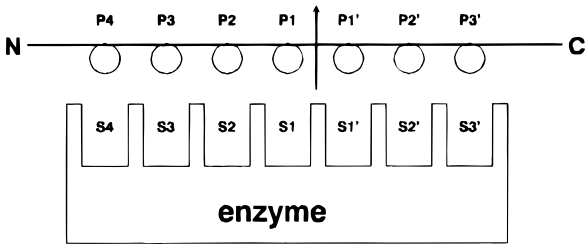


Figure 1. Terminology of protease specificity. (According to Berger and Schechter, ref 41.)

diagram developed by Berger and Schechter,[41] according to which papain has seven subsites at the active site which can each bind to an amino acid residue of the substrate. Papain is particularly good at cleaving peptides at Arg and Lys residues (P1), but this S1 subsite has a much less well-defined specificity than the S2 subsite, which prefers hydrophobic site chains such as Phe. The S2 site of the enzyme is a hydrophobic pocket, formed by the amino acid residues Trp69, Tyr67, Phe207, Pro68, Ala160, Val133, and Val157.[42] Papain is isolated as an inactive form, in which the cysteine residue at the active site is blocked by a disulfide bond with Cys22 and Cys63 has a free thiol group instead.[42] Activation is possible by intramolecular disulfide exchange with thiol reagents or with reducing agents. Papain is formed from a classical zymogen (propapain).[44] This proregion has been shown to be essential for correct folding and processing[505] and can act as a high-affinity inhibitor of the enzyme.[505] Other plant cysteine proteases whose known fragment sequences indicate a relationship to papain[43] are e.g. the chymopapains A and B,[97c] also from *Carica papaya*, ficin[97a] (EC 3.4.22.3) from the latex of various *Ficus* species (Moraceae), the bromelains[97b,d] (EC 3.4.22.4) from the latex of the pineapple tree steam (Bromeliaceae),[43] and the protease CC-III[378] isolated from another *Carica* species. Other recently characterized plant enzymes which also belong to the papain family are aleurain[419] (related to cathepsin H), phytolacain R,[417] and a ginger protease.[418] The above-mentioned legumains[498] are asparaginyl endopeptidases and constitute a unique group of cysteine proteases distinct from the papain family. Papain, cathepsin B,[61,406] the viral 3C proteinases, cruzain, and the bleomycin hydrolase,[473] mentioned above, as well as actinidin[45] (= actinidain) from kiwi fruit (chinese gooseberry, *Actinidia chinesis*), ICE[401] (see section 2.6.2), and caricain[495] (= papaya protease Ω, EC 3.4.22.30) are the only cysteine proteases for which the complete amino acid sequence as well as the tertiary structure are known. Although the sequences of actinidin and papain are only 48% identical, their structures are remarkably similar. Thus the specificity of protein cleavage of actinidin is similar to that of papain; peptides with hydrophobic amino acids at P2 are preferentially hydrolyzed although aromatic residues are bound 10−100 times weaker by the smaller hydrophobic S2 site. A detailed comparison of both structures is given in refs 42 and 45. It is noteworthy that the tertiary structure of calotropin DI from *Calotropis gigantea* has been elucidated although the amino acid sequence is not yet complete.[46,47] The molecular architecture is similar to the other two proteases; the peptide chain is also folded into two domains which are separated by a cleft containing the active site. The Cys residue involved in hydrolysis is on one site of the cleft (L domain) and the corresponding His residue is on the opposite site (R domain).

2.6. Mammals and Humans

The most important cysteine proteases in mammals are the cytoplasmic calpains and the lysosomal cathepsins. The name "cathepsin" was introduced by Willstätter and Bamann in 1929 for a protease which is active at acid pH but can be differentiated from pepsin.[48] In 1941, Fruton *et al.* were able to identify at least three enzymes in cathepsin preparations, cathepsin I, II, and III.[49] The cathepsins were reclassified on the basis of their specificity by Tallan *et al.* in 1952 and the names cathepsin A, B, and C were suggested for the intracellular counterparts of pepsin, trypsin, and chymotrypsin.[50] In 1957, Greenbaum and Fruton showed that cathepsin B is a cysteine protease.[52] In 1971, Otto and co-workers discovered that cathepsin B shows two enzymatic activities.[53] Cathepsin B1 is an endopeptidase[54] and is known today as cathepsin B (EC 3.4.22.1, synonym cathepsin II) and cathepsin B2 is the lysosomal carboxypeptidase B[54] (EC 3.4.18.1, synonym cathepsin IV). Today the term "cathepsin" is used to refer to intracellular proteases, mostly localized in the lysosomes, which are active at weakly acid pH values (the lysosomal pH is about 5).[55] Lysosomes are organelles, discovered by de Duve in 1949, which form by pinching off from the Golgi apparatus and which contain a multitude of hydrolytic enzymes.[57] Most of the lysosomal proteases are cysteine proteases, with the exception of cathepsin G[58,60a] (EC 3.4.21.20) and the exopeptidase cathepsin A[59] (EC 3.4.16.1, synonym cathepsin I, catheptic carboxypeptidase A, lysosomal carboxypeptidase A, LCA), which are both serine proteases, and the homologous as-

Table 1. Cathepsins and Families of Cysteine Proteases

a. Cathepsins[a]

name	EC no.	type	MW (kD)	MW_{sub} (kD)	p*I*	comments	synonyms	ref(s)
A	3.4.16.5	Ser	100−400	20−55	4.7−5.8	L	Cath I, LCA carboxypeptidase C	59,203
B	3.4.22.1	Cys	29 27[b]	24 +5	4.5−5.5	S G L X	Cath II, B1	58,59 61,82
B2	3.4.18.1	Cys	50	25 ×2	5	L	Cath IV, LCB	59
C	3.4.14.1	Cys	200	24 ×8	5.0−6.0	G Hal L	DAP I	59
D	3.4.23.5	Asp	42 36[b]	26 +10	5−7	G S L		58,60b
E	3.4.23.34	Asp	40−90	45 ×2	4.1−4.4	M		58
F		?	50			L? M?		58,62
G	3.4.21.20	Ser	17−30			G L		58,60a
H	3.4.22.16	Cys	26 24[b]	22 +4	6.0−7.1	S L G	Cath B_a,B_3,III, BANA-hydrolase	58,59 65,78
I		Cys	26		5.9−6.5		identical to Cath H	63
J		Cys	230		5.8−6.1	L?		64
(K		Cys	650		5.3	C?		64)
K	3.4.22.38	Cys	27/29			L S G	Cath O, O2, X	489−491
L	3.4.22.15	Cys	29	24 +5	5.0−6.1	S G L		72,78 80,81
M		Cys	30			M L		63,88
N		Cys	20−34		5−7	L	collagenolytic Cath	58,63 86
(O		Cys	23				S G	407)
P		Cys	32			L	Cath B variants	73
R	3.4.21.52	Ser	30			R		58
S	3.4.22.27	Cys	14−30		6.3−6.9	N L S		63,86 91,494
T		Cys	34			G L		63,87
(X		Cys					possibly identical to Cath L	92,93,94)
Z								493
III	3.4.11.1	Met	326−360	54 ×6		S G C	cytosolic amino peptidase	96

b. Families of Cysteine Proteases[c]

family	representative enzymes	family	representative enzymes
clan A/C1	papain	C12	deubiquitinating peptidase Yuh 1
clan A/C2	calpain	C13	hemoglobinase
clan A/C10	streptopain	C14	ICE
clan B/C3	picornain	C15	pyroglutamyl-peptidase I
clan B/C4	tobacco etch virus NIa endopeptidase	C16	mouse hepatitis virus endopeptidase
clan C/C6	tobacco etch virus HC-proteinase	C17	microsomal ER60 endopeptidase
clan C/C7	chestnut blight virus p29 endopeptidase	C18	hepatitis C virus endopeptidase 2
C5	adenovirus endopeptidase	C19	deubiquitinating peptidase Ubp 1
C8	chestnut blight virus p48 endopeptidase	C20	type IV prepilin leader peptidase
C9	sindbis virus nsP2 endopeptidase	C21	turnip yellow mosaic virus endopeptidase
C11	clostripain		

[a] Abbreviations for part a: MW, approximate molecular weight of the quaternary structure; MW_{sub}, approximate molecular weight of the subunits. [b] MW without carbohydrate portion; S, amino acid sequence known; N, amino acid sequence of N terminal known; Hal, requires halide ions; M, membrane-bound enzyme; R, ribosomal enzyme; G, glycoprotein; L, lysosomal enzyme; C, cytoplasmic enzyme; X, X-ray structure known; LC, lysosomal carboxypeptidase; BANA, *N*-benzoylarginine-*β*-naphthylamide; DAP, dipeptidyl-aminopeptidase; Ser, serine protease; Cys, cysteine protease; Met, metalloprotease; Asp, aspartate protease. [c] According to ref 399.

partate proteases cathepsins D and E[58,60b] (EC 3.4.23.5). The ribosomal cathepsin R is also a serine protease (EC 3.4.21.52).[58] Table 1a gives an overview of the enzymes which are or have been referred to in the literature as cathepsins.

2.6.1. Lysosomal Cysteine Proteases

Cathepsins. Of all the lysosomal cysteine proteases, cathepsin B has been the most extensively investigated. This enzyme has been isolated from various mammalian tissues; the enzymes from different species do not differ to any great extent.[51] Recently the enzyme was discovered in fungi.[3] The three-dimensional structure of the enzyme was first obtained in 1991;[61] recently the structure of recombinant rat cathepsin B was elucidated.[406] Cathepsin B is a glycoprotein which has a species-specific carbohydrate residue[75] bound to a Asn residue[61] (Asn110/113) (papain/human cathepsin B numbering). The sequence of cathepsin B is known for the enzyme from rat liver,[65] bovine liver,[66] and human liver[67] (254 amino acids) and fragmental sequences are known for the enzyme from various other mammalian tissues.[63] The molecular weight of the gly-

Table 2. Cysteine Protease Inhibitors and K_I Values (nM) for Inhibition of Cysteine Proteases by Cystatins

a. Cysteine Protease Inhibitors[a,b]

CPI	Synonyms, Species-Specific Variants	CPI	Synonyms, Species-Specific Variants
		Family 1	
cystatin A	ACPI, stefin A, rat cystatin α, rat epidermal TPI	stefin C	
		cystatin B	NCPI, stefin B, rat cystatin β, rat liver TPI
		Family 2	
cystatin C	gamma-trace, post-gamma-globulin, human cystatin, chicken cystatin, bovine colostrum cystatin	cystatin SN	cystatin SA-1, cystatin SU
		cystatin SA	
		cystatin D	
cystatin S	SAP-1		
		Family 3	
LMW kininogens	L-kininogen, α_2-CPI, α_2-TPI	T kininogens	MAP, thiostatin, rat α_1-CPI, rat-LMW-kininogen
HMW kininogens	H-kininogen, α_1-CPI, α_1-TPI		(only known in rat at present)

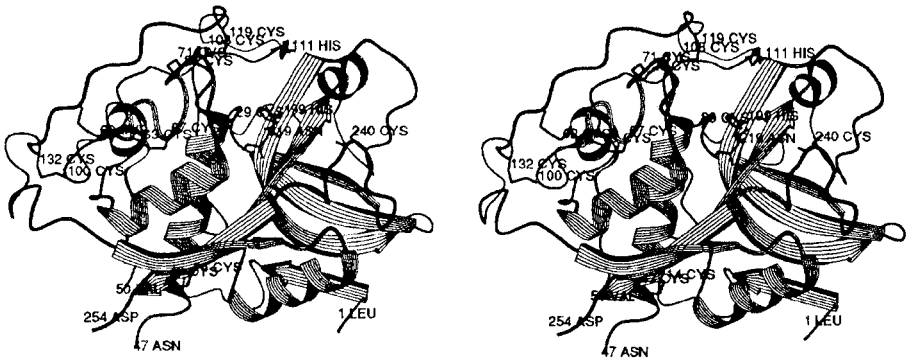
b. K_I Values [nM] for Inhibition of Cysteine Proteases by Cystatins[c,d]

enzyme	cystatin A	cystatin B	cystatin C	chicken cystatin	HMW kininogens	LMW kininogens	segment 1	segment 2	segment 3
papain	0.019	0.12	0.005	0.005	*a*	0.015	>100	0.083	0.030
Cath B	8.2	73	0.25	1.7	400	600	*a*	*a*	*a*
Cath H	0.31	0.58	0.28	0.064	1.1	1.2	*a*	*a*	*a*
Cath L	1.3	0.23	0.005	0.019	0.019	0.017	>100	0.14	0.005
Cath C	33	0.23	3.5	0.35	*a*	>130 *b*	*a*	*a*	*a*
CANP	>10 000	>10 000	>10 000	>10 000	*a*	1.0	>100	1.0	>100

[a] Abbreviations for part a: CPI, cysteine protease inhibitor; TPI, thiol protease inhibitor; ACPI, acidic CPI; NCPI, neutral CPI; SAP, human salivary acidic protein; LMW, low molecular weight; HMW, high molecular weight; MAP, major acute phase α_1-protein, major acute phase globulin. [b] According to refs 195 and 506. [c] Abbreviations for part b: a, not determined; b, mixture of H- and L-kininogens. [d] According to ref 196.

coprotein is ca. 29 000, or 27 500 without the carbohydrate portion. Cleavage between Asn43/47 and Val44/48 produces a light chain (MW 5000) and a heavy chain (MW 24 000) which are bound together by a disulfide bridge.[61] The active site is a part of the smaller chain (Cys25/29). Cathepsin B is formed as a 39 kDa N-glycosylated proenzyme[514] which is cleaved to the double-chain enzyme on its path through the Golgi apparatus to the lysosomes.[68] There is evidence that cathepsin B activates procathepsin B itself.[74] Autocatalytic activation by the propeptide might also be possible. Recently the crystal structure of procathepsin B was solved.[513,519] The main difference between propeptide and native enzyme is that the occluding loop region (Ile105-Pro126) in procathepsin B is lifted away from the enzyme surface, building a wall that interacts with the propeptide, thus supporting its structure and shielding the active site from exposure to solvent.[513] A synthetic peptide constructed corresponding to the proregion of cathepsin B was found to be a potent inhibitor of cathepsin B itself.[515] According to recent results inhibition occurs by blocking access to the active site.[519] A part of the proregion enters the cleft between the two domains in a reverse orientation to natural substrates.[519] As for all lysosomal enzymes, the sugar portions of the molecule are responsible for sorting, i.e. separation of lysosomal enzymes from secretory proteins. A phosphomannosyl residue functions as a recognition marker, which interacts with the mannose 6-phosphate receptor in the Golgi membrane and thus steers transport in the direction of the lysosomes.[69] Cathepsin B has seldom been detected in the extracellular medium, at least in nonpathological tissue, due to its low stability in neutral to alkaline pH.[63,70] It is interesting that the alkali-stable enzyme forms that have been found in the extracellular medium mostly have a higher molecular weight than the lysosomal enzymes and were mainly found in tumor tissue[42,63,71] (section 5.3). In addition, cathepsins are inhibited by specific endogenous extracellular proteins (section 6) and thus their activity is difficult to detect. In contrast to lysosomal cathepsin B, the high molecular weight extracellular forms are not inhibited by the nonspecific endogenous inhibitor α2-macroglobulin.[71] Comparison of the sequences of cathepsins B and H, papain and actinidin, and the known partial sequences of bromelain shows that the tertiary structures of all these proteases must be similar.[45,72] This was confirmed by the X-ray structural analysis of the two-chain form of cathepsin B, published in 1991[61] (Chart 3, from ref 61). One hundred and sixty-six of the amino acid residues are topologically equivalent to papain but large structural changes result from insertions (106−124) in the middle part of the chain building an occluding loop. This leads to significant changes in the surface of the cleft between the domains and explains the difference in specificity. Although the S2 site is made up of similar residues to that of papain (Tyr75, Pro76, Ala77, Ala173, and Ala200), it lacks a residue equivalent to Val157 of papain which means that the S2 site is larger and, due to an additional Glu245 residue, can accept positively charged amino acids.[61] Because of the extreme dipeptidyl peptidase activity of cathepsin B it is difficult to draw precise conclusions about its specificity.[42,76] This exopeptidase activity results from the insertion loop which blocks the primed site of the active-site cleft. Two positively charged His111 and His110 residues with relatively low pK_a's located at the occluding loop fix the carboxylate group of the C terminal of the substrate so that a dipeptide can be cleaved off at the C terminal end in the active site.[61] The P1 amino acids can vary widely; P2 amino acids tend to be Gly, Arg, and hydrophobic residues.

Chart 3. Three-Dimensional Structure of Cathepsin B. (Reprinted from ref 61. Copyright 1991 Oxford University Press.)



The S1-subsite specificity is modulated by two in the papain family highly conserved Gly residues (23/65, papain numbering) (see also section 6.2). Cathepsin B has less activity for substrates with Pro or Arg at P1′. The reason is a S1′ site which only can accept extended hydrophobic site chains (Leu, Phe, Tyr, Trp).[61,523] Endopeptidase activity increases with pH, due to deprotonation of the His111 residue. Cathepsin H[58,59,77] is composed of 220 (rat liver)[65] or 230 amino acids (human kidney).[78] The molecular weight is ca. 26 000, including the carbohydrate portion (on Asn110, papain numbering). Like cathepsin B, cathepsin H can be cleaved into a heavy and a light chain; however, cleavage occurs between Asn169a and Gly169b, so that the active site is a part of the large chain. This is no longer active after separation from the light chain because the amino acids Asn-Ser-Trp (175−177), which are a part of the cleft that contains the active site, belong to the light chain.[63] Biosynthesis of the enzyme is similar to that of cathepsin B.[68b,79] Cathepsin H is also similar to cathepsin B in that it has not yet been possible to determine the exact specificity of its endopeptidase activity.[76] Due to its equally strong aminopeptidase activity, cathepsin H is also classed as an endoaminopeptidase.[42,63] Cathepsin L[78,80,81,85] differs from cathepsins B and H in that it lacks exopeptidase activity and has higher proteinase activity: it is the enzyme with the highest proteolytic activity in the lysosomes.[82] Cathepsin L hydrolyzes extracellular matrix proteins such as collagen and elastin more effectively than collagenase and neutrophilic elastase, the enzymes which are better known for their activity on these substrates.[83] Cleavage to the two-chain form takes place much faster than with the other two cathepsins. A further difference is that relatively large amounts (up to 40%) of procathepsin L are secreted.[84] Procathepsin L itself has proteolytic activity in the presence of surface materials, and this is the first evidence that the proenzyme of a cysteine protease shows catalytic activity.[516] The proregion of procathepsin L is a potent and selective inhibitor of cathepsin L too.[518] A new C3-cleaving cysteine protease mainly located at the cell surface (p39)[517] shares sequence identities with procathepsin L. Cathepsin L has a broad substrate tolerance; substrates with hydrophobic P2 and P3 residues are preferred.[63,82] Cathepsin C[59,63] is made up of eight subunits, each of 24 kDa, and it cleaves dipeptides from the N terminal of peptide chains. The enzyme is inactive for substrates that have Arg, Lys, or Pro residues at this position. In contrast to other cysteine proteases, cathepsin C requires halide ions for full activity. Cathepsin S[91,494] is unevenly distributed between organs. The enzyme has been detected in high concentrations in spleen and lung.[494] The stability of the enzyme above pH 7 is a remarkable property. Cathepsin N has only been found in lymphatic cells (lymph nodes, spleen), and at present, this enzyme is together with cathepsin K,[409] the only known cathepsin which is tissue specific.[63] Due to their similarity to cathepsin L, it was uncertain for a long time whether cathepsins S and N were simply tissue or species specific variants of cathepsin L; however in 1988, all three enzymes were detected simultaneously and their separation was achieved.[86] Cathepsin K (OC2,[409] cathepsin O,[408] X,[492] O2[491]) is a recently discovered cathepsin homologous to cathepsins S and L expressed selectively in osteoclasts.[409,489,490] The abundant expression of this enzyme in osteoclasts suggests that it plays a specialized role in bone resorption.[409,489,490] Further unrelated sequences also have been referred to as cathepsin O,[407] cathepsin X,[92 − 94] and cathepsin K[64] (see Table 1a). The only known natural substrate of cathepsin T is tyrosine aminotransferase. Cathepsin T catalyzes cleavage of the native 53 kDa subunit of the transferase into a smaller subunit of 48 kDa.[87] Cathepsin M[63,88] and fructose-1,6-diphosphatase-converting enzyme[63,89,90,95] are the only lysosomal proteases that are known to be membrane bound. In contrast to other lysosomal cathepsins, both enzymes are most active at neutral pH and are stable at high pH, at least in the membrane-bound form. The fru-P2ase C-enzyme (MW ca. 70 000) converts fructose-1,6-diphosphatase into the form which is active at pH 9.2, while cathepsin M inactivates this enzyme.

Other Lysosomal Cysteine Proteases. Two cysteine proteases (TP-1 and TP-2) have been isolated from the lysosomes of the thyroid gland, which release the thyroid gland hormone thyroxine (T4) from thyroglobulin. Both these enzymes are similar to cathepsins H and B (TP-1, MW 28 kDa, p*I* 6.3; TP-2, MW 23 kDa, p*I* 5.5−5.8).[98] The human insulin-degrading enzyme, insulysin (EC 3.4.24.56)[203] ("insulinase") was sequenced in 1988 via its cDNA.[14,99] No homology with known sequences of other enzymes has been observed until now. However, the sequence is similar to that of an *E. coli* protease which is not thiol dependent. Insulysin, which occurs in the lysosomes and in the cytosol, also cleaves glucagon and seems to be responsible for deactivation of these hormones

in vivo. According to recent results, this enzyme represents a new metallopeptidase family.[100] It is mentioned, however, because it was classed as a thiol protease for a long time.

2.6.2. Non-Lysosomal Cysteine Proteases

Calpains. The calpains (EC 3.4.22.17, calcium-activated neutral proteinase CANP, receptor transforming factor RTF, kinase activating factor KAF, calcium activated factor CAF), discovered in 1964,[102] are the cytoplasmic cysteine proteases which have been most extensively investigated. The name "calpain", introduced in 1981, refers to the activation of these enzymes by calcium ions and to the analogy of the cysteine proteases with papain.[101] They occur, together with their specific endogenous inhibitors, the calpastatins,[103] in almost all mammalian and avian cell types.[104,107] They were also discovered in fungi[105,500] and in a *Drosophila* strain,[106] but they have not been detected in plants. Two types of calpain have been isolated which differ in their calcium requirements.[363,370−372] Calpain I (μCANP) requires micromolar calcium concentrations (1−100 μM) whereas calpain II (mCANP) is only activated by millimolar calcium concentrations (0.1−1 mM).[108,363] Further isoenzymes were discovered recently (CANP 3,[120] μ/m-calpain,[469] stomach-specific nCANP,[121,469] and muscle-specific p94 = nCL1[469]). The presence of calcium is required for activation of the calpains rather than for proteolytic activity. Calpains are cytoplasmic enzymes, 7−30% are membrane bound.[107] They are heterodimers, made up of a 80 kDa catalytic and a 30 kDa regulatory subunit. The complete sequence of both subunits has been known since 1984.[109,110] All the calpains of a particular organism use the same regulatory subunit,[111] and differences in specificity and calcium sensitivity arise from variation in structure of the catalytic unit. On the basis of sequence homologies, the 80 kDa unit can be divided into four domains (I−IV).[107] Domain II contains a sequence similar to the active center of papain.[369] The amino acids of the active site have been identified by site-directed mutagenesis[436] (Cys105, His262, Asn286). Domain IV contains several EF-hand sequences similar to calmodulin.[109] The N-terminal domain I (propeptide domain[115]) and domain III do not have similarity with any known sequences. Two domains can be distinguished within the regulatory unit: an N-terminal domain with unusually long polyglycyl sequences, which is possibly responsible for binding to membrane lipids, and a C-terminal domain similar to calmodulin.[110] The calmodulin domains are the calcium binding sites.[112] Due to the fact that, in physiological conditions, the calcium concentration of <1 μM cannot be sufficient to activate the calpains, the enzymes which have been isolated are assumed to be inactive procalpains *in vivo*.[113,114] The active forms, i.e. the forms that are active at physiological calcium concentrations, have not yet been isolated, probably due to their instability.[114] μCANP can be activated by binding to membrane lipids: to be precise, by binding to phosphatidyl inositol (PIP_2, phosphatidyl 4,5-diphosphate). Binding to the membrane enables autocatalytic processes to take place at the N-termini of both subunits and the calcium requirement is thereby reduced to physiological concentrations (0.1−1 μM).[114] Knowledge of which factors play a role in transport to the membranes, and thus influence *in vivo* activity, is incomplete at present. Possibly a calcium influx into the cell, which often occurs in response to an extracellular signal, results in binding of μCANP to the membrane. Recent results suggest that the activation of calpain corresponds to the dissociation into subunits in the presence of calcium and that calpain functions as a monomer of the 80 kDa subunit *in vivo*.[435] The relationship of the calpains to one another is unclear (see section 2.7). It was assumed that calpain I is produced from calpain II;[108,117,120] however some authors considered that the differences in the heavy subunits are too great.[118] Calpain preferentially cleaves peptides with Tyr, Met, Lys, or Arg at P1 and hydrophobic amino acids such as Leu and Val at the P2 position and both isoenzymes are similar in this respect.[368] However, other calpain specificities have been observed with larger proteins:[124]

$$P9-P1/P1'-P9' = X(+,P)XXXX(+,-)XX/HXPPX(-,P) + XX$$

(X, any amino acid; P, proline; +, basic amino acid; −, acidic amino acid; H, hydrophilic amino acid; /, cleavage point).

The pH optimum, which is neutral to weakly alkaline, corresponds to that of the cytosol.[104]

Calpain Inhibitors: The Calpastatins. Calpain can be inactivated by the endogenous specific inhibitor calpastatin, which always occurs together with the enzyme, or at higher calcium concentrations by further autocatalytic degradation.[119] Since calpastatin is completely specific as an inhibitor, it is discussed here with respect to the calpains. All of the calpastatins that have been discovered are extremely heat stable proteins.[104] They have similar amino acid compositions, specificity and activity. However, information about their molecular weight (50−400 kDa) varies considerably.[122] A high molecular weight (HMW) and a low molecular weight (LMW) inhibitor can be distinguished.[122] The LMW inhibitor is a heterodimer with two subunits of 24 and 26 kDa. An intact inhibitor molecule can bind two CANP molecules. The HMW inhibitor is made up of two identical subunits each of molecular weight 110 kDa. There have also been reports of tetrameric high molecular weight forms.[103] The LMW inhibitor is probably formed by proteolysis of the HMW inhibitor. Four repeat sequences of 140 amino acids are present in a HMW monomer and these are independent of one another in their inhibitory activity.[123,374,375] The sequence TIPPXYR, which is responsible for calpain binding, occurs in the center of each domain.[123,124,373] Each of these sequences can bind one calpain molecule. It is not certain whether cleavage of calpastatin by calpain is necessary for enzyme inhibition, which would mean that calpain acts as a suicide substrate,[125a] or whether inhibition occurs without any interaction with the enzyme's active site. However, calpastatin seems to be a substrate for calpain.[125b] Since no data are available about the tertiary and quaternary structure of calpain, the interaction between enzyme, calcium, membrane, calpastatin, and possible activators (e.g. calcium-

dependent proteinase regulator CDPR[126]) is not well understood. Recent results indicate that control of calpains by calpastatins is associated with reversible phosphorylation of the inhibitor.[56]

Physiological Functions of Calpains and Their Roles in Different Diseases. Like the cathepsins (section 4), the calpains cleave a variety of proteins *in vitro*;[42,107] however, since their endogenous substrates are not known, a role for calpains in organisms is only speculation at present. Especially short-lived PEST proteins (proteins with abundant Pro-Glu-Ser-Thr-rich sequences) are good substrates for calpains.[114,434] In contrast to cathepsins, calpains perform only limited proteolysis on many proteins which suggests that they actually have a rather specific proteolytic role.[107] One of the first calpain reactions to be discovered was the activation of different kinases (phosphorylase B kinase, pyruvate kinase) (KAF kinase-activating factor).[127] In addition, calpains cleave myofibrillar proteins[128] (e.g. myofibril Z disk) as well as various other proteins of the cytoskeleton such as MAPs (microtubule associated proteins), fodrin, actin, lamin, tubulin, and vimentin.[129] Consequently they may play a role in degradation of muscle proteins and cytoskeletal proteins. Calpains also cleave the uterine estrogen receptor (RTF receptor transforming factor)[130] and the EGF receptor[362] (EGF epidermal growth factor). Degradation of the erythropoietin receptor is discussed too.[501]

At the moment, the extent to which these enzymes influence different pathological processes is speculation,[107] e.g. muscle degradation in muscular dystrophy may be at least partly attributed to calpain activity[469] (section 5). This is supported by the observation that degradation of the Z disk, troponins I and C and the myosin heavy chain occurs where the calcium concentration is high.[131] PTH (parathyroid hormone)-induced osteoblast retraction may also be mediated through hydrolysis of intracellular cytoskeletal proteins (e.g. vinculin) by calpains.[467] Thus these enzymes would be involved in bone resorption. Formation of cataracts by degradation of soluble lens proteins (crystallins) to insoluble fragments by calpains is also discussed[534] (see section 7.2.10). A review published in 1994[437] gives a summary of the possible contribution of calpains in various pathological processes. Table 3 taken from this reference gives an overview of therapeutic areas in which overactivation of calpains may be involved. Recently, a possible regulatory effect on the transcription factors c-*jun* and c-*fos*, which also belong to the PEST proteins, has been discussed in connection with cleavage of protein kinase C (PKC) by calpain (which is probably activated simultaneously on the membrane).[114] Recent experiments on *Plasmodium falciparum* show that invasion of erythrocytes by the protozoa is associated with an increase in the calcium concentration in the cell and this may be used by calpain as a signal to degrade the cytoskeletal proteins of the erythrocytes (spectrins)[5] (see also Table 17b).

Other Cytoplasmic Cysteine Proteases. Of the proline-specific endopeptidases (PEP, EC 3.4.21.26, post-proline-cleaving enzyme)[14,132a] classified as serine proteases,[132b] there are many types which differ in their sensitivity to specific cysteine protease inhibitors[132c] (peptidyl diazomethyl ketones; section 7.2.7). Enzymes which are probably cysteine proteases are also involved in activation of many peptide hormones by cleavage of the prohormone, mostly at double basic residues (e.g. Lys-Arg, Arg-Arg). The calcium-dependent proinsulin-processing endopeptidases I and II[14] belong to this group as well as various other enzymes which are involved in formation of dynorphins,[133] somatostatin,[134] and enkephalin (PTP = prohormone thiol protease).[135,416,421] For a review about proteases in prohormone processing see ref 420. A dimeric cysteine protease, which releases the inflammation mediator interleukin-1β from its precursor (IL-1β converting enzyme ICE) was isolated and characterized in 1989[136] (EC 3.4.22.36[203]). The enzyme is a heterodimer, composed of 20 and 10 kDa subunits. The sequence and the tertiary structure[401] are known and have no homology with other cysteine or serine proteases. The enzyme is unusual in that it is inhibited by a protein of the serpin superfamily;[204] these proteins are known to be specific inhibitors of serine proteases. The IL-1β proprotein is cleaved by ICE between amino acid residues Asp116 and Ala117 and thus the active cytokine is released. ICE is related to proteins responsible for apoptosis (e.g. CED-3 = *Caenorhabditis elegans* cell-death protein,[481] Nedd-2 = ICH1,[485] Mch-2,[486] Yama = CPP-32 = apopain,[482] CMH-1,[483] TX = ICH2 = ICE rel II,[487] ICE-Lap-3,[484] ICE rel III[487]) and is probably involved itself in regulation of programmed cell death.[402,403,433,477,478] For this reason and the fact that inhibitors of ICE may be a new group of antiphlogistic drugs this enzyme is a research subject of immense current interest. For a review abou ICE see ref 462. References 479 and 480 are reviews about ICE and apoptosis. Pyroglutamyl peptide hydrolase (EC 3.4.11.8)[14] removes pyroglutamyl residues from the N terminal of various pyroglutamyl peptides such as the hypothalamus hormones TRH (thyroliberin, thyrotropin-releasing hormone) or LHRH (gonadoliberin, luteinizing hormone-releasing hormone). Macropain[139] from human erythrocytes is an enzyme made up of eight subunits with MW between 21000 and 32000 (600 kDa in total); it probably has at least two catalytic centers since complete inhibition cannot be achieved with classical cysteine protease inhibitors. The pH optimum is in the neutral to alkaline region (pH 7.5−11).

2.7. Structural Relationships

The cysteine proteases are divided into at least 21 families (C1−C21) on the basis of the sequences or tertiary structures known for these enzymes (see Table 1b).[399] Nearly half of the known families are represented in viruses (C3−C9, C16, C18, C21). Of these the picornain family (C3) is the best characterized. Most of the enzymes known at present belong to the papain family (C1): proteases from protozoa, plant proteases, and lysosomal cathepsins. The families C1, C2 (calpain family), and C10 (streptopain family) can be described as "papain-like" and form clan A. According to Berti[476] the papain superfamily consists of three members: papain group, calpain

group, and bleomycin hydrolase. It is plausible that the first cysteine proteases in this family existed in the feeding vacuoles of protozoa and developed in the course of evolution into enzymes in plant vacuoles or mammalian lysosomal enzymes[2] (divergent evolution). Thus the origin of the papain superfamily could have been early during eukaryote evolution and may have occurred before the eukaryote/prokaryote divergence.[476] The phylogeny of the papain group implies that after an early divergence of the cathepsin B class, many enzymes diverged almost simultaneously.[476] The cytoplasmic calpains belong to the second family. Homology of the amino acid sequences of the domains containing the vital Cys residue to the corresponding sequence in papain suggests a genetic relationship. It is possible that the calpains arose by gene fusion of a calmodulin gene, a cysteine protease gene, and further sequences of unknown origin.[140] The m- and μ-type calpains diverged probably about 300 million years ago.[476]

Analogous to the serine protease subtilisin bacteria contain cysteine proteases which have developed independently[2,14] (convergent evolution), e.g. clostripain (C11). The extent of any similarities within the bacterial enzymes is unknown due to lack of data.

The viral proteases have no homology to any of the above enzymes. The tertiary structure of picornains is similar to those of the serine protease chymotrypsin. This is the only instance so far discovered in which an evolutionary relationship crosses the boundary of catalytic types and it may have arisen from a single base change that converted Ser to Cys.[399]

As mentioned earlier, there are also human cysteine proteases whose genetic origin is unknown, e.g. the ICE (family C14).

3. Mechanism of Proteolysis

Unlike the mechanism of the serine proteases, the molecular basis of the mechanism of hydrolysis catalyzed by cysteine proteases is not known.[400] It is certain that Cys25 and His159 form the active site of papain,[141] but it is questionable whether these two residues are sufficient for full catalytic activity. The imidazole group of the histidine polarizes the SH group of the cysteine and enables deprotonation even at neutral to weakly acidic pH;[151,152] a thiolate/imidazolium ion pair is thereby produced (Figure 2a) which is highly nucleophilic. The existence of this ion pair is hardly disputed any more but whether the ion pair has actually been shown to exist is the subject of much controversy in the literature.[42,142] The ion pair mechanism does explain the unusually high reactivity of cysteine proteases toward electrophilic reagents in comparison to the nucleophilicity of the sulfur of cysteine or glutathione, especially in slightly acidic environments.[143] The thiolate anion attacks the carbonyl carbon of the peptide bond to be cleaved (Figure 2b) and a tetrahedral intermediate is produced (Figure 2c). The oxyanion is fixed by a oxyanion hole, as for serine proteases. Stabilization of this transition state occurs by H bonding to the backbone NH of Cys25 and to the NH_2 group of the Gln19 side chain. This is supported by the X-ray

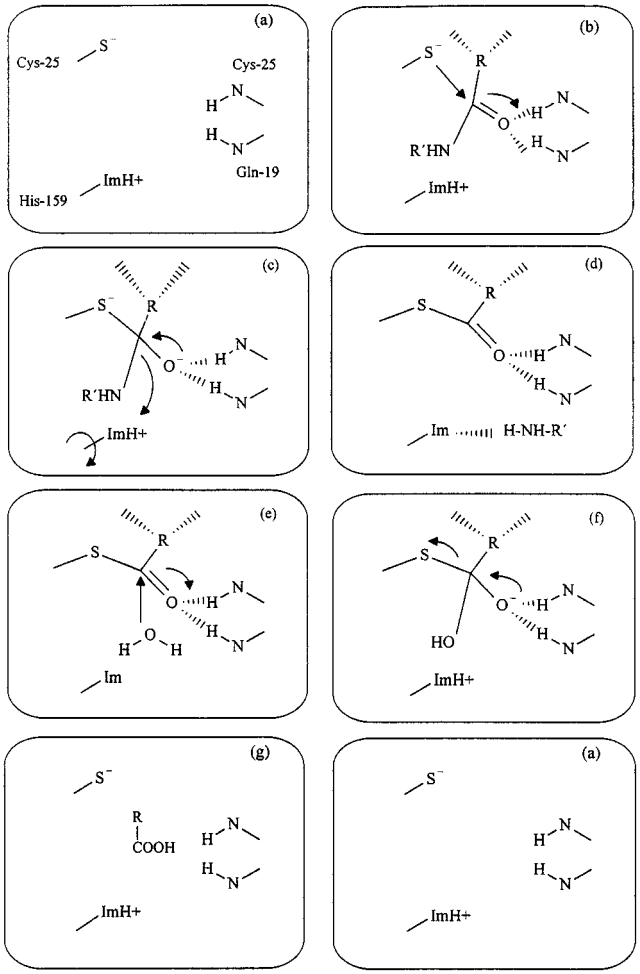


Figure 2. Mechanism of proteolysis using papain as an example. Explanation: (a) Active form of papain with the ion pair Cys-S^- (25)/His-ImH^+ (159); (b) noncovalent Michaelis complex formation; (c) first tetrahedral intermediate, stabilization by the oxyanion hole formed by backbone-NH of Cys25 and side chain NH of Gln19; (d) acylation of the enzyme, rotation of His159, and protonation of the leaving amine (general acid catalysis); (e) reaction of the acyl enzyme with water, nucleophilic attack of a water molecule at the acyl enzyme, protonation of His159 (general base catalysis); (f) second tetrahedral intermediate, (g) release of the second product R-COOH and regeneration of the free enzyme (a). (According to refs 42 and 400.)

structure of the enzyme−inhibitor complexes of papain with BPACK[144] and papain with leupeptin.[375] However, it was thought to be contradicted by the fact that thiono esters, in which the oxygen of the carbonyl group has been replaced by sulfur, can only be hydrolyzed by cysteine proteases and not by serine proteases.[145] Sulfur reduces the ability to form H bonds compared to oxygen and thus the tetrahedral transition state cannot be stabilized by serine proteases. But the inability of papain to hydrolyze peptidyl thioamides clearly shows that the oxyanion hole plays an important role in hydrolysis[455] because of the poor H-bond-accepting properties of sulfur. The mentioned facile hydrolysis of thiono esters can easily be explained by differences in electronegativity of O vs N in thioesters vs thioamides, respectively.[455]

Esterification of the thiol makes the imidazolium ion sufficiently acidic ($pK_a = 4$, general acid catalysis) to protonate the nitrogen of the leaving group and the acyl enzyme is produced[146] (Figure 2d). In order

to donate its proton to the amide nitrogen, rotation of the active site histidine is necessary.[400] Deacylation may occur via a general base-catalyzed mechanism, whereby the imidazole nitrogen polarizes a water molecule which then attacks the acyl enzyme at the carbonyl carbon (Figure 2e). The cleaved substrate (as free acid) and the regenerated enzyme (Figure 2, parts g and a) are produced via a second tetrahedral intermediate (Figure 2f). There is much discussion about whether Cys25 and His159 are sufficient for the hydrolysis or whether a negatively charged amino acid stabilizes the ion pair imidazolium cation/negative transition state by a symmetrical charge distribution (− + −), as for the serine proteases.[147] In papain, Asp158 was thought to be the negatively charged amino acid;[42,43,148] however, this is no longer accepted due to the discovery that the aspartate residue is located at a large distance from the active site.[149] In addition, an amino acid comparable to Asp158 is missing in other cysteine proteases. However, kinetic measurements lead to the conclusion that in papain, as well as in cathepsin B, actinidin, and cathepsin H, a further deprotonizable group with a pK_a of 4−5.5 influences the reactivity of the ion pair.[42,147] It is also possible that the dipole moment of the α-helix in the vicinity of the active site contributes to stabilization of the ion pair.[42,147,150] Of possible importance is also the proximity of the Asn175 side chain to His159. H bonding between these residues has led to the proposal that the role of Asn175 might be to direct the imidazole in optimal positions for the different steps of hydrolysis and/or to stabilize the ion pair by keeping the imidazole ring in a favorable position.[494]

This role of an additional amino acid, the existence of a further deprotonizable group and the existence of tetrahedral intermediates are still under discussion.[400] These are also points at which the mechanism differs from that of the serine proteases; the differences result from the higher acidity of thiols in comparison to alcohols. The pK_a values of the nucleophile and the histidine are closer to one another: pK_a SH = 8.5; pK_a imidazolium = 7. As these values show, the SH group in thiols is normally more basic than an imidazolium cation; thus, the unusually high acidity of the cysteine in papain[141a,151,152] (pK_a = 4; pK_a ImH^+ = 8.5) can only be explained by stabilization of the resulting ion pair, either by other functional groups or by the α-helix. Assuming an ion pair is formed, it follows that general base catalysis, as for serine proteases, is not necessary for acylation of the enzyme—the acylation is a simple nucleophilic attack of the thiolate and formation of a tetrahedral intermediate is not necessary, the acylation is a concerted process.[400] However, according to the principle of microscopic reversibility, general acid catalysis also cannot be involved in the deacylation.[147] Ion pair formation not only makes the thiol a good nucleophile but also a good leaving group during deacylation due to the low pK_a value. Deacylation can also therefore take place without acid catalysis because C−S bonds are weaker than C−O bonds (272−360 kJ/mol).[147] For serine proteases, stabilization of the tetrahedral intermediate by a further negatively charged amino acid and an oxyanion hole are essential to favor formation of the ion pair imidazolium cation/oxyanion from an initial uncharged ground state. In cysteine proteases, an ion pair already exists and formation of the tetrahedral intermediate state only serves to redistribute charge. It follows that a cysteine protease would be more activated in the initial ground state than a serine protease or, in other words, serine proteases overcome the activation energy by stabilization of the tetrahedral intermediate, whereas cysteine proteases start from high-energy ground state.[147]

4. Function of the Lysosomal Cathepsins in the Organism

Although there is plentiful evidence that cathepsins play a role in a large number of physiological processes, it has not yet been possible to determine the exact function of individual enzymes. Many proteins are good substrates *in vitro* for the different cathepsins; whether however these reactions are of physiological importance cannot definitely be confirmed.[42] Protein degradation can take place via two pathways in the cell: a lysosomal path, by which most cellular proteins are nonselectively degraded,[379] and a non-lysosomal path (20−30% of the total protein degradation), by which short-lived and abnormal proteins are preferentially broken down[153] and in which the multicatalytic proteinase complex of the cytoplasm ("proteasome")[137,138] (700 kDa) probably plays the most important role. Thiol-dependent cathepsins form the main enzyme activity in the lysosomal pathway. Exogenous and endogenous proteins, which are taken up by the cell via endocytosis or autophagocytosis, transported in the form of endosomes or autophagosomes and finally directed to the lysosomal degradation path by fusion of these vesicles with lysosomes, both undergo similar degradation by this pathway. Proteins can be degraded down to amino acids in the lysosomes by interaction of both endo- and exopeptidases.[63,137] Due to the high concentrations of cathepsins in the lysosomes (10−40 mg/mL),[63,137] it is understandable that the cell has to protect itself from this enormous hydrolytic potential. The cell is successful in this respect because although cathepsins have activity for synthetic substrates at neutral pH,[161] the enzymes are autolyzed or denatured in the physiological conditions of the cytosol,[63,137] and they are also controlled by specific inhibitors in the cytosol and the extracellular space[137] (section 6). In addition to this nonspecific protein degradation, cathepsins may play a role in specific processing of proteins, e.g. in activation and deactivation of enzymes and hormones. Cathepsin B catalyzes, for example, conversion of proalbumin to albumin,[154] prorenin to renin,[155] and trypsinogen to trypsin.[156] Along the same lines, it is thought that pancreatitis is associated with premature activation of the pancreatic zymogens by lysosomal proteases.[157] Formation of insulin from proinsulin is catalyzed by cathepins B and H together,[158] and cathepsin L can activate nucleotide phosphodiesterase.[159] Both cathepsins B and L can regulate the activity of aldolase.[160] However, whether these reactions take place *in vivo* seems questionable. Either the proteins to be cleaved must be taken up by the lysosomes, where they would

Table 3. Calpain in Pathological Processes[437]

disorder	proposed mechanism
stroke	degradation of cytoskeletal proteins leading to neuronal cell death and permanent tissue damage
brain trauma	similar mechanism to stroke
subarachnoid haemorrhage	activation of protein kinase C leading to sustained cerebral vasospasm
Alzheimer's disease	abnormal processing of amyloid precursor protein
spinal cord injury	degradation of myelin proteins
cardiac ischaemia	breakdown of myofibril proteins causing cardiac myocyte shrinkage, cell death, and tissue damage
muscular dystrophy	breakdown of myofibril proteins
cataract	lens protein (crystallins) breakdown leading to precipitation that causes lens opacity
thrombotic platelet aggregation	proteolysis of aggregin, promoting platelet aggregation
restenosis	renarrowing of blood vessels after angioplasty owing to calpain-mediated proliferation and migration of smooth muscle cells
arthritis	breakdown of cartilage and extracellular matrix component proteoglycan

probably be completely degraded by the many enzymes (ca. 50 hydrolases) present, or the cathepsins must exist in the cytosol, a neutral to alkaline medium in which they are not very stable. It is possible that extralysosomal degradation is achieved by membrane-bound cathepsins[162] or via high molecular weight cathepsin forms such as cathepsin P[73] which are more stable in alkaline medium (Table 1a; see also section 5). Another probable function of the cathepsins, especially cathepsins B, L, and K[489,490] and a 70 kDa proteinase similar to cathepsin L,[163d] is the degradation of collagen[163,460] in the bone matrix of the osteoclasts. In addition, activation of collagenase,[164] which is also involved in bone degradation, is catalyzed by cathepsin B. Cathepsins B, L, and H are also able to degrade histones[165] and cathepsin B cleaves fibrinogen.[166] Recent evidence supports a role for cathepsin L in reproduction.[444] The cathepsins are probably also involved in cell differentiation and proliferation,[167] and a role for macrophage cathepsin B in antigen processing is being discussed.[209]

5. Role of Cathepsins in Different Medical Conditions

The possible roles of calpains in various disease stages have been summarized in section 2.6.2 and Table 3. This section describes a few examples of pathological conditions in which the cathepsins are involved.

5.1. Inflammatory and Traumatic Processes

A common factor in both of these processes is that the equilibrium between lysosomal enzymes released by macrophages or neutrophilic granulocytes and their endogenous inhibitors in the extracellular space is disturbed.[161] This imbalance may originate from reduced inhibitor activity (proteolytic degradation by lysosomal enzymes;[161,169,170] inactivation by reactive oxygen species, which are produced by reduction of H_2O_2 catalyzed by a myeloperoxidase released by neutrophilic granulocytes;[161,169,170] saturation of the inhibitors by excess release of lysosomal enzymes;[161,169] a change in the binding properties of the inhibitors and thus easier dissociation of the enzymes from the enzyme−inhibitor complexes[168]) and/or increased stability of lysosomal cathepsins which are normally inactive in the extracellular space (protection of the enzyme from inactivation by binding to membrane systems of the cell;[161] creation of microenvironments with low pH by the inflammation process). Free lysosomal proteases, in particular the neutrophilic elastase and cathepsin G, inactivate plasma factors, proteinase inhibitors, immunoglobulins, and transport proteins by non-substrate-specific, uncontrolled proteolysis and thereby destroy proteins of the cell membrane and of connective and supportive tissues. Toxic peptides are produced by this process which inhibit the enzymes of the blood system.[169] Neutrophilic elastase was previously used as a marker enzyme for inflammation;[170] however, raised concentrations of cysteine proteases have also been found in bodily fluids from patients with inflammatory diseases.[171] For example, the cathepsin B level is much higher in the joint fluid of patients with rheumatoid arthritis.[172] Cathepsin L seems to be partly responsible for degradation of cartilage and joints in osteoarthritis.[173,393] There is also evidence that cathepsin B in alveolar macrophages is involved in causing pulmonary emphysema.[174] High cathepsin B levels are also found in various body fluids of polytraumatized patients and of patients with septic shock. The cathepsin B activity in blood plasma correlates with the extent of organ malfunction here.[171] In addition monocyte-derived macrophages mainly involved in tissue damage in chronic inflammatory diseases have been shown to secrete fully processed and active forms of cathepsins B, L, and S into extracellular milieu.[445] Cathepsin S could also be a candidate responsible for destruction of tissue proteins since it retains proteolytic activity after prolonged exposure to neutral pH.[503]

5.2. Duchenne Muscular Dystrophy (DMD)

DMD is an X chromosomal recessive hereditary degenerative muscle disease, which leads to an early death (age 20−25) by progressive muscular atrophy in those afflicted; due to the sex-linked recessive inheritance, it only occurs in males. The condition is caused by deletions in the gene coding for the muscle protein dystrophin.[175] Dystrophin belongs, like α-actinin of the Z disk, to the rod-shaped proteins and is associated with the plasma membrane of muscle cells. The progressive muscle degradation of this and other related diseases is explained by membrane defects—caused by missing dystrophin—which lead to an increased calcium influx into the muscle cells and depletion of proteins in these cells. Cathepsins B, H, and L[176] and the calpains[177] are probably involved in degradation of the muscle

proteins, and calcium influx into the cells could lead to activation of calpains.[179] Migration of macrophages, which remove destroyed muscle fibers by phagocytosis, is thought to be the cause of raised cathepsin levels in the skeletal muscle.[178]

5.3. Tumors

The largest structural barrier to formation of metastases and invasive growth of malignant tumors is the connective tissue of the extracellular matrix (ECM); the most important part is the basal membrane, which provides an immunological separation of different tissues and surrounds the blood and lymph vessels.[180] The basal lamina is composed of type IV collagen, proteoglycans, and the cell surface proteins fibronectin, laminin, and entactin.[180] A characteristic of malignant tumors is the destruction of this lamina.[180] It is now certain that the degradation of the ECM, which is necessary for metastasis formation and invasion of tumors into neighboring tissue, involves cathepins B[189,191,383,447,472] and L[180,191,446,447,472] and collagenase.[181] Thus the cathepsin B activity of a tumor tissue correlates with the aggressiveness of the tumor[180,183] and elevated cathepsin B levels in lung tumor can even be correlated to poor survival prognosis[502] thus introducing cathepsin B as an independent prognostic factor. Most cathepsin B-like proteases released by tumor cells have a higher molecular weight[181,187] and unusual stability at neutral to alkaline pH.[181,185,187] For example, a latent form has been found with a MW of 41 kDa and an active form with MW 33 kDa.[186] The latent form can be activated by aspartate proteases such as pepsin or cathepsin D[380] or by neutrophilic elastase.[381] Tumor cathepsins do not differ from normal lysosomal cathepsins in their immunological and kinetic characteristics.[188] The relationship of tumor proteases to the nonpathological forms is unclear. The question is whether tumor enzymes are incorrectly processed proenzymes[187,188] or products of other genes.[382] Further causes of raised cathepsin activity in the extracellular space were mentioned in section 5.[192] For example, alterations in the balance between endogeneous inhibitors (CPI's, see section 6) and the cathepsins have been postulated to contribute to malignant progression.[448] In various cancers, the level of cathepsin in the plasma membrane fraction is up to 30 times higher than in nonpathological cells,[180] which means that these enzymes are protected from endogenous CPI's and from denaturation, by binding to the membrane.[180,190] In addition, the presence of large protein substrates such as the ECM proteins seems to reduce inactivation of cathepsin B at pH 7.[180,182] A further characteristic of malignant tumors is increased platelet aggregation and blood coagulation. Following on from this, the isolation of a cysteine protease (cancer procoagulant, MW 68 kDa; pI 4.8) from different tumor tissues should be mentioned here as this enzyme can activate factor X of the blood-clotting cascade. This is the only nonserine protease which has been found to be associated with blood clotting.[189,509] There is also evidence that cathepsin B plays an important role in platelet aggregation of tumor cells.[184]

There are also indications that cathepsins may be involved in Alzheimer's disease,[395,396,463] protein degradation in myocardial infarction,[394] and osteoporosis.[163c]

6. Inhibitors with Protein Structure

6.1. Endogenous Inhibitors: The Cystatin Superfamily

On the basis of sequence homologies, the cysteine protease inhibitors (CPIs) from mammals, the cystatin superfamily,[193−196] are divided into three families: family 1, the stefins; family 2, the cystatins; and family 3, the kininogens. Table 2a gives an overview of the different inhibitors. Common to all CPIs is their enormous stability at high temperatures (up to 100 °C)[384] and at extreme pH (LMW-CPIs pH 2−12, kininogens pH 5−12) as well as their specificity for cysteine proteases.[195]

The three-dimensional structures of human cystatin B (= stefin B)[201] and CEW-cystatin[200] have been elucidated.

6.1.1. The Stefin Family[193−196,506]

The proteins of family 1 have a molecular weight of about 11 kDa (ca. 100 amino acids), and they lack disulfide bridges and carbohydrate residues. Like biosynthesis on free ribosomes and lack of a signal peptide, these are also characteristics of intracellular proteins. Cystatin A and B are found in human tissue, whereas cystatin α and β are species-specific variants from rat. Cystatin A (p*I* 4.5−5.0) is found mainly in epithelial cells and neutrophilic granulocytes while cystatin B (p*I* 6.0−6.6) occurs in almost all cells and tissue. A third cystatin of this family, stefin C, has been isolated from bovine thymus[506,507] but no human homolog has been reported yet.

6.1.2. The Cystatin Family[193−196,506]

These cystatins, together with the inhibitors of family 1, belong to the LMW-CPIs (LMW, low molecular weight). With 110−120 amino acids and molecular weights from 12 to 13 kDa, they are not much bigger than the stefins. They also have no carbohydrate residues (with the exception of cystatin C from rat[197]) but do have two disulfide loops at the C terminal. To this family belong cystatins C, D, S, SN, and SA. Cystatin C (p*I* 8.0−9.5) is widely distributed in the extracellular space and has also been found within some cells, e.g. in cortical neurons, in pancreatic islet cells, in the thyroid glands[195] and in parotid glands.[504] Since the concentration in the cerebrospinal fluid is relatively high in comparison with that in the blood plasma, their synthesis may take place in the CNS.[195] Cystatin S (p*I* 4.7) was first discovered in human saliva and has since been isolated from different salivary glands.[195,443] The protein has also been found in tear fluid, serum, gall, urine, pancreas, and bronchi.[195] The three S-type cystatins (S, SN, SA) have similar primary structures.[506] Cystatin D was also isolated from saliva.[508] The first CP inhibitor with a protein structure, chicken egg white cystatin[198,199,385] (chicken cystatin, egg white cystatin, CEW cystatin), was found in 1968, and this also belongs to family 2; it inhibits plant cysteine proteases and cathepsins.

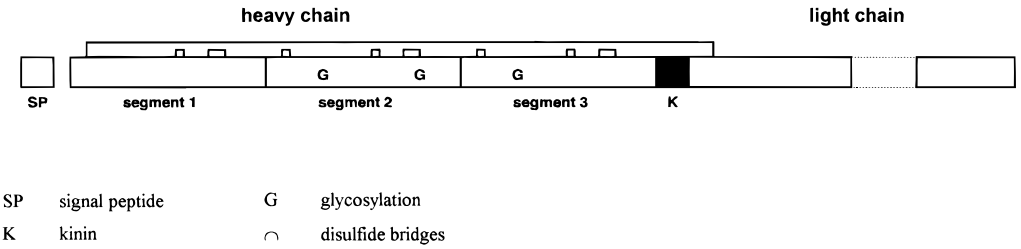


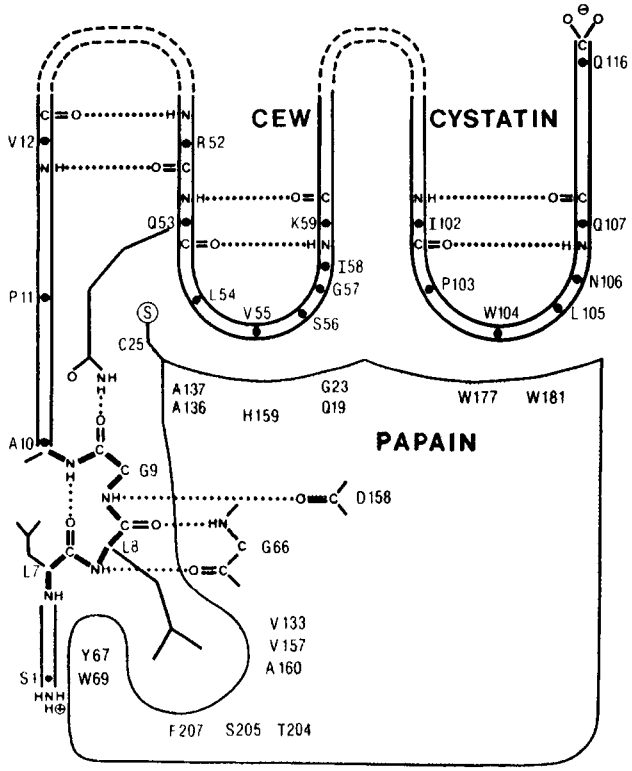
Figure 3. Scheme of kininogene structure. (According to refs 195 and 196.)

6.1.3. The Kininogen Family[193−196]

These proteins form the HMW-CPI group (HMW, high molecular weight). A HMW kininogen (MW ca. 120 kDa) and a LMW kininogen (MW 50−80 kDa) are known, but a protein corresponding to the T kininogen from rat (MW ca. 68 kDa) has not yet been found in humans. The kininogens contain three sequences which each correspond to the polypeptide chains of cystatins (type 2). These three domains, together with the 10 amino acids of kinin, form the N-terminal heavy chain from which a signal peptide of up to 18 amino acids is cleaved after translation. This part of the protein is identical in both kininogens while the C-terminal light chains differ significantly in both sequence and size. In addition to the two disulfide loops in each segment, domains 2 and 3 each have a disulfide bridge at their N terminals and a ninth disulfide bond links segment 1 with the light chain (Figure 3). Each molecule has three carbohydrate residues. These characteristics of the kininogens (carbohydrate residues, signal peptide, disulfide bridges) are typical for extracellular proteins. In addition to their properties as CPIs, these proteins are precursors for the vasodilatatory peptides bradykinin and kallidin. Kallikrein releases the peptides from the kininogens, giving the heavy and light chains bound together by just a single disulfide bridge. Plasma kallikrein releases bradykinin, and tissue kallikrein releases kallidin. H-kininogen is also involved in the blood clotting cascade: in the activation of prokallikrein and Factors XI and XII. A kininogen also exists in rat plasma which has no kininogen characteristics. T-kininogen is one of the proteins whose concentration rises drastically during inflammation (major acute phase protein). Kininogens can exist as monomers or as oligomeric aggregates.

In 1989 the primary structure of a pig leucocyte cysteine protease inhibitor (PLCPI) was determined.[510] The possibility that this new inhibitor introduces a new family of CPI's was discussed and the name cathelin was proposed. Its amino acid sequence exhibited remarkable similarity to the proregion of several antibacterial peptides. In 1993 however, it was found that this cathelin sample was composed of two proteins, a stefin-type CPI (PLCPI) and a noninhibitory protein similar to proregions of antibacterial peptides.[511] Therefore the name cathelin should be excluded from CPI terminology. Nevertheless the proregion of cathelicidins, a novel family of antimicrobial proteins, shows sequence similarity to members of the cystatin superfamily, especially to the cystatin-like domains of kininogens.[512]

Chart 4. Interaction between CEW-Cystatin and Papain. (Reprinted from ref 194. Copyright 1991 Elsevier.)



6.2. Inhibition of Cysteine Proteases

All CPIs only inhibit cysteine proteases, including the exopeptidase cathepsin C.[195] Exceptions are bromelain[195] and glycyl endopeptidase (papaya proteinase IV, PPIV, EC 3.4.22.25)[439,496] which are not inhibited by any of the proteins. The resistance of PPIV to inhibition by cystatins is probably due to changes in the S1 subsite of the enzyme:[520] Gly23 and Gly65 are replaced by Glu and Arg, respectively, leading to a reduced size of the S1 subsite. Calpain is only inhibited by kininogens; segment 2 is responsible for the inhibition.[195,441] Clostripain and the polio virus proteases are the only enzymes that do not belong to the papain family but are nevertheless inhibited by cystatins.[195] A model based on the three-dimensional structure of chicken cystatin[200] describes the interaction of papain and cystatin (Chart 4, from ref 194). According to this model, the N terminal of the inhibitor which has the Gly9 residue conserved in all cystatins[386] (CEW cystatin numbering), interacts with the active site and the S1−S3 sites of the enzyme, while two hairpin loops, which contain the conserved sequences QVVAG and Pro-Trp, are bound to the S1′−S2′ sites. Segment 1 of the kininogens,

which lacks these sequences, has no inhibitory activity unlike the other two domains. The model was confirmed by X-ray structural analysis of the enzyme−inhibitor complex of papain and recombinant human stefin B.[201] Inactivation of the cysteine proteases occurs by competitive, noncovalent, reversible inhibition. Unlike the inhibitors of the serine proteases (a comparison of the binding mechanism is given in ref 202), CPIs also form complexes with cysteine proteases whose active site has reacted with a thiol-blocking reagent. Kinetic constants for the different inhibitors and enzymes are given in Table 2b.

A squamos cell carcinoma antigen (SSCA) which is a member of the serine protease inhibitors serpin family was shown to inhibit cathepsin L.[449] This is beside the mentioned inhibition of ICE by a serpin (see section 2.6.2), another example of "crossclass" interaction between a proteinase and an inhibitor.

7. Low Molecular Weight Synthetic Inhibitors

7.1. Classification of Enzyme Inhibitors

Before the low molecular weight inhibitors are discussed, a few terms used in the literature in characterization of enzyme inhibition will be explained, which are by no means obvious in meaning. What are "active-site-directed", "mechanism-based", "tight-binding", and "slow-binding" inhibitors and how do they differ from "affinity labels", "suicide substrates", "transition-state analogs", and "dead-end" inhibitors? An initial classification is based on whether the inhibitor interacts with the active site ("active site directed") or whether it attacks another site on the enzyme (allosteric effectors).[210] The inhibitors which attack the active site can be divided according to the type of interaction into covalent/noncovalent and irreversible/reversible inhibitors. Reversible and irreversible inhibitors can be differentiated by lowering the inhibitor concentration, by diluting the reaction preparation, or by gel filtration or dialysis. In the case of a reversible inhibitor, the enzyme activity will increase again. Usually reversible inhibition involves a noncovalent interaction between enzyme and inhibitor but cases are also known in which covalently bound inhibitors result in reversible inhibition because of hydrolytically labile bonding. Examples are peptidyl aldehydes or nitriles as inhibitors of serine and cysteine proteases (sections 7.2.1 and 7.2.5). Irreversible inhibitors (= inactivators) always bind to the enzyme covalently. In practice it is often difficult to differentiate between reversible and irreversible inhibitors, for example if a reversible inhibitor binds to the enzyme with such a high affinity that the enzyme−inhibitor complex only dissociates very slowly and thus appears irreversible. This type of inhibitor is known as "tight binding".[211] Normally a rapid equilibrium is observed with reversible inhibitors, whereas reactions which result in modification of the enzyme take place relatively slowly. However, there are also reversible inhibitors which only inhibit enzyme activity very slowly due to conformational changes following enzyme−inhibitor complex formation ("slow binding") as well as irreversible inhibitors whose reaction with the enzyme occurs via a noncovalent transition state and thus leads to rapid reduction of enzyme activity.[211] A subdivision of the inhibitors which undergo noncovalent interactions with the enzyme are the substrate analogs, which are similar in structure to the substrate ground state. These are the classical, reversible, fast-binding, competitive inhibitors.[212] An enzyme−inhibitor complex is formed which does not react any further. The second group are the "tight-binding" inhibitors mentioned above. This group includes the transition-state analogs, which are similar in structure to an energy-rich intermediate, as well as all inhibitors which can bind to several domains on the enzyme ("multidomain inhibitors").[212] Covalent inhibitors can be subdivided according to whether they react with the active site by the normal process of catalysis ("mechanism based")[213] or by another chemical path which does not correspond to the catalytic mechanism of the enzyme ("affinity labeling", affinity marking). The "normal" catalytic process within the first group mentioned can result in various products and the inhibitors can be further subdivided according to these products:[212]

(1) Reaction of the inhibitor with the active site results in a product analogous to the transition state which cannot react further ("transition-state analogs").

(2) Only by reaction with the active site is a reactive intermediate produced, which subsequently reacts with the enzyme by a reaction that is not part of the normal catalytic path. These are "enzyme-activated", "suicide", or better, "Trojan Horse inhibitors" (k_{cat} inhibitors[213]), whereby the term "mechanism based" is often used synonymously.[211,214−216]

(3) This group includes all inhibitors which react with the enzyme to form a covalent enzyme−inhibitor complex which cannot react further ("dead end"). Of course, the transition-state analogs of group 1 also belong to this group and should be termed accordingly "mechanism-based/dead-end/transition-state analogs. According to an earlier definition however an "dead-end" inhibitor does not have to form a covalent enzyme−inhibitor complex but just a catalytically inactive complex.[524]

(4) Inhibitors of this group, unlike "dead-end" inhibitors, can react further by the normal catalytic pathway to corresponding products; however, these reactions occur so slowly that they are hardly measurable ("alternate substrate inhibitors").

The term "affinity label", also referred to in the literature as "active-site-directed inhibitors",[214,217] means a reagent that has a chemical group that reacts with the active site of the enzyme to form an adduct which cannot be separated again by either gel filtration or dialysis, i.e. it is without doubt an irreversible inhibitor. The reaction path is nonspecific and does not correspond to the normal catalytic mechanism. If this substance reacts with other molecules that have functional groups corresponding to those of the active site, it is known as a "chemically reactive affinity label". If this is not the case and the reagent has no activity for nonenzymatic molecules, it is known as a "quiescent affinity label".[212]

As the individual substance classes described in the following section show, classification of an inhibitor into one of these groups depends on the reaction path of inhibition. Since the pathway is well-defined in

active site directed inhibitors (⟷ allosteric effectors)

Inhibitor	Example	Ref.
I. non-covalent interactions		
I.1. substrate analogs	malonate/succinate dehydrogenase	[218]
I.2. tight binding inhibitors		
- transition state analogs	Proscar/5-α-reductase	[219]
	phosphonyl peptides/metallo proteases	[220]
- multi domain inhibitors	phosphono acetyl-L-aspartate/carbamoyl transferase	[533]
II. covalent interactions		
II.1. mechanism based inhibitors		
- transition state analogs	peptidyl aldehydes/cysteine & serine proteases	[221]
- enzyme activated inhibitors	haloenollactones/serine proteases	[222]
	Sulbactam/ß-lactamases	[223]
- dead end inhibitors	peptidyl nitriles/cysteine proteases	[224]
- alternate substrate inhibitors	Physostigmine/acetylcholinesterase	[225]
II.2. affinity labels		
- chemical reactive affinity labels	chloromethylketones/cysteine & serine proteases	[226]
- quiescent affinity labels	acyloxymethylketones/cysteine proteases	[227]

Figure 4. Classification of enzyme inhibitors.

relatively few cases, it may well be questionable to attempt a differentiated inhibitor classification as well. Figure 4 gives an overview of the different inhibitor classes. The classification used follows in most parts the suggestion made by A. Krantz in 1992.[212]

A very good characterization of enzyme inhibition from the kinetic viewpoint can be found in ref 211.

7.2. The Inhibitors

Most of the inhibitors presented in the next sections follow the usual structural scheme for development of protease inhibitors. This comprises a peptide segment for recognition by the enzyme, corresponding to the sequence of a good substrate; it must contain two or more amino acids to achieve good affinity, at least for endopeptidases. The peptide segment is bound to a nucleophilic attackable/substitutable group which can react with the cysteine residue of the active site.

7.2.1. Aldehydes

Development of peptidyl aldehydes as inhibitors of cysteine and serine proteases is based on two independent research strategies: (1) The assumption that a tetrahedral intermediate is involved in enzymatic hydrolysis has led to investigation of the effect of carbonyl compounds on these proteases, with the intention of developing analogs of this transition state (e.g. Ac-Phe-Glyal).[228,387] (2) During screening of culture filtrates of different *Streptomyces* strains, a number of peptidyl aldehydes were isolated with inhibitor activity toward cysteine and serine proteases.[229] These are the leupeptines,[230,231,233] chymostatins,[230,231] antipain,[230,231] elastinal,[230,231] and *β*-MAPI[231] (Figure 5). Peptidyl aldehydes are not

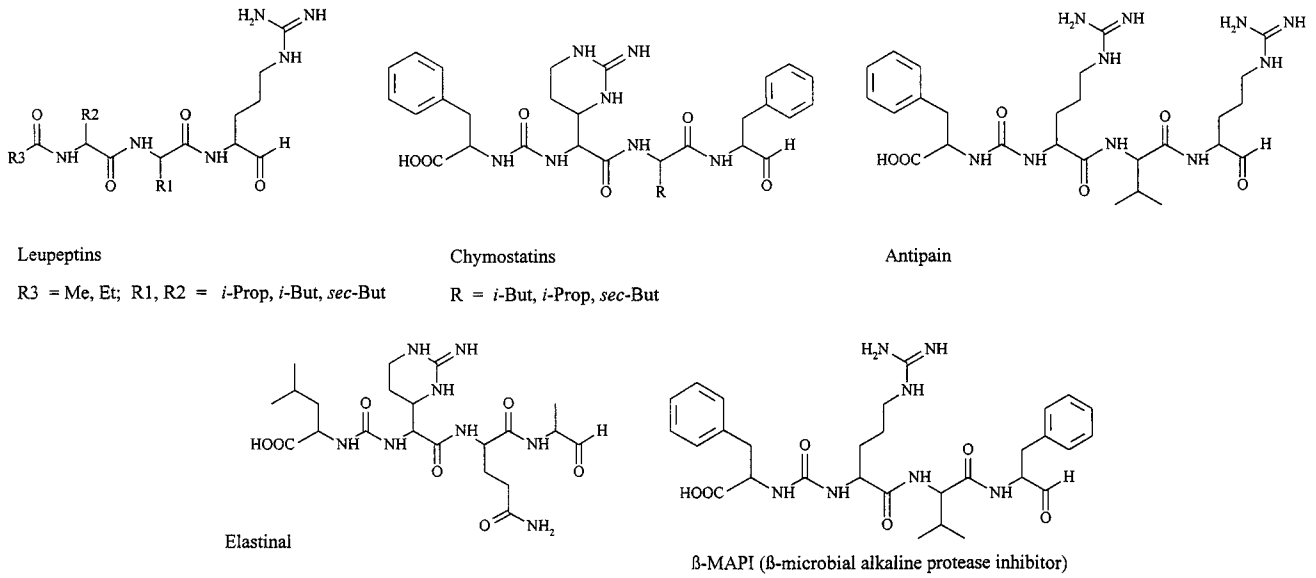


Figure 5. Microbial protease inhibitors with aldehyde structure.

Table 4. Inhibitory Activity of Microbial Peptidyl Aldehydes

	IC_{50}/K [(μg mL^{-1})/K_I/(nM)]		chymostatin: IC_{50} (μg mL^{-1})
enzyme	leupeptin	antipain	
papain	0.5[230]/1.12[232] 0.053[235,c]/2.2[2,a]	0.16[230]/nd[e]	7.5[230]
cat. B	0.44[230]/4[63] 0.31[233,c]/6[2,234,b]	nd	2.6[230]
calpain	0.1[239,c]/5[239] 1.8[235,c]/400[2]	nd/1400[2]−2000[14]	
trypsin	2[230]/35[234] 8.1[233,c]/340[231]	0.26[230]/ca.100[128]	>250[230]
a-CTR[d]	>500[230]/>1000[234,c]	>250[230]/nd	0.15[230]
plasmin	8[230]/3400[234] 3.7[233,c]	93[230]/nd	>250[230]

[a] $K_I = 2.2 \times 10^{-11}$ M relative to the true concentration of free aldehyde (ref 232). [b] $K_I = 10^{-10}$ M relative to the true concentration of free aldehyde (ref 232). [c] [μM]. [d] CTR, chymotrypsin. [e] nd, not determined.

selective inhibitors *per se*. They inhibit both cysteine and serine proteases (Table 4). Certain selectivities result from variation of the amino acids in P1 and P2, corresponding to the preferred substrates of the individual enzymes. Peptidyl aldehydes are reversible inhibitors despite binding covalently to the enzyme. A tetrahedral hemithioacetal, whose formation has been shown by NMR studies,[236,237] is formed by nucleophilic attack of the thiolate (Figure 6). Thus the peptidyl aldehydes are so-called transition-state analogs. Recent results indicate, however, that the hemithioacetal formed cannot be considered a good model of the transition state.[466] Site-directed mutagenesis has been used to investigate the role of the oxyanion hole in the binding of peptidyl aldehydes and nitriles.[466] For the peptidyl aldehydes mutation of Gln19 resulted in a small but significant increase in stability of the tetrahedral hemithioacetal adduct while the thioimidate adducts formed with nitriles (see section 7.2.5) are less stable. Thus the inhibition of papain by peptidyl nitriles is a process closer to that of substrate hydrolysis than is the inhibition by aldehydes.[466] "Slow binding" observed in the inhibition by aldehydes, i.e. the slow attainment of steady state during inhibition with a lag phase with a half-life of several minutes, is not due to induction of conformational changes in the enzyme ("slow binding" in the usual sense) but is rather a result of the low concentration of free aldehyde in solution.[232] In the case of the leupeptins, 42% exists as hydrate, 56% as cyclic aminol and only 2% exists as free aldehyde (Figure 6). The aldehyde is essential for inhibition; inhibition by the hydrate, which is theoretically possible, does not take place.[236] Many dipeptidyl and tripeptidyl aldehydes have been synthesized with the aim of improving selectivity.[233,238,465] This has had limited success both within the cysteine protease (Table 5) and serine protease groups. Most of the inhibitors in Table 5, especially those with PheH, LeuH, and MetH at P1[238] have significant activity toward α-chymotrypsin, and likewise those with ArgH at P1[233] have considerable activity for trypsin, plasmin, thrombin, and kallikrein. Derivatives with LysH instead of ArgH[233] are reasonably selective for cathepsin B; whether a higher inhibitory activity is associated with stronger binding or with an increased concentration of free aldehyde is still being investigated.[233] A second goal for the synthesis of new inhibitors is the development of membrane-permeable substances.[234,235] Charged derivatives such as leupeptins have poor activity *in vivo* since they cannot pass through the cell membrane,[235,239,279] a fact that was forgotten in many experiments. This led to the synthesis of calpeptin[234,235] (Z-Leu-nLeuH) and MDL 28170[234,235] (Z-Val-PheH) as well as many other derivatives with LeuH and MetH at P1.[235,238] Their activity toward cysteine proteases increases in the following order: cathepsin H ≪ cathepsin B < calpain II = calpain I < cathepsin L (Table 5). Concentrations of 50 μM still inhibit α-chymotrypsin by 95−40 %.[238] This lack of selectivity is also the reason for many side reactions of peptidyl aldehydes *in vivo*.[14] Likewise, it is difficult to attribute the activity of inhibitors, for example in degradation of proteins in experiments on cell cultures or animals, to inhibition of specific enzymes.[14]

7.2.2. Semicarbazones

Peptidyl semicarbazones have been used as intermediates in the synthesis of aldehydes. It has thereby been shown that they are inhibitors of cysteine proteases themselves although much weaker than the corresponding aldehydes (Table 6). Rather than taking place by hydrolysis of the semicarbazone

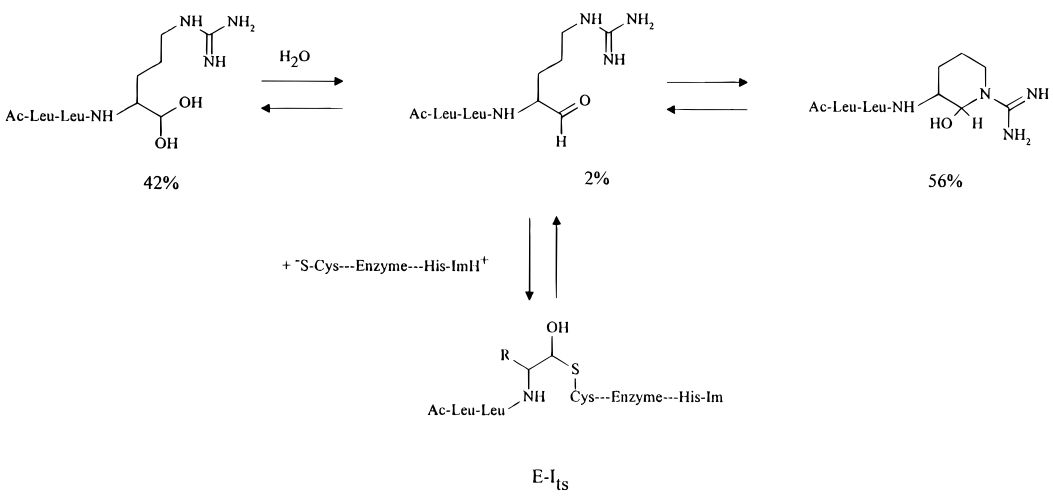


Figure 6. Inhibition of cysteine proteases by peptidyl aldehydes.

Table 5. Inhibitory Activity of Synthetic Peptidyl Aldehydes:[f] K_I Values (μM) and IC_{50} Values (μM)[e]

	papain	cathepsin B	CANP	cathepsin L
Bz-GlyH[228]	25[a]			
Z-GlyH[228]	7.2			
Ac-Phe-GlyH	0.046[b]			
Z-Phe-GlyH[2]	<0.05	1.25		
Z-Phe-AlaH		0.021[14]/0.045[3,465]		0.016[e,465]
Z-Phe-PheH		0.07[e,465]	0.1[e,465]	0.0007[e,465]
Z-Ile-PheH[2]	0.044	0.13		
Z-Leu-PheH[238]		2.4	0.08	0.02
PB-Leu-PheH[238]		0.43	0.06/0.08[e]	0.0014
Z-Val-PheH[234,239,d]		0.025	0.01/0.1[e]	
Z-Leu-NleH[235,238,c]	0.14[e]	0.13	0.065/0.04[e]	0.0034
Z-Leu-MetH[235,238]	0.2[e]	0.25	0.05/0.03−0.4[e]	0.013
PB-Leu-NleH[235,238]	0.015[e]	0.22	0.066/0.04−0.3[e]	0.0033
PB-Leu-MetH[235,238]	0.02[e]	0.16	0.045/0.06[e]	0.0045
Ac-Leu-Leu-NleH[238]		0.15	0.2	0.0005
Ac-Leu-Leu-MetH[238]		0.1	0.2	0.0006
Ac-Xaa-Xaa-ArgH[233]		100−130,[e] Xaa = Leu,Val		
Ac-Xaa-Xaa-ArgH[233]		1.1−1.6,[e] Xaa = Leu, Phe, or Tyr		
Ac-Xaa_1-Leu-Xaa_2H[233]		18−28,[e] Xaa_1 = Tyr,Val,Phe; Xaa_2 = Arg,Lys		
Ac-Leu-Xaa_1-Xaa_2H[233]		0.1−0.2,[e] Xaa_1 = Leu,Ile,Phe; Xaa_2 = Arg,Lys		
Ac-Phe-Val-ArgH[233]		0.04[e]		
Ac-Leu-Val-LysH[233]		0.004[e]		
Z-Gly-Phe-GlyH[2]	0.0052	0.51		
Z-Arg-Ile-PheH[2]	0.0010	0.011		
Ac-Gly-Phe-NleH[2]	0.0005	0.013		
Mns-$(Gly)_n$-Phe-GlyH[2]	0.005−0.01(n = 0−2)			

[a] K_I = 2 μM relative to the actual concentration of free aldehyde of 7.8%.[236] [b] K_I = 5.2 nM relative to the actual concentration of free aldehyde of 11.2%.[236] [c] Calpeptin. [d] MDL 28170. [e] IC_{50} (μM). [f] Bz, benzoyl; Z, benzyloxycarbonyl; PB, phenylbutyryl; Mns, mansyl or 6-(*N*-methylanilino)-2-naphthalene-1-sulfonyl.

Table 6. Inhibitory Activity of Peptidyl Semicarbazones[a]

	K_I value (nM)	
	papain	cathepsin B
Z-Arg-Ile-Phe-Sc	23	500
Z-Ile-Phe-Sc	2 700	1 200
Z-Phe-Gly-Sc	nd[b]	20 000
Z-Gly-Phe-Gly-Sc	9.2	1 000

[a] According to ref 2. [b] nd, not determined.

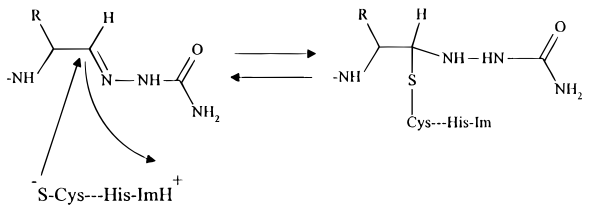


Figure 7. Inhibition of cysteine proteases by peptidyl semicarbazones.

to the aldehyde, inhibition is due to formation of a tetrahedral adduct by attack of the thiolate on the protected carbonyl carbon[2,240] (Figure 7). This reaction is also reversible which is why semicarbazones, like aldehydes, are suitable as ligands for purification of serine and cysteine proteases by affinity chromatography.[2,388]

7.2.3. Methyl Ketones and Trifluoromethyl Ketones

Investigations of inhibition of the serine protease elastase by peptidyl aldehydes *in vivo* have shown that rapid loss of activity occurs if the aldehyde is oxidized to a carboxylic acid. For this reason, the aldehyde group was replaced by the metabolically more stable trifluoromethyl ketone function (TFMK).[241] This produced significantly more potent inhibitors of serine proteases, also *in vitro*. Here, it has also been shown that a tetrahedral hemiketal is formed as covalent enzyme−inhibitor adduct.[242] One possible explanation for the increased stability of the adduct is a stronger binding of the hydroxy group of the hemiketal, which is relatively acidic due to the TFMK function, to the oxyanion hole of the serine proteases.[244] Many derivatives have been developed which inhibit different serine proteases,[244−246,251] metalloproteases and aspartate proteases such as pepsin,[247] renin,[248,390] carboxypeptidase A[247] and ACE,[247] and esterases such as acetylcholinesterase.[247,249] However, for the metalloproteases and aspartate proteases, it is the hydrated form that binds (noncovalently) to the enzyme.[250] In contrast, TFMK and difluoromethyl ketone peptides, and also the corresponding methyl ketones, are much weaker inhibitors of cysteine proteases (Table 7). Methyl ketone and TFMK derivatives are, like the aldehydes, reversible inhibitors of cysteine proteases so that here the formation of a tetrahedral hemithioketal is assumed too.[243] The low inhibitory activity of these compounds toward cysteine proteases has been suggested to be due to steric hindrance.[252,253] However, according to recent investigations, this can be explained as a result of increased thermodynamic stability of the hydrates and hemiketals of fluoro ketones in comparison to the corresponding hemi*thio*ketals.[389]

7.2.4. α-Keto Acids, α-Keto Esters, α-Keto Amides, and Diketones

Since the synthesis of TFMK and methyl ketone peptides did not bring about any improvement in inhibitory activity toward cysteine proteases, the TFMK group was replaced by other electron-attracting groups. Peptidyl α-keto carboxylic acid deriva-

Table 7. Inhibitory Activity of Difluoro- and Trifluormethyl Ketone Peptides (TFMK) and Methyl Ketone Peptides

	K_I (μM)			
	cathepsin B	papain	CANP	α-chymotrypsin
Z-Phe-Ala-CH_3[243]	31			
Z-Phe-Ala-CF_3[243]	300−470			
Ac-Phe-Gly-CH_3[14]		1550		
Z-Phe-CH_3[254]			>2000	
Bz-Phe-CHF_2[254]			>1000	7.7
Bz-Phe-CF_3[254]			>1000	2.3
Z-Val-Phe-CF_3[254]			>180	2.4
Z-Val-D-Phe-CF_3[254]			>180	45

Table 8. Inhibitory Activity of α-Ketocarboxylic Acid Peptides and α-Diketones

	K_I (μM)			
	papain	cathepsin B	CANP II/I	α-CTR[e]
Bz-Phe-COOMe[254]			150	0.15
Z-Phe-COOEt[254]			92	0.13
Z-Phe-$COCH_3$[254]			250	1.2
Z-Val-Phe-COOMe[254]			0.4	0.06
Z-Val-Phe-$COCH_3$[254]			0.7	0.2
Z-Phe-Gly-COOH[258]	7	2		
Z-Phe-Gly-$COOCH_3$[258]	1	5		
Ac-Phe-Gly-$COOCH_3$[258]	2			
Ac-Phe-Gly-$COCH_3$[258]	23	5		
Z-Phe-Gly-CONHEt[258]	1.5	4		
Z-Phe-Gly-CO-LeuOMe[258]	1.5			
Z-Phe-Gly-COO*n*-But[258]	0.8	1.5		
Z-Gly-Phe-Gly-COO*n*-But[258]	1.5	1.3		
Z-Phe-Gly-$CO_2CH_2CO_2Et$[258]	1	0.2		
Z-Phe-Gly-$CO_2(CH_2)_3CO_2Me$[258]	1	3		
Z-Leu-Phe-$CONH_2$[257]		32	0.9	
Z-Leu-Abu-$CONH_2$[257]	190	3.4	0.02/0.3	
Z-Leu-Phe-CONHR[257,a]	45	3.0−9.3	0.02−0.07[b]	
Z-Leu-Abu-CONHR[257,a]	93	2−13	0.02−0.4	
Z-Leu-Phe-$CONEt_2$[257]			76	
Z-Leu-Abu-$CONR_2$[257,c]		32 to >400	not soluble	
Z-Leu-Phe-COOH[257]	7	4.5	0.007	
Z-Leu-Abu-COOH[257]	13.5	1.5	0.06	
Z-Leu-Phe-COOEt[257]	75	340	ca. 1	
Z-Leu-Abu-COOR[257,d]	40−220	4−30	0.4/2−9.5	
Z-Leu-nLeu-COOEt[257]	190	20	0.2	
Z-Leu-Met-COOEt[257]	140	55	ca. 1	
Z-Leu-Abu-CO-NHEt-SO_2Et[e]			0.13[458]	
$(CH_3)_2$N-CO-Leu-Abu-CONHEt[e]			0.3[458]	
MPl-CO-Leu-Abu-CONHEt[e]			0.1[458]	

[a] R = Et, n-Prop, n-But, i-But, Bzl, CH_2CH_2Ph. [b] CANP II, values for CANP I: R = CH_2CH_2Ph, 0.052; R = n-Prop, 15.0. [c] R = Et, n-But, Bzl. [d] R = Me, Et, n-But, Bzl. [e] CTR, α-chymotrypsin; Abu, α-aminodimethylacetic acid; Mpl, morpholino.

tives were also developed as inhibitors of serine proteases and aminopeptidases.[251,254−256] While the activity for serine proteases increases in the order aldehyde < TFMK < α-diketone < keto ester,[254] the order of increasing activity for cysteine proteases is TFMK < methyl ketone ≪ α-keto carboxylic acid derivative = α-diketone < aldehyde.[254,257,258] The order of activity within the keto acid derivative group is different from enzyme to enzyme (Table 8). Many derivatives have been synthesized with Leu at P2 and tested for their activity toward calpains, cathepsin B, and papain.[257] In general, keto acids are better inhibitors of calpains than the corresponding unsubstituted or monosubstituted amides, followed by esters and disubstituted amides.[257] Z-Leu-Phe-COOH and the corresponding monosubstituted amides[458] were found to be highly active and relatively selective inhibitors of the calpains. Good inhibition is due to stabilization of the tetrahedral adduct by electronic interactions or H bonds with the histidine residue of the active site (Figure 8) and

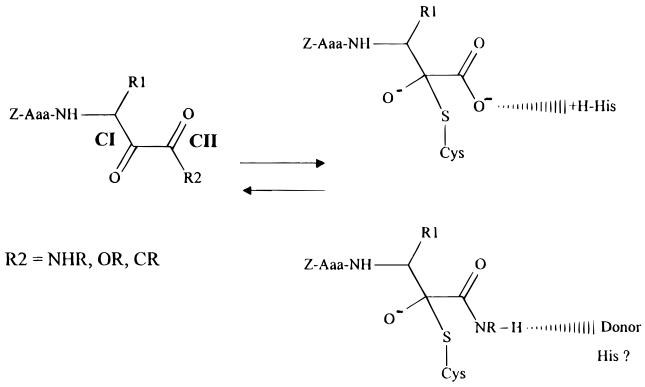


Figure 8. Inhibition by α-keto carboxylic acid derivatives and diketones.

possible stabilization of the oxyanion in calpains by calcium.[257] The early inhibitors were very hydrophobic and poorly soluble in aqueous solutions. New compounds were prepared with polar groups at the

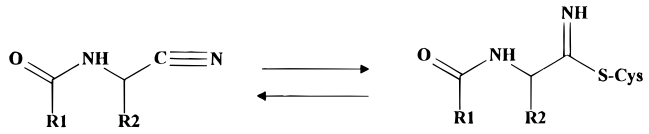


Figure 9. Inhibition by peptidyl nitriles.

Table 9. Inhibition by Peptidyl Nitriles and Thioamides

	K_I (μM)	
	papain	cathepsin C
Z-NH-CH(CH_2OH)-CN[260]	20	
Ac-NH-CH_2-CN[259]	40 000	
Bz-NH-CH_2-CN	140[2]/ca. 400[14,259]	
Ac-Phe-NH-CH_2-CN	6.8[260]/0.73[224]	
CH_3-O-CO-Phe-NH-CH_2-CN	1.8[259]	
Ac-Phe-NH-CH(*i*-But)-CN	5.8[260]	
Gly-NH-CH(CH_2PH)-CN		1.1[224]/2.7[14]
Ac-NH-CH_2-$CSNH_2$	30 000[259]	
Bz-NH-CH_2-$CSNH_2$	2 300[259]	
CH_3-O-CO-Phe-NH-CH_2-$CSNH_2$	610[259]	

C- and N-termini.[458] These modifications resulted in more soluble compounds that were still potent inhibitors of calpain.[458] As expected, keto acid derivatives are less active in whole-cell systems due to their lower membrane permeability.[257] The difference between the two calpains can be surprisingly large, with the K_I values for CANP II generally 10−30× lower.[257] Since hydrate formation and attack of other nucleophiles always occurs at C-I and reduction of this carbonyl group also leads to complete loss of inhibition, attack of the thiolate of the active site at the C-I atom is likely for all derivatives[254,257,258] (Figure 8), corresponding to the "normal" chemical reactivity of α-keto carboxylic acid derivatives.

7.2.5. Nitriles

Peptidyl nitriles, which were discovered to be inhibitors of papain fairly early on,[259] are also reversible inhibitors of cysteine proteases. The isothioamides, which are produced by nucleophilic attack of the thiolate on the nitrile carbon, are thought to be the "dead-end" adducts, on the basis of NMR spectroscopic investigations[224,260] (Figure 9). Hydrolysis of the thioamide does not take place[14,224] and the thioamides are themselves weak inhibitors[259,455] (Table 9). Nitriles which have been investigated are weaker inhibitors than the corresponding aldehydes (Table 9). Investigations with serine proteases have produced inconsistent results.[14] A peptide nitrile hydratase activity has been engineered into papain by a single mutation (Gln19Glu).[521] The role of Glu introduced at position 19 is to participate in the hydrolysis of the thioimidate formed.[521]

7.2.6. Halomethyl Ketones

TPCK (1-(tosylamino)-2-phenylethyl chloromethyl ketone) has been known as a reagent for affinity labeling of the histidine residue of the chymotrypsin active site for a long time.[262,391] Cysteine proteases are also inhibited by this reagent; however, X-ray structural analysis of papain−inhibitor adducts show that instead of the histidine, it is the cysteine residue of the active site that is irreversibly alkylated,[39,406] as for the reaction with iodoacetamide (Figure 10).

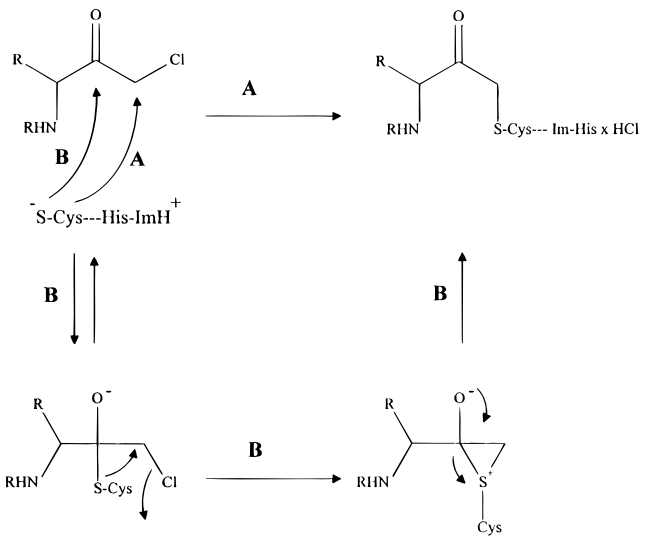


Figure 10. Inhibition by chloromethyl ketones (according to ref 2).

There are two possible pathways:[2] (A) the thiolate anion can react directly with the carbon of the chloromethyl group in a nucleophilic substitution; (B) the sulfur attacks the carbonyl carbon and the product is formed via a tetrahedral intermediate and a sulfonium ion in a 3-center reaction. According to this second mechanism, the peptidyl chloromethyl ketone would be a "mechanism-based inhibitor" instead of an "affinity label", since the first step, the formation of the tetrahedral intermediate, is part of the "normal" catalytic pathway.[2] The second path B seems more likely,[2,14] in analogy to the inhibition of trypsin by Z-Lys chloromethyl ketone, in which formation of a tetrahedral adduct occurs between the serine−OH and the C=O from the inhibitor; this has been demonstrated by NMR spectroscopy.[262] Many peptidyl choromethanes have been synthesized and a certain amount of selectivity can be achieved within the group of cysteine proteases by variation of the P1 and P2 amino acids, as for the inhibitors already described (Table 10). A disadvantage of the chloromethyl ketones is their high reactivity and therefore lack of selectivity. Not only do they react with serine proteases, but also with other SH-containing molecules, such as glutathione or nonproteolytic enzymes,[14] which means that they are not suitable for *in vivo* application because they would be too toxic. This led to the development of monofluoromethyl ketone derivatives in which replacement of chlorine with fluorine could have the potential for lower activity during alkylation. This expectation was fulfilled and reactivity for glutathione was reduced to 0.2% of that of chloro derivatives.[14] In spite of this, the fluoro derivatives are still relatively good inhibitors of cysteine proteases, whereas inhibition of serine proteases is not as good.[14] The rate of formation of the covalent adduct E-I is slower (k_i is smaller) but binding of the inhibitor to the enzyme is stronger (dissociation constant of the enzyme−inhibitor complex K_I is smaller) which results in a similar second order rate constant k_i/K_I[14] (Table 10). Among the derivatives presented in Table 17b, substances have been found which inhibit growth of *Plasmodium falciparum* in erythrocytes to about the same order of magnitude as chloroquine.

Table 10. Inhibition by Peptidyl Halomethyl Ketones

	k_{2nd} (M^{-1} s^{-1})[a]			
	Cath B	CANP I/II	Cath L	papain
TLCK[c]	520[63]	35[b]/64[b,263]	780[63]	750[294]
Z-Phe-Phe-CH_2Cl[266]	9 000			
Z-Phe-Phe-CH_2F[266]	4 000			
Z-Phe-Ala-CH_2Cl[264]	45 000			
Z-Phe-Ala-CH_2F	53 000[266]/16 000[264]	350[283]		
Z-Leu-Tyr-CH_2F	640[14]	17 000[265]	12 000[265]	
Z-Ala-Phe-CH_2F	70[14]			
Z-Leu-Leu-Tyr-CH_2F[265]		29 000	680 000	
Z-Tyr-Ala-CH_2F[265]			7 400 000	
Z-Leu-Leu-Phe-CH_2Cl[283]	190 000	>100 000		
Pro-Phe-Arg-CH_2Cl	200 000[2]/5 100 000[294]		30 000 000[63]	
Leu-Leu-Phe-CH_2Cl[b,263]		0.2	21 500 000[14]	2
Leu-Leu-Lys-CH_2Cl[b,263]		0.65		1.1
Ala-Phe-Lys-CH_2Cl	550 000			
Ala-Phe-Lys-CH_2F[14]	110 000			
Z-Leu-Gly-CH_2Cl[340]	520 000	30 000		
Z-Leu-Gly-CH_2Br[340]	190 000	4 000		
Z-Leu-Ala-CH_2Cl[340]	90 000	6 000		
Z-Leu-Phe-CH_2Cl[340]	3 000	5 000		

[a] Second-order rate constant, $k_{2nd} = k_i/K_I$. [b] IC_{50} [μM]. [c] TLCK, Tos-Lys-CH_2Cl; Cathepsin H: 180.[294]

7.2.7. Diazomethanes

The development of diazomethanes is based on the observation that the antibiotic azaserine[267] inhibits cellular growth by alkylation of a thiol group on the amidotransferase involved in purine synthesis.[268] At the same time it was known that diazomethyl ketones used in synthesis of chloromethyl ketones have no activity toward serine proteases.[268] Thus diazomethyl ketones could be suitable as potential selective inhibitors of cysteine proteases. With Z-Phe-CHN_2 one first active and irreversible inhibitor of papain was discovered.[269] The fact that serine proteases are not inhibited by this substance class and these substances do not react with simple thiols like mercaptoethanol or dithiothreitol led to synthesis of a multitude of derivatives (see, for example, ref 281) some of which have noteworthy selectivity within the cysteine protease group[525] (Table 11). The exact mechanism of inhibition is not known at present. It is thought that the carbonyl carbon undergoes nucleophilic attack by the thiolate to form a hemithioketal. The diazomethyl carbon is subsequently protonated by the imidazolium ion of the histidine—the rate-determining step of the reaction—and the thioether end product is formed via a three-membered transition state with concomitant cleavage of nitrogen[14,268] (Figure 11). The pH dependence of the reaction is the reverse of that of the chloromethyl ketones, for which nucleophilic attack is favored at neutral to weakly basic pH by the presence of the thiolate. In contrast, the diazomethyl ketones are more effective at weakly acidic pH since protonation is required for formation of the unstable diazonium ion.[14] The resonance system of the diazomethyl ketone is disrupted by the preceding hemithioketal formation, and so protonation can take place more easily. According to the definition in section 7.1, the diazomethyl ketones should be classed as suicide inhibitors rather than "affinity labels".[270] There is no plausible explanation at present for the inactivity toward serine proteases but it has been suggested that the geometry of the enzyme inhibitor complex is unfavorable,[268] so that after protonation to the diazonium ion, slow inactivation of the inhibitor takes place by cleavage of the C-terminal C−C bond without alkylation of the serine[14,268,271,274] The only known example of inhibition of a serine protease by this substance class is the weak inhibition of plasma kallikrein by Z-Phe-Arg-CHN_2 (k_{2nd} = 250 M^{-1} s^{-1}).[14,274] Due to their selectivity and inactivity

Table 11. Inhibition by Peptidyl Diazomethanes

inhibitor	enzyme	k_{2nd} (M^{-1} s^{-1})[a]
Z-Phe-Ala-CHN_2	Cath B	1 100[14,268]−1 250[281] 500−630[280]
	Cath C	18[281]
	Cath L	620 000[280]/136 000[14]
	Cath H	0.6[280]
	clostripain	<0.05[281]
	strept prot[b]	7 666[281]
	CANP	<10[283]
	papain	35 000[294]
Z-Phe-Thr(OBzl)-CHN_2	Cath B	300 000[14,268]
Z-Ala-Ala-Pro-CHN_2	Cath B	0.2/1.2[281]
	prolyl end[c]	5 833[268]
	strep prot	6.1[281]
Z-Gly-Pro-CHN_2[278]	prolyl end	10 000−60 000
Z-Lys-CHN_2[281]	Cath B	300
	clostripain	467
Z-Ala-Phe-Ala-CHN_2	Cath B	1 175[281]
	strept prot	30 000[268]
Z-Pyro[d]-Glu-CHN_2	pyroglutam[e]	683[268]
Pyro-CHN_2[277]	pyroglutam	153 333
Z-Leu-Val-Gly-CHN_2[273]	Cath B	3 300
	papain	600 000
	strept prot	102
Z-Phe-Tyr(O-*t*-But)-CHN_2[14]	Cath B	10.3
	Cath L	200 000
	CANP	−[f]
Ser(OBzl)-CHN_2[14]	Cath H	2 600
	Cath C	−
Gly-Phe-CHN_2	Cath B	−[281]
	Cath C	17 333[281]
	Cath H	6 700[14]
Z-Leu-Leu-Tyr-CHN_2[14]	Cath B	1 300
	Cath L	1 500 000
	CANP	230 000
Z-Tyr(I)-Ala-CHN_2[14]	Cath B	27 800
	Cath L	1 128 000
	CANP	<10

[a] Second-order rate constants. [b] Streptococcal protease. [c] Prolylendopeptidase. [d] Pyroglutamyl. [e] Pyroglutamyl peptidase. [f] −, no inhibition.

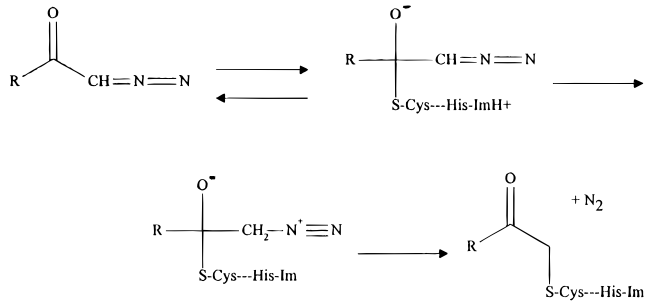


Figure 11. Inhibition by diazomethyl ketones.

toward simple thiols, which is essential for enzyme assays with cysteine proteases, the diazomethyl ketones have been useful in many experiments on cell cultures (see, for example, refs 14 and 275−279). Experiments with radioactively labeled inhibitors on various human tissues indicate that cysteine proteases seem to be the sole targets of the inhibitors.[272] To some authors it seems to be doubtful whether diazomethyl ketones could be used as drugs due to the instability and chemical reactivity of the diazomethyl group and the resultant possibility of toxicity;[240] however, there are very few reports of investigations in animals. The diazomethyl ketone Z-LVG-CHN_2 (Z-Leu-Val-Gly-CHN_2), which corresponds to the proteinase-binding sequence of cystatin, inhibits growth of many *Streptococcus* species *in vitro* and *in vivo*. Doses 10 times higher than that required to cure a fatal streptococcal infection in mice had no toxic effects.[273] E-64, an inhibitor of the purified streptococcal protease, is not active in cell culture or *in vivo* due to its low lipophilicity.[273] Recent results however, indicate that peptidyl diazomethanes are embryotoxic.[471]

7.2.8. Acyloxymethyl Ketones

The peptidyl acyloxymethyl ketones are another group of inhibitors whose development was based on a similar concept to that of the peptidyl monofluoro ketones.[284] Reduced chemical activity is achieved by a space-filling leaving group which is only weakly active in S_N2 reactions.[184] Wide variation of the structure is possible via both the peptide sequence and the structure of the leaving group. Thus, both selectivity for cysteine proteases, corresponding to the S1′ subsites, and reactivity can be controlled (Table 12a).[282,286] On the basis of NMR spectroscopic comparisons with peptidyl chloromethyl ketones, it is likely that the same enzyme−inhibitor adduct is formed.[284] If the chloro derivatives are classical, chemically reactive "affinity labels", the acyloxymethyl ketones must be classed as "quiescent affinity labels" since with their discovery selective (only very weak inhibition of elastase by Z-Ala-Ala-Pro-Val-CH_2-O-CO-(2,6-(CF_3)-Ph), k_{2nd} < 15 M^{-1} s^{-1})[14] and chemically inert inhibitors of the cysteine proteases have been found.[284] An important requirement for a good inhibitor is the pK_a value of the leaving group, which should not be higher than 4 (Table 12a).[282,284] This is one reason why alkanoyl oxymethyl ketones are only weak inhibitors. There is also some speculation about the reaction pathway for acyloxymethyl ketones.[282] If alkylation occurs via a tetrahedral hemithioketal, as for chloro derivatives, which is probable according to general basic chemical principles and because of the resultant end product, the acyloxymethyl ketones should be classed as suicide inhibitors.[282] The substances are only relatively weak inhibitors of the calpains, possibly because peptides with aromatic amino acids in P1′ are not among the preferred substrates of the calpains.[283] Incorporation of polar or charged functional groups in the inhibitor structure afforded significantly enhanced *in vivo* cathepsin B inhibition.[459] Since there is no correlation between *in vitro* and *in vivo* activities differences in bioavalibility or metabolism might be of importance. Derivatives with Asp at position P1 (section 2.6.2, Table 12b) are found to be effective inhibitors of ICE (interleukin-1β converting enzyme).[285,452] It is unusual that Z-Asp-CH_2-O-CO-2,6-Cl_2Ph is a relatively strong inhibitor with activity of 7100 M^{-1} s^{-1} although it only has one amino acid residue; normally a recognition sequence of two amino acids is necessary to attain inactivation rates of >1000 M^{-1} s^{-1} with cysteine endopeptidases.[285]

7.2.9. Methylsulfonium Salts

S-Adenosylmethionine transfers its methyl group to other molecules, but in some cases, it is the decarboxylated methionine side chain that is transferred.[287] Peptidyl sulfonium salts were developed as potential alkylating inhibitors on this basis.[288] It was discovered that for cathepsin B and radioactively labeled Z-Phe-Ala-$CH_2S^+(Me)_2$, it is the peptide chain and not the methyl group that is transferred to the enzyme.[288] Peptidyl methylsulfonium salts in solution are in equilibrium with a ylide structure and the equilibrium tends toward this ylide form with increasing pH (pH 7, 50% ylide; Figure 12).[288] Since raising the pH results in increased inhibitory activity, this suggests that the ylide is the reactive species for inhibition.[288] Sulfonium salts also offer the possibility of controlling the selectivity through an affinity for the S′ site by variation of the substituents on the sulfur atom;[14,288] however, this possibility has barely been investigated. The selectivity of the sulfonium salts for serine and cysteine proteases and their inhibitory activity toward cathepsin B lies between that of the chloromethyl and diazomethyl ketones. The activity generally increases in the following order for a given peptide recognition sequence:[283] diazomethyl ketone < sulfonium methyl ketone < monofluoromethyl ketone ≪ acyloxymethyl ketone. The order is reversed for calpain:[283] sulfonium methyl ketone > fluoromethyl ketone, diazomethyl ketone ≫ acyloxymethyl ketone. Sulfonium methyl ketones are the most potent inhibitors of the calpains known.[283] It has been observed for serine proteases that changes in the substitution at the sulfur atom can lead to a change in the nature of the inhibitor.[289] *S*-Dimethylsulfonium salts are irreversible inhibitors whereas *S*-methyl-*S*-benzyl derivatives are reversible inhibitors of serine proteases.[289] Kinetic data for inhibition by sulfonium salts is given in Table 13. Of interest in this inhibitor group are some effective inhibitors of the clostripain (Table 17a).[289,290]

Table 12. Inhibition by Acyloxymethyl Ketones[a]

a. Inhibition by Acyloxymethyl Ketones

inhibitor	pK_a[b]	enzyme	k_{2nd}[c] (M^{-1} s^{-1})
Z-Phe-Ala-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph	0.58	CANP	–[283,e]
		Cat B	1 600 000[282]
		Cat L	332 000[286]
		Cat S	364 000[286]
Z-Phe-Ala-CH_2-O-CO-(2,5-$(CF_3)_2$)-Ph	2.63	Cat B	38 000[282]
		Cat L	2 400[286]
		Cat S	4 700[286]
Z-Phe-Ala-CH_2-O-CO-(2,6-Me_2-4-COOMe)-Ph	2.67	Cat L	3 600[286]
		Cat S	42 000[286]
Z-Phe-Ala-CH_2-O-CO-4-NO_2-Ph	3.43	CANP	–[283]
		Cat B	610[282]
		Cat L	44 000[286]
		Cat S	3 300[286]
Z-Phe-Ala-CH_2-O-CO-(2,4,6-Me_3)-Ph	3.45	CANP	–[283]
		Cat B	14 000[282]
		Cat L	4 200[286]
		Cat S	500[286]
Z-Phe-Ala-CH_2-O-CO-CMe_3	5.03	Cat B	330[282]
		Cat L	7 800[286]
		Cat S	2 700[286]
Z-Phe-Ala-CH_2-O-C_6F_5	5.53	Cat B	134 000[284]
Z-Phe-Lys-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph		CANP	200[283]
		Cat B	>2 000 000[283]
Z-Phe-Lys-CH_2-O-CO-(2,4,6-Me_3)-Ph		Cat B	230 000[283]
		Cat L	71 000[286]
		Cat S	120 000[286]
Z-Phe-Ser(OBzl)-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph		Cat B	2 600 000[282]
		Cat L	4 290 000[286]
		Cat S	52 000[286]
Z-Phe-Cys(SBzl)-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph		Cat B	2 900 000[282]
		Cat L	10 700 000[286]
		Cat S	1 550 000[286]
Z-Leu-Leu-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph		CANP	–[283]
		Cat B	270 000[283]
Z-Val-Phe-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph		CANP	–[283]
		Cat B	600[283]
Z-Leu-Leu-Phe-CH_2-O-CO-(2,6-$(CF_3)_2$)-Ph		CANP	<1 000[283]
		Cat B	2 500[283]

b. Inhibition by Acyloxymethyl Ketones (According to Ref 285)

k_{2nd}[c] (M^{-1} s^{-1})	ICE[d]	Cat B
Z-Asp-CH_2-O-CO-(2,6-Cl_2-Ph)	7 100	<10
Z-Val-Asp-CH_2-O-CO-(2,6-Cl_2-Ph)	41 000	380
Z-Val-Ala-Asp-CH_2-O-CO-(2,6-Cl_2-Ph)	407 000	2 250
Z-Glu-CH_2-O-CO-(2,6-Cl_2-Ph)	–	

[a] For abbreviations see 12b. [b] pK_a of the leaving group. [c] Second order rate constant. [d] Interleukin-1β converting enzyme. [e] –, no inhibition.

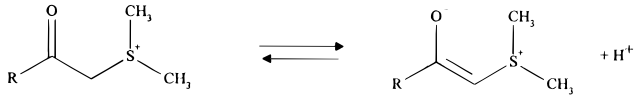


Figure 12. Peptidyl sulfonium salts: ylide form (according to ref 288).

7.2.10. Epoxysuccinyl Derivatives

In 1978, Hanada *et al.* succeeded in isolating a highly active, irreversible inhibitor of papain from culture extract of *Aspergillus japonicus*.[292] The substance was identified as 1-[[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]amino]-4-guanidinobutane,[293] E-64 (Figure 13). Systematic studies were carried out to investigate the role of the different structural components of the inhibitor in enzyme inhibition and the *trans*-L-(*S*,*S*)-epoxysuccinic acid was discovered to be the reactive group essential for inhibition.[295] A change of configuration of the epoxide residue or the neighboring amino acids reduces the activity by a factor of 10−100.[294] At this point, the importance of kinetic constants in determination of inhibitory activity should be explained. IC_{50} values are obtained from fixed assays. The rate of inhibition and dissociation of the enzyme−inhibitor complex are neglected and so IC_{50} values are only suitable for a rough comparison of reversible inhibitors.[211] Misleading results can occur with irreversible inhibitors and for reversible inhibitors where dissociation of the enzyme−inhibitor complex is slow (tight binding). If the reaction between inhibitor and enzyme is incomplete at the time of measurement, the value depends on the incubation time and is of no use.[211] On the other hand, for irreversible inhibition in which the inhibitor reacts with the active center, the IC_{50} value corresponds to approximately half of the total enzyme concentration if the reaction is completed before measurement takes place.[211] However, inhibitors can be differentiated with a continuous enzyme assay since the reaction rate (k_i) and enzyme affinity (K_I)

Table 13. Inhibition by Peptidyl Sulfonium Salts

inhibitor	enzyme	k_{2nd} (M^{-1} s^{-1})
$(CH_3)_3$-S^+	papain	—[288,c]
Z-Phe-CH_2-S^+-$(Me)_2$	papain	31, 7[288]
Z-Phe-CH_2-S^+-(Me)(Bzl)	papain	233[288]
Z-Phe-CH_2-S^+-$(Et)_2$	papain	16[288]
Z-Phe-Ala-CH_2-S^+-$(Me)_2$	papain	4 667[288]
	Cath B	*b*
	calpain	1 100[283]
Z-Phe-Lys-CH_2-S^+-$(Me)_2$[289]	kallikrein	31
	plasmin	20
	trypsin	5
Z-Phe-Lys-CH_2-S^+-(Me)(Bzl)	kallikrein[289]	K_I 1.9 μM[a]
	plasmin[289]	K_I 10 μM[a]
	clostripain[289]	1.1×10^5
Z-Lys-CH_2-S^+-$(Me)_2$	clostripain	1.7×10^5 [289]
Ala-Lys-Lys-CH_2-S^+-$(Me)_2$	Cath B	50[290]
	clostripain	5.3×10^6 [290]
Ala-Lys-Arg-CH_2-S^+-$(Me)_2$	Cath B	69[290]
	clostripain	1.6×10^6 [290]
Ala-Arg-Lys-CH_2-S^+-$(Me)_2$	Cath B	52[290]
	clostripain	7.7×10^5 [290]
Ala-Arg-Arg-CH_2-S^+-$(Me)_2$	Cath B	25[290]
	clostripain	5.5×10^5 [290]
Bz-Phe-Arg-CH_2-S^+-$(Me)_2$	clostripain	1.3×10^6 [290]
	kallikrein	—[290]
Z-Leu-Leu-Phe-CH_2-S^+-$(Me)_2$	Cath B	3 500[283]
	calpain	≫200 000[283]

[a] Reversible, slow binding. [b] pH 5.4, 3167; pH 6.5, 6333; pH 7.0, 15216.[288] [c] —, no inhibition.

of the inhibitors can be compared[211,376,377]

$$E + I \overset{K_I}{\rightleftharpoons} [EI] \overset{k_i}{\rightleftharpoons} E{-}I$$

$$k_{2nd} = k_i \times K_I^{-1} \; [M^{-1}\ s^{-1}]$$

Thus, in the first experiments of different isomers of E-64 on papain, equivalent activity was found for all isomers ($IC_{50} = 0.3$ nM),[295] whereas later continuous assays clearly showed that the L configuration of both the epoxide and the leucine have higher activity[294] (Table 14a). In contrast to other microbial inhibitors, epoxysuccinyl peptides only inhibit cysteine proteases.[292,294] Similarly, simple thiols do not react with these substances, at least in physiological conditions.[294] NMR spectroscopic investigations show that the active-site thiolate attacks at C-3 of the oxirane ring (Figure 13), and the epoxide ring is opened with inversion of the configuration at C-3.[296] On the basis of observations that esters of the epoxysuccinic acid also have significant activity, especially the benzyl ester (ca. 50% of the nonesterified substance), it was thought that the peptide part of the inhibitor does not bind to the S1−S2 site of the enzyme like other inhibitors but rather to the S′ site, and thus the benzyl residue binds to the S2 site which favors hydrophobic aromatic amino acids.[294,303] However, X-ray structural analyses of papain−E-64[40] and papain−E-64c complexes[297] (the agmatine residue is replaced by an isoamylamine residue in E-64c) have shown this assumption to be false, at least for this enzyme. The epoxide residue interacts with the S1 site and the leucyl residue is bound to the S2 site. The direction of the peptide chain is thus reversed to that of a substrate (Figure 14). However, binding to the S′ site was found for inhibition of cathepsin B by epoxysuccinyl-(iso)leucyl-proline derivatives[405]

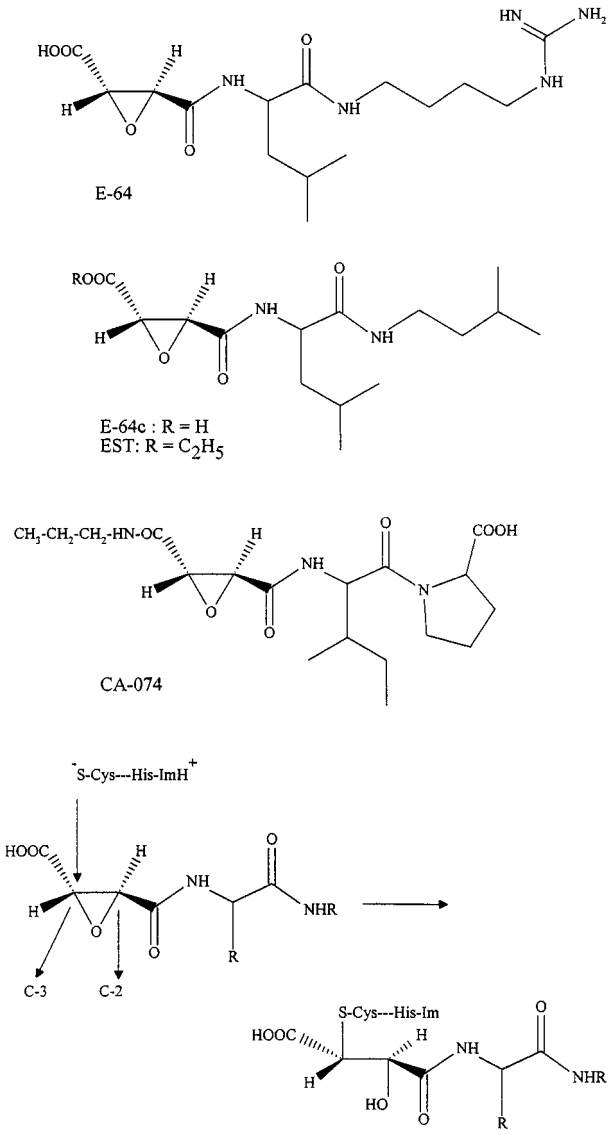


Figure 13. Inhibition by epoxysuccinyl derivatives.

(Chart 5, from ref 405). These derivatives inhibit cathepsin B relatively selectively if the C-terminal proline residue is not esterified.[298−301] Interactions of the C-terminal carboxylate are possible with the histidinium residues 110 and 111, responsible for the exopeptidase activity of this enzyme (section 2.6.1).[298,405] Derivatives of R-Eps-Ile(Leu)-Pro have been synthesized with different amide and ester substituents (R) on the epoxide ring.[298−300] Of these, CA-074 (Figure 13) is the most selective inhibitor of cathepsin B; this inhibitor was developed by molecular modeling as a selective inhibitor of cathepsin B which binds to the S subsite of the enzyme, before the three-dimensional structure of cathepsin B was elucidated.[299] Derivatization of the carboxylate residue of the epoxide makes the two ring carbons more similar so that attack at C-2 is possible.[405] Many derivatives of E-64 have been synthesized (see, for example, refs 294, 298, 300, 303, 304, 305, and 307) including some recent examples;[307] among these are epoxides which are not derived from succinic acid (*erythro*-α-amino epoxide,[306] no kinetic data at present). As shown in Table 14, parts a and b, the selectivity achieved within the cathepsin group has been rather low, with the exception of the Eps-Ile-

Table 14. Inhibition by Epoxysuccinyl Peptides[g]

a. k_{2nd}

	k_{2nd} (M^{-1} s^{-1})					
	papain	Cath B	Cath L	Cath H	CANP[a]	Cath S[b]
E-64 L	638 000[e]	89 400[e]	96 250[e]	4 000[e]	7 500	99 000
	572 000[c]	27 000[d]	218 000[b]			
	374 000[d]					
E-64 D	60 900[e]	1 900[e]	2 700[e]	65[e]	1 070	
Ep 459 (E-64a)	205 000[c]	69 500[e]	27 500[e]	3 080[e]	2 790	
Ep 460 (E-64aZ)	471 000[e]	175 000[e]	231 000[e]	778[e]	23 340	
Ep 459-Ac					3 040	
Ep 479	874 000[e]	339 000[e]	142 600[e]	2 070[e]	4 990	
Ep 174	207[e]	388[e]				
EpsLeuOBzl[b]			791 000			171 000
Ep 47 LL[e]	357 000	298 000	206 000	2 018	7 450	
(E-64c) LD[e]	32 500	3 790		28		
DL[e]	38 000	6 900		6		
DD[e]	110	24				
Ep 420[e]	11 340	49 400	12 660	1 970	440	
Ep 429 (E-64b)	122 000[e]	64 500[e]				
	98 000[c]					
EpsLeuProOBzl	176 000[f]	8 700[f]				
EtO-EpsLeuProOBzl	110[f]	30[f]				
i-ButNH-EpsLeuProOBzl	2 070[f]	206[f]				
EpsLeuPro	30 000[f]	2 200[f]				
Et-EpsLeuPro	760[f]	13 800[f]				
i-ButNH-EpsLeuPro	558[f]	52 000[f]				
EpsPheOBzl	13 000[d]	910[d]	434 000[b]			61 000[b]
EpsArgOBzl	3 000[d]	8 300[d]	73 000[b]			820[b]
EpsIleOBzl			239 000[b]			60 000[b]
EpsPheNBzl	43 000[d]	9 000[d]	27 765 000[b]			501 000[b]
EpsLeuNBzl	90 000[d]	37 600[d]	4 224 000[b]			54 200[b]

b. IC_{50}

	IC_{50} (nM)				
	papain	Cath B	Cath L	Cath H	CANP I/II
E-64	290[k]	35[l]			1 000/2 600[k]
E-64	0.22[n]	240[j]	110[j]		7 000[l]
E-64c		240[j]	130[j]		
E-64c		3.36[m]	0.09[m]	1 640[m]	3 000[m]
E-64d[h]	13[i]	3.4[i]			
CA-074	57 400[i]	2.2[m]	172 000[m]	420 000[m]	200 000[m]
CA-028		30.4[m]	530[m]	15 400[m]	82 000[m]
CA-030	16 000[i]	2.3[m]	32 000[m]	240 000[m]	200 000[m]
CA-030-OMe[m]		4 100	18 300	10 900	200 000
EtO-Eps-Pro-Pro	>100 000[i]	25[m]/50[i]	47 000[m]	1 000 000[m]	200 000[m]
EtO-Eps-Thr-Ile		13.5[m]	540 000[m]	1 000 000[m]	3 000[m]
EtO-Eps-Ile-Ala[i]	5 800	23			
EtO-Eps-Gly-Pro[i]	95 400	15 300			
HO-Eps-Leu-OEt	0.37[n]				
HO-Eps-Leu-OBzl	0.51[n]				
Bzl-O-Eps-Leu-OEt	0.4[n]				
Ho-Eps-Ile-OBzl	0.48[n]				
HO-Eps-Phe-OEt	0.52[n]				
HO-Eps-Orn(Z)-OEt	0.47[n]				
HO-Eps-Arg(NO_2)-OMe	0.41[n]				
HO-Eps-Arg-OMe	2.91[n]				
HO-Eps-Arg	76.2[n]				
HO-Eps-Lys(Z)-OBzl	1.88[n]				

[a] Reference 315. [b] Reference 307. [c] Reference 304. [d] Reference 522. [e] Reference 294. [f] Reference 298. [g] Eps, 2(*S*),3(*S*)-*trans*-epoxysuccinate; Ep 460, HO-Eps-Leu-NH-$(CH_2)_4$-NH-Z; Ep 479, HO-Eps-Leu-NH-$(CH_2)_7$-NH_2; Ep 459, HO-Eps-Leu-NH-$(CH_2)_4$-NH_2; Ep 420, Bzl-DL-Eps-Ile-TyrOMe; Ep 429, HO-Eps-Leu-Leu; Ep 174, HO-Eps-Leu; CA-028, HO-Eps-Ile-Pro; CA-030, Et-O-Eps-Ile-Pro; CA-074, nPr-NH-Eps-Ile-Pro. [h] Ethyl ester of (*E*)-**64c**. [i] Reference 299. [j] Reference 309. [k] Reference 263. [l] Reference 354. [m] Reference 300. [n] Reference 303.

(Leu)-Pro derivatives mentioned above. However, their selectivity for cysteine proteases and chemical inactivity continue to make this substance class indispensable as reagents in the search for the function of cysteine proteases (see, for example, refs 308−314), and since they are stable in solution longer than diazomethyl ketones, for example, they seem to be the most suitable substances for development of pharmaceuticals.[316−318] Their cell permeability can be improved by replacement of the guanidino function by uncharged substituents.[313] Derivatives esterified on the carboxyl residue of the epoxide ring, which have 100−1000-fold worse activity *in vitro*, are more easily resorbed *in vivo* due to their increased lipophilicity and the acid is released by hydrolysis as the active form.[314] The activity of E-64c derivatives with

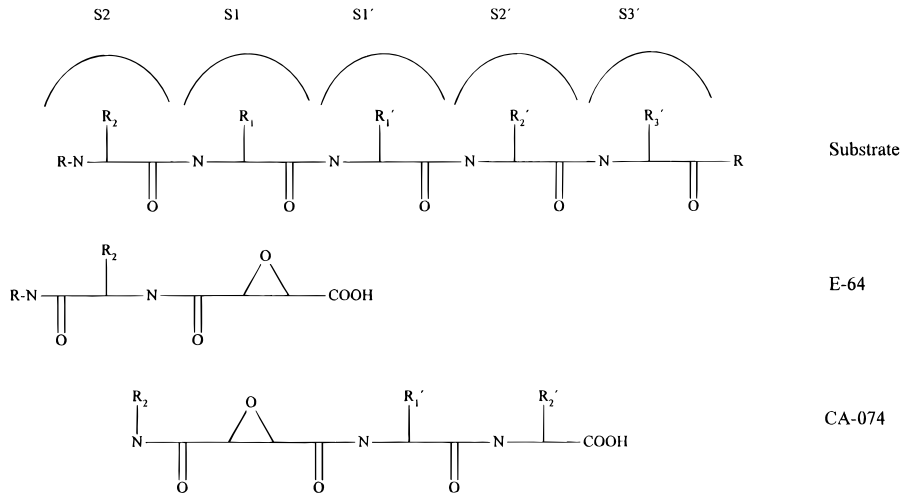
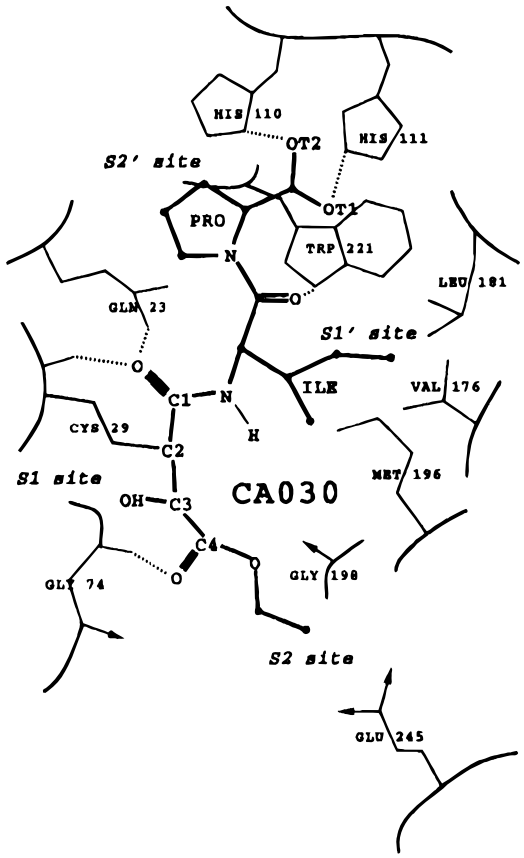


Figure 14. Binding of substrates and various epoxysuccinyl peptides to cysteine proteases.

Chart 5. Schematic Presentation of CA030 Binding in the Cathepsin B Environment. (Reprinted from ref 405. Copyright 1995 American Chemical Society.)



various ester substituents on calpain has been investigated in lyzed and intact platelets; however, ease of ester hydrolysis has no influence on activity in intact cells.[305] The ethyl ester of E-64c (aloxistatin, loxistatin, estate, EST, E-64d, Ep 453) was tested in clinical trials in Japan in 1986 as a prodrug for treatment of muscular dystrophy,[316] but development was stopped in 1992 in phase III because the efficacy did not fulfill expectations.[319] In addition, it was shown that the inhibitor can also covalently bind to proteins other than cysteine proteases.[291,302] This inhibitor in the form of eye drops has also been shown to be effective in the prevention and treatment of cataracts[451] probably by inhibition of calpain.[534] In addition aloxistatin has been shown to inhibit the replication of a mouse hepatitis virus strain.[488]

The *cathestatins* from *Penicillium citrinum* are further examples of microbial metabolites related to E-64 with specific cysteine protease inhibiting properties.[463]

7.2.11. Unsaturated Derivatives

After E-64 was discovered to be an alkylating agent, the next obvious step was to replace the epoxide group with a Michael system which can undergo nucleophilic attack.[294] In DC-11, the epoxysuccinyl group of E-64c is replaced with a fumarate residue.[294] Although the reaction rates of *trans*-epoxysuccinate and fumarate with cysteine are similar, DC-11 is at least 100 times weaker than E-64c but is much more active than *N*-ethylmaleimide, a classical cysteine protease inhibitor.[294] Similar results were obtained with unsaturated analogs of substrates of papain and cathepsin C[323,324] (Table 15). The compounds are irreversible inhibitors and it is assumed that nucleophilic addition takes place at the activated double bond since derivatives without electron-attracting substituents are not active. Although affinities of the inhibitors for the enzyme are relatively high (K_I = 26−220 μM), the inhibitory activities are very low, due to extremely slow alkylation (k_i = 0.0018−0.015 s^{-1}).[323,324] Other related substances (Table 15) developed as inhibitors of α-chymotrypsin and the leucine aminopeptidases (metallopeptidases) are weak, competitive, reversible inhibitors (K_I = 4−60 mM).[324] (*Z*)-Benzylidenemalonic acid mono ethyl ester[332a] is also a weak inhibitor of papain.[333] Here, inhibition by Michael addition is also assumed since the inhibitor reacts with both cysteine[333] and mercaptoethanol[334] under assay conditions. Serine proteases and esterases are not inhibited by this or other similar substances.[332] With the peptidyl vinyl sulfones a new and highly potent class of irreversible and specific cystein protease inhibitors with activated double bonds was developed[474,526] (Table 15). Some of them display second-order rate constants higher than 10^7 M^{-1} s^{-1}.

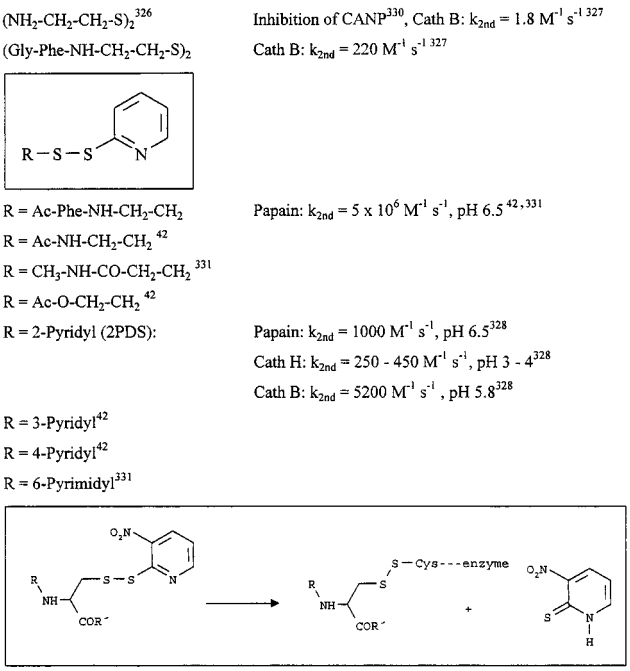
Table 15. Inhibition by Michael Systems

inhibitor	enzyme	k_{2nd} (M^{-1} s^{-1})
DC-11	Cath B	625[294]
	Cath H	11[294]
	Cath L	2 272[294]
	CANP	6[315]
Fum-Phe-OBzl	papain	0.5[530a]
Fum-Ile-NH-*n*Bu	papain	47[527]
Fum-Ile-Pro-OBzl	papain	80[527]
N-ethylmaleimide	papain	6[322]/2.7[321]
	Cath B	1[294]
N-butylmaleimide	papain	7[322]/4.3[321]
N-hexylmaleimide	papain	9[322]/12[321]
N-decylmaleimide	papain	358[321]
Ac-Phe-NH-CH_2-CH=CH-COOMe[a]	papain	70[323,324]
Ac-Phe-NH-CH_2-CH=CH	papain	9.2[d,f,324]
Gly-NH-CH(Bzl)-X[b]	Cath C	68[324]
Gly-NH-CH(Bzl)-Y[c]	Cath C	56[324]
Ac-NH-CH(Bzl)-X	α-CTR	31[d,f,324]
Ac-NH-CH(Bzl)-Y	α-CTR	29[d,f,324]
NH_2-CH(Bzl)-X	LAP[e]	24−61[d,f,324]
NH_2-CH(Bzl)-Y	LAP	4−9[d,f,324]
Mu-Phe-Hphe-VsPh[474]	Cath B	16 400
	Cath L	224 000
	Cath S	7 700 000
	Cath K	10 164
	cruzain	203 000

[a] Trans. [b] X = CH=CHCOOMe trans. [c] Y = CH=CHSO_2Me trans. [d] K_I (mM). [e] Leucine aminopeptidases. [f] Reversible inhibition. [g] Fum = EtOOCCH=CH-CO trans; Mu = morpholine urea; Hphe = homophenylalanine; VsPh = (vinyl sulfonyl) benzene CH=CHSO_2Phe.

7.2.12. Disulfides

It has been known for a long time that cystamine can inactivate papain by blocking the cysteine residue at the active site.[326] The inhibition is stronger if cystamine derivatives are used with hydrophobic peptide residues which correspond to the P2 specificity of the protease[327] (Figure 15). However, these symmetrical disulfides only react very slowly. If the leaving group is replaced by the smaller 2-pyridyl residue, much more effective inhibitors are obtained (factor 1000).[327] These inhibitors have applications in affinity chromatography[2,42,327,328] since blocking of the active site can be overcome with an excess of thiol. These substances are also used for determination of the active-site concentration in cysteine proteases ("active-site titration")[42,328] and for detection and characterization of the residues localized at the active site ("reactivity probes").[42,328,329,331] The 2-pyridyl disulfides have been particularly useful since the basic pyridine nitrogen has two electrophilic forms depending on the pH which both have different characteristics ("two-protonic state reactivity probes"). In contrast to other reagents used for determination of the number of cysteine residues such as DTNB (Ellman's reagent, 5,5′-dithiobis(2-nitrobenzoic acid))[320,335] or cystamine, in acid medium these derivatives only react with the thiolate of the active site, which is a great advantage in investigations of cysteine proteases with other free thiol groups.[42,328] 2-PDS (2,2′-dipyridyl disulfide) is the inhibitor of choice for cathepsin H since this enzyme is only weakly inhibited by the more usual E-64. Figure 15 shows some of the disulfides that have been used.



a Values were obtained in the presence of cysteine; true activities are about 100 times higher since the inhibitors are partly degraded on reaction with cysteine; according to ref 325 .

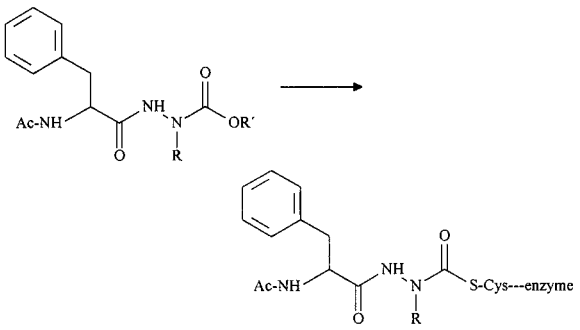
Figure 15. Inhibition by disulfides.

7.2.13. Azapeptides

In azapeptides, an α-carbon atom is replaced by nitrogen[336] (Figure 16). It has been known for a long time that serine proteases are inhibited by azapeptides which have a good leaving group (*p*-nitrophenol) at the C terminal.[337] Probably an acylenzyme is formed which cannot be hydrolyzed further. Derivatives which are esterified with alkyl residues cannot inhibit serine proteases.[337,338] A disadvantage of the phenol derivative is its instability in water (oxadiazolone formation).[337e] Due to the greater nucleophilicity of the sulfur at the active site of the cysteine proteases, these enzymes can be inhibited by the less reactive alkyl derivatives.[339] However, the activity of the derivatives which have been investigated is modest, as is that of various azapeptide analogs α-halomethyl ketones[340] (Figure 16).

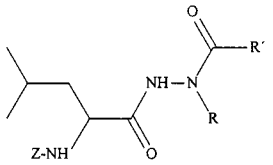
7.2.14. Azobenzenes, Photoregulation of Activity

It is possible to control the activity of papain with the use of irradiation with light of different wavelengths by covalently linking azobenzenes to the enzyme.[341,342] Azobenzoic acids (Figure 17) form amides with lysine residues of the enzyme.[341] The enzyme activity is reduced dependent on the position of the carboxylate residues and the loading of the enzyme with this substance. On irradiation with UV light of 320 nm, the enzyme-bound inhibitor is partly



Inhibition of papain according to ref 339 :

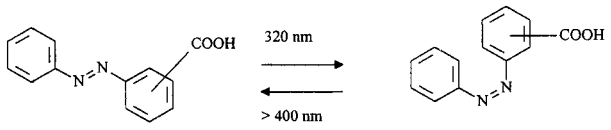
R = H, R′= Me k_{2nd} = 13 $M^{-1}s^{-1}$
R = H, R′= *i*-But k_{2nd} = 18 $M^{-1}s^{-1}$
R = Me, R′= *i*-But k_{2nd} = 0.02 $M^{-1}s^{-1}$
R = H, R′= Ph k_{2nd} > 11000 $M^{-1}s^{-1}$



Inhibition of cathepsin B and calpain according to ref 340 :

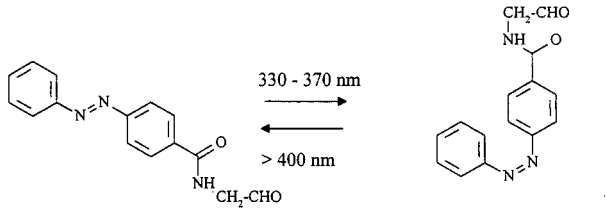
	cath B	CANP
R = H, R′= CH_2Cl	k_{2nd} = 184	536 $M^{-1}s^{-1}$
R = H, R′= CH_2Br	k_{2nd} = 875	1500 $M^{-1}s^{-1}$

Figure 16. Inhibition by azapeptides.



Activity of papain according to ref 341 :

Papain	100%	
Papain - azobenzene adduct:		
trans		cis/trans = 0.9
4-COOH	80%	30%
3-COOH	36%	21%
2-COOH	1%	1%



Inhibition of papain according to ref 342 :

K_I = 2.1 μM 83% cis K_I = 8.8 μM

Figure 17. Azobenzenes.

converted to the cis form and the enzyme activity is further reduced (Figure 17). Renewed irradiation >400 nm gives the trans form again and enzyme activity increases. The reason for these changes in enzyme activity is probably conformational changes in the main chain. The *trans*-*N*-[[4-(phenylazo)-benzoyl]amino]acetaldehyde,[342] in contrast, is known to bind to the active site as a transition-state analog and is a reversible inhibitor of papain. In this case, the cis isomer is the weaker inhibitor; on irradiation with light of 330−370 nm, the inhibitor exist as 83% cis isomer and the activity of the enzyme increases by 500%. Further irradiation at >400 nm reduces the activity to the original value.

7.2.15. O-Acylhydroxamates

These substances were initially developed as inhibitors of the serine protease DP IV (dipeptidyl peptidase IV).[343] However, they are much more effective inhibitors of cysteine proteases[344,345] (Table 16). Following reversible formation of a tetrahedral intermediate, two stable covalent enzyme−inhibitor products are, in addition to hydrolysis to peptide and O-acylhydroxylamine, possible via the 1,2 anionotropic rearrangement analogous to the Lossen rearrangement.[14] If the peptide residue leaves, the thiocarbamate is formed, whereas if the enzyme residue leaves, the sulfenamide results (Figure 18). Since it is usually the more stable anion that leaves, the resulting product is the sulfenamide and this has been shown by NMR spectroscopy to be the case.[346] In the absence of reducing agents normally used for enzyme assays with cysteine proteases (e.g. smaller thiols), the inhibitors are hydrolyzed and the enzyme is inactivated by oxidation by the *O*-acylhydroxylamine which is produced. This inactivation is reversible and can be abolished with reducing agents. In the presence of reducing thiols, hydrolysis/oxidation/reduction runs in a catalytic, cyclic process and irreversible formation of the sulfenamide occurs concomitantly ($k_{cat}/k_i = 6-12$).[346] Unlike the results obtained by NMR spectroscopy for inhibition of papain with Z-Phe-Gly-NHO-Mes,[346] the enzyme−inhibitor adduct for inactivation of the serine protease subtilisin by BOC-Ala-Pro-Phe-NHO-Nbz was found by X-ray structural analysis to be the carbamate.[347]

7.2.16. Lysosomotropic Bases

Lysosomotropic bases are weakly basic substances which accumulate in high concentrations in the lysosomes since the lysosomal membrane is, like the plasma membrane, impermeable to dissociated compounds.[349] The activity of all hydrolases is inhibited by influencing the pH value in the lysosomal space.[349] Examples of such substances are ammonium chloride, methylamine, tributylamine, nigericin, gramicidin, and the antimalarial chloroquine.[349] Chloroquine itself is also a reversible inhibitor of cathepsins B, H, and L (K_I = 1.8, 0.6, and 1.5 mM, respectively).[63] In addition to its application as an antimalarial, chloroquine is also used as a basic therapy

Table 16. Inhibition by Peptidyl *O*-Acyl Hydroxamates

inhibitor[a]	enzyme	k_{2nd} (M^{-1} s^{-1})
Boc-Ala-Pro-NHO-Nbz	Cath S	20[b]
	Cath L	50[b]
	Cath B	10[b]
	PSE	2.5[b]
Boc-Ala-Phe-NHO-Nbz	Cath S	30[b]
	Cath L	600[b]
Boc-Phe-Ala-NHO-Nbz	Cath S	42 000[b]
	Cath L	437 000[b]
	Cath B	14 000[b]
	Cath H	21[b]
Z-Phe-Ala-NHO-Mes	Cath B	640 000[14]
Z-Phe-Gly-NHO-Mes	Cath B	580 000[345]
Boc-Phe-Gly-NHO-Bz	Cath S	19 000[b]
	Cath L	136 000[b]
	Cath B	2 600[b]
Boc-Gly-Phe-NHO-Bz	thermitase	652[348]
	subtilisin	1 020[348]
Z-Phe-Phe-NHO-Bz	Cath S	5 600[b]
	Cath L	63 000[b]
	Cath B	600[b]
Z-Phe-Phe-NHO-Ma	Cath S	21 000[b]
	Cath L	1 222 000[b]
	Cath B	2 800[b]
	Cath H	19[b]
Boc-Ala-Ala-NHO-Nbz	elastase	12[348]
	thermitase	166[348]
	subtilisin	226[348]
Ala-Pro-NHO-Nbz	DP IV	1.9[343b]
Boc-Phe-Pro-Ala-NHO-Nbz	Cath S	1 100[b]
	Cath B	200[b]
Boc-Ala-Phe-Leu-NHO-Nbz	Cath S	229 000[b]
	Cath L	696 000[b]
	Cath B	12 000[b]
	Cath H	32[b]
Boc-Gly-Phe-Phe-NHO-Nbz	Cath S	267 000[b]
	Cath L	800 000[b]
	Cath B	8 300[b]

[a] Nbz, 4-nitrobenzoyl; Ma, methacroyl; Mes, mesitoyl; Bz, benzoyl; PSE, proline-specific endopeptidase; DP, dipeptidyl peptidase. [b] Reference 344.

for treatment of chronic polyarthritis, whereby its efficacy is probably associated with inhibition of the release of lysosomal enzymes by stabilization of the lysosomal membrane.

Table 17. Inhibition of Clostripain and *Plasmodium falciparum*

a. Inhibition of Clostripain

inhibitor	k_{2nd} (M^{-1} s^{-1}) × 10^6	ref
D-Phe-Pro-Arg-CH_2Cl	0.045	14
Z-Phe-Arg-CHN_2	0.085	14
Bz-Phe-Arg-CH_2F	1.1	14
Bz-Phe-Arg-$CH_2S^+(CH_3)_2$	1.3	290
Z-Lys-$CH_2S^+(CH_3)_2$	0.17	289
Z-Phe-Lys-$CH_2S^+(CH_3)(C_7H_7)$	0.11	289
Ala-Lys-Lys-$CH_2S^+(CH_3)_2$	5.3	290
Ala-Lys-Arg-$CH_2S^+(CH_3)_2$	1.5	290
Ala-Arg-Lys-$CH_2S^+(CH_3)_2$	0.77	290
Ala-Arg-Arg-$CH_2S^+(CH_3)_2$	0.55	290

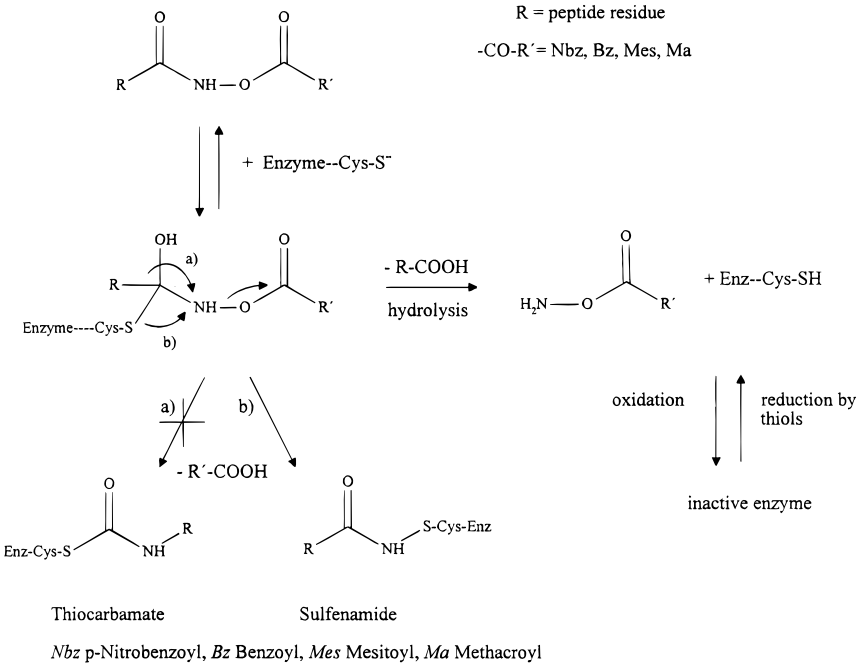
b. *Plasmodium falciparum*

inhibitor	inhibition of growth/invasion in erythrocytes IC_{50} (nM)	
	growth	invasion
chloroquin[b]	12.8	
Z-Leu-Tyr-CH_2F[b]	59	
Z-Phe-ImNva[a]-CH_2F[b]	80	
Z-Phe-Ala-CHN_2[b]	8 790	
Z-Phe-Ala-$CH_2S^+(CH_3)_2$[b]	1 950	
leupeptin[c]		22 000
bestatin[c]		25 000
chymostatin[c]		15 000
Ac-Leu-Leu-Nle-CHO[c]		130
Ac-Leu-Leu-Met-CHO[c]		160

[a] ImNva, imidazolyl norvalin. [b] Reference 35a. [c] Reference 35b.

7.2.17. Calmodulin Antagonists

It is known that some pharmaceuticals, e.g. phenothiazines, can inhibit the ability of calmodulin to bind to its target enzymes and thereby to influence their activity; this calmodulin inhibition occurs via interactions with hydrophobic regions on the calmodulin surface.[350] Since calpains contain the relevant calmodulin sequence, it was logical to test these substances for their effect on calpains.[351,352] Calmid-



Nbz p-Nitrobenzoyl, *Bz* Benzoyl, *Mes* Mesitoyl, *Ma* Methacroyl

Figure 18. Inhibition by *O*-acyl hydroxamates.

azole (CDZ, 1-[bis(4-chlorophenyl)methyl]-3-[2-[(2,4-dichlorophenyl)methoxyl]ethyl]-1*H*-imidazolium chloride) inhibits calpain II at micromolar concentrations[352] and is thereby less active than toward various calmodulin-dependent enzymes. Trifluoroperazine, one of the phenothiazines mentioned above which are used as neuroleptics, and the naphthylsulfonamide W-7 (*N*-(6-aminohexyl)-5-chloro-1-naphthylsulfonamide) only have a slight effect on the proteolytic activity of the calpains.[351] W-13 (*N*-(4-aminobutyl)-5-chloro-2-naphthylsulfonamide) activates the enzyme at concentrations of 10−40 μM.[351] Since inhibition of calpains by calpastatin is reversed by TFP, W-7, or W-13, it is assumed that all of these substances act at the same binding site.[351]

7.2.18. Aziridines and Thiiranes

(*S*)-Aziridine-2-carboxylic acid is an irreversible inhibitor of papain (k_{2nd} = 17 M^{-1} s^{-1}).[356] On the basis of this result and the fact that aziridine-2-carboxylates and -2,3-dicarboxylates can be hydrolyzed by serine proteases and esterases[357] and do not inactivate the enzymes, it seemed useful to use an aziridine bound to a peptide as an irreversible inhibitor of cysteine proteases, analogous to the epoxide derivatives. Since derivatizations can be carried out at the nitrogen, more possibilities are opened up for influencing the reactivity and selectivity. Attempts to incorporate the (*S*)-aziridine-2-carboxylic acid as the C or N terminal of the relevant peptide (Figure 19c) were successful but the substances were no longer stable once the protective groups have been removed.[358] For (*S*)-aziridin-2-carboxylic acid (AzyOH) containing peptides first kinetic data are given in ref 461 (e.g. Z-Phe-Azy-NH-$(CH_2)_3$- Phe, k_{2nd} = 262 M^{-1} s^{-1} papain). The aziridine analogs of Ep-475 (active form of aloxistatin) and Ca-O74 have been tested.[457] The inhibition was found to be strongly pH dependent, with maximum activity at pH 4. At this pH the aziridines showed inhibition rates similar to the epoxides. In contrast to the epoxides, the diastereomers with *R*,*R*-configurated ring are the preferred isomers for inhibition. Another difference is the weak inhibition of cathepsin B by the Ca-074 analog (13 M^{-1} s^{-1}). These differences are attributed to the presence of the protonated aziridine ring modifying the binding mode at the active site.[457] Aziridine-2,3-dicarboxylates N-acylated with amino acids have been tested in the author's laboratory[530a] and found to be weaker inhibitors than aziridines with free N-atom. Aziridine-2,2-dicarboxylates were found to be weak reversible inhibitors.[530b]

(*S*)-Methylthiirane-2-carboxylate is a reversible inhibitor of papain, too[529] (K_i = 0.2 mM). Experiments are also underway to replace the epoxysuccinate unit with epithiosuccinate.[359]

7.2.19. Inhibitors with Various Other Structures

In this section, some inhibitor structures will be discussed, which have been developed just recently or for which only single examples have been investigated. The cyclopropenone derivative bound to a peptide unit shown in Figure 19a is a competitive inhibitor of papain.[353] The configuration of the 1′ position influences the activity. The substance has no effect at concentrations of ca. 100 μM on the serine

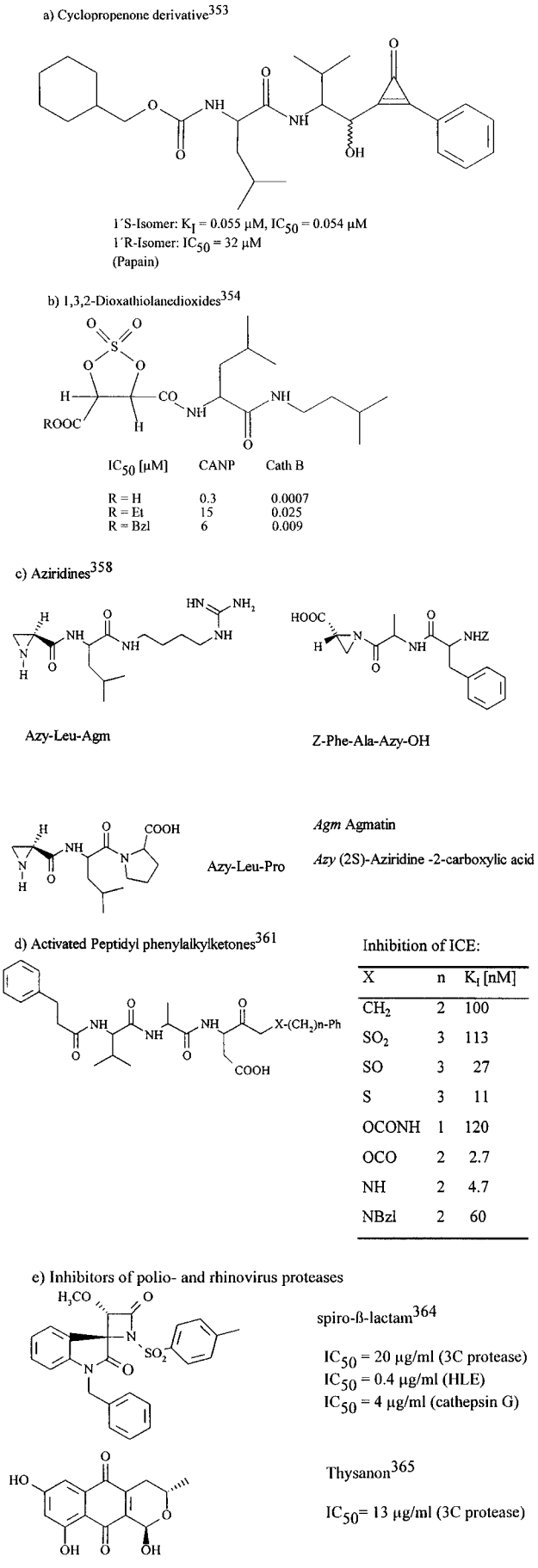
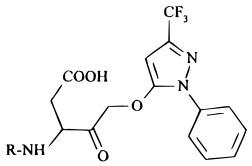
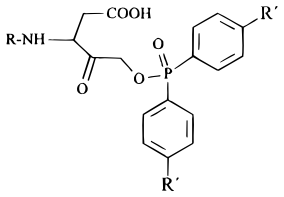


Figure 19. Inhibitors with various structures.

a) Peptidyl-(1-phenyl-3-(trifluoromethyl)-pyrazol-5-yl)oxy)methylketones (PTP-methylketones) [453]
as ICE-Inhibitors (e.g. R = Z-Val-Ala k_{2nd} = 280000 $M^{-1}s^{-1}$)



b) Diphenylphosphinyloxymethylketones (DPP-methylketones) [454]
as Inhibitors of ICE, cath B and CANP I



e.g. R = Z-Val-Ala, R′= Cl
k_{2nd} (ICE) = 230000 M^{-1} s^{-1}

Figure 20. Novel classes of ICE inhibitors.

proteases thrombin and cathepsin G, or on the carboxypeptidase cathepsin D, and could be a representative of a new selective and potent class of cysteine protease inhibitors. The mechanism of inhibition is not known but the complex reactivity of cyclopropenones offers various suitable points of attack.

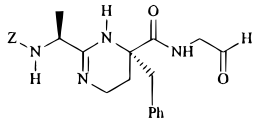
In the 1,3,2-dioxathiolane dioxide derivatives[354] in Figure 19b, the epoxide residue of E-64c is replaced by a cyclic sulfate residue which can also undergo nucleophilic attack. The enzyme−inhibitor adduct for this substance class should be the same as for epoxysuccinyl derivatives.[355] Serine proteases are not inhibited by these substances.

The tripeptidyl alcohol Ac-Gly-Phe-Nle-OH (α-carboxyl replaced by OH) is a reversible inhibitor of papain (K_I = 6.5 μM).[2] Replacing the alcohol group with a methylsulfonate residue (Ac-Gly-Phe-Nle-O-SO_2-CH_3, α-carboxyl group replaced with -O-SO_2-CH_3) gives an irreversible but weak papain inhibitor (k_{2nd} = 17 M^{-1} s^{-1}).[2]

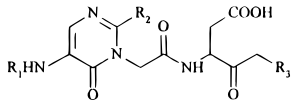
Various activated peptidyl phenylalkyl ketones,[360] in which the β'-C atom is replaced with heteroatoms or electron-attracting groups,[361] are reversible inhibitors of ICE (Figure 19d). Novel classes of potent irreversible ICE inhibitors were found with the aspartyl α-[(1-phenyl-3-(trifluoromethyl)pyrazol-5-yl]-oxy]methyl ketones (PTP-methyl ketones)[453] (Figure 20a) and the aspartyl α-[(diphenylphosphinyl)oxy]-methyl ketones (DPP-methyl ketones)[454] (Figure 20b).

The spiro-β-lactam[364] shown in Figure 19e is an inhibitor of the 3C proteases of polio- and rhinoviruses; as mentioned, these enzymes have no sequence homology to other known proteases and are not inhibited by cysteine protease inhibitors such as leupeptin, E-64, and trifluoromethyl ketones. The substance also inhibits human leucocyte elastase (HLE) and cathepsin G. The naphthoquinone thysanon[365] has been isolated from culture extract of the fungus *Thysanophora penicilloides*, and it inhibits the 3C protease of rhinovirus.

a) Tetrahydropyrimidin derivative [456]
as a conformationally restricted papain inhibitor
K_I = 790 nM



b) 5-Aminopyrimidin-6-one derivatives as peptidomimetic ICE inhibitors [528]



R_1 = Z, $PHSO_2$, H, $PhCH_2NHCO$, (2-furanyl)CO, $Me_2N(CH_2)_5CO$
R_2 = 4-F-Ph, 2-thienyl, 3-pyridinyl, Me
R_3 = PTP, DPP, DCB (= 2,6-dichlorobenzoyl)oxy

e.g. k_{2nd} = 268000 M^{-1} s^{-1} for R_1 = Z, R_2 = 4-F-Ph, R_3 = DCB

Figure 21. Peptidomimetic inhibitors.

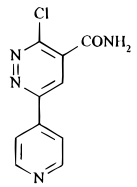
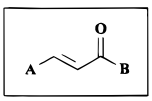
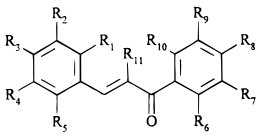


Figure 22. Pyridazine-4-carboxylic acid derivatives as ICE inhibitors,[464] e.g. 3-chloro-6-(4-pyridyl)pyridazine-4-carboxamide.

a) Chalcone derivatives [532]



A, B = aryl, heteroaryl e.g.:



R_1 - R_{10} = H, F, Cl, Br, NO_2, CF_3 OH, OCH_3, $N(CH_3)_2$

R_{11} = H, CH_3

b) Oxalic bis[(2-hydroxy-1-naphthylmethylene)hydrazide] [531]

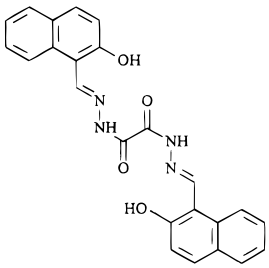


Figure 23. Inhibitors of malarial cysteine proteases.

Two recent publications deal with peptidomimetic pyrimidine-based inhibitors[456,528] (Figure 21, parts a and b). One of the new inhibitors is an aldehyde, and it inhibits papain reversibly,[456] the others are derived from PTP, DPP, or (2,6-dichlorobenzoyl)oxy

(DCB) methylketones and are potent irreversible inhibitors of ICE.[528] New ICE inhibitors were also found in *pyridazine-4-carboxylic acid derivatives*[464] (Figure 22). Further nonpeptidic inhibitors of malarial cysteine protease were found in a series of *chalcones*[532] and in an *oxalic bis hydrazide*[531] (Figure 23, parts a and b).

8. Summary

Apart from the very few examples mentioned in section 7.2.19 all substances developed at present fit into the general structural scheme: peptide recognition sequence + reactive group. According to the properties of this reactive group, the cysteine residue of the active center is either irreversibly alkylated or acylated, or a reversible reaction occurs with the transition-state tetrahedral hemiacetal/ketal as covalent product. The latter is so well stabilized that the reverse reaction is very slow (tight binding). This hemiacetal/ketal is also the first covalent intermediate for irreversible inhibitors which have a reactive carbonyl group. Due to their similar proteolytic mechanisms, many inhibitors react with both serine and cysteine proteases.[392] These include the reversible aldehyde and ketone derivatives and the chemically reactive chloromethyl ketones. In order to achieve selective inhibition for cysteine proteases, it is necessary to make use of the greater nucleophilicity of the SH group compared to the OH group; the unusually high reactivity of the active-site cysteine is due to its existence in the thiolate form. Among these inhibitors are the diazomethyl ketones, acyloxymethyl ketones, epoxysuccinyl derivatives, and to a limited extent, the *O*-acylhydroxamates, monofluoromethyl ketones, and sulfonium derivatives. The peptide portion of the inhibitors is important for the rate of formation of the noncovalent enzyme−inhibitor complex, the first reversible phase of inhibition, and for the stability of this complex. During alkylation or acylation of the active site, the irreversible phase is usually independent of this portion of the inhibitor; this step of the inhibition, the rate-determining step, is influenced by the electrophilicity of the reactive group and the properties of the leaving group and does not differ much between the individual enzymes. If the leaving group of the inhibitor can be varied, it is possible to control selectivity within the cysteine protease group via the specificity of the S′ site of the enzyme. This possibility has not been made use of to any great extent yet. Since mammalian and human cysteine proteases, especially the cathepsins and calpains, unlike the serine proteases, are very similar in their specificity, it has only been possible to achieve specific inhibition of an individual protease in a few cases. A starting point is the additional exopeptidase activity of some proteases which may enable reduction in size of the peptide portion without loss of activity, as has been achieved once for ACE inhibitors.[366] If the inhibitors are to be used for experiments in cell culture or *in vivo*, in addition to selectivity, further problems occur because of the more complex environment. Since cysteine proteases normally exist within the cell as lysosomal or cytoplasmic enzymes, the inhibitor must be uncharged in order to pass rapidly through the cell membrane. In addition, the reactivity of the inhibitors must be sufficiently low that they are inert toward catalytically irrelevant thiol groups or essential SH groups of nonhydrolytic enzymes. Since lower reactivity of irreversible inhibitors is associated with increased selectivity between cysteine and serine proteases, the inhibitors of choice are the epoxides, and the diazo- and acyloxymethyl ketones, which can be classed as suicide inhibitors or "quiescent affinity labels".

Cysteine protease inhibitors may be suitable as drugs for treatment of tumors, inflammatory diseases, pathological processes which involve increased degradation of bone, cartilage or muscle, infections with viruses, and bacterial and protozoa infections. A pharmacologically effective protease inhibitor should fulfill the following conditions: The interaction between enzyme and inhibitor must be irreversible, or if reversible, the dissociation constant of the enzyme−inhibitor complex must be <0.1 nM in order to maintain activity for a longer period. The problem of selectivity and reduced chemical reactivity, which are both required for low toxicity, has been mentioned already. On the other hand, inhibition of individual enzymes is of little use in treatment of conditions such as DMD in which several enzymes are involved. As well as development of membrane-permeable substances, a further criterion for oral application is the resorption and stability in the gastrointestinal tract, whereby the latter can present particular problems for substances with peptide structures. Synthesis of prodrugs, for example by esterification of the epoxysuccinyl derivative E-64c or by linking with lysosomotropic polymers[367] which only release the pharmacologically active substance in the lysosomes on cleavage by lysosomal enzymes, is of particular importance.

In addition to continued investigation of recently developed inhibitors, the quest for new possibly nonpeptidal basic structures via screening of microbial components or known substances should be given increased emphasis.

Enzymatic assays (substrates, active-site titrations) and the processes of isolating, purifying, and cloning enzymes have not been discussed here. Information on these topics can be found in the comprehensive monograph in *Methods in Enzymology* published in 1994[397] and in the book *Proteolytic Enzymes*, [398] which provides a very helpful practical introduction to this area.

Many cysteine proteases have already been cloned, and thus new results concerning the three-dimensional structures of the enzymes can certainly be expected in the near future.

Also not mentioned are the cysteine protease inhibitors with protein structures which could be isolated from various plants.

9. Notes and Abbreviations

Amino acids are L configured, unless otherwise specified.

Bz, benzoyl; Bzl, benzyl; Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; But, butyl; Prop, propyl; Me, methyl; Et, ethyl; Ph, phenyl; Eps, epoxysuccinyl; Nle, norleucine; Nva, norvaline; Im, imidazole; Mes,

mesitoyl; Ma, methacryl; TLCK, Tos-Lys-CH_2Cl; TPCK, Tos-Phe-CH_2Cl; Nbz, *p*-nitrobenzoyl; BPACK, Z-Phe-Ala-CH_2Cl; Tos, tosyl.

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