Mini Reviews in Medicinal Chemistry, 2003, *3,* 585-596

Cysteine Protease Inhibitors Containing Small Rings

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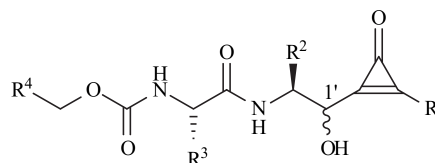
Abstract: Since the discovery of E-64 in 1978 as potent cysteine protease inhibitor a variety of inhibitors containing small rings as electrophilic building blocks responsible for enzyme inhibition have been developed. In this review we summarize new aspects concerning epoxysuccinyl peptides derived from E-64 and discuss inhibition potency, selectivity and mechanisms of peptidic and peptidomimetic inhibitors containing epoxide, aziridine, thiirane, cyclopropane, ß-lactam and ß-lactone rings as electrophilic fragments.

INTRODUCTION

One of the intensively studied fields in protease inhibitors research is the development of cysteine protease inhibitors as potential new antiinfective, antidegenerative and antiinvasive drugs. No drug which targets a cysteine protease is currently on the market, some inhibitors, however, are in the industrial pipelines [1-7]. The hydrolysis mechanism of cysteine proteases comprises attack of a negatively charged cysteinate residue at the carbonyl carbon of the peptide bond leading to an acyl enzyme which is hydrolyzed in the second hydrolysis step. Therefore, reactive building blocks which mimick the partially positively charged carbonyl carbon can serve as electrophilic traps. Attack of the active site´s cysteine leads to a covalently modified enzyme which in contrast to the acyl enzyme of the "normal" hydrolysis cannot further be hydrolyzed. This inhibition mechanism is also known in serine dependent enzymes with ß-lactam antibiotics as well known examples of inhibitors. One of the first cysteine protease inhibitors which exploited the electrophilicity of small heterocyclic rings was the epoxysuccinyl peptide E-64 (Fig. **6**), isolated from *Aspergillus japonicus* in 1978. But maybe, it was the other way round, and the isolation and discovery of E-64 as cysteine protease inhibitor gave the impetus for the development of a new concept in inhibitor design. During the last years an immense number of new cysteine protease inhibitors have been developed. Thus, a complete review is nearly impossible to write. We therefore decided to summarize and review several topically successional parts [8]. In this chapter we will focus on new cysteine protease inhibitors, published since 1997 [9], containing small rings as reactive electrophilic units which are responsible for enzyme inhibition.

CYCLOPROPENONES

Various analogs of the competitive and reversible diastereomeric papain inhibitors **1a** (*1´S*-configuration) and **1b** (*1´R*-configuration) (Fig. **1**) with cyclopropenone moiety, first published in 1993 [10], have been synthesized and tested against papain, m-calpain, and cathepsins B and L [11].



R^1 = H, CH_3, $CH_3CH(CH_3)_2$, (*Z*)-1-hexenyl, Ph, 4-F-Ph, 2-Me-Ph, 5-TMS-2-thienyl

R^2 = $CH(CH_3)_2$, *n*-C_4H_9

R^3 = CH_3, $CH(CH_3)_2$, $CH_3CH(CH_3)_2$, Bn

R^4 = cyclohexyl, Ph

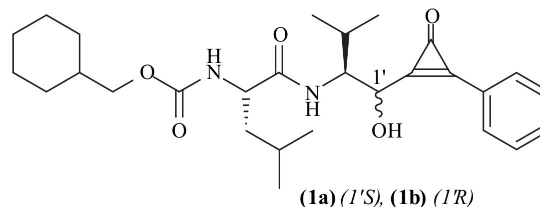


Fig. (1). Cyclopropenones as cysteine protease inhibitors.

None of the new compounds, however, showed improved inhibitory activity against papain, cathepsin B and L in comparison to the original inhibitors **1a** and **1b**. Better activity against m-calpain could only be achieved by replacing the bulky R^1 phenyl residue with hydrogen (table 1)(cpds 2 and 3). Methyl, *i*-butyl and *(Z)*-1-hexenyl substituents in this position led to substantially higher IC_{50} values. Despite their bulkiness some R^1-aromatic substituted compounds (Fig. **1**) showed quite good activities leading to the assumption that the planarity of the aryl cyclopropenone unit is essential for good calpain inhibiting activity. Simple cyclopropenone derivatives are inactive against calpain. Thus, according to the known substrate specificity of calpain, the hypothetical binding sites of the peptidic compounds to calpain may be cyclohexylmethyl (-CH_2-R^4) in S3, *i*-butyl (R^3) in S2, R^2 in S1 and R^1 in S1´. Additionally, the 1´-hydroxy group seems to play an important role since 1´-methoxy, 1´-acetoxy and 1´-oxo derivatives showed no or only weak activity against calpain. No general rule for the stereospecificity of inhibition could be found. The *(1´S)* isomer **1a** showed strong activities against all tested cysteine proteases. On the contrary, the *(1´R)* isomer **1b** inhibited cathepsins B and L strongly, but

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Table 1. Inhibition of Cysteine Proteases by Cyclopropenones[a,b]

cpd	R[1]	R[2]	IC_{50} [µM]*(1´S)*	IC_{50} [µM]*(1´R)*	enzyme
1a *(1´S)*, **1b** *(1´R)*	Ph	$CH(CH_3)_2$	0.054 1.62 0.71 0.00086	22 3.25 0.044 0.0013	papain calpain CB CL
2[c]	H	$CH(CH_3)_2$	0.50		calpain
3[d]	H	n-C_4H_9	0.81		calpain

[a] See fig. 1; [b] R^4 = cyclohexyl, R^3 = $CH_2CH(CH_3)_2$; [c] diastereomeric ratio *(1´S):(1´R)* = 7:3; [d] diastereomeric ratio *(1´S):(1´R)* = 8:2.

showed only weak activity against papain. The mechanism of inhibition by cyclopropenone derivatives remains unclear at the moment. According to the instability of the compounds in basic media the formation of a covalent adduct of the active site´s thiol residue and the cyclopropenone could be possible.

ß-LACTONES

The ß-lactone ring is known to acylate serine, threonine and cysteine side chains of various enzymes [12-17]. This led to the assumption that cysteine proteases, too, may be inactivated by properly substituted ß-lactones. In this context, N-Cbz-*(S)*-serine-ß-lactone (**1a**) (scheme 1) was shown to irreversibly inactivate the HAV 3C cysteine protease (k_i = 0.7 min^{-1}; K_i = 1.84 10^{-4} M; k_{2nd} = 3800 M^{1-} min^{-1}; IC_{50} = 35 µM) [18]. The open chain serine and homoserine analogs as well as the γ-lactones were inactive at 100 µM. Mass spectrometric and NMR spectroscopic studies with ^{13}C labeled **1a** confirmed ring opening of the ß-lactone by the active site cysteine. The inactivation takes place by nucleophilic attack at C-4 of the oxetanone ring (scheme 1). Interestingly, the enantiomeric *(R)* isomer **1b** is a truely competitive and reversible HAV 3C protease inhibitor (K_i = 1.5 10^{-6} M; IC_{50} = 6 µM).

ß-LACTAMS

Bicyclic and monocyclic ß-lactams are long known as inhibitors of bacterial transpeptidases. Their mechanism of action comprises the inactivation of these enzymes by acylation of the serine residue of the active site. They are also known as inhibitors of ß-lactamases, of which some are using a serine within their active site, too. This inhibition mechanism first led to the development of ß-lactams as serine protease inhibitors [19-24]. Due to the similar hydrolysis mechanisms of serine and cysteine proteases ß-lactams have recently also been discovered as cysteine protease inhibitors. Thus, several patents claim monocyclic and bicyclic ß-lactams as inhibitors or regulators of cysteine proteases.

Table 2 shows a selection of 3-substituted 4-oxa-1-azabicyclo[3.2.0]heptan-7-ones (oxapenams) (Fig. **2**) claimed by Synphar Lab. for which IC_{50} values against papain and CB have been determined [25].

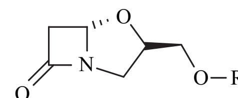


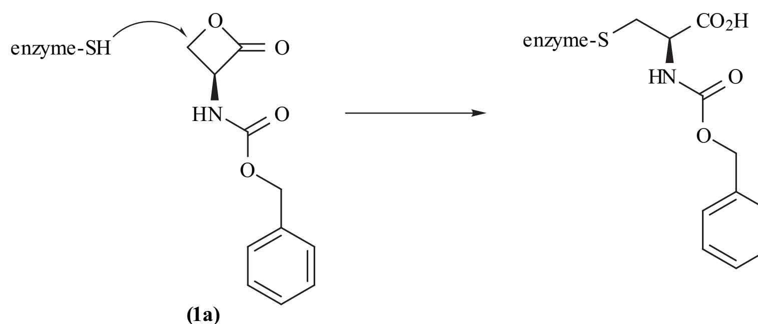
Fig. (2). 3-Substituted oxapenams as cysteine protease inhibitors.

Table 2. Oxapenams as Cysteine Protease Inhibitors

cpd	R [a]	papain IC_{50} [µM]	CB IC_{50} [µM]
1	Cbz-*(S)*-Phe	0.24	0.59
2	Cbz-*(S)*-Pro	0.49	0.44
3	Cbz-*(S)*-Ile	0.38	0.51
4	Cbz-*(S)*-Ile-*(S)*-Pro	0.74	4.50
5	Cbz-*(S)*-Phe-Gly	3.51	3.11

[a] See fig. 2.

Molecular modeling studies with these inhibitors suggest that the N-1 atom of the oxapenam ring can be involved in hydrogen-bonding to a protonated histidinium residue in the



Scheme 1. Inhibition of HAV 3C protease by a *(S)*-ß-lactone.

active site. This may weaken the lactam bond and activate the four membered ring towards acylation of the proteases´ cysteine residue. Additionally, a substitution of the 6-position was found to possibly enhance the S2 subsite interaction with papain. On the basis of these findings a series of 6-substituted oxapenams have been developed [26]. The most potent one for cathepsin L is shown in Fig. **3**.

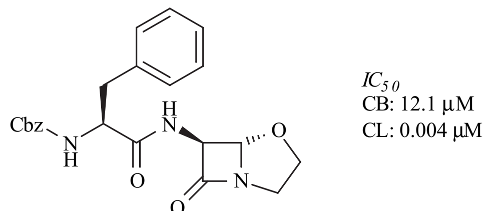


Fig. (3). A 6-substituted oxapenam as potent cathepsin L inhibitor.

By simplifying the oxapenam structure to the essential inhibitory part more potent CL inhibitors could be developed. Thus, two patents of the same company [27,28] describe 3-amino substituted monobactams (Fig. **4**). In general, ß-lactams with *cis* configuration of the hydrogens at C-4 and C-3 of the ß-lactam ring (e.g. **6b**) are the better inhibitors (table 3). Since only IC_{50} values, obtained by determination of residual enzyme activity after 10 min incubation of enzyme and inhibitor, are given in all patents only a crude evaluation of the inhibitors is allowed. No inhibition mechanisms are discussed.

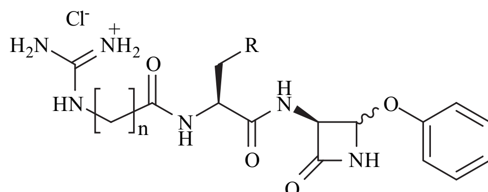


Fig. (4). Monobactams as cysteine protease inhibitors.

A series of 1-peptidyl-4-phenyl substituted monocyclic ß-lactams have also been synthesized and tested as papain inhibitors. While 1-acylated derivatives didn´t show any activity against papain an 1-alkylated compound showed weak irreversible inhibition (Fig. **5**) [29]. No significant difference was found between the diastereomeric *(4S)* and *(4R)* derivatives.

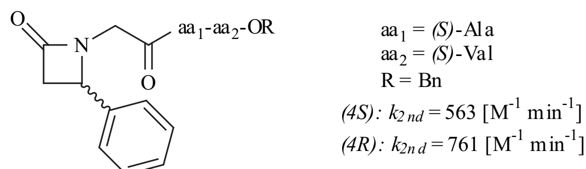


Fig. (5). 4-Phenyl substituted ß-lactams as papain inhibitors.

EPOXIDES

Epoxysuccinyl Peptides

E-64 from *Aspergillus japonicus* (Fig. **6**) [30-32] has been the first cysteine protease inhibitor containing *trans* epoxysuccinic acid as electrophilic key element found in nature. In the meantime a number of structurally related microbial metabolites with similar inhibition properties have been discovered. Examples are the cathestatins [33,34] produced by *Penicillium citrinum* or *Microascus longirostris*, WF14861 from *Colletotrichum sp. No. 14861*, [35,36] WF14865A and B from *Aphanoascus fulvescens* [37], the estatins [38] from *Myceliophthora sp.*, TMC-52 A to D from *Gliocladium sp.*, [39] and circinamide from the cyanobacterium *Anabaena circinalis* [40]. With the exception of the latter all compounds are of fungal origin. Their common feature is the *trans* epoxysuccinic acid coupled on one side to an amino acid amide (e.g. Phe, Leu, Ile, Tyr). The amine component of this amide comprises the polyamines agmatine, homospermidine, spermidine, 1,4-diamino butane, and 1-H-imidazole-2-ylamine, respectively. All these compounds are selective inhibitors of cysteine proteases of the papain super family but with little or no selectivity between the individual members of this enzyme clan.

CB Selective Epoxysuccinyl Peptides

Since the discovery of E-64 a multitude of synthetical epoxysuccinyl peptides have also been developed with the aim to improve inhibition potency and selectivity within the papain super family proteases. First success has come with the elucidation of the three dimensional enzyme structures and binding modes of epoxysuccinyl peptides to several cysteine proteases by X-ray crystallography of enzyme

Table 3. Inhibition of Cathepsins by Peptidyl Monobactams

cpd	R [a]	n	C-4	CB IC_{50} [µM]	CL IC_{50} [µM]	CK IC_{50} [µM]	CS IC_{50} [µM]
1	phenyl	3	(S)	> 50	0.081	2.5	> 2.5
2	2-naphthyl	3	(S)	37	0.37	50	0.47
3	2-naphthyl	2	(S)	48	0.38	50	0.393
4	2-naphthyl	4	(S)	9.04	0.014	1.14	0.201
5	2-naphthyl	5	(S)	8.81	0.0028	27.7	0.073
6a	cyclohexyl	5	(S)	48	1.91	> 2.5	0.0329
6b	cyclohexyl	5	(R)	0.57	0.08	0.10	0.017

[a] See fig. 4.

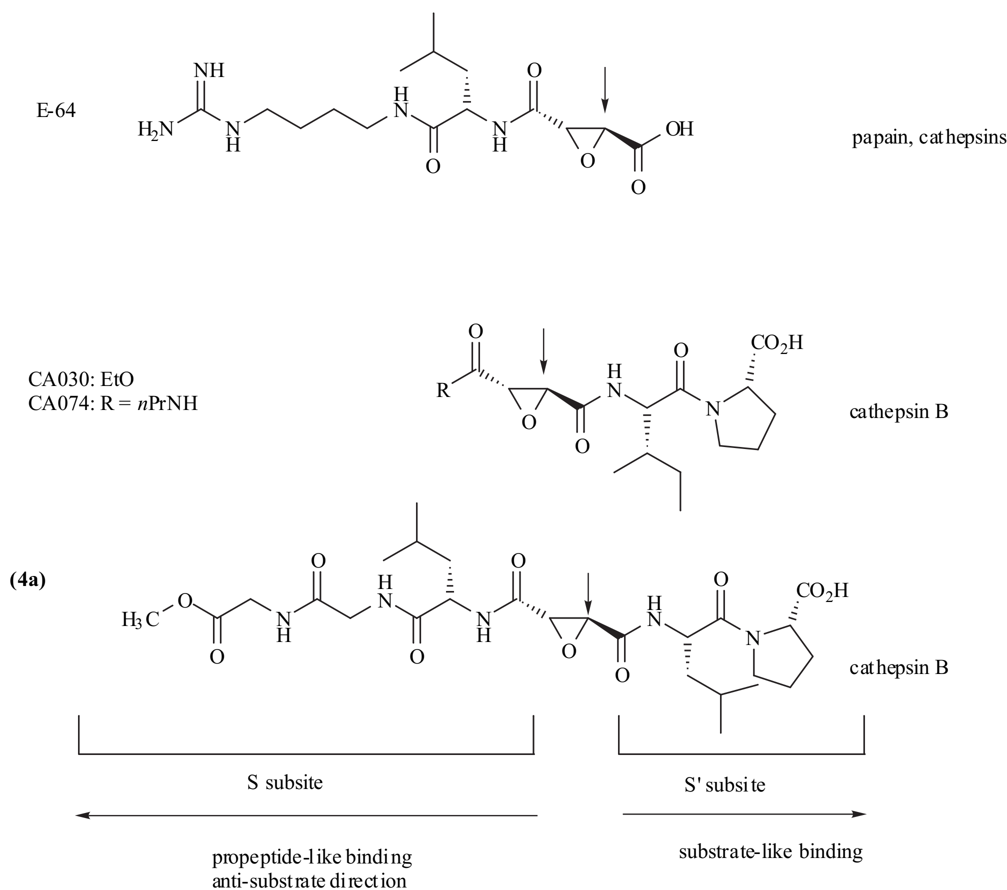


Fig. (6). Binding modes of epoxysuccinyl peptides to cysteine proteases, the arrow indicates the oxirane carbon attacked by the cysteine residue of the active site.

inhibitor complexes. CA030 and CA074 (Fig. **6**) have been the first cathepsin B-selective inhibitors. They exploit the dipeptidyl peptidase activity unique to cathepsin B. In contrast to E-64 which interacts with the non-primed subsites they bind to the S′ subsite of CB which consists of an occluding loop solely existent in CB. Two protonated His residues are responsible for binding of the inhibitors′ C-terminal carboxylate.

These different binding modes of unselective derivatives with a free acid at the epoxide ring [41-44] (e.g. Fig. **1**: E-64; table 4: cpds **1**, **7**, **8**) and the cathepsin B-selective ones containing the Leu(Ile)-Pro-OH (table 4, cpds **2**, **3**; Fig. **6**: CA030, CA074) or Leu-Arg-OH (table 4: cpd **6**) sequence [42,45-50] have led to the development of bispeptidyl derivatives which address both substrate binding sites of cathepsin B (Fig. **6**; table 4: cpds **4**, **5**) [51,52]. MeO-Gly-Gly-Leu-*(2S,3S)*-Eps-Leu-Pro-OH, (cpd **4a**, Fig. **6**) e.g., contains the inhibitory propeptide domain (amino acids 46-48) [53] of CB and the known CB selective P1′-P2′ (Leu(Ile)-Pro-OH) sequence. This compound is one of the most potent CB inhibitors known so far and comprises a very good selectivity for CB over CL. The diastereomeric *(2R,3R)* derivative showed significantly lower inhibition potency. This observation agrees with previous findings that interactions with the S subsites are favored by epoxysuccinic acid derivatives with *(2S,3S)* configuration and interactions with the S′ subsites are favored by those with *(2R,3R)* configuration (table 4). Besides **4a** and the *n*-propylamide derivative **3a** which are in their *(2S,3S)* configurations the more selective CB inhibitors the *(2R,3R)* diastereomers **2b**, **5b**, and **6b** are now the most promising lead compounds in terms of selectivity for CB over CL.

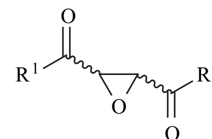


Fig. (7). New epoxysuccinyl peptides.

The rates of inhibition of CB with the selective inhibitors **2a**-**6b** were found to be strongly affected by pH. This can be explained by the electrostatic interaction of the C-terminal acid group with the two protonated His residues (110, 111) of CB′s occluding loop. In contrast the pH effect on the E-64 binding is much smaller. This leads to a comparable or even lower reactivity of all new selective CB inhibitors at pH 7.4 than that of E-64 [52], which means that the potency of CB selective inhibitors in the physiological pH range is strongly limited.

Epoxysuccinyl Peptides as Drug Carrier Systems and Chemical Probes for Cysteine Proteases

According to modeling studies the propeptide portion of inhibitor **4a** (table 4, Fig. **6**) bridges the whole non-primed subsite whereas the terminal Gly residue is located on the

Table 4. Inhibition of Cysteine Proteases by New Epoxysuccinyl Peptides

cpd	$R^{1\ a}$	$R^{2\ a}$	C-2,C-3	papain k_{2nd} [$M^{-1}s^{-1}$]	CB k_{2nd} [$M^{-1}s^{-1}$]	CL k_{2nd} [$M^{-1}s^{-1}$]	CB/CL
1a (E-64)	HO	Leu-Agm	2S,3S	869.000	81.400	43.800	1.9
1b	HO	Leu-Agm	2R,3R	86.000	1.170	4.930	0.2
2a	EtO	Leu-ProOH	2S,3S	6.130	44.400	170	260
2b	EtO	Leu-ProOH	2R,3R	56	567.000	26	21.800
3a	*n*PrNH	Leu-ProOH	2S,3S	103	153.000	22	6.960
3b	*n*PrNH	Leu-ProOH	2R,3R	5	29.400	46	640
4a	MeO-Gly-Gly-Leu	Leu-ProOH	2S,3S	14.800	1.520.000	1.204	1.262
4b	MeO-Gly-Gly-Leu	Leu-ProOH	2R,3R	870	214.600	269	798
5a	Agm-Orn	Leu-ProOH	2S,3S	6.220	197.000	250	790
5b	Agm-Orn	Leu-ProOH	2R,3R	225	63.300	26	2.440
6b	EtO	Leu-ArgOH	2R,3R	92	291.000	81	3.590
7b	HO	Leu-ArgOH	2R,3R	3.890	520	680	0.8
8b	HO	Leu-Pro-OH	2R,3R	3.270	270	74	3.6

[a] See fig. 7.

surface of the enzyme and thus allows further functionalization with effector groups without affecting its inhibition potency [54]. This working assumption was confirmed by the conjugate of ß-cyclodextrin with **4a** as a cathepsin B-selective drug carrier system targeting for tumor cells (Fig. **8**, table 5) [55]. With the cytotoxic drug methotrexate, known to form a 1:1 complex with ß-cyclodextrin, inclusion complex formation with the **4a** ß-cyclodextrin conjugate (cpd **1**, table 5) was proven by ESI-MS and UV/vis spectroscopy. Additionally, two affinity labels derived from **4a** (rhodamine B and biotin, cpds **2**, **3**, table 5) have been developed. They represent a novel tool kit that facilitates studies of extracellular CB activity in inflammation, tumor invasion and metastasis [54].

Table 5 Inhibition of Papain, CB and CL by Effector Substituted Epoxysuccinyl Peptides

cpd	R^a	CB k_{2nd} [$M^{-1}s^{-1}$]	CL k_{2nd} [$M^{-1}s^{-1}$]
1	ß-cyclodextrin-HN-CO-	1.050.000	393
2	rhodamine-B-NH-CH_2-	1.530.000	323
3	biotin-NH-CH_2-	1.726.000	256

[a] See fig. 8.

Other works on targeting cathepsin B have been published recently [56,57]. By replacement of the *n*-propyl moiety of CA074 (Fig. **6**) by a tryptamine (derived from the inhibitor JPM-565, Fig. **10**) to allow labeling with ^{125}I the epoxysuccinyl peptide MB074 was obtained [56]. At reduced inhibition potency (Fig. **9**, table 6) the selectivity against CB is preserved as could be shown by cell lysate assays. Studies on CB inhibition *in vitro*, in cell lysates and in whole cells with these inhibitors point out the current major problem concerning specificity of cathepsin inhibitors. Neither CA074 or MB074 (table 6) nor **4a** (table 4) or the effector substituted derivatives **2** and **3** (table 5) are cell-permeable and are therefore only useful for extracellular CB detection. Esterification at the C-terminal Pro residue (CA074-OMe) [58] leads to a highly cell-permeable inhibitor, but with dramatically reduced CB selectivity *in vivo* [56]. Obviously, inactivation of other cathepsins occurs faster than hydrolysis of the methyl ester group by unspecific esterases to yield the CB selectivity determinant (Pro-OH). Interestingly marginal changes in substituents can have large effects on specificity. Thus, the *i*-butyl derivative of CA074 (CA074b) (Fig. **9**, table 6) is equipotent to CA074 towards CB but shows activity against other cathepsins in cell lysates. These data suggest that although the Ile(Leu)-Pro-OH motive contributes some degree of specificity to an inhibitor addressing the dipeptidyl peptidase activity of CB, this is clearly not the only

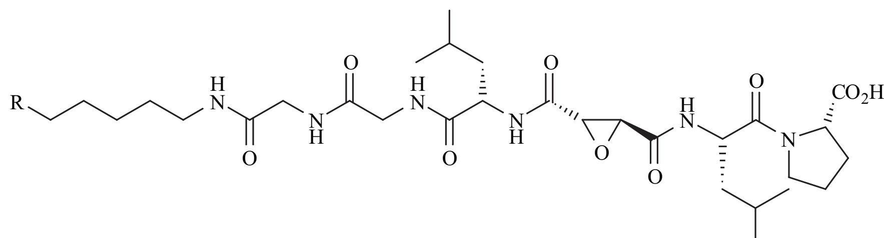


Fig. (8). Effector substituted CB-selective epoxysuccinyl peptides.

important specificity element.

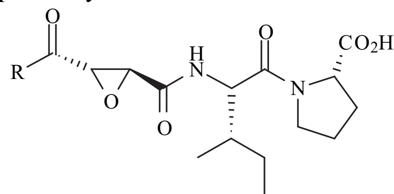


Fig. (9). CA074 and derivatives.

Table 6. Inhibition of CB by CA074 and Derivatives

cpd	R[a]	CB k_{2nd} [$M^{-1}s^{-1}$]
1 (CA074)	*n*PrNH	15.800
2 (MB074)	(4-OH)-Ph-$(CH_2)_2$-NH	2.750
3 (CA074b)	*i*ButNH	29.000

[a] See fig. 9.

JPM-565 (Fig. **10**) has also been the lead structure for the design of chemical probes for the detection of a broad set of cysteine proteases in crude cell extracts [57]. DCG-03 and DCG-04 as well as ^{125}I labeled derivatives (Fig. **10**) have been used to profile these proteases during the progression of a normal skin cell to a carcinoma. A library of DCG-04 derivatives in which the Leu residue was replaced with all natural amino acids was constructed and used to obtain inhibitor activity profiles for multiple protease targets. Additionally, the affinity tag of DCG-04 allowed purification and identification of inactivated proteases by mass spectrometry.

CL Selective Epoxysuccinyl Peptides

The known X-ray crystallographic structures of the enzyme-inhibitor complexes of papain/E-64 [59], CB/CA074 [47,49,50], CB/CA030 [45], CK/E-64 [60] and CL/E-64 [44] have been used as a basis for the molecular modeling aided design of a series of specific CL inhibitors, named CLIK (cathepsin L inhibitor Katunuma) [61]. The major difference in the primed site between CL and CB is the above already mentioned occluding loop which is missing in all other cathepsins except CB. This top domain is completely open in CL. Therefore, an inhibitor specific for CL over CB should have substituents too bulky for the partially covered S1´-S2´ pocket of CB. CL and CK differ in that region only slightly: Gln-142 in CK is replaced by Leu-142 in CL. In the non-primed site, especially in the hydrophobic S2 pocket, common in all cysteine proteases of the papain super family, minor differences have to be exploited to design selective inhibitors. The Ala and Ser residues within the S2 pockets of CL and papain, respectively, allow binding of a large Phe, whereas the

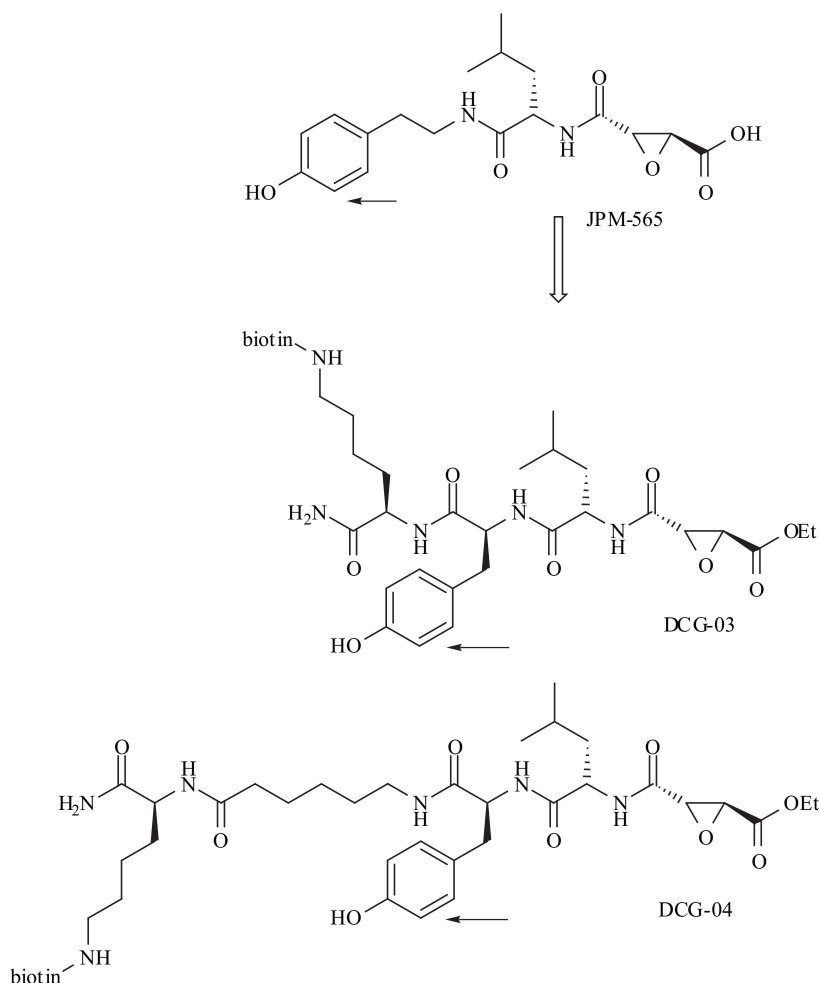


Fig. (10). Epoxysuccinyl peptides as chemical probes, the arrows indicate the iodination sites.

smaller S2 pocket in CK, occupied by Leu-205, only allows binding of less bulkier amino acids (e.g. Leu). Thus, an inhibitor which shall be specific for CL over CK should be substituted with a rigid and bulky phenyl ring. The combination of these two aspects (CL vs. CB and CL vs. CK) have led to the design of a series of disubstituted epoxysuccinyl peptides which probably cover both substrate binding sites of CL (Fig. **11**, table 7). The proposed binding mode could, until now, only be proven in a papain/CLIK-148 complex (Fig. **11**) [62]. The aromatic Phe residue is located in the hydrophobic S2 pocket and the pyridine ring interacts with Trp-177 of papain´s S2´ pocket. The obtained inhibition constants (table 7, Fig. **12**), the known structural similarities between papain and CL as well as additional modeling studies support the CL/CLIK binding hypothesis. These studies show that CLIK-079 which lacks the pyridine better fits into CB. CLIK inhibitors containing a Lys residue (CLIK-066, e.g.) should be able to form a hydrogen bridge to CK´s Gln-142 in S1´. This is supported by the considerable inhibition of CK (and CC) by Lys containing CLIKs [63].

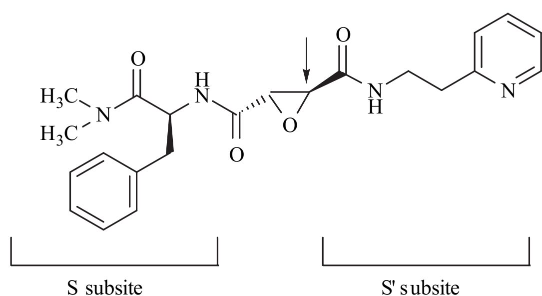


Fig. (11.). Binding of CLIK-148 to papain, the arrow indicates the oxirane carbon attacked by the cysteine residue of the active site.

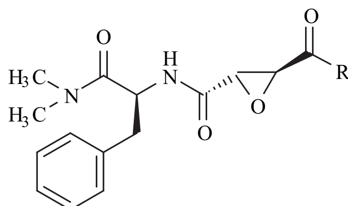


Fig. (12). CL specific inhibitors of the CLIK series.

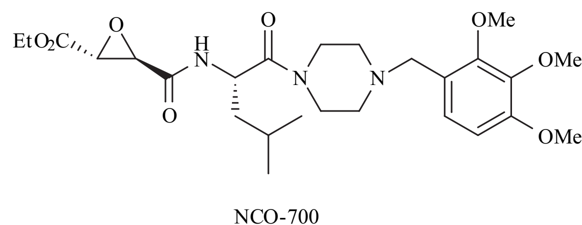
Table 7. CL Specific Inhibitors of the CLIK Series, % Inhibition at 1 µM Inhibitor Concentration

cpd	R[a]	CB	CL	CK	CS	CC
1 CLIK-066	Lys-NH-Ph	0	100	11	0	0
2 CLIK-088	NH-Ph-(4-H_2N-CH_2)	0	79	15	0	0
3 CLIK-112[*b*]	NH-Ph-(4-Et)	0	100	30	14	0
4 CLIK-121	NH-Ph-(4-*t*But)	0	100	17	72	0
5 CLIK-148	NH-$(CH_2)_2$-2-pyr	0	100	0	30	0
6 CLIK-195	Phe-$N(CH_3)_2$	0	100	0	25	0
7 CLIK-079[*c*]	HO					

[a] See fig. 12; [b] no inhibition by the *(2R,3R)* isomer CLIK-141; [c] CLIK-079 is the enzymatic degradation product of cpds **2**, **3**, **4** produced in liver and small intestine homogenates and shows reduced potency and selectivity for CL.

New Pharmacological Properties of Epoxysuccinyl Peptides

NCO-700 and TOP-008 (Fig. **13**) are piperazine containing *(2R,3R)* configured epoxysuccinyl peptides which have originally been developed as calpain and cathepsin B inhibitors for the treatment of cardiovascular diseases [64-71]. Recently, a new previously unrecognized pharmacological activity has been found. Both compounds show anti-cancer activity against human breast and prostate tumor cell lines [72]. This activity was additionally confirmed *in vivo*. Since extensive toxicology studies in rodents and dogs, as well as a preliminary safety study in humans have shown NCO-700 to be a relatively safe and well-tolerated compound, phase II studies with NCO-700 as new oncolytic drug are underway [4]. The exact target for these new anti-cancer drugs remains unclear at the moment. First studies showed apoptosis inducing activities [72] which are not correlated with the cysteine protease inhibiting potencies of the compounds.



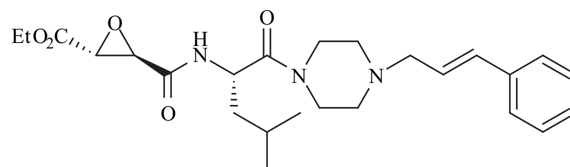


Fig. (13). Piperazine containing epoxysuccinyl peptides with anti-cancer activity.

Peptidomimetics Containing an Epoxide Ring

The potency of the epoxysuccinyl peptides as cysteine protease inhibitors has led to a variety of strategies which utilize an epoxide as surrogate for the scissile peptide bond or the peptide´s C- and N-terminus, respectively (Fig. **14**). A well known exemplar for such compounds is the proteasome inhibitor epoxomicin [73,74], originally isolated from an *Actinomycetes* strain. According to Fig. **14** epoxomicin and the related antitumor agents eponemycin and epopromycins are type IV peptides with an additional residue R^3 (methyl or hydroxymethyl) at the epoxide ring.

Type IV peptidomimetics (table 8) have been designed as cysteine protease inhibitors by combining the features of E-64 and dipeptidyl fluoromethyl ketones (fmk) [75]. In contrast to E-64 but in common to the fmks the peptide backbone is supposed to bind in a substrate-like direction to cysteine proteases.

Both compounds shown in table 8 are potent irreversible inhibitors of the trypanosomal papain like cysteine protease

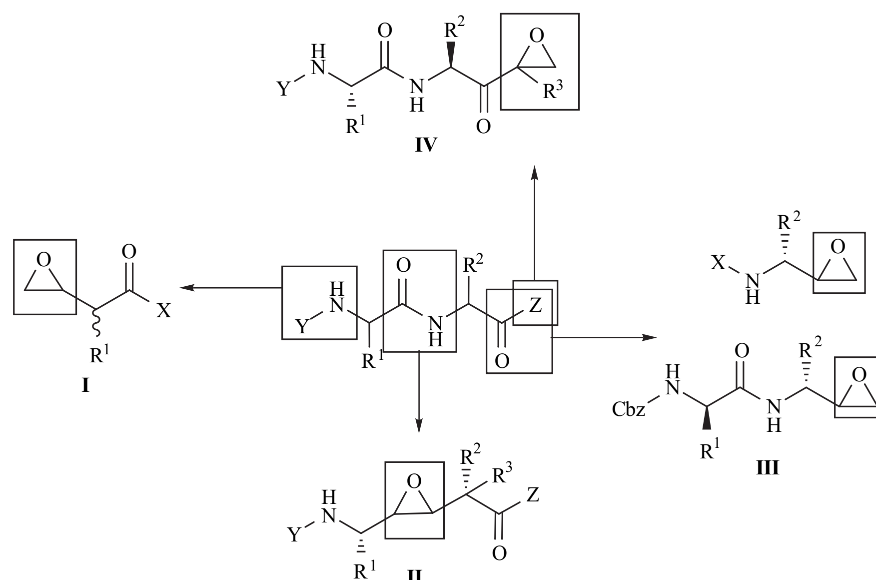


Fig. (14). Epoxide containing peptidomimetics type I – IV.

cruzain and even exceed the inhibitory activity of E-64. Interestingly the *(2S)* epimers are the better inhibitors. Thus, these α′,ß′-epoxy ketones differ from epoxomicin not only in their specificity for cysteine proteases but also in their stereospecificity: *(2R)* epoxomicin is a quite good irreversible inhibitor of the chymotrypsin like activity of the 20S proteasome (k_{2nd} = 37.200 $M^{-1}s^{-1}$), whereas *(2S)* epi-epoxomicin only shows k_{2nd} = 246 $M^{-1}s^{-1}$. Papain, CB and calpain are not inhibited at 50 μM by epoxomicins.

Table 8. Inhibition of Cruzain by Type IV Epoxides[a]

cpd	R^1	R^2	Y	k_{2nd} [$M^{-1}s^{-1}$]
1	Me	$(CH_2)_2$-Ph	Cbz	128.000
2	Bn	$(CH_2)_2$-Ph	Cbz	330.000
E-64				70.600

[a] See fig. 14, *(2S)* epimers, R^3 = H; [b] *(2R)* epimers: IC_{50} ≥ 1 μM.

2-Benzyl-3,4-epoxybutanoic acid methyl ester (BEBA-OMe) (Fig. **15**) is a type I amino acid mimetic (Fig. **14**) which has been tested in all four possible configurations as inhibitor of α-chymotrypsin. The free acid BEBA is long known as highly efficient and fast acting inactivator of the zinc peptidase CPA [76,77]. Upon binding to CPA the oxirane ring is activated by the zinc ion and nucleophilic attack of the Glu-270 carboxylate leads to ring opening and covalent enzyme modification. Only the two *(2R,3S)* and *(2S,3R)* enantiomers are active against CPA. In contrast, both *(2R)* diastereomers of BEBA-OMe are substrates for the serine protease α-chymotrypsin releasing the free acid as hydrolysis product [78]. The two *(2S)* diastereomers, however, are inhibitors. The *(2S,3R)* isomer is a weak irreversible one (k_{2nd} = 27 $M^{-1}s^{-1}$) whereas the *(2S,3S)* isomer only inhibits α-chymotrypsin reversibly with K_I = 9.95 mM. The reverse stereospecificity can be observed with papain [79]: *(2S,3R)* BEBA-OMe is the reversible inhibitor (K_I = 0.71 mM), whereas *(2S,3S)* BEBA-OMe is the irreversible one (k_{2nd} = 18 $M^{-1}s^{-1}$; K_I = 132 μM; k_i = 0.14 min^{-1}). Since the benzyl ester *(2S,3S)* BEBA-OBn shows three fold increased inhibition activity binding of the ester moiety into the hydrophobic S2 pocket is assumed. The difference in stereospecificity at C-3 of the oxirane ring, representing the scissile peptide bond, between the serine protease α-chymotrypsin and the cysteine protease papain leads to the inference that the nucleophilic attack of the active sites′ serine and cysteine, respectively, might occur on the *si* and *re* face, respectively, of the scissile amide carbon.

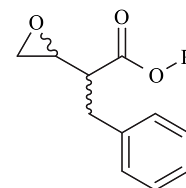
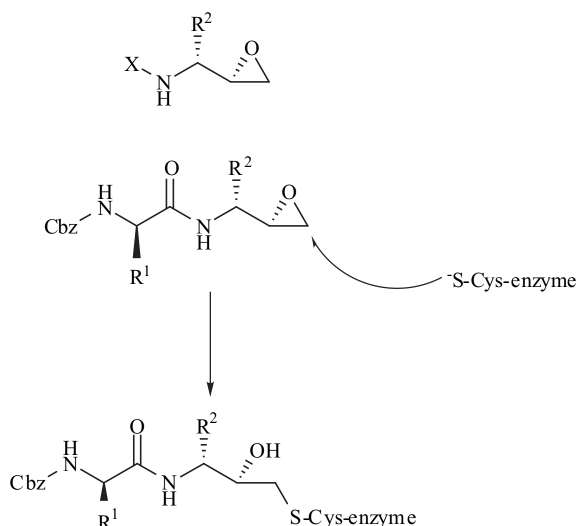


Fig. (15). BEBA esters and acids as inhibitors of α-chymotrypsin, papain and CPA.

α-Amino epoxides and peptidyl epoxides of type III (scheme 2) have been designed and investigated by A. Albeck and coworkers [80-82]. These compounds exhibit time- and concentration-dependent inhibition of cysteine proteases and do not inhibit serine proteases. The inhibition is strongly stereospecific: only peptidyl epoxides derived from *(S)* amino acids bearing an *erythro* relative configuration inactivate cysteine proteases. Examples are BOC-nitro-arginyl epoxide (X = BOC, R^2 = $(CH_2)_3NHC(NH)NHNO_2$) (*erythro/threo* = 6/1; k_{2nd} = 0.52 $M^{-1}s^{-1}$ CB, 0.1 $M^{-1}s^{-1}$ papain) and Cbz-lysyl epoxide (X = Cbz, R^2 = $(CH_2)_4NH_2$) (k_{2nd} = 0.34 $M^{-1}s^{-1}$ CB, 2.22 $M^{-1}s^{-1}$ papain). Assays with radioactively labeled inhibitors like Cbz-Phe-Ala-epoxide (R^1 = Bn, R^2 = Me) and BOC-Cys-OEt as a model for the active site thiol revealed the inactivation taking place by nucleophilic attack on the *exo* carbon of the epoxide ring (scheme 2) leading to a secondary alcohol without inversion of configuration at the epoxide carbon.

Recently, di-, tri- and tetrapeptide mimetics [83] containing an epoxide ring instead of the peptidic bond (type



Scheme 2. Type III α-amino epoxides and peptidyl epoxides as cysteine protease inhibitors.

II) (Fig. **16**) have been synthesized by solid phase synthesis. These compounds are weak time-dependent, but reversible papain inhibitors (table 9). The activity is strictly dependent on the presence of the epoxide as shown by the lacking activity of a respective peptide analogue. Test results with other protease types, inhibition mechanism and stereospecificity of inhibition still have to be investigated.

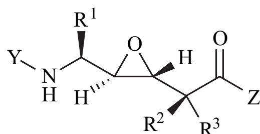


Fig. (16). Type II epoxide peptidomimetics.

Table 9. Inhibition of Papain by Type II Epoxide Peptidomimetics[a]

cpd	Y	R^1	R^2, R^3	Z	K_I [µM]
1	Fmoc	Me	$R^2 = R^3 = Me$	S-*t*But	24
2	Fmoc	Me	$-(CH_2)_5-$	S-*t*But	106
3[b]	H	Me	$R^2 = R^3 = Me$	NH_2	2
4	Val	Me	$R^2 = R^3 = Me$	Ala	48

[a] See fig. 16; [b] diastereomeric ratio 2/1.

Comparison of inhibition constants of epoxides lacking an electron withdrawing substituent at the epoxide ring (type I, II, III inhibitors, Fig. **14**) to epoxysuccinyl peptides or type IV inhibitors (Fig. **14**) shows that the activation of the epoxide ring by appropriate groups ($-CO_2R$, -CONHR, -CO-R) is an essential requirement for high inhibition activity.

AZIRIDINES

The inhibition mechanism of epoxysuccinyl peptides with the epoxide ring as „quiescent“ electrophilic trap led to the development of peptides containing the aza analogue aziridine ring. According to the „normal“ chemical reactivity of aziridines and epoxides against nucleophiles aziridinyl peptides containing the same peptide sequence are the weaker inhibitors (Fig. **17**, table 10) [84,85]. They exhibit inhibition constants in the range of the type III (scheme 2), type I (Fig. **15**) and type II (table 9) epoxides. However, reduction of the pH of the reaction medium leads to an dramatic increase in inactivation activity until pH 4 (cpd **2**, table 10), whereas the contrary applies for epoxides, which are the more active ones at pH 6-7 [86]. Interestingly, N-unsubstituted aziridines show an inverted stereoselectivity and are more active with *(R,R)* configured aziridine ring (the data of the less potent *(S,S)* diastereomers are not shown in table 10). The CA074 analgue aziridines, either in *(S,S)* or in *(R,R)* configuration (cpd **4**, table 10), are not very active and do not inhibit CB selectively. Due to the lack of suitable crystallized enzyme inhibitor complexes the reasons remain unclear at the moment. Structural properties of aziridines vs. epoxides which can partially explain the differences in inhibition behaviour comprise decreased ring strain, enhanced basicity and potential H-bond donation. An advantage of replacement of oxygen against nitrogen in the three memberd ring is the additional possibility of derivatization. A second peptide chain cannot only be attached at the second carboxylic acid function but also at the aziridine nitrogen. Peptides [87,88] and peptidomimetics [89] of this type have been studied in the author´s group. We found that acylation of CA074 analogue aziridines (cpd. **4**, table 10) with BOC-Phe (cpds. **4a,b**, table 11) leads to an increase in inhibition activity and selectivity against CB which maybe due to binding to both substrate binding sites. N-unsubstituted aziridines are the more active ones with a free carboxylic acid function at the aziridine ring. This is comparable to epoxysuccinyl peptides. N-acylated ones, however, show an increase in inhibition potency going from the monoesters to the diacids (table 11). First molecular modeling results have shown that a peptide bond attached at the aziridine nitrogen leads to a rather folded inhibitor conformation in contrast to the streched conformation of epoxysuccinyl and N-unsubstituted aziridinyl peptides [85]. As a consequence a second carboxylic acid maybe necessary for ionic interaction between inhibitor and the histidinium ion of the active site.

The electrophilic building block of these inhibitors, the aziridine-2,3-dicarboxylic acid, can be isolated as *(S,S)* isomer from a *Streptomyces* strain [90]. A peptidyl derivative similar to E-64 and other naturally occurring epoxysuccinyl peptides has been found in nature, too [91]. Miraziridine A (Fig. **19**) is a cysteine protease inhibitor isolated from the marine sponge *Theonella* aff. *mirabilis*. An IC_{50} value of 1.4 µg mL^{-1} (= 2.1 µM) was determined against CB. Interestingly, and in contrast to the natural epoxysuccinyl peptides and the aziridine-2,3-dicarboxylic acid, miraziridine A shows *(R,R)* configuration at the three-membered ring. Besides the aziridine ring the activated

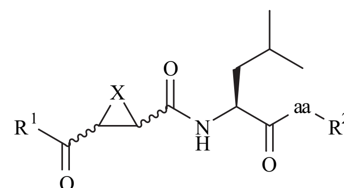


Fig. (17). Analogue epoxides and aziridines.

Table 10. Inhibition by Analogue Peptides with Epoxide and Aziridine Ring[a,b]

cpd	ring conf.	R^1	X	a[a]	R^2	papain k_{2nd} [$M^{-1}s^{-1}$]	CB k_{2nd} [$M^{-1}s^{-1}$]	CL k_{2nd} [$M^{-1}s^{-1}$]
1	S,S	HO	O	-	NH*i*Am	35.700	298.000	206.000
2	R,R	HO	NH	-	NH*i*Am	14.400	nd[d]	nd
2[c]	R,R	HO	NH	-	NH*i*Am	720.000	nd	nd
3	S,S	*i*ButNH	O	Pro	OH	558	52.000	nd
4	R,R	*i*ButNH	NH	Pro	OH	ni[e]	13	50
5	S,S	HO	O	Pro	OBn	176.000	8.700	nd
6	S,S+R,R	HO	NH	Pro	OBn	128	65	282

[a] See fig. 17 [b] pH = 6.5; [c] pH = 4; [d] not determined; [e] no time-dependent inhibition.

double bond of the vArg residue may be another electrophilic moiety responsible for inactivation of cysteine proteases. With the Sta residue miraziridine A contains a typical aspartate protease inhibiting moiety.

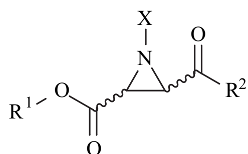


Fig. (18). N-acylated aziridines as cysteine protease inhibitors.

THIIRANES

Besides epoxides and aziridines, thiiranes are also known to react with nucleophiles. Thus, the thiirane ring is a useful building block for cysteine protease inhibitors. Synthesis of epithiosuccinyl peptides failed and *(S,S)* diethyl epithiosuccinate is not stable enough for further reactions. We therefore chose *(S)* thiirancarboxylic acid as readily available building block for peptidic inhibitors [92]. While the esters are weak reversible papain inhibitors the free acid shows irreversible inhibition (Fig. **20**). Compared to the aza analogue *(S)* aziridine carboxylic acid (k_{2nd} = 17 [$M^{-1}s^{-1}$]) [93], the inhibition constant is about four fold smaller. Inhibition potency, however, can be improved by attaching amino acids [94]. The observed order of decreasing reactivity (oxirane > aziridine > thiirane) is in accordance with decreasing ring strain and decreasing electronegativity of the heteroatoms (O > N > S).

A common feature of all inhibitors with three membered heterocycles bearing carboxylic acids or derivatives at the ring is the selective inhibition of cysteine proteases. Proteases of other types, as tested so far, are not inhibited.

Table 11. Inhibition by N-Acylated Aziridines[a]

cpd	ring conf.	R^1	X	R^2	papain k_{2nd} [$M^{-1}s^{-1}$]	CB k_{2nd} [$M^{-1}s^{-1}$]	CL k_{2nd} [$M^{-1}s^{-1}$]
1	S,S	Et	BOC-Phe	OEt	7	2.1	1.1
2	S,S+R,R	H	BOC-Phe	OEt	8.4	21	3.1
3	S,S+R,R	H	BOC-Phe	OH	60	14	635
4a	S,S	Et	BOC-Phe	Leu-ProOH	3	109	11
4b	R,R	Et	BOC-Phe	Leu-ProOH	12	114	3.5

[a] See fig. 18.

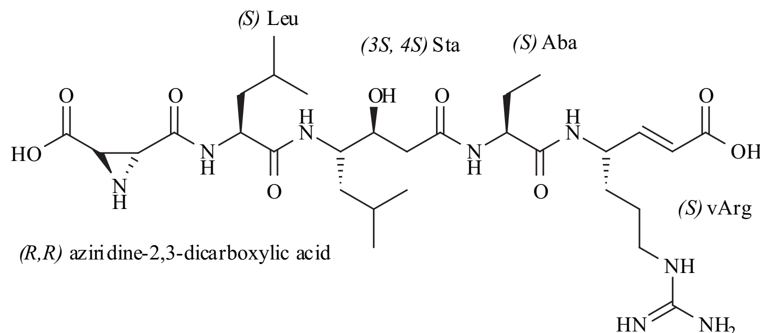


Fig. (19). Miraziridine A..

This changes if the acid as an electron withdrawing group is removed. Examples are BEBA and its aza and sulfur analogues [76] as inhibitors of carboxypeptidase A, a metallo protease, BEBA esters as serine protease inhibitors, and EPNP as an aspartate protease inhibitor [95].

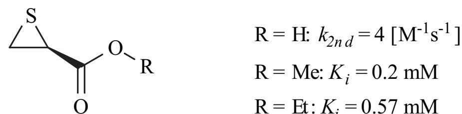


Fig. (20). Inhibition of papain by *(S)* thiirancarboxylic acid and esters.

SUMMARY

With this review we wanted to summarize the state of the art in cysteine protease inhibitors design concerning the use of small electrophilic cyclic building blocks as essential structural feature. Inhibitors published before 1997 have only been included if new aspects have appeared in the meantime. For completion the reader is referred to our former review [9]. As shown, the most promising inhibitor class still comprises the epoxysuccinyl peptides, originally derived from E-64, the inhibitor, the „small ring story“ in cysteine protease inhibitors design has started with.

ABBREVIATIONS

Amino acids are written in the three letter code and are L-configured except otherwise indicated.

aa = Amino acid
Aba = α-amino butanoic acid
BEBA = 2-benzyl-3,4-epoxybutanoic acid
Bn = Benzyl
BOC = *t*-butoxycarbonyl
CB = Cathepsin B
Cbz = Benzyloxycarbonyl
CC = Cathepsin C
CK = Cathepsin K
CL = Cathepsin L
CPA = Carboxypeptidase A
cpd = Compound
EPNP = 1,2-epoxy-3-(p-nitrophenoxy)propane
Eps = Epoxysuccinic acid
Et = Ethyl
fmk = Fluoromethyl ketone
HAV = Hepatitis A virus
*i*But = *i*-butyl
Me = Methyl
*n*Pr = *n*-propyl
Ph = Phenyl
Pyr = Pyridine
Sta = Statine
TMS = Trimethylsilyl
vArg = Vinyl arginine

ACKNOWLEDGEMENTS

Financial support of our research concerning the development of new cysteine protease inhibitors by the DFG (Deutsche Forschungsgemeinschaft) and the FCI (Fonds der Chemischen Industrie) is gratefully acknowledged.

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