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Aziridinyl Peptides as Inhibitors of Cysteine Proteases: Effect of a Free Carboxylic Acid Function on Inhibition

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Abstract—Peptides containing aziridine-2,3-dicarboxylate (Azi) as electrophilic building block are evaluated as inhibitors of the cysteine proteases papain, cathepsin B, cathepsin L and clostripain. The influence of a free carboxylic acid as functional group at different positions of the inhibitor molecule on inhibition is analyzed. Structure–activity relationships and binding mode hypotheses are discussed. In contrast to the bacterial enzyme clostripain, the papain like mammalian proteases (cathepsins) are irreversibly inactivated by aziridinyl peptides. *N*-Unsubstituted aziridines are much more potent inhibitors of papain and cathepsins if they contain the free carboxylic acid attached to the aziridine ring (HOAzi-Leu-ProOBzl). Two free carboxylic acid functions at the aziridine ring are necessary for good inhibition of these enzymes by *N*-acylated aziridinyl peptides (BOC-Phe-Azi$(OH)_2$). Chimeric bispeptidyl derivatives are selective CB inhibitors if the free acid is located at the C-terminus of the peptide (BOC-Phe-(EtO)Azi-Leu-ProOH). Clostripain is only inhibited by aziridinyl peptide esters.

Introduction

Until today more than 10 lysosomal papain-like cysteine proteases have been characterized, including the well known cathepsins B,[1] C,[2] H,[3] K,[4] L,[5] and S[6] as well as the recently discovered cathepsins F,[7] O,[8] V,[9] W,[10] X,[11] and Z.[12] They are supposed to be key enzymes responsible for disregulated protein turnover in a variety of diseases. Examples are muscular dystrophy,[13] chronic inflammatory diseases,[14] tumour progression,[15] and osteoporosis.[16] Inhibitors of these enzymes could therefore be promising therapeutic agents. Epoxysuccinyl peptides[17] (Table 1) and aziridinyl peptides (peptide derivatives of *trans*-aziridine-2,3-dicarboxylic acid)[18] have previously been shown to be irreversible and selective inhibitors of cysteine proteases. Inactivation of enzymes by both inhibitor classes proceeds from nucleophilic ring opening of the epoxide and aziridine ring, respectively, by the enzyme's active site cysteine residue. This leads to alkylation and therefore irreversible inhibition of the proteases. Besides this common mechanism of enzyme inactivation epoxysuccinyl and aziridinyl peptides show remarkable differences in their respective behaviour as cysteine protease inhibitors. They concern inhibition activity, stereospecificity, pH-dependency of inhibition, and selectivity between the different cathepsins.[18] First results have indicated that there are also differences in the influence exerted on inhibition properties by a free carboxylic acid function within the inhibitor structure.[18a,c] This is very well investigated and elucidated in the case of the epoxide derived inhibitors with the results shown in Table 1. Epoxides containing only Leu as amino acid (**I–V**) are much more potent with a free carboxylic acid function at the epoxide ring and, simultaneously, an ester or amide as C-terminal functional group (**II–IV** versus **I, V**). These inhibitors do not discriminate between CB and CL and are known to interact in an anti-substrate orientation with the S-subsite of the enzymes.[4,5,27] The reverse holds true for epoxides containing the dipeptide Leu(Ile)-Pro (**VI–X**). These compounds are selective and highly potent inhibitors of CB if they contain an ester or amide function at the epoxide ring and, simultaneously, a free carboxylic acid function at the C-terminus (**IX, X**). X-ray structure analysis showed these inhibitors to interact with the S′-subsite of CB in a substrate-like direction.[28] In contrast, this influence of a free carboxylic acid function on inhibition of cysteine proteases

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Abbreviations: Amino acids are written in the three letter code and are L configured. Azi aziridine-2,3-dicarboxylate, AMC aminomethyl coumarine, CB cathepsin B, CL cathepsin L, CL(Pt) CL from *Paramecium tetraurelia*, CL(h) CL from human liver, DCC dicyclohexylcarbodiimide, DMAP 4-(dimethylamino)pyridine, DPPA diphenyl phosphorazidate, DPSI diphenylsulfimine, DTT dithiothreitol, EEDQ 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, TEA triethylamine, TFA trifluoro acetic acid, iAm isoamyl.

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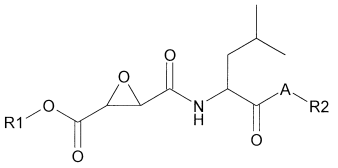
Table 1. Epoxysuccinyl peptides as inhibitors of cysteine proteases



No.	R1	A[a]	R2	Ring configuration	Papain k_{2nd} (M^{-1} s^{-1})	CB k_{2nd} (M^{-1} s^{-1})	CL k_{2nd} (M^{-1} s^{-1})	Ref
I	Et	—	NHiAm	*S,S*	750			19
II	H	—	NHiAm	*S,S*	357,000	298,000	206,000	23
III	H	—	NHiAm	*R,R*	38,000	6900		23
IV	H	—	OBzl	*S,S*			791,000	24
V	H	—	OH	*S,S*	207	388		23
VI	Et	Pro	OBzl	*S,S*	110	30		25
VII	H	Pro	OBzl	*S,S*	176,000	8700		25
VIII	H	Pro	OH	*S,S*	30,000	2200		25
IX	Et	Pro	OH	*S,S*	760	13,800	100	25
X	Et	Pro	OH	*R,R*		567,000	26	26

[a]A: amino acid Pro or none.

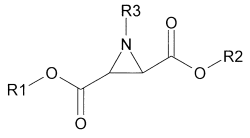
has not been studied systematically with aziridinyl peptides, especially with *N*-acylated ones, since up to now only few aziridine derivatives containing a free carboxylic acid function have been synthesized and tested. The present paper provides a systematic study concerning the influence of a free carboxylic acid as functional group at different positions of the inhibitor molecule on inhibition of cysteine proteases. Syntheses and inhibition profiles are described and structure–activity relationships are discussed comparing the inhibition constants obtained with the acids to those obtained with the recently published corresponding esters.[18]

Results

Syntheses

Starting materials for the syntheses of aziridinyl peptides are the aziridine building blocks **1–4** (Table 2, Scheme 1). Diethyl aziridine-2,3-dicarboxylates **1a** and **1b** were synthesized as pure (*R,R*) (=a-series) and (*S,S*) (=b-series) *trans* enantiomers by the literature known five step synthesis starting from (*S,S*) and (*R,R*) diethyl tartrate, respectively.[18c] The building blocks **2a+b**,[18c] **3a+b**, and **4a+b** were obtained as racemic mixtures of *trans* (*R,R*) and (*S,S*) enantiomers by Michael type addition of diphenylsulfimine to the corresponding fumarates (Scheme 1). Selective alkaline hydrolysis of one ester group of diethyl esters **1a** and **1b**, respectively, lead to the monoesters **5a** and **5b** (Table 2).[18c] The dipeptides **6a**, **6b** and the tripeptides **10a**, **10b** (Table 3) were synthesized by DPPA mediated peptide coupling of the monoesters with LeuOBzl and Leu-ProOBzl, respectively, as published recently.[18c] Compound **7a** (Table 3) with a free C-terminal carboxylic acid function was obtained by hydrogenolytic cleavage of the benzyl ester of **6a**.[18c] Compounds **8a,b**, **9a,b** and **11a,b** (Table 3) have been synthesized and tested by Martichonok et al.[18a] Since these inhibitors complete the list of acid/ester pairs of *N*-unsubstituted aziridinyl peptides their inhibition constants are included for discussion. Compounds **14a+b** with the free carboxylic acid function attached to the aziridine ring were synthesized as a diastereomeric mixture starting from building blocks **4a+b**. The crude monoesters **12a+b**, obtained by alkaline hydrolysis, were coupled to Leu-ProOBzl via the DPPA procedure leading to tripeptides **13a+b**. Subsequent acid mediated cleavage of the *tert*butyl esters lead to **14a+b** (Scheme 2).

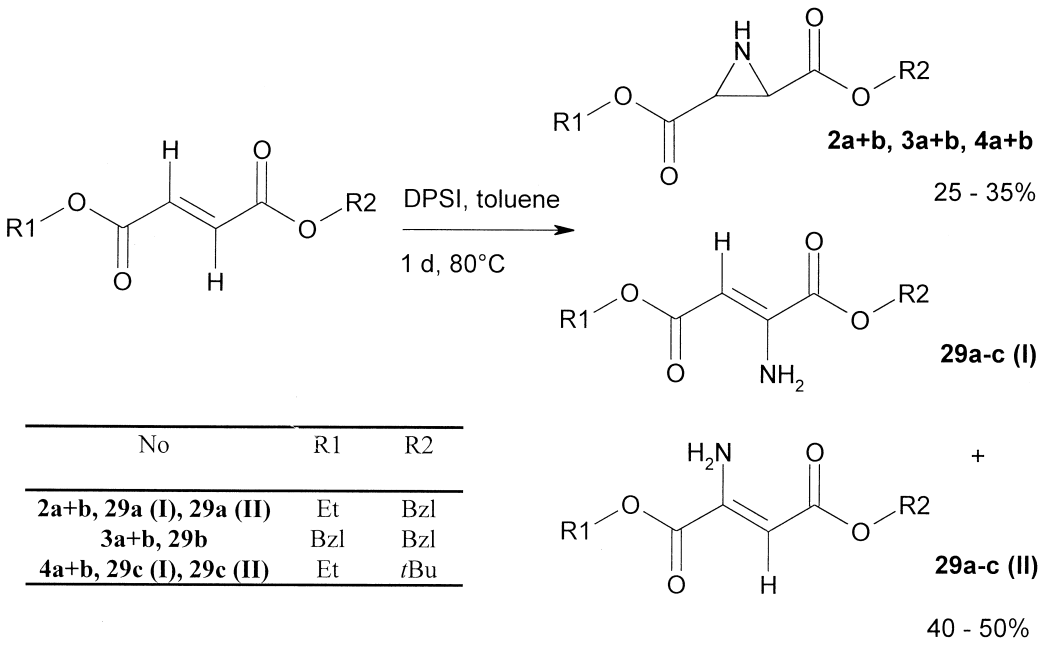
Table 2. Inhibition of papain by aziridine building blocks



No.	R1	R2	R3	k_{2nd} (M^{-1} min^{-1})	Ref
1a	Et	Et	H	11 ± 0.5[a]	18b
1b	Et	Et	H	12 ± 0.5[a]	18b
2a+b	Et	Bzl	H	141[a,b]	
3a+b	Bzl	Bzl	H	273[a]	
5b	Et	H	H	4692 ± 1000[a]	18c
27b	Et	Et	CH_2CO_2Bzl	8 ± 2.5[a]	
28b	Et	Et	CH_2CO_2H	98 k_i 0.033 ± 0.0012 (min^{-1}) K_i 0.34 ± 0.058 (mM)	

[a]Measurements were limited to the linear range, with $[I] \leq K_i$, therefore only the second-order rate constant could be obtained.
[b]CB: $k_{2nd} = 204$, $k_i = 0.037\ min^{-1}$, $K_i = 0.18$ mM.

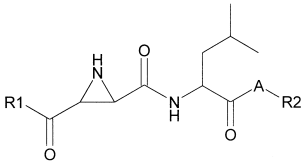
The syntheses of the *N*-acylated aziridinyl dipeptides **15–17** (Table 4) (Scheme 3) with BOC-Phe as N-terminal amino acid were performed by DMAP catalyzed acylation[18b,c] of the building blocks **1–3** with BOC-Phe anhydride. The *N*-acylated tripeptides **20, 21** and **23** were synthesized by EEDQ mediated peptide coupling[18c] of BOC-Phe-Ala or BOC-Leu-Gly to the aziridine building blocks **1** and **2**, respectively.



No	R1	R2
2a+b, 29a (I), 29a (II)	Et	Bzl
3a+b, 29b	Bzl	Bzl
4a+b, 29c (I), 29c (II)	Et	*t*Bu

Scheme 1.

Table 3. Inhibition of papain and cathepsins by *N*-unsubstituted aziridinyl peptides

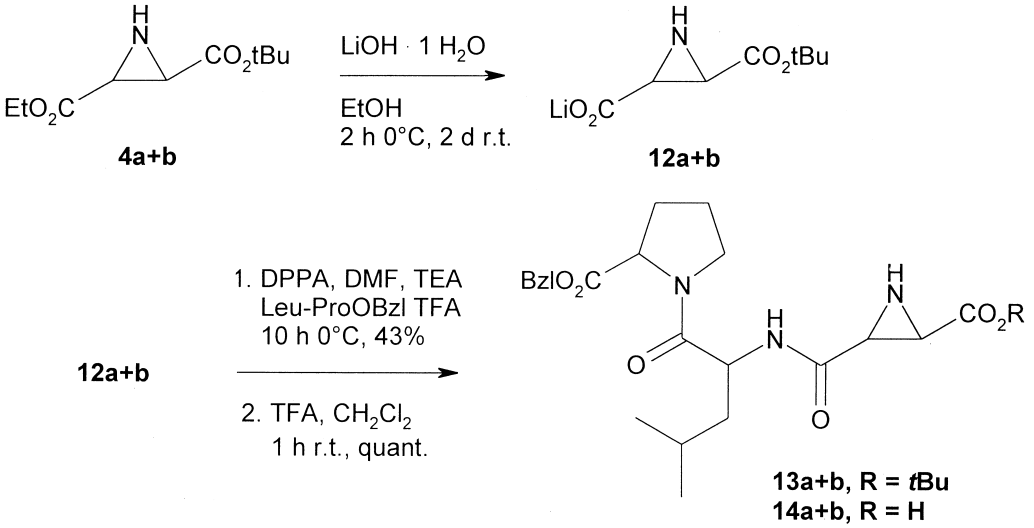


No.	R1	A[a]	R2	Papain k_{2nd} (M^{-1} min^{-1})	CB k_{2nd} (M^{-1} min^{-1})	CL(Pt) k_{2nd} (M^{-1} min^{-1})	Ref
6a	EtO	—	OBzl	1533 ± 41[b]	1607 ± 395[b]	16,261 k_i 0.44 ± 0.0004 (min^{-1}) K_i 0.027 ± 0.0001 (mM)	18c
6b	EtO	—	OBzl	214 k_i 0.036 ± 0.0015 (min^{-1}) K_i 0.17 ± 0.02 (mM)	41 ± 8[b]	3130 k_i 0.055 ± 0.009 (min^{-1}) K_i 0.018 ± 0.0021 (mM)	18c
7a	EtO	—	OH	63 ± 18[b]	nd	nd	18c
8a	EtO	—	NHiAm	3300	nd	nd	18a
8b	EtO	—	NHiAm	180	nd	nd	18a
9a	HO	—	NHiAm	864,000 ± 42,000	nd	nd	18a
9b	HO	—	NHiAm	108,000 ± 6000	nd	nd	18a
10a	EtO	Pro	OBzl	466 ± 37[b]	188 k_i 0.025 ± 0.0001 (min^{-1}) K_i 0.13 ± 0.0011 (mM)	1728 k_i 0.043 ± 0.0033 (min^{-1}) K_i 0.025 ± 0.057 (mM)	18c
10b	EtO	Pro	OBzl	227 k_i 0.021 ± 0.0002 (min^{-1}) K_i 0.094 ± 0.0034 (mM)	405 k_i 0.039 ± 0.02 (min^{-1}) K_i 0.096 ± 0.01 (mM)	2142 k_i 0.047 ± 0.005 (min^{-1}) K_i 0.022 ± 0.0085 (mM)	18c
11a	iBuNH	Pro	OH	ni	780 ± 180	3000 ± 60	18a
11b	iBuNH	Pro	OH	108 ± 6	ni	402 ± 6	18a
14a + b	HO	Pro	OBzl	7660 k_i 0.24 ± 0.025 (min^{-1}) K_i 0.031 ± 0.0009 (mM)	3870 k_i 0.066 ± 0.047 (min^{-1}) K_i 0.017 ± 0.004 (mM)	16,930[c] k_i 0.51 ± 0.13 (min^{-1}) K_i 0.03 ± 0.011 (mM)	

[a]A: amino acid Pro or none; nd: not determined; ni: no time-dependent inhibition.
[b]Measurements were limited to the linear range, with $[I] \leq K_i$, therefore only the second-order rate constant could be obtained.
[c]CL(h).

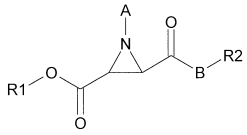
Again, the peptides **15a, 20a** and **15b, 20b** derived from the building blocks **1a** and **1b**, respectively, were synthesized as pure isomers, whereas peptides **16a + b**, **17a + b**, **21a + b** and **23a + b** are derivatives of the racemic building blocks **2a + b** or **3a + b** and are therefore diastereomeric mixtures.

The corresponding monoesters **18a + b**, **22a + b** and **24a + b** as well as the diacids **19a + b** were obtained by hydrogenolysis of the benzyl esters (Scheme 3). This synthetic pathway, which leads to the *N*-acylated aziridines with one or two carboxylic acid functions attached to the aziridine ring, had to be chosen because



Scheme 2.

Table 4. Inhibition of papain and cathepsins by *N*-acylated aziridinyl peptides



No.	R1	A[a]	B[b]	R2	Papain k_{2nd} (M^{-1} min^{-1})	CB k_{2nd} (M^{-1} min^{-1})	CL(Pt) k_{2nd} (M^{-1} min^{-1})	Ref
15a	Et	BOC-Phe	—	OEt	42 ± 9[d]	18 ± 6[d]	16 ± 6[d]	18b
15b	Et	BOC-Phe	—	OEt	408 ± 16[d]	125 ± 19[d]	65 ± 12[d]	18b
16a + b	Bzl	BOC-Phe	—	OEt	76 ± 9[d]	nd[c]	nd	18c
18a + b	H	BOC-Phe	—	OEt	505 k_i 0.21 ± 0.013 (min^{-1}) K_i 0.41 ± 0.07 (mM)	1230 k_i 0.83 ± 0.019 (min^{-1}) K_i 0.68 ± 0.03 (mM)	188 ± 6[d]	18c
19a + b	H	BOC-Phe	—	OH	3544 k_i 0.2 ± 0.014 (min^{-1}) K_i 0.057 ± 0.015 (mM)	807 ± 81[d]	38,081[e] k_i 1.02 ± 0.08 (min^{-1}) K_i 0.027 ± 0.005 (mM)	
20a	Et	BOC-Phe-Ala	—	OEt	91 ± 17[d]	nd	nd	
20b	Et	BOC-Phe-Ala	—	OEt	1370 k_i 0.028 ± 0.0026 (min^{-1}) K_i 0.02 ± 0.009 (mM)	455 ± 15[d]	469 k_i 0.051 ± 0.019 (min^{-1}) K_i 0.11 ± 0.08 (mM)	18c
21a + b	Bzl	BOC-Phe-Ala	—	OEt	1232 k_i 0.020 ± 0.001 (min^{-1}) K_i 0.016 ± 0.001 (mM)	433 ± 71[d]	281 ± 2[d]	18c
22a + b	H	BOC-Phe-Ala	—	OEt	1456 k_i 0.67 ± 0.059 (min^{-1}) K_i 0.46 ± 0.046 (mM)	676 ± 82[d]	843 ± 42[d,e]	
23a + b	Bzl	BOC-Leu-Gly	—	OEt	149 k_i 0.01 ± 0.001 (min^{-1}) K_i 0.069 ± 0.0012 (mM)	240 ± 61[d]	3237 k_i 0.028 ± 0.00015 (min^{-1}) K_i 0.0088 ± 0.0015 (mM)	18c
24a + b	H	BOC-Leu-Gly	—	OEt	575 k_i 0.021 ± 0.0003 (min^{-1}) K_i 0.037 ± 0.0046 (mM)	766 k_i 0.062 ± 0.0032 (min^{-1}) K_i 0.081 ± 0.021 (mM)	13,030[f] k_i 0.030 ± 0.002 (min^{-1}) K_i 0.0022 ± 0.001 (mM)	
25a	Et	BOC-Phe	Leu-Pro	OBzl	768 ± 125[d]	1938 ± 440[d]	5869 ± 409[d]	18c
25b	Et	BOC-Phe	Leu-Pro	OBzl	321 k_i 0.016 ± 0.0019 (min^{-1}) K_i 0.05 ± 0.029 (mM)	450 k_i 0.037 ± 0.0022 (min^{-1}) K_i 0.082 ± 0.013 (mM)	1210 ± 260[d]	18c
26a	Et	BOC-Phe	Leu-Pro	OH	176 ± 29[d]	6538 k_i 0.85 ± 0.064 (min^{-1}) K_i 0.13 ± 0.018 (mM)	655 ± 76[d,e]	
26b	Et	BOC-Phe	Leu-Pro	OH	704 k_i 0.052 ± 0.0054 (min^{-1}) K_i 0.073 ± 0.03 (mM)	6859 k_i 0.78 ± 0.053 (min^{-1}) K_i 0.11 ± 0.026 (mM)	212 k_i 0.027 ± 0.0031 (min^{-1}) K_i 0.12 ± 0.04 (mM)	

[a]A: amino acid BOC-Phe or dipeptides BOC-Phe-Ala and BOC-Leu-Gly, respectively.
[b]B: dipeptide Leu-Pro or none.
[c]nd: not determined.
[d]Measurements were limited to the linear range, with [I]≤K_i, therefore only the second-order rate constant could be obtained.
[e]CL(h).
[f]CL(h): k_{2nd} = 10,950 (M^{-1} min^{-1}), k_i = 0.0220 ± 0.0025 (min^{-1}), K_i = 0.002 ± 0.0007 (mM).

of the lability of the aziridide bond towards alkaline hydrolysis.[18c]

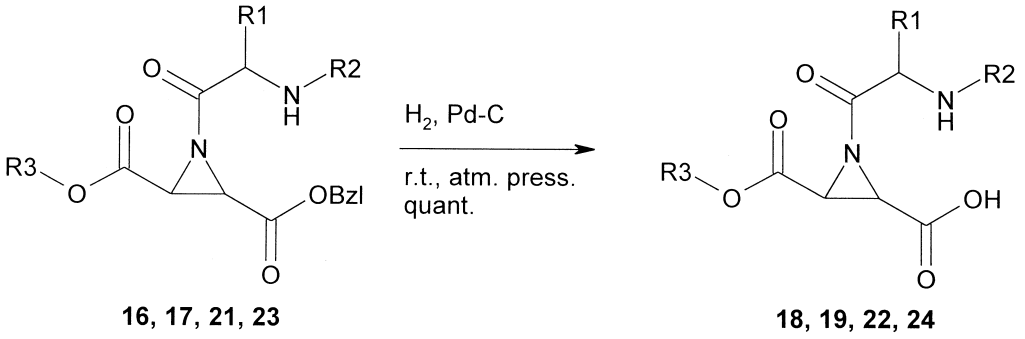
The *N*-acylated tetrapeptide esters **25a, 25b**[18c] and acids **26a, 26b** result from *N*-acylation of **10a** and **10b**, respectively, via the symmetric anhydride procedure and subsequent hydrogenolytic benzyl ester cleavage (Scheme 4).

N-Alkylated aziridine building block esters **27a**, **27b**[20] and acids **28a**, **28b** were obtained by alkylation with benzyl bromoacetate followed by hydrogenolysis (Scheme 5).

Inhibition of cysteine proteases

Aziridine building blocks and aziridinyl peptides were tested against papain and the papain-like cathepsins B, L(Pt) and L(h), all belonging to the C1 cysteine protease family. As a cysteine protease non-related to the papain clan the bacterial enzyme clostripain[21] isolated from *Clostridium histolyticum* was included in the test series. This protease which belongs to the C11 cysteine protease family is one of few known bacterial cysteine proteases and does not share any sequence homology with the papain-like enzymes. Inhibitors of clostripain may be of therapeutic interest since this protease is supposed to act as a virulence factor in infections.

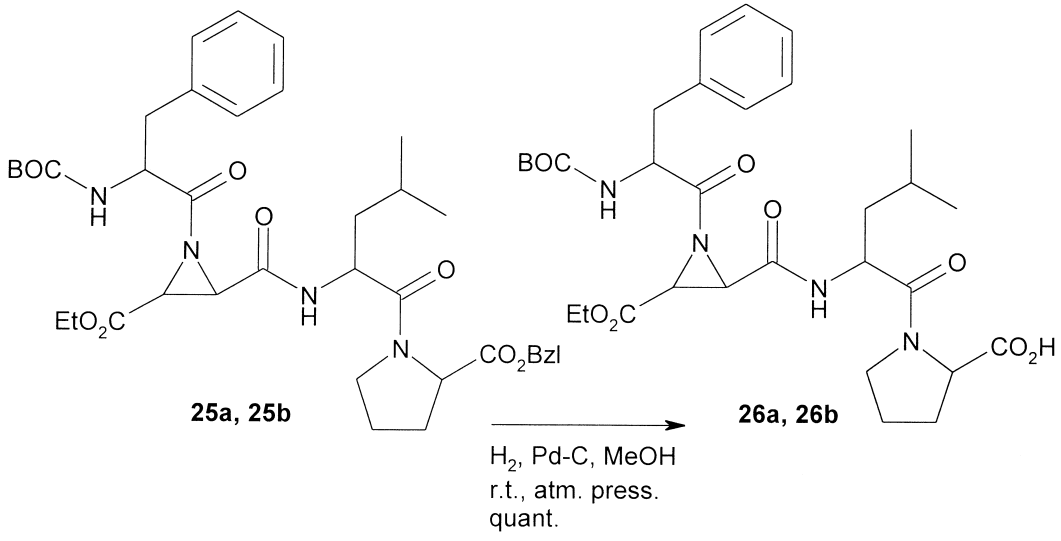
The second-order rate constants of inhibition k_{2nd} (M^{-1} min^{-1}) ($k_{2nd} = k_i/K_i$) for irreversible inactivation of papain and cathepsins B and L were determined by continuous[22a] or dilution[22b] assays as described previously.[18c] This irreversibility is evident from the time-dependency of inhibition and was confirmed by dialysis experiments.[18c] In most cases both individual inhibition constants, the dissociation constant K_i (mM) and the first-



16, 17, 21, 23 **18, 19, 22, 24**

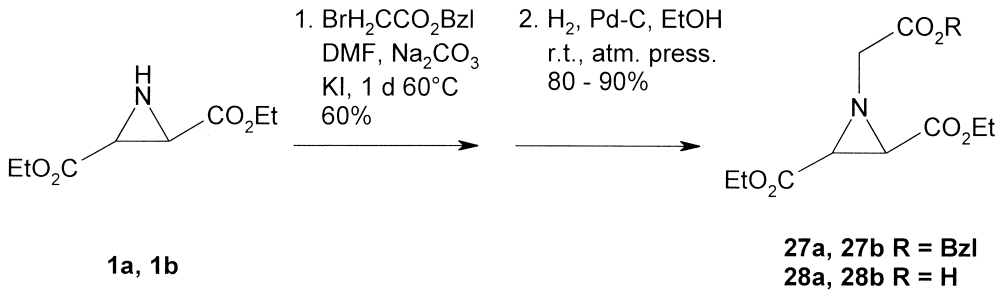
No	R1	R2	R3
16a+b, 18a+b	Bzl	BOC	Et
17a+b	Bzl	BOC	Bzl
19a+b	Bzl	BOC	H
21a+b, 22a+b	Me	BOC-Phe	Et
23a+b, 24a+b	H	BOC-Leu	Et

Scheme 3.



25a, 25b **26a, 26b**

Scheme 4.



Scheme 5.

Table 5. Inhibition of clostripain by aziridinyl peptides

No.	Compound	K_i (mM)
5a	EtOAziOH	5.05 ± 0.38
6a	EtOAzi-LeuOBzl	0.056 ± 0.0043
18a + b	BOC-Phe-(EtO)AziOH	1.07 ± 0.09
23a + b	BOC-Leu-Gly-(EtO)AziOBzl	0.068 ± 0.018
26b	BOC-Phe-(EtO)Azi-Leu-ProOH	0.066 ± 0.0019

order rate constant k_i (min^{-1}), could be determined. Dissociation constants K_i (mM) for the reversible inhibition of clostripain were obtained by Dixon plots as described in previous experiments.[18c] The results of the assays are listed in Tables 2–5.

Discussion

In earlier publications,[18b,c] we have reported on the structure–activity relationships concerning activity, stereoselectivity, pH-dependency of inhibition, and selectivity between different cathepsins. Herein we will focus the discussion on inhibition of cysteine proteases by the new aziridinyl peptides containing a free carboxylic acid function.

N-Unsubstituted aziridinyl peptides (Table 3) containing a Leu derivative as the only amino acid exhibit very high inhibition constants if they contain a free carboxylic acid function at the aziridine ring (**9a, 9b**). Esterification at this position (**6, 8**) or a free acid at the C-terminus (**7a**) lead to a dramatic decrease in inhibition potency. These results are similar to those obtained with epoxysuccinyl peptides (Table 1). The results obtained with the Leu-Pro derivatives, however, are contrary to those of the epoxides. A free carboxylic acid at the C-terminus of these inhibitors neither leads to higher inhibition constants nor to higher selectivity between CB and CL (**11** versus **10**). For these derivatives, however, an inhibition improvement (but not an increase in selectivity) can be reached with an additional free acid function at the aziridine ring (**14**). These findings apply also to the aziridine building blocks (**5** versus **1**–**3**) (Table 2). An acid/ester ratio of 10 is found for the *N*-alkylated inhibitors **28**/**27** with the free acid attached to a methylene spacer at the aziridine nitrogen.[29]

In the case of the *N*-acylated inhibitors (Table 4) only a slight inhibition improvement with an acid/ester ratio of 2–10 is caused by one free carboxylic acid at the aziridine ring (**18, 22, 24** versus **15, 16, 20, 21, 23**). No difference is found between CL(Pt) and CL(h) (compound **24**). A second acidic function, however, leads to the desired high increase of inhibition constants with acid/ester ratios up to 2000, especially for CL (**19** versus **15, 16, 18**). The main reason for this good inhibition of CL by the diacid **19** is the high k_i value of 1 min^{-1}. This is in contrast to compound **24** for which the high inhibition constant is mainly caused by a low K_i value (2 μM).

An inhibition improvement is also reached by *N*-acylation of the Leu-Pro derivatives **10a, 10b** (Table 3) with BOC-Phe to yield the bispeptidyl derivatives **25a, 25b** (Table 4). For these compounds the free acid at the C-terminal Pro residue (**26a, 26b**) (Table 4) leads to higher inhibition constants for CB but not for CL or papain. That means, the increase in selectivity, which one could have expected for the Leu-Pro derivatives **11a, 11b** without *N*-substituent, is achieved by introduction of a third amino acid at the N-terminus of the peptide.

Opposite acid/ester ratios of 0.01–0.05 are found for inhibition of clostripain (Table 5). Dissociation constants in the micromolar range are only reached with aziridine esters. In contrast to cysteine proteases of the papain family the inhibition of clostripain by aziridinyl peptides is reversible. This is comparable to results obtained with epoxysuccinyl peptides.[30]

These results, in combination with the proved binding modes of epoxysuccinyl peptides to cysteine proteases of the papain family, allow first hypotheses on the binding of aziridinyl peptides to these enzymes. One reason for the good inhibition of cysteine proteases by epoxysuccinyl peptides containing a Leu amide or ester on the one hand and a free carboxylic acid at the epoxide ring on the other is an ionic interaction between the histidinium cation of the active site and the carboxylate anion of the inhibitor.[27] This interaction could also be possible with the carboxylate of *N*-unsubstituted aziridinyl peptides and aziridine building blocks (**5, 9, 14**) and would explain the similar behaviour of these inhibitors compared to the epoxides. Obviously this ionic bond is not possible with the one carboxylate of *N*-acylated aziridines (**18, 22, 24**). Previous molecular modeling results[18c] indicated that the aziridide structure leads

to a less favourable inhibitor conformation in which the free carboxylate is possibly turned away from the histidinium ion. Therefore, we reasoned that this ionic interaction which is the main factor for good inhibition will only become possible if the aziridine bears a second carboxylate at the three-membered ring (**19**).

In contrast, the inhibition improvement by a free acid attached to the methylene spacer of *N*-alkyated aziridines (**28**) should have another explanation. As shown earlier,[18a,c] the aziridine-*N* exhibits a very low basicity leading to increased inhibition constants at pH 4. A similarly low basicity could be expected for the *N*-alkylated aziridine-*N*. We could prove this by determination of pH-dependency of inhibition of papain by compound **27b**.[29] A free carboxylic acid function at the methylene spacer (**28b**) should now lead to enhancement of basicity compared to the ester (compare pK_a values of GlyOH/GlyOMe: 9.8/7.8) and as a consequence to a higher degree of protonation and activation towards nucleophilic ring opening under the assay conditions (pH 6.5).

The reasons for the increase in selectivity between CB and CL by epoxides containing Leu-ProOH are ionic interactions with the C-terminal carboxylate of the inhibitors and two protonated histidinium residues located at the occluding loop specific for CB.[28] A corresponding interaction between the C-terminal Pro carboxylate of the *N*-unsubstituted aziridinyl peptides **11** containing Leu-ProOH and these His residues in CB seems, based on the obtained inhibition constants, very unlikely. It may, however, be possible for the *N*-acylated derivatives **26** since these inhibitors exhibit a selective improvement in inhibition of CB. The N-terminal BOC-Phe residue of these peptides may additionally bind to the hydrophobic S2-subsite of CB as shown by Schaschke et al.[26] for chimeric bispeptidyl derivatives of the epoxysuccinic acid which are highly selective and potent CB inhibitors.

Unlike other cysteine protease inhibitors (e.g., peptidyl sulfonium salts)[31] aziridines and epoxides are only reversible clostripain inhibitors. In contrast to the papain-like cysteine proteases, however, no X-ray data are available for clostripain. Therefore, at the moment no discussion is possible on binding modes or inhibition mechanism.

To finally prove the binding mode hypotheses presented herein, X-ray analyses of enzyme inhibitor complexes are underway.

Experimental

General methods

Enzymes were purchased from the following companies and used without further purification: papain from Fluka, CB (bovine spleen) from Sigma, CL(Pt) and CL(h) from Calbiochem, clostripain from Sigma. The substrate Z-Phe-Arg-AMC, the dipeptide BOC-Leu-GlyOH and all amino acids were purchased from Bachem. Leu-ProOBzl TFA and BOC-Phe-AlaOH were prepared by well established literature procedures.[32] Reagent grade chemicals were purchased from the following companies: EEDQ from Novabiochem, DPSI, LiOH from Merck, DCC, DMAP from Fluka, DPPA from Aldrich, Pd–C 10% type E 10 N/D from Degussa. Preparative flash column chromatography was performed using silica gel 60, 40–63 μm, from Merck. Melting points are uncorrected and were obtained on Mel-Temp II capillary melting point apparatus. Optical rotations were measured on a Perkin–Elmer 241 polarimeter. IR spectra were determined on a Perkin–Elmer 841 IR spectrometer. Mass spectra were measured on a Finnigan MAT 312 by the Chemisches Laboratorium of the University of Freiburg. Elemental analyses were determined on a Perkin–Elmer Elemental Analyzer 240 by the Chemisches Laboratorium of the University of Freiburg. NMR spectra were recorded on Varian Unity 300 (300 and 75.43 MHz). ^{1}H NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak at $\delta = 7.26$. ^{13}C NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak at $\delta = 77.00$. All ^{1}H NMR assignments were supported by homonuclear decoupling experiments or by 2-D COSY experiments and all ^{13}C NMR assignments by 2-D HETCOR experiments.

Syntheses

The syntheses of the following compounds have been published previously: (**1a**, **1b**, **15a, 15b**),[18b] (**2a + b**, **5a**, **5b**, **6a**, **6b**, **7a**, **10a**, **10b**, **16a + b**, **18a + b**, **20b**, **21a + b**, **23a + b**, **25a**, **25b**, **29a(I,II)**).[18c]

General procedures

Synthesis of aziridine building blocks and enamino esters starting from fumarates. DPSI (5.2 g, 24 mmol) and 20 mmol of the corresponding fumarate were dissolved in 60 mL toluene and heated at 80 °C for 24 h. The mixture was evaporated in vacuo. Column chromatography (silica gel, cyclohexane:ethyl acetate, 4:1) yielded the aziridines (30–35%) **3a + b** and **4a + b**, respectively, and the enamino esters (40–50%) **29b** and **29c(I,II)**, respectively.

Catalytic hydrogenolysis of benzyl esters. An evacuated solution of benzyl ester in MeOH or EtOH and Pd–C (10%) was vigorously stirred at rt and atmospheric pressure for 0.5–3 h (TLC control) under a slow stream of hydrogen. The catalyst was removed by filtration over Celite and washed with MeOH or EtOH. The filtrate was evaporated in vacuo and the residue was recrystallized.

(2*R*,3*R*) + (2*S*,3*S*) Dibenzyl aziridine-2,3-dicarboxylate (3a + b). The general procedure A with 5.9 g dibenzyl fumarate yielded 1.8 g (30%) **3a + b** as a yellowish solid. R_f 0.27; mp 60–61 °C. IR (ethyl acetate): $\nu = 3276$, 3064, 2956, 1733, 1497, 1455, 1384, 1340 cm^{-1}. ^{1}H NMR ($CDCl_3$): $\delta = 1.90$ (bs, 1H, NH), 3.00 (s, 2H, ring-H), 5.20 (2×d, $J = 12.0$ Hz, 2×2 H), 7.40 (m, 10H). ^{13}C NMR ($CDCl_3$): $\delta = 35.68$, 67.48, 127.27, 128.31, 128.48, 134.28, 170.0. Anal. ($C_{18}H_{17}NO_4$): calcd: C 69.44, H 5.50, N 4.50; found: C 69.26, H 5.47, N 4.42.

(2*R*,3*R*) + (2*S*,3*S*) 2-*tert*Butyl-3-ethyl aziridine-2,3-dicarboxylate (4a + b). The general procedure A with 4.0 g *tert*butyl ethyl fumarate yielded 1.5 g (35%) **4a + b** as a yellowish liquid. R_f 0.18. IR (ethyl acetate): ν = 3279, 2982, 2939, 1725, 1477, 1460, 1444, 1394, 1371, 1344 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.27 (t, 3H, *J* = 7.0 Hz), 1.35 (s, 9H), 1.67 (bs, 1H), 2.60 and 2.62 (2×d, *J* = 2.2 Hz, 2H), 4.06 (q, *J* = 7.0 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ = 13.60, 27.43, 35.03, 35.97, 61.19, 82.07, 168.85, 169.76. MS (CI, C_4H_{10}): *m*/*z* (%) = 215 (28) [M^+], 158 (100). Anal. ($C_{10}H_{17}NO_4$): calcd: C 55.80, H 7.96, N 6.51; found: C 55.18, H 7.71, N 6.75.

(2*R*,3*R*) + (2*S*,3*S*) 3-*tert*Butoxycarbonyl aziridine-2-carboxylate, Li salt (12a + b). 4a + b (280 mg, 1.3 mmol) were dissolved in 20 mL EtOH. LiOH monohydrate (54 mg, 1.3 mmol) was added. After 2 h at 0 °C the mixture was stirred at rt for 2 days. The residue remaining after evaporation of the solvent was used without further purification: mp 178 °C. ^1H NMR (pyridine-d_5): δ = 1.36 (s, 9H), 3.0–3.12 (m, 2H, ring-H), 4.9 (bs, 1H). MS (EI, 70 eV): *m*/*z* (%) = 186 (32.85) [M^+], 85 (100).

(2*R*,3*R*) + (2*S*,3*S*) *N*-{[3-*tert*Butoxycarbonyl aziridine-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline benzyl ester (*R*,*R*/*S*,*S* = 1:1) (13a + b). Crude **12a + b** (193 mg, 1.0 mmol) and 432 mg (1.0 mmol) Leu-ProOBzl TFA were dissolved in 10 mL DMF abs. under N_2 atmosphere. At 0 °C 240 μL (1.1 mmol) DPPA and 170 μL (1.2 mmol) TEA were added. The solution was stirred for 10 h at 0 °C and for further 12 h at rt. Ethyl acetate (100 mL) was added. The solution was washed with water (3×25 mL), 5% $NaHCO_3$ (1×25 mL) and brine (2×25 mL). The organic layer was dried with $MgSO_4$ and evaporated in vacuo. Flash column chromatography (silica gel, cyclohexane:ethyl acetate, 1:1) gave 210 mg (43%) **13a + b** as a colourless viscous liquid. R_f 0.43. $[\alpha]_D^{20} = -59.6$ (c 0.51, EtOH). IR (ethyl acetate): ν = 3268, 2957, 1739, 1636, 1549, 1447 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 0.80–1.0 (m, 6H, Leu), 1.32–1.60 (m, 11H, butyl, βH Leu), 1.60–1.80 (m, 2H, NH azi, γH Leu), 1.85–2.05 (m, 3H, βH Pro, 2×γH Pro), 2.15–2.25 (m, 1H, βH Pro), 2.58, 2.65, 2.75, 2.85 (d each, *J* = 1.9 Hz, together 2H, ring-H), 3.45–3.60 (m, 1H, δH Pro), 3.62–3.80 (m, 1H, δH Pro), 4.55 (m, 1H, αH Pro), 4.70 (m, 1H, αH Leu), 5.12 (m, 2H, benzyl), 6.80 and 6.92 (2×bs, together 1H, NH Leu), 7.10–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 21.7, 23.27 (δC Leu), 24.62 (γC Leu), 24.84 (γC Pro), 27.91 (*t*butyl), 28.91 and 29.63 (βC Pro), 35.78, 36.30 (double peak), 37.09 (ring-C), 41.25 and 41.57 (βC Leu), 46.81 (δC Pro), 48.27 (αC Leu), 58.85 and 58.89 (αC Pro), 66.88 (OCH_2 benzyl), 83.0 (*t*butyl), 126.88, 128.09, 128.11, 128.26, 128.50, 135.51, 168.07, 170.77, 170.86, 171.62. MS (EI, 70eV): *m*/*z* (%) = 487.1 (2.2) [M^+], 69.9 (100). HREIMS: calcd: 487.2682; found: 487.2667. Anal. ($C_{26}H_{37}N_3O_6$): calcd: C 64.05, H 7.65, N 8.62; found: C 63.96, H 7.67, N 8.81.

(2*R*,3*R*) + (2*S*,3*S*) *N*-{[3-Carboxy aziridine-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline benzyl ester, trifluoro acetate (*R*,*R*/*S*,*S* = 1:1) (14a + b). 13a + b (500 mg, 1.02 mmol) was dissolved in 5 mL CH_2Cl_2 abs. at 0 °C. TFA (5 mL) was added and the solution was stirred at rt for 1 h. The solvent was removed in vacuo, 5 mL CH_2Cl_2 were added and the solvent was evaporated again. This was repeated two times. The residue was dried in vacuo. Yield: 556 mg (100%), colourless viscous liquid. $[\alpha]_{20}^{D} = -42.5$ (c 1.13, EtOH). IR (ethyl acetate): ν = 3987, 3784, 2956, 1732, 1634, 1444, 1180 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 0.8–1.0 (m, 6H, Leu), 1.25–1.60 (m, 2H, βH Leu), 1.60–1.80 (m, 1H, γH Leu), 1.90–2.10 (m, 3H, βH Pro, 2×γH Pro), 2.15–2.25 (m, 1H, βH Pro), 2.95 (d, *J* = 1.9 Hz, 1H, ring-H), 3.05 (d, *J* = 1.9 Hz, 1H, ring-H), 3.50–3.70 (m, 1H, δH Pro), 3.70–3.90 (m, 1H, δH Pro), 4.42–4.60 (m, 1H, αH Pro), 4.65–4.75 (m, 1H αH Leu), 5.05–5.20 (m, 2H, benzyl), 7.00–7.45 (m, 6H, arom., NH Leu), 8.80 (bs, 3H, NH_2^+, CO_2H). ^{13}C NMR ($CDCl_3$): δ = 21.21, 22.89 (δC Leu), 23.69 (γC Leu), 24.60 (γC Pro), 28.73, 29.61 (ßC Pro), 38.68, 40.0 (ring-C), 40.05 (βC Leu), 47.26 (δC Pro), 49.50 (αC Leu), 59.48 (αC Pro), 67.24 (benzyl), 115.06 (Q, *J* (C,F) = 286 Hz, CF_3), 126.27, 126.29, 128.17, 128.54, 129.47, 130.05, 130.06, 135.13, 159.90 (Q, ^2J (C,F) = 40 Hz, C=O, TFA), 171.13, 171.85. MS (ESI): *m*/*z* (%) = 478 (100) [$M^+ + 2Na$], 432 (2.5) [$M^+ + 1$]. Anal. ($C_{22}H_{29}N_3O_6 \cdot C_2F_3HO_2$): calcd: C 52.84, H 5.54, N 7.70; found: C 53.87, H 5.68, N 7.57.

(2*R*,3*R*) + (2*S*,3*S*) Dibenzyl-1-[*N*-(*tert*butoxycarbonyl)-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (*R*,*R*/*S*,*S* = 1:1) (17a + b). BOC-Phe (3.2 g, 12 mmol) and 1.24 g (6 mmol) DCC were dissolved in 15 mL CH_2Cl_2 at 0 °C and stirred for 45 min at rt. Insoluble dicyclohexyl urea was filtered off and 930 mg (3 mmol) **3a + b** were added together with a few crystals of DMAP (10 mol%). The solvent was removed after 1 d at rt and the residue was purified by flash column chromatography (silica gel, cyclohexane:ethyl acetate, 10:1). Yield: 810 mg (48.4%), yellowish viscous liquid. R_f 0.1. $[\alpha]_{18}^{D} = -5.8$ (c 0.86, EtOH). IR (ethyl acetate): ν = 3371, 2928, 1739, 1604, 1497, 1447, 1369 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.40 (s, 9H, BOC), 3.06 (mc) and 3.23 (mc) (together 2H, βH Phe), 3.41 (s) and 3.55 (s) (together 2H, ring-H), 4.51 (m) and 4.63 (m) (together 1H, αH Phe), 5.00 (d) and 5.13 (d) (*J* = 8.2 Hz, together 1H, NH Phe), 5.20 (m, 4H, OCH_2), 7.13–7.43 (m, 15H). ^{13}C NMR ($CDCl_3$): δ = 28.02, 28.07 (BOC), 38.01, 39.00, 39.76, 40.08 (ring-C, βC Phe), 55.92, 56.38 (αC Phe), 67.97, 67.99 (OCH_2), 79.53, 79.71 (BOC), 126.58, 126.62, 128.23, 128.41, 128.44, 128.47, 128.52, 128.59, 129.43, 134.92, 134.33, 135.92, 136.06, 154.59, 154.75 (BOC), 165.59, 165.63, 178.73, 178.86. MS (EI, 70 eV): *m*/*z* (%) = 558 (0.3) [M^+], 164 (100). HREIMS: calcd: 558.2366; found: 558.2374. Anal. ($C_{32}H_{34}N_2O_7$): calcd: C 68.80, H 6.13, N 5.01; found: C 69.36, H 5.56, N 4.41.

(2*R*,3*R*) + (2*S*,3*S*) 1-[*N*-(*tert*Butoxycarbonyl)-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylic acid (*R*,*R*/*S*,*S* = 1/1) (19a + b). The general procedure B with 160 mg (0.28 mmol) **17a + b**, 40 mL EtOH and 100 mg Pd-C gave after 1.5 h 106 mg (100%) **19a + b** as colourless crystals: mp 77 °C. $[\alpha]_{D}^{18} = -15.6$ (c 0.50, EtOH). IR (ethyl acetate): ν = 3432, 2076, 1740, 1700, 1643, 1370 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.10–1.42 (m, 9H, BOC), 3.60–3.76 (m, 4H, βH Phe, ring-H), 4.40 and 4.62 (2×m, together 1H, αH Phe), 5.32 (bs, 1H, NH Phe, disappears with D_2O), 7.0–7.4 (m, 7H, 2×CO_2H, disappears with

D_2O, 5H, arom.). ^{13}C NMR ($CDCl_3$): δ = 28.21 (BOC), 38.42, 38.74, 40.34 (βC Phe, ring-C), 56.46 (αC Phe), 80.46 (BOC), 126.83, 127.02, 128.38, 128.58, 129.34, 129.62, 136.08, 155.71 (BOC), 166.11, 169.17, 174.74. MS (EI, 70 eV): *m/z* (%) = 378.2 (0.8) [M^+], 57 (100). HREIMS: calcd: 378.1427; found: 378.1437. Anal. ($C_{18}H_{22}N_2O_7 \cdot C_2H_5OH$): calcd: C 55.80, H 7.80, N 5.42; found: C 55.60, H 7.61, N 5.56.

(2*R*,3*R*) Diethyl-1-[*N*-(*tert*butoxycarbonyl)-(*S*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylate (20a). 1a (101 mg, 0.54 mmol) and 180 mg (0.54 mmol) BOC-Phe-Ala were dissolved in 5 mL DMF abs. EEDQ (142 mg, 0.57 mmol) was added and the mixture was stirred at 50 °C. After 2 days 50 mL ethyl acetate were added and the solution was washed with 5% $NaHCO_3$ (1×50 mL) and water (1×50 mL). The organic layer was dried with $MgSO_4$ and removed in vacuo. The residue was purified by flash column chromatography (silica gel, cyclohexane:ethyl acetate, 3:1). Yield: 100 mg (40%), colourless viscous liquid. R_f 0.13. $[\alpha]_{20}^{D} = -12.6$ (c 0.46, EtOH). IR (ethyl acetate): ν = 3785, 2924, 1741, 1663, 1595 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.15–1.21 (m, 18H, CH_3 ester, βH Ala, BOC), 3.05 (m, 2H, βH Phe), 3.42 (s, 2H, ring-H), 4.15–4.4 (m, 5H, ester, αH Phe), 4.50 (m, 1H, αH Ala), 4.91 (bd, *J* = 8.5 Hz, 1H, NH Phe), 6.42 (bd, *J* = 8.6 Hz, 1H, NH Ala), 7.2 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 13.98 (CH_3 ester), 18.31 (βC Ala), 28.19 (BOC), 38.31 (βC Phe), 40.10 (ring-C), 48.08, 49.49 (αC Phe, Ala), 62.63 (OCH_2 ester), 80.05 (BOC), 128.64, 129.33, 129.36, 136.37, 155.27 (BOC), 165.88, 170.72, 179.31. MS (CI, C_4H_{10}): *m/z* (%) = 506 (7) [$M^+ + 1$], 319 (100). Anal. ($C_{25}H_{35}N_3O_8$): calcd: C 59.39, H 6.98, N 8.31; found: C59.22, H 7.08, N 8.85.

(2*R*,3*R*) + (2*S*,3*S*) 1-[N-(*tert*Butoxycarbonyl)-(*S*)-phenylalanyl - (*S*) - alanyl] - 3 - ethoxycarbonyl aziridine - 2 - carboxylic acid (*R*,*R*/*S*,*S* = 1:1.3) (22a + b). The general procedure B with 160 mg (0.28 mmol) **21a + b**, 20 mL EtOH and 50 mg Pd–C gave after 1 h 130 mg (100%) **22a + b** as colourless crystals: mp 73 °C. $[\alpha]_{18}^{D} = -3.4$ (c 0.5, EtOH). IR (ethyl acetate): ν = 3296, 2976, 1735, 1524, 1449 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.03–1.50 (m, 15H, BOC, CH_3 ester, βH Ala), 2.85–3.10 (m, 2H, βH Phe), 3.40–3.70 (m, 2H, ring-H), 4.12–4.28 (m, 2H, OCH_2), 4.40 (m, 1H, αH Phe), 4.68 (m, 1H, αH Ala), 5.30 and 5.62 (2×bs, together 1H, NH Phe), 6.80 (bs, 2H, NH Ala, CO_2H), 7.03–7.35 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 13.77, 13.90 (CH_3 ester), 17.89, 17.97 (βC Ala), 28.08 (BOC), 37.72, 38.63, 39.50, 39.64, 41.67 (βC Phe, ring-C), 49.58, 49.77, 52.30, 55.57 (αC Phe, αC Ala), 62.16, 62.22 (OCH_2), 80.24 (BOC), 126.74, 128.46, 128.61, 129.20, 129.28, 136.53, 155.60, 155.74 (BOC), 166.49, 166.61, 169.49, 171.49, 179.98. MS (EI, 70 eV): *m/z* (%) = 477.3 (0.9) [M^+], 120 (100). HREIMS: calcd: 477.2111; found: 477.2121. Anal. ($C_{23}H_{31}N_3O_8 \cdot C_2H_5OH$): calcd: C 57.35, H 7.12, N 8.03; found: C 57.42, H 7.08, N 7.37.

(2*R*,3*R*) + (2*S*,3*S*) 1-[*N*-(*tert*Butoxycarbonyl)-(*S*)-leucyl-glycyl] - 3 - ethoxycarbonyl aziridine - 2 - carboxylic acid (*R*,*R*/*S*,*S* = 1:1.5) (24a + b). The general procedure B with 340 mg (0.65 mmol) **23a + b**, 50 mL MeOH and 100 mg Pd–C gave after 3 h 280 mg (100%) **24a + b** as colourless crystals: mp 79 °C. $[\alpha]_{20}^{D} = -20.0$ (c 0.66, EtOH). IR (ethyl acetate): ν = 3984, 3784, 3312, 2959, 1739, 1522, 1443, 1369 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 0.85–0.95 (m, 6H, δH Leu), 1.27 (t, *J* = 7.15 Hz, 3H, ester), 1.37–1.55 (m, 11H, BOC, βH Leu), 2.60–2.76 (m, 1H, γH Leu), 3.47 (m, 2H, ring-H), 3.95–4.08 (m, 1H, Gly), 4.10–4.28 (m, 4H, ester, αH Gly, αH Leu), 4.85 (bs, 1H, NH Leu), 6.70 (bs, 1H, NH Gly). ^{13}C NMR ($CDCl_3$): δ = 13.97, 21.77, 22.99 (δC Leu), 24.74 (γC Leu), 28.28 (BOC), 39.52, 39.62, 39.85 (ring-C), 41.32 (βC Leu), 43.80 (Gly), 53.26, 53.29 (αC Leu), 62.23, 62.79 (OCH_2), 80.33 (BOC), 155.64 (BOC), 165.94, 166.41, 166.47, 172.68, 176.49, 176.53. MS (ESI): *m/z* (%) = 428 (66) [M^+], 287 (100). Anal. ($C_{19}H_{31}N_3O_8 \cdot 1/2$ MeOH): calcd: C 52.60, H 7.46, N 9.44; found: C 52.49, H 7.48, N 9.79.

(2*R*,3*R*) *N*-{1-[*N*-(*tert*Butoxycarbonyl)-(*S*)-phenylalanyl]-3-ethoxycarbonyl aziridine-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline (26a). The general procedure B with 40 mg (0.057 mmol) **25a**, 20 mL MeOH and 20 mg Pd–C gave after 0.5 h 35 mg (100%) **26a** as colourless crystals: mp 109 °C. $[\alpha]_{20}^{D} = -32.6$ (c 1.0, EtOH). IR (ethyl acetate): ν = 3278, 2960, 1740, 1701, 1630, 1530, 1448 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 0.95 (m, 6H, Leu), 1.30–1.40 (m, 12H, BOC, CH_3), 1.40–2.10 (m, 7H, βH, γH Leu, Pro), 3.10–3.30 (m, 4H, βH Phe, ring-H), 3.40–3.60 (m, 1H, βH Pro), 3.60–3.80 (m, 1H, δH Pro), 4.20 (m, 2H, OCH_2), 4.40 (m, 1H, H Pro), 4.60 (m, 1H, αH Phe), 4.75 (m, 1H, αH Leu), 5.30 (bs, 1H, NH Phe), 6.90 (bs, 1H, NH Leu), 7.09–7.20 (m, 5H, arom.). ^{13}C NMR ($CDCl_3$): δ = 13.90 (CH_3), 21.37, 23.41 (δC Leu), 24.74 (γC Leu), 24.86 (γC Pro), 28.14 (BOC), 28.90 (βC Pro), 36.37 (βC Phe), 38.39 (ring-C), 40.42 (βC Leu), 41.02 (ring-C), 47.23 (δC Pro), 49.11 (αC Leu), 56.06 (αC Phe), 58.10 (αC Pro), 62.26 (OCH_2), 79.53 (BOC), 126.94, 128.20, 129.09, 136.38, 154.89 (BOC), 162.46, 164.70, 171.10. MS (EI, 70 eV): *m/z* (%) = 616 (0.2) [M^+], 90.9 (100). Anal. ($C_{31}H_{44}N_4O_9 \cdot MeOH$): calcd: C 59.24, H 7.46, N 8.64; found: C 59.09, H 7.21, N 8.84.

(2*S*,3*S*) N-{1-[N-(*tert*Butoxycarbonyl)-(*S*)-phenylalanyl]-3-ethoxycarbonyl aziridine-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline (26b). The general procedure B with 400 mg (0.57 mmol) **25b**, 50 mL MeOH and 100 mg Pd–C gave after 1 h 350 mg (100%) **26b** as colourless crystals: mp 119 °C. $[\alpha]_{23}^{D} = -33.4$ (c 1.05, EtOH). IR (ethyl acetate): ν = 3287, 2980, 1740, 1712, 1637, 1543, 1529, 1498, 1454 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 0.92 (d, *J* = 6.6 Hz, 6H), 1.29 (t, *J* = 7.0 Hz, 3H, CH_3), 1.38 (s, 9H, BOC), 1.46–1.52 (m, 3H, βH, γH Leu), 2.0–2.20 (m, 3H, βH, 2×γH Pro), 2.20–2.32 (m, 1H, βH Pro), 3.0–3.40 (m, 4H, βH Phe, ring-H), 3.50–3.64 (m, 1H, δH Pro), 3.68–3.80 (m, 1H, δH Pro), 4.20 (m, 2H, OCH_2), 4.40 (mc, 1H, αH Pro), 4.60 (mc, αH Phe), 4.75 (mc, αH Leu), 5.31 (bs, 1H, NH Phe), 6.81 (bs, 1H, NH Leu), 7.12–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 13.85 (CH_3), 21.59, 23.21 (δC Leu), 24.52 (γC Leu), 24.80 (γC Pro), 28.15 (BOC), 28.70 (βC Pro), 39.15 (βC Phe), 39.74 (ring-C), 40.95 (βC Leu), 41.61 (ring-C), 47.07 (δC Pro), 49.23 (αC Leu), 56.75 (αC Phe), 59.51 (αC Pro), 62.34 (OCH_2), 79.38 (BOC), 126.56, 128.23, 129.61, 136.61, 154.73 (BOC), 162.82, 164.65, 166.45, 171.17, 179.62. MS

(ESI): m/z (%) = 655 (66) [$M^+ + K$], 639 (100) [$M^+ + Na$], 617 (3) [M^+]. Anal. ($C_{31}H_{44}N_4O_9 \cdot MeOH$): calcd: C 59.24, H 7.46, N 8.64; found: C 59.99, H 7.05, N 8.60.

(2*R*,3*R*) Diethyl-1-(benzyloxycarbonylmethyl)aziridine-2,3-dicarboxylate (27a). 1a (1.0 g, 5.3 mmol), 555 mg (5.3 mmol) Na_2CO_3, a few crystals of KI and 1.7 mL (10.5 mmol) benzyl bromoacetate were stirred at 60 °C in 20 mL DMF abs. for 2 days. Ice water (100 mL) was added and the mixture was extracted with ethyl acetate (4×100 mL). The organic layer was washed with water (1×100 mL) and brine (1×100 mL), dried with $MgSO_4$ and removed in vacuo. The residue was purified by flash column chromatography (silica gel, cyclohexane:ethyl acetate, 5:1). Yield: 890 mg (52.3%), yellowish solid. R_f 0.2; mp 45–47 °C. $[\alpha]_{20}^{D} = +20.1$ (c 0.76, EtOH). IR (ethyl acetate): ν = 2984, 1750, 1732, 1498, 1455 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.30 (t, J = 7.1 Hz, 6H), 2.84 (bs) and 3.15 (bs) (together 2H, ring-H), 3.80 (d) and 3.95 (d, J = 16.8 Hz, together 2H, NCH_2), 4.19 (q, J = 7.1 Hz, 2H), 5.20 (s, 2H, OCH_2), 7.21–7.41 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 13.95, 39.89 and 43.65 (ring-C), 51.56 (NCH_2), 61.69 (OCH_2), 66.59 (OCH_2), 128.21, 128.23, 128.41, 135.31, 167.5 and 168.5 (CO_2Et), 169.18 (CO_2Bzl). MS (EI, 70eV): m/z (%) = 334.9 (0.83) [M^+], 261.9 (100). MS (ESI): m/z (%) = 374 (70) [$M^+ + K$], 358 (100) [$M^+ + Na$], 336 (7.4) [$M^+ + 1$]. Anal. ($C_{17}H_{21}NO_6$): calcd: C 60.89, H 6.31, N 4.18; found: C 60.98, H 6.31, N 3.98.

(2*S*,3*S*) Diethyl-1-(benzyloxycarbonylmethyl)aziridine-2,3-dicarboxylate (27b). 1b (1.6 g, 8.5 mmol), 880 mg (8.5 mmol) Na_2CO_3, a few crystals of KI and 2.6 mL (16 mmol) benzyl bromoacetate were stirred at 60 °C in 40 mL DMF abs. for 2 days. Ice water (120 mL) was added and the mixture was extracted with ethyl acetate (4×120 mL). The organic layer was washed with water (1×120 mL) and brine (1×120 mL), dried with $MgSO_4$ and removed in vacuo. The residue was purified by flash column chromatography (silica gel, cyclohexane:ethyl acetate, 5:1). Yield: 1.5 g (52.6%), yellowish solid. R_f 0.2; mp 49–50 °C. $[\alpha]_{20}^{D} = -20.7$ (c 0.92, EtOH). IR (ethyl acetate): ν = 2984, 1750, 1732, 1498, 1455 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.30 (t, J = 7.1 Hz, 6H), 2.84 (bs) and 3.15 (bs) (together 2H, ring-H), 3.80 (d) and 3.95 (d, J = 16.8 Hz, together 2H, NCH_2), 4.19 (q, J = 7.1 Hz, 2H), 5.20 (s, 2H, OCH_2), 7.21–7.41 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 13.95, 39.89 and 43.65 (ring-C), 51.56 (NCH_2), 61.69 (OCH_2), 66.59 (OCH_2), 128.21, 128.23, 128.41, 135.31, 167.5 and 168.5 (CO_2Et), 169.18 (CO_2Bzl). MS (CI, C_4H_{10}): m/z (%) = 336 (100) [$M^+ + 1$]. HREIMS: calcd: 335.1368; found: 335.1380. Anal. ($C_{17}H_{21}NO_6$): calcd: C 60.89, H 6.31, N 4.18; found: C 60.82, H 6.39, N 3.69.

(2*R*,3*R*) 2-[2,3-(Bis(ethoxycarbonyl)aziridine-1-yl]acetic acid (28a). The general procedure B with 810 mg (2.4 mmol) **27a**, 100 mL EtOH and 150 mg Pd–C gave after 1 h of hydrogenolysis and recrystallization with water/EtOH 488 mg (83%) **28a** as colourless crystals; mp 151 °C. $[\alpha]_{18}^{D} = -9.4$ (c 0.51, EtOH). IR (ethyl acetate): ν = 2955, 1740, 1676, 1440 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.20 (t, J = 7.1 Hz, 6H), 2.90 (s, 2H, ring-H), 3.51–3.90 (m, 2H), 4.18 (q, J = 7.1 Hz, 4H), 9.0 (bs, 1H, CO_2H). ^{13}C NMR ($CDCl_3$): δ = 13.32, 36.04, 51.22, 61.20, 169.19, 172.33. MS (CI, C_4H_{10}): m/z (%) = 258 (39) [$M^+ - CO_2 + C_4H_9$], 244 (22) [M^+]. Anal. ($C_{10}H_{15}NO_6 \cdot \frac{1}{2}H_2O$): calcd: C 47.31, H 6.34, N 5.52; found: C 47.04, H 5.94, N 5.28.

(2*S*,3*S*) 2-[2,3-(Bis(ethoxycarbonyl)aziridine-1-yl]acetic acid (28b). The general procedure B with 900 mg (2.7 mmol) **27b**, 100 mL EtOH and 300 mg Pd–C gave after 1 h of hydrogenolysis and recrystallization with water/EtOH 590 mg (89%) **28b** as colourless crystals; mp 158–160 °C. $[\alpha]_{24}^{D} = +4.8$ (c 1.33, EtOH). IR (ethyl acetate): ν = 3459, 3000, 2956, 2850, 1738, 1676, 1440 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.20 (t, J = 7.1 Hz, 6H), 2.85 (s, 2H, ring-H), 3.51–3.90 (m, 2H), 4.18 (q, J = 7.1 Hz, 4H), 9.0 (bs, 1H, CO_2H). ^{13}C NMR ($CDCl_3$): δ = 13.32, 36.04, 51.22, 61.20, 169.19, 172.33. MS (CI, C_4H_{10}): m/z (%) = 258 (100) [$M^+ - CO_2 + C_4H_9$], 244 (23) [M^+]. Anal. ($C_{10}H_{15}NO_6 \cdot H_2O$): calcd: C 45.63, H 6.51, N 5.32; found: C 45.30, H 6.02, N 5.82.

Dibenzyl-2-amino fumarate (29b). The general procedure A with 5.9 g dibenzyl fumarate yielded 2.5 g (40%) **29b** as a yellowish liquid. R_f 0.60. IR (ethyl acetate): ν = 3483, 3352, 3091, 3066, 3035, 2956, 2892, 1728, 1679, 1620, 1554, 1498, 1455, 1380 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 5.21 (s, 2H), 5.30 (s, 2H), 5.66 (s, 1H), 6.51 (bs, 2H, NH_2), 7.25–7.50 (m, 10H). ^{13}C NMR ($CDCl_3$): δ = 65.0, 67.5, 88.0, 127.5–128.4 (6 peaks), 134.1, 136.1, 146.1, 162.5, 168.5. Anal. ($C_{18}H_{17}NO_4$): calcd: C 69.44, H 5.50, N 4.50; found: C 69.70, H 5.56, N 4.41. GC-MS: R_t = 15.0 min, m/z (%) = 311 (5) [M^+], 91 (100).

1-*tert*Butyl-4-ethyl-2-amino fumarate (29c(I))/1-ethyl-4-*tert*butyl-2-amino fumarate (29c(II)), I/II = (1:1.6). The general procedure A with 4.0 g *tert*butyl ethyl fumarate yielded 2.1 g (49%) **29c(I) + 29c(II)** as a yellowish liquid. R_f 0.63. IR (ethyl acetate): ν = 3492, 3349, 2983, 1724, 1677, 1619, 1581, 1477, 1459, 1394 cm^{-1}. ^1H NMR ($CDCl_3$): δ = 1.20 and 1.22 (2×t, J = 7.1 Hz, together 3H), 1.42 and 1.44 (2×s, together 9H), 4.09 and 4.20 (2×q, J = 7.1 Hz, together 2H), 5.35 and 6.70 (2×s, together 1H). ^{13}C NMR ($CDCl_3$): δ = 13.95, 14.27, 27.66, 27.79, 59.20, 60.97, 81.61, 83.01, 87.94 (2 peaks), 147.62 (2 peaks), 162.40, 163.96, 164.98, 169.85. MS (EI, 70 eV): m/z (%) = 215 [M^+], 87 (100). Anal. ($C_{10}H_{17}NO_4$): calcd: C 55.80, H 7.96, N 6.51; found: C 56.16, H 7.59, N 6.75. Assignment of NMR signals was carried out by INEPT long range NMR spectroscopy.

Enzyme assays

Kinetic measurements were carried out for papain, CB and CL(Pt) as described previously.[18c] The assay buffer for clostripain (3.0–5.9 μg/mL) was 50 mM phosphate buffer, pH 7.6, 200 mM NaCl, 2.5 mM DTT, and for CL(h) (0.005 μg/mL) 50 mM citrate buffer, pH 6.0, 5 mM EDTA, 2.5 mM DTT, 0.005% Brij 35, 200 mM NaCl. Z-Phe-Arg-AMC (0.011–0.094 mM) was used as substrate for both enzymes. The following K_m values were used: CL(h) 3.8 μM, clostripain 0.023 mM. The kinetic constants were obtained by non-linear or linear regression analysis using the program GraFit.[33]

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