New Peptidic Cysteine Protease Inhibitors Derived from the Electrophilic α-Amino Acid Aziridine-2,3-dicarboxylic Acid

Tanja Schirmeister*

Department of Pharmaceutical Chemistry, Pharmaceutical Institute, Albert-Ludwigs-University of Freiburg, Hermann-Herder-Strasse 9, D-79104 Freiburg, Germany

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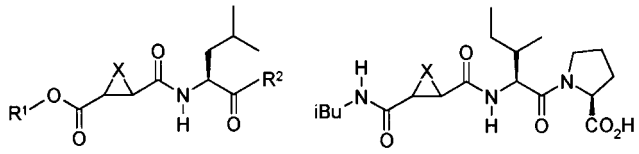
Three different types of peptides containing aziridine-2,3-dicarboxylic acid (Azi) as an electrophilic α-amino acid at different positions within the peptide chain (type I, N-acylated aziridines with Azi as C-terminal amino acid; type II, N-unsubstituted aziridines with Azi as N-terminal amino acid; type III, N-acylated bispeptidyl derivatives of Azi) have been synthesized and tested as inhibitors of the cysteine proteases papain, cathepsins B, L, and H, and calpains I and II, as well as against several serine proteases, one aspartate, and one metalloprotease. All aziridinyl peptides are specific cysteine protease inhibitors. Papain and cathepsins B and L are inhibited irreversibly, whereas cathepsin H and calpains are inhibited in a non-time-dependent manner. Some compounds turned out to be substrates for serine proteases and for the metalloprotease thermolysin. Remarkable differences can be observed between the three different types of inhibitors concerning stereospecificity, pH dependency of inhibition, selectivity between different cysteine proteases, and the importance of a free carboxylic acid function at the aziridine ring for inhibition. Above all type II inhibitors, aza analogues of the well-known epoxysuccinyl peptides, are potent cysteine protease inhibitors. With the exception of BOC-Leu-Gly-(*S,S*+*R,R*)-Azi-$(OEt)_2$ (**28a**+**b**), a highly selective and potent cathepsin L inhibitor, N-acylated aziridines of type I are weaker inhibitors than type II or type III compounds. The observed results can be explained by different binding modes of the three types of inhibitors with respect to their orientation in the S- and S′-binding sites of the enzymes. Furthermore, the presence of a protonated aziridine N modifies the binding mode of type II inhibitors.

Introduction

The papain superfamily of cysteine proteases includes a variety of enzymes with closely related amino acid sequences and overall folding structures.[1] Among them are vacuolar plant enzymes (e.g., papain),[2] protozoen enzymes[3] (e.g., cruzipain, falcipain), and mammalian lysosomal cathepsins[4] (e.g., cathepsins B, L, H) and cytoplasmatic calpains.[5] The mammalian cysteine proteases are involved in a variety of pathological processes including dysregulated protein turnover such as muscular dystrophy,[6] bone resorption,[7] growth and malignancy of tumors,[8] and myocardial infarct.[9] Therefore these enzymes are promising targets for the development of inhibitors as therapeutic agents.

A number of irreversible and selective cysteine protease inhibitors have been developed. Most of them exhibit a peptide segment for recognition by the enzyme and an electrophilic building block for reaction with the cysteine residue of the enzyme's active site as common structural features. Examples are diazomethyl ketones,[10] fluoromethyl ketones,[11] acyloxymethyl ketones,[12] *O*-acylhydroxamates,[13] vinyl sulfones,[14] and epoxysuccinic acid derivatives.[15] With regard to the requirements for drugs, the latter are one of the most promising inhibitor classes. Inactivation of cysteine proteases by epoxysuccinyl peptides proceeds from a nucleophilic opening of the epoxide ring leading to alkylated enzymes.[16] Development of these inhibitors was based on the discovery of E-64 (**1**) (Chart 1) isolated from an *Aspergillus japonicus* culture by Hanada et al. in 1978.[17] Cell permeability was improved by replacing the agmatine by uncharged alkyl groups and by esterification of the carboxylate function, e.g., Loxistatin (**2**) (Chart 1).[18] Even if the esters are 100−1000 times less active than the free acids in vitro, they are used as prodrugs which are easily resorbed and subsequently hydrolyzed to their active forms.[19] These epoxides have been shown to interact in an antisubstrate orientation with the S-subsite of papain and cathepsin B.[20] Cysteine proteases of the papain superfamily prefer substrates

Chart 1



1 X = O; R^1 = H; R^2 = Agm
E - 64 (HO-(S,S)-Eps-Leu-Agm)

2 X = O; R^1 = Et; R^2 = NH - iAm
Loxistatin (Et-(S,S)-Eps-Leu-NH-iAm)

3 X = O; R^1 = H; R^2 = NH - iAm
E-64c (HO-(S,S)-Eps-Leu-NH-iAm)

5a/b X = NH; R^1 = Et; R^2 = NH - iAm
(a) Et-(S,S)-Azi-Leu-NH-iAm
(b) Et-(R,R)-Azi-Leu-NH-iAm

6a/b X = NH; R^1 = H; R^2 = NH - iAm
(a) HO-(S,S)-Azi-Leu-NH-iAm
(b) HO-(R,R)-Azi-Leu-NH-iAm

4 X = O
iBu-NH-(S,S)-Eps-Ile(Leu)-ProOH

7a/b X = NH
(a) iBu-NH-(S,S)-Azi-Leu-ProOH
(b) iBu-NH-(R,R)-Azi-Leu-ProOH

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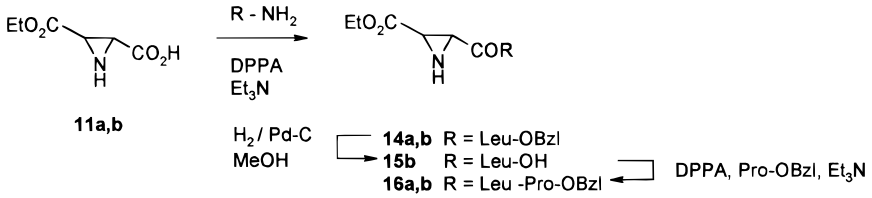
Scheme 1



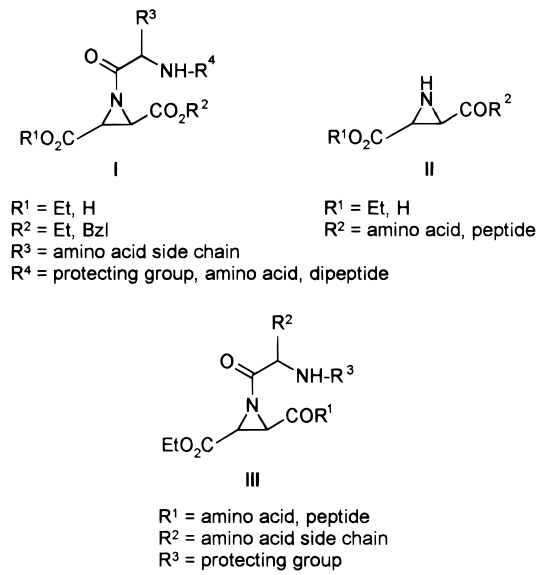
with Phe in the P2-position due to a hydrophobic binding pocket built up by the amino acids Trp67, Phe207, Pro68, Ala160, Val133, and Val157 (papain numbering).[21] Epoxysuccinyl peptides such as **1**−**3** (Chart 1) bind with their Leu residue into this hydrophobic binding pocket. Epoxides such as **4** interact with the S′-subsite of cathepsin B in a substrate-like mode. In contrast to inhibitors **1**−**3**[22] they are significantly more potent in the *R*,*R* configuration[23] and exhibit a very good selectivity against cathepsin B.[24] This selectivity results from interaction of the proline carboxylate of **4** with two histidine residues (His110, His111) which are located at the occluding loop of cathepsin B and are responsible for its dipeptidyl peptidase activity.[25] A chimeric epoxysuccinyl peptide inhibitor has been prepared by Moroder et al.[23] by combining the S′-recognition element of cathepsin B (Leu-Pro) with the inhibiting propeptide sequence of cathepsin B (Leu-Gly-Gly, sequence portion 46−48 of the propeptide) which spans the S-binding pockets in an antisubstrate orientation. This inhibitor is the most potent cathepsin B inhibitor known so far and exhibits a very good selectivity between cathepsin B and cathepsin L.

The three-membered aziridine ring is closely related in structure to the epoxide and is also susceptible to ring opening by nucleophiles.[26] Jones et al.[27] have synthesized and tested the aziridine analogues **5**−**7** (Chart 1) of the well-known cysteine protease inhibitors EtO-Eps-Leu-NHiAm (**2**), HO-Eps-Leu-NHiAm (**3**), and ^{i}BuNH-Eps-Ile(Leu)-ProOH (**4**). Even though these aziridine analogues are also irreversible inhibitors of the cysteine proteases papain, cathepsin B, and cathepsin L, remarkable differences between the epoxides and the aziridines have been found with respect to reactivity, selectivity, stereospecificity, and pH dependency of inhibition.[27] Recently a variety of aziridinyl peptides in which the Leu residue of compounds **5** and **6** has been replaced by Phe have been patented by Takeda Chem. Ind. as powerful cathepsin L inhibitors.[28,29] For these inhibitors naturally occurring epoxide analogues, called cathestatins, are known.[30]

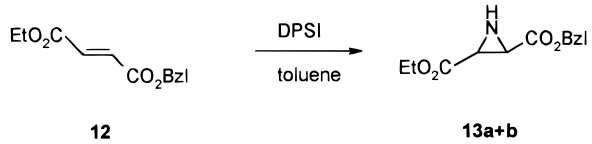
In contrast to the epoxide the aziridine ring can additionally be derivatized at its heteroatom. This offers a greater variability for structure−activity studies. Chimeric inhibitors can not only be built by derivatization of the two carboxylates but also by variations at the aziridine nitrogen. Even though some N-alkylated aziridinyl peptides are described in the above-mentioned patents[28,29] and some results concerning N-acylated derivatives could already be obtained in the author's laboratory[31] this field has yet to be explored systematically.

In the present paper, both the syntheses and the inhibition profiles of peptides containing aziridine-2,3-dicarboxylic acid as the electrophilic α-amino acid at different positions within the peptide chain (types I−III, Chart 2) are described. These are classified according to their structural differences: Type I aziridinyl peptides contain the aziridine moiety as the C-terminal amino acid. They are aziridines N-acylated with amino acids or peptides. Type II peptides are aza analogues of the known epoxysuccinyl peptides and contain the aziridine-2,3-dicarboxylic acid as the N-terminal amino acid. In type III peptides the aziridine is located in the middle of the peptide chain. Although type I and III peptides are both N-acylated aziridines, experimental results show differences between these two types which support their classification as two different inhibitor types. The particular peptide moieties used for preparation of the inhibitors have been selected with respect to the substrate specificities[21] of hydrolysis and to known inhibiting sequences,[23] respectively.

Chart 2



Scheme 2

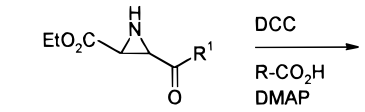


Preparation of Inhibitors

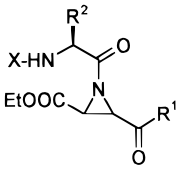
The syntheses of the compounds were performed as depicted in Schemes 1−5. Stereoselective synthesis of (*S*,*S*)- and (*R*,*R*)-diethyl aziridine-2,3-dicarboxylate (**10a**,**b**) was carried out according to previously[31,32] described procedures using the reverse configured epoxides (*R*,*R*)-**8b** and (*S*,*S*)-**8a**, respectively, as starting materials. The latter were prepared from (*R*,*R*)- and (*S*,*S*)-diethyl tartrate, respectively.[33,34]

Half-esters **11a,b** of the aziridine building blocks were prepared by alkaline hydrolysis with 1 equiv of LiOH

Scheme 3

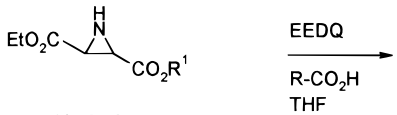


10a,b, 13a+b, 14a,b, 16a,b

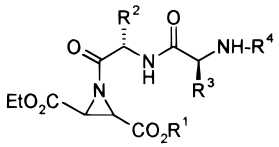


17a,b	R^1 = OEt; R^2 = Bzl; X = BOC
18a	R^1 = OEt; R^2 = Me; X = Z
19a	R^1 = OEt; R^2 = Me; X = BOC
20a	R^1 = OEt; R^2 = iBu; X = Fmoc
21a,b	R^1 = Leu-OBzl; R^2 = Bzl; X = BOC
22a,b	R^1 = Leu-Pro-OBzl; R^2 = Bzl; X = BOC
23a+b	R^1 = OBzl; R^2 = Bzl; X = BOC

Scheme 4

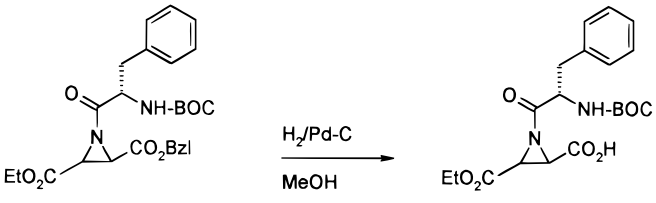


10a,b, 13a+b



24a	R^1 = Et; R^2 = H; R^3 = H; R^4 = Z
25a,b	R^1 = Et; R^2 = Bzl; R^3 = Me; R^4 = Z
26a	R^1 = Et; R^2 = Me; R^3 = Bzl; R^4 = BOC
27a+b	R^1 = Bzl; R^2 = Me; R^3 = Bzl; R^4 = BOC
28a+b	R^1 = Bzl; R^2 = H; R^3 = iBu; R^4 = BOC
29a	R^1 = Et; R^2 = Me; R^3 = Bzl; R^4 = Z -Gly

Scheme 5



23a+b **30a+b**

monohydrate in ethanol and subsequent protonation by ion exchange.[27] Peptide coupling at the free carboxylate function of the aziridine leading to the type II inhibitors **14a,b** and **16a,b** could be achieved by the DPPA method, whereby N-protection was not necessary.[27] Both stepwise and fragment condensations were carried out (Scheme 1).

Racemic *trans*-benzylethyl aziridine-2,3-dicarboxylic acid (**13a**+**b**) was synthesized by Michael type addition of diphenylsulfimine to the corresponding fumarate **12** (Scheme 2). Most of the aziridinyl peptides derived from aziridines **10a**,**b** were prepared as single diastereomers with *S*,*S* (**a** series) as well as *R*,*R* (**b** series) configured aziridine ring. Peptides derived from the racemic aziridine **13a**+**b** (Scheme 2) were synthesized as diastereomeric mixtures (**a**+**b** series).

Acylation of the aziridine nitrogen with amino acids was carried out via symmetric anhydrides, prepared from 2 equiv of N-protected amino acid and 1 equiv of DCC, under DMAP catalysis (Scheme 3). This yielded the type I compounds **17a,b**,[31] **18a**,[31] **19a**,[31] **20a**, and **23a**+**b** and the type III inhibitors **21a,b** and **22a,b**.

Nitrogen acylation with di- or tripeptides could be performed by fragment condensation using mixed anhydrides of peptide and isobutyl chloroformiate.[35] Since this method leads to racemization within the peptide chain, the one-pot EEDQ[36] procedure was chosen to prepare the type I inhibitors **24a**, **25a**,**b**, **26a**, **27a**+**b**, **28a**+**b**, and **29a** (Scheme 4).

A free carboxylate function at the aziridine ring has been shown to be essential for inhibition by N-unsubstituted derivatives.[27] To examine the role of a free carboxylate group for inhibition by N-acylated derivatives, selective hydrolysis of one ester function of the N-acylated diethyl ester **17a** by alkaline or enzymatic methods was investigated. For the alkaline procedure either 1 equiv of LiOH/EtOH or 1 equiv of NaOH/CH_3-CN was used. The enzymatic methods were carried out using the following enzymes:[39] PLE, chymotrypsin, trypsin, CCL, PPL, LPR, LRA, and LAN. Alkaline hydrolyses and enzymatic procedures with the serine hydrolases PLE, trypsin, and chymotrypsin, respectively, led to preferential amide hydrolysis. The reasons for the sensitivity of the amide bond to alkaline hydrolysis are disturbed amide resonance[37] and high acidity of the leaving group. Amides containing an aziridine as the amino component cannot form mesomeric structures due to increased ring tension. The pK_a of the aziridinium ion has been determined by Jones et al.[27] with pK_a 3.6 for compound **6a**. In the author's laboratory a pK_a of 3.8 ± 0.2[73] was found for compound **11a**. PLE is a serine esterase which can also cleave amide bonds and which prefers aromatic residues.[38] Chymotrypsin and trypsin are serine proteases preferring hydrophobic and basic amino acids, respectively, in the P1-position. In both cases, amide and simultaneous ester hydrolysis could be observed. Compound **17a** was not hydrolyzed by the above-mentioned lipases. Due to the failure of the selective hydrolysis of one ester function with maintenance of the aziridide structure, another synthetic path had to be chosen. The aziridine building block **13a**+**b** was used as a starting material to allow ester cleavage by nonhydrolytic procedures. The N-acylated aziridinyl peptide **30a**+**b** which contains one free carboxylate function was obtained by N-acylation of building block **13a**+**b** with BOC-Phe via the symmetric anhydride (Scheme 3, compound **23a**+**b**) and subsequent hydrogenolysis of the benzyl ester (Scheme 5).

Results

The second-order rate constants for the inactivation of papain and cathepsins B and L by the aziridinyl peptides of type I, **17**−**20** and **23**−**30**, which contain the aziridine-2,3-dicarboxylate as the C-terminal amino acid, are shown in Table 1. Since proteases of the papain family prefer Phe in the P2-position, compounds **17a,b**, **25a,b**, and **26a** with a Phe residue at different positions

Table 1. Second-Order Rate Constants for Inactivation of Papain,[a] Cathepsin B,[b] and Cathepsin L[b] by Type I Aziridinyl Peptides

inhibitor	k_i/K_i (M^{-1} min^{-1})		
	papain	cathepsin B	cathepsin L
BOC-Phe-(*S*,*S*)-Azi-$(OEt)_2$ (**17a**)[c]	408 ± 16	125 ± 19	65 ± 12
BOC-Phe-(*R*,*R*)-Azi-$(OEt)_2$ (**17b**)[c]	42 ± 9	18 ± 6	16 ± 6
Z-Ala-(*S*,*S*)-Azi-$(OEt)_2$ (**18a**)[c]	73 ± 2	67 ± 17	305 ± 26
BOC-Ala-(*S*,*S*)-Azi-$(OEt)_2$ (**19a**)	43 ± 5[c]	nd[d]	nd
Fmoc-Leu-(*S*,*S*)-Azi-$(OEt)_2$ (**20a**)	65 ± 9[c]	nd	99 ± 10[c]
BOC-Phe-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OBzl) (**23a**+**b**)[c]	76 ± 9	nd	nd
BOC-Phe-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OH) (**30a**+**b**)	505 k_i[e] = 0.21 ± 0.013 K_i[f] = 0.41 ± 0.07	1230 k_i = 0.83 ± 0.019 K_i = 0.68 ± 0.03	188 ± 6[c]
Z-Gly-Gly-(*S*,*S*)-Azi-$(OEt)_2$ (**24a**)	297[c] ± 55	389[c] ± 11	317 k_i = 0.087 ± 0.011 K_i = 0.27 ± 0.06
Z-Ala-Phe-(*S*,*S*)-Azi-$(OEt)_2$ (**25a**)	177[c] ± 10	125 k_i = 0.011 ± 0.001 K_i = 0.090 ± 0.001	29 ± 10[c]
Z-Ala-Phe-(*R*,*R*)-Azi-$(OEt)_2$ (**25b**)[c]	21 ± 1	22 ± 1	33 ± 4[c]
BOC-Phe-Ala-(*S*,*S*)-Azi-$(OEt)_2$ (**26a**)	1370 k_i = 0.028 ± 0.0026 K_i = 0.020 ± 0.009	455 ± 15[c]	469 k_i = 0.051 ± 0.019 K_i = 0.11 ± 0.08
BOC-Phe-Ala-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OBzl) (**27a**+**b**)	1232 k_i = 0.020 ± 0.001 K_i = 0.016 ± 0.001	443 ± 71[c]	281 ± 2[c]
BOC-Leu-Gly-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OBzl) (**28a**+**b**)	149 k_i = 0.01 ± 0.001 K_i = 0.069 ± 0.0012	240 ± 61[c]	3237 k_i = 0.028 ± 0.0015 K_i = 0.0088 ± 0.0015
Z-Gly-Phe-Ala-(*S*,*S*)-Azi-$(OEt)_2$ (**29a**)[c]	523 ± 25	421 ± 100	315 ± 48

[a] pH 6.5. [b] pH 6.0. [c] Measurements were limited to the linear range, with [I] ≤ K_i, due to solubility problems. Therefore, only the second-order rate constant could be obtained. [d] Not determined. [e] k_i (min^{-1}). [f] K_i (mM).

within the peptide chain were synthesized to evaluate the most favorable distance between the electrophilic building block and P2-recognition element. **20a**, **24a**, and **28a**+**b** are peptides which contain fragments of the inhibiting procathepsin B sequence Leu-Gly-Gly. These type I inhibitors are irreversible inhibitors of cathepsins B and L and papain. This irreversibility is evident from the time dependency of inhibition in the continuous or dilution assays which were performed to determine the second-order rate constants of inhibition. It was confirmed by dialysis experiments with papain and inhibitors **17a** and **11a** as well as with cathepsin L and inhibitor **28a**+**b**.

Second-order rate constants for these type I inhibitors are very low and are similar to those found by Albeck et al.[49] for several *erythro*-peptidyl epoxides. Especially cathepsin L is inactivated very weakly by type I aziridines. An interesting and surprising exception in this regard is the inhibition of cathepsin L by mixture **28a**+**b**. Although it is still a weak inhibitor, the second-order rate constant for this diastereomeric mixture is about 10 times higher than all other values found for type I inhibitors. This increase in inhibition results from a 10 times lower binding constant (K_i) and not from a higher first-order rate of inactivation (k_i). A difference between the overall foldings of cathepsins B and L and papain which may be a criterion to explain this outstanding value is the structure of the S2-pocket of cathepsin L.[47] An additional Met161 residue makes its pocket more shallow and narrow compared to those of cathepsin B and papain.

The highest inhibition constants for type I derivatives containing Phe within the peptide moiety are found for compounds **26a** and **27a**+**b** with BOC-Phe-Ala as the N-terminal peptide chain.

In contrast to N-unsubstituted aziridines of type II, **14a,b, 5a,b**,[27] and **6a,b**,[27] for which the second-order rate constants are reported in Table 2, the *S*,*S* diastereomers **17a** and **25a** are about 7−10 times more active than their *R*,*R* isomers **17b** and **25b**. On the other hand this stereospecificity corresponds to the one observed for the epoxysuccinyl peptides exemplified by compounds **1**−**3**. Another difference between type I aziridines and aziridines without N-derivatization and epoxides **1**−**3** is the low inhibition improvement by the free carboxylic acid function at the three-membered ring. While **6a,b**[27] (Table 2) and even the single aziridine building block **11a** (Table 5) exhibit an about 300−600 times higher second-order rate constant for the inhibition of papain than their corresponding esters, values for the inhibition by **30a**+**b** are only 3−10 times higher in comparison to those for **17a**,**b** or **23a**+**b**. Interestingly this inhibition improvement is a result of a higher first-order rate of alkylation (k_i) and not of a lower binding constant (K_i). Taking into consideration that **30a**+**b** is a free acid mixture of diastereomers with *S*,*S* and *R*,*R* configured aziridine ring, the inhibition constant for the eutomer may be somewhat higher but nevertheless cannot reach the rates found for N-unsubstituted derivatives.

Prolongation of the peptide chain is normally known to improve inhibition of endoproteinases.[40] However, this is not the case for N-acylated aziridinyl peptides of type I (inhibitor **29a**) as well as for the N-unsubstituted ones of type II (inhibitors **16a**,**b**). The highest inhibition constants within the type II inhibitors are observed for *R*,*R* configured aziridines coupled to a Leu derivative (**14b** and **5b**[27]).

As shown by Jones et al.,[27] N-unsubstituted aziridines are much more potent at low pH values due to protonation of the aziridine nitrogen. The data[27] showed that the neutral form must be at least 100 times less reactive than the protonated form. The true inhibition constants

Table 2. Second-Order Rate Constants for Inactivation of Papain,[a] Cathepsin B,[b] and Cathepsin L[b] by Type II Aziridinyl Peptides

	k_i/K_i (M^{-1} min^{-1})		
inhibitor	papain	cathepsin B	cathepsin L
EtO-(*S*,*S*)-Azi-Leu-OBzl (**14a**)	214 k_i [e] = 0.036 ± 0.0015 K_i [f] = 0.17 ± 0.02	41 ± 8[c]	3130 k_i = 0.055 ± 0.009 K_i = 0.018 ± 0.021
EtO-(*R*,*R*)-Azi-Leu-OBzl (**14b**)	1533 ± 41[c]	1607 ± 395[c]	16261 k_i = 0.44 ± 0.0004 K_i = 0.027 ± 0.0001
EtO-(*R*,*R*)-Azi-Leu-OH (**15b**)	63 ± 18[c]	nd	nd[d]
EtO-(*S*,*S*)-Azi-Leu-Pro-OBzl (**16a**)	227 k_i = 0.021 ± 0.0002 K_i = 0.094 ± 0.0034	405 k_i = 0.039 ± 0.02 K_i = 0.096 ± 0.01	2142 k_i = 0.047 ± 0.005 K_i = 0.022 ± 0.0085
EtO-(*R*,*R*)-Azi-Leu-Pro-OBzl (**16b**)	466 ± 37[c]	188 k_i = 0.025 ± 0.0001 K_i = 0.13 ± 0.0011	1728 k_i = 0.043 ± 0.0033 K_i = 0.025 ± 0.0057
EtO-(*S*,*S*)-Azi-Leu-NHiAm (**5a**)[g]	180		
EtO-(*R*,*R*)-Azi-Leu-NHiAm (**5b**)[g]	3300		
HO-(*S*,*S*)-Azi-Leu-NHiAm (**6a**)[g]	(108 ± 6) × 10^3		
HO-(*R*,*R*)-Azi-Leu-NHiAm (**6b**)[g]	(864 ± 42) × 10^3		
^{i}BuNH-(*S*,*S*)-Azi-Leu-Pro-OH (**7a**)[g]	108 ± 6	ni[h]	402 ± 6
^{i}BuNH-(*R*,*R*)-Azi-Leu-Pro-OH (**7b**)[g]	ni[h]	780 ± 180	3000 ± 60

[a] pH 6.5. [b] pH 6.0. [c] Measurements were limited to the linear range, with [I] ≤ K_i, due to solubility problems. Therefore, only the second-order rate constant could be obtained. [d] Not determined. [e] k_i (min^{-1}). [f] K_i (mM). [g] Taken from Jones et al.[27] [h] No time-dependent inhibition.[27]

Table 3. Second-Order Rate Constants for Inactivation of Papain,[a] Cathepsin B,[b] and Cathepsin L[b] by Type III Aziridinyl Peptides

	k_i/K_i (M^{-1} min^{-1})		
inhibitor	papain	cathepsin B	cathepsin L
BOC-Phe-(*S*,*S*)-(EtO)-Azi-Leu-OBzl (**21a**)[c]	171 ± 40	122 ± 12	219 ± 15
BOC-Phe-(*R*,*R*)-(EtO)-Azi-Leu-OBzl (**21b**)	225 ± 70[c]	257 ± 29[c]	314 k_i[d] = 0.0099 ± 0.0023 K_i[e] = 0.031 ± 0.0023
BOC-Phe-(*S*,*S*)-(EtO)-Azi-Leu-Pro-OBzl (**22a**)	321 k_i = 0.016 ± 0.0019 K_i = 0.05 ± 0.029	450 k_i = 0.037 ± 0.0022 K_i = 0.082 ± 0.013	1210 ± 260[c]
BOC-Phe-(*R*,*R*)-(EtO)-Azi-Leu-Pro-OBzl (**22b**)	768 ± 125[c]	1938 ± 440[c]	5896 ± 409[c]

[a] pH 6.5. [b] pH 6.0. [c] Measurements were limited to the linear range, with [I] ≤ K_i, due to solubility problems. Therefore, only the second-order rate constant could be obtained. [d] k_i (min^{-1}). [e] K_i (mM).

Table 4. Inhibition Constants for the Non-Time-Dependent Inhibition of Cathepsin H,[a] Calpain I,[b] and Calpain II[b]

	K_i (μM)		
inhibitor	cathepsin H	calpain I	calpain II
BOC-Phe-(*S*,*S*)-Azi-$(OEt)_2$ (**17a**)	210 ± 19	ni[c]	nd[d]
Z-Ala-(*S*,*S*)-Azi-$(OEt)_2$ (**18a**)	ni	160 ± 31	nd
(EtO)-(*S*,*S*)-Azi-Leu-OBzl (**14a**)	260 ± 35	nd	nd
(EtO)-(*R*,*R*)-Azi-Leu-OBzl (**14b**)	ni	ni	nd
BOC-Phe-(*S*,*S*)-(EtO)-Azi-Leu-OBzl (**21a**)	93 ± 8	nd	nd
BOC-Phe-(*R*,*R*)-(EtO)-Azi-Leu-OBzl (**21b**)	137 ± 11	19 ± 7	42 ± 14

[a] pH 6.5. [b] pH 7.5. [c] No inhibition. [d] Not determined.

Table 5. Second-Order Rate Constants for Inactivation of Papain at Different pH Values[a]

	k_i/K_i (M^{-1} min^{-1})		
inhibitor	pH 4	pH 6.5	pH 8
10a[b]	61 ± 5	11 ± 0.5	nd[c]
11a[b]	24774 ± 7000	4692 ± 1000	780 ± 85
14a	786 ± 39[b]	168[d]	24 ± 5[b]
17a[b]	72 ± 10	424 ± 32	410 ± 21

[a] Substrate, 1.4−1.5 mM L-BAPA; [E] = 0.5 mg mL^{-1}; [I]: **10a**, 0.58−4.7 mM; **11a**, 6.6 μM−0.3 mM; **14a**, 0.27−1.36 mM; **17a**, 0.092−0.37 mM. [b] Measurements were carried out at the linear range, where [I] ≤ K_i. Therefore, only the second-order rate constant was obtained. [c] Not determined. [d] k_i = 0.033 ± 0.002 (min^{-1}), K_i = 0.20 ± 0.033 (mM).

for the completely protonated inhibitor would be much higher at pH values below pH 4 which cannot be tested because of loss of enzyme activity.[27] The maximum activity pH of 4 determined by Jones et al.[27] for N-unsubstituted derivatives can be confirmed as shown in Table 5. In contrast to N-unsubstituted aziridines the N-acylated aziridine **17a** shows nearly identical inhibition constants at pH 6.5 and pH 8. Rich et al.[41] investigated the pH dependency of inhibition of papain by a nonionizable amide of **3**. This inhibition was found to depend on two acidic dissociation constants (pK_a's 3.93 and 4.09) of papain. This is in contrast to the inhibition of papain by E-64 (**1**) for which an acidic dependency and an alkaline dependency were found.[27] The latter result supports the assumption that His159 may play an essential role for inhibition. Since **17a** is sensitive to alkaline media, determination of second-order rate constants at pH values above pH 8 is useless. Table 6 reports the pH dependency of inhibition of papain by **17a**. These values, obtained within a pH range from 5.2 to 8.0, indicate, however, that inhibition

Table 6. pH Dependency of Inhibition of Papain by **17a**

pH	activity (mU mL^{-1}) without **17a**[a]	activity (mU mL^{-1}) with **17a**[a,b]	% inhibition
5.2	6.54 ± 0.2	1.86 ± 0.03	71.6
6.0	7.33 ± 0.14	1.38 ± 0.1	81.1
6.5	7.38 ± 0.21	0.98 ± 0.09	86.7
7.0	7.33 ± 0.15	0.75 ± 0.15	89.8
8.0	7.31 ± 0.15	0.75 ± 0.2	89.7

[a] Mean values from five assays. [b] [I] = 17 μM; incubation time 30 min; [S] = 1.49 mM L-BAPA; [E] = 0.5 mg mL^{-1}.

by this N-acylated aziridine corresponds to the one found for the amide of **3** rather than to that found for E-64 (**1**).

Activation of aziridines toward nucleophilic ring opening is possible not only by protonation of the aziridine nitrogen but also by N-acylation.[42] In Table 3 inhibition constants are reported for inhibitors of type III (**21a,b, 22a,b**). These are bispeptidyl derivatives which bear the aziridine moiety in the middle of the peptide chain and which can be regarded as chimeric inhibitors combining the P1′ and P2′ (Leu-Pro) specificity of cathepsin B with its P2 specificity (Phe). The N-acylation of tripeptide **16b** with BOC-Phe to tetrapeptide **22b** leads only to a weak inhibition improvement in the case of cathepsin L and papain, but to a 10-fold improvement of inhibition in the case of cathepsin B. No or only a slight increase of inhibition is obtained by N-acylation of the type II inhibitors **14a**,**b** and **16a.** Again, in accordance with the results found for type II inhibitors, the *R*,*R* diastereomers are the more potent, but with a very low eudismic ratio.

When the selectivity between cathepsins B and L and papain was compared, the inhibitors of types II and III which bear peptide chains at one of the carboxylate functions exhibit higher activities against cathepsin L than against cathepsin B or papain. The reverse holds for the type I inhibitors which are, with the one exception of mixture **28a**+**b** already mentioned, only weak cathepsin L inhibitors. Cathepsin H and calpains are not inhibited time dependently by all types of inhibitors. If a reduction in enzyme activity can be observed, K_i values in the upper micromolar range are also observed (Table 4). This non-time-dependent inhibition may be due to extremely low alkylation rates which are also known for the inhibition of these enzymes by epoxysuccinyl peptides.[22,43] For a better inhibition of cathepsin H, inhibitors without an N-terminal protecting group may be required.

To evaluate the selectivity between proteases with different mechanisms of hydrolysis (cysteine − serine − aspartate − metallo) the aziridines **10a**, **14b**, **17a**, **18a**, and **21b** were tested against the serine proteases chymotrypsin, trypsin, and elastase, the aspartate protease pepsin, and the metalloprotease thermolysin, respectively. As shown by the above-mentioned hydrolysis assays of **17a** with several serine hydrolases, this compound does not react as an inhibitor but as a substrate for these enzymes. BOC-Phe and compounds **10a** and **11a** could be isolated and identified by TLC and IR and NMR spectroscopy. This is in agreement with earlier reports on ester hydrolysis of aziridinecarboxylates by these enzymes.[44,45] In contrast to **21b** compound **14b** is a substrate for thermolysin which prefers Leu in the P1′-position.[46] In this case **11b** and LeuOBzl could be identified as hydrolysis products. Neither **10a** nor **21b** could inhibit pepsin.

Summary and Discussion

Even though several similarities, including the exclusive inhibition of cysteine proteases and the irreversibility of inhibition of papain and cathepsins B and L, exist between aziridines and epoxides, their respective behavior as cysteine protease inhibitors shows remarkable differences. These differences may first of all be due to the possible protonation of the aziridine nitrogen in the case of the type II aziridines, thus leading to a different binding mode with an additional water molecule not being necessary and with the positioning of the inhibitor being disturbed by the positively charged group. Second, aziridines of type II are analogues of the epoxides concerning their chemical reactivity, but they are not bioisosters. The epoxide oxygen is a H-bond acceptor, whereas the aziridine nitrogen in most cases reacts as a H-bond donor. Thus totally different interactions with the enzyme could be possible. On the other hand, there exist noteworthy differences between the different types of aziridinyl peptides. These differences concern pH dependency, stereospecificity, and selectivity of inhibition. In contrast to the type II inhibitors H-bonds cannot be built under participation of the aziridine N by the N-acylated type I and III inhibitors. Type I inhibitors which are more active with the *S*,*S* configured aziridine ring and which in this regard resemble the epoxysuccinyl peptides **1**−**3** could bind to the S-binding site in a substrate-like mode. Comparison of inhibition constants obtained for the Phe-containing series of inhibitors (compounds **17a,b** and **25a,b** with **26a** and **27a**+**b**) leads to the assumption that the Phe residue of the latter is probably located in the hydrophobic S2-pocket as known from the peptidyl chloromethyl ketone BPACK.[48] The aziridide structure may possibly lead to a more unfavorable inhibitor conformation which could impede the attack of the active site cysteine residue at the aziridine ring carbons and therefore lead to low alkylation rate constants. A superimposition of minimized conformations of the epoxide **2**, the type II aziridine **14b**, and the type I inhibitor **26a** supports this supposition (Figure 1A).[76] However, first results of docking experiments[77] performed with papain and **26a** indicate that the flexibility of the molecule nevertheless allows a conformation in which the Phe residue of this inhibitor can bind into the hydrophobic S2-pocket and the N-terminal BOC group can be located within the S3-subsite (Figure 1B).

A comparison of type II compounds **14a,b** and **16a,b** with the N-acylated type III compounds **21a,b** and **22a,b** shows that the activation of the aziridine ring to nucleophilic ring opening by N-acylation does in general not lead to an inhibition improvement. Therefore, the main reason for the remarkable increase in inhibition by protonation in the case of type II inhibitors should be due to improved binding rather than to improved nucleophilic ring opening. Type III inhibitors are new chimeric inhibitors, and similar to epoxides with two peptide chains,[23] a tendency for similar second-order rate constants for the *R*,*R* and *S*,*S* configured isomers can be observed. Thus, one can assume that these inhibitors, like the chimeric epoxides, bind to both the S- and S′-binding sites. A new aspect which should be taken into consideration is the possibility that the aziridine ring can be opened by nucleophiles in two different ways. Not only is cleavage of the C−N bond possible but also the cleavage of the C−C bond.[50] C−N cleavage would lead to aspartic acid derivatives, while C−C cleavage would form glycine derivatives. These two

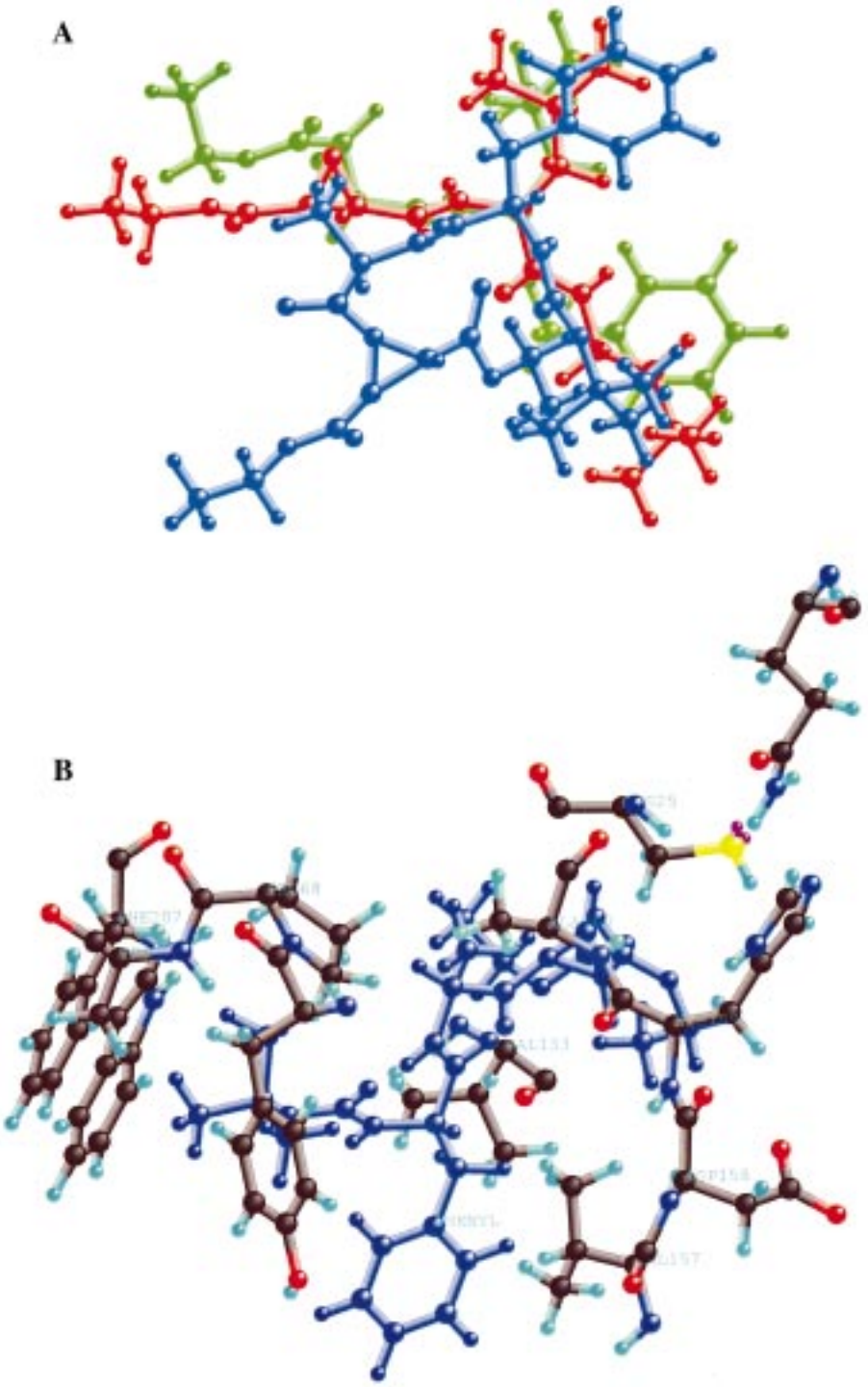


Figure 1. (A) Superimposition of minimized conformations of epoxide **2**, type II aziridine **14b**, and type I aziridine **26a**.[76] (B) Possible binding mode of **26a** to the active site of papain.[77]

possibilities may also play a role in the observed differences between epoxides and aziridines on the one hand and between different types of aziridinyl peptides on the other hand.

The exclusive inhibition of cysteine proteases could be explained by the enhanced nucleophilicity of the active site compared to serine proteases as a result of the thiolate−imidazolium ion pair[74] even though the nucleophilicity of serine and cysteine proteases cannot be compared that easily. The "hard" nucleophile OH of the serine proteases attacks the "hard" electrophilic C═O center, while the "soft" S^- nucleophile attacks the "soft" ring carbon.[75] Another reason for this selectivity may be the opposite active site geometry of serine proteases[51] which could convert aziridinyl peptides from inhibitors into substrates for serine proteases. Starting points to improve the selectivity between different cysteine proteases are the good activities of type II inhibitors and the type I mixture **28a**+**b** against cathepsin L and the remarkable inhibition improvement by the N-acylation of **16b** with BOC-Phe to **22b** in the case of cathepsin B. A better understanding of the observed differences between epoxysuccinyl peptides and aziridinyl peptides on the one hand and between the three types of aziridines on the other hand will require more

detailed molecular modeling studies as well as X-ray analysis of enzyme−inhibitor complexes. These studies are in progress. Nevertheless, the present study shows that N-unsubstituted aziridinyl peptides and in special cases N-acylated ones, too, can be highly selective and potent inactivators of cysteine proteases.

Experimental Section

General Methods. Enzymes were purchased from the following companies: papain from Carica papaya from Fluka (BioChemika USP, 76220), cathepsin B from bovine spleen from Sigma (C 6268), cathepsin H from human liver (219404), calpain I from porcine erythrocytes (208712) and calpain II from porcine kidney (208715) from Calbiochem, chymotrypsin from bovine pancreas (102307), trypsin from bovine pancreas (124579) and pepsin (7185) from Merck, elastase from porcine pancreas (20929) from Serva. Cathepsin L from *Paramecium tetraurelia*[56] was a gift from Prof. J. Schultz, Department of Pharmaceutical Chemistry, University of Tuebingen, Germany. Pig liver esterase PLE (46063), lipase from *Rhizopus arrhizus* LRA (62305), lipase from *Aspergillus niger* LAN (62294), lipase from *Penicillium roqueforti* LPR (62308), and lipase from hog pancreas PPL (62300) were from Fluka. *Candida cylindraceae* lipase type VII CCL (L1754) was from Sigma. All enzymes were used without further purification. All substrates, protected amino acids, and di- and tripeptides were purchased from Bachem. Leu-Pro-OBzl trifluoroacetate and BOC-Phe-AlaOH were prepared by well-established literature procedures.[24,52] Buffer substances were biochemical grade and were purchased from Merck. *N*-Ethylmaleimide was from Aldrich; E-64 was from Boehringer-Mannheim. Reagent grade chemicals were purchased from the following companies and were used without further purification: EEDQ from Novabiochem, DPSI, NaN_3, $LiOH \cdot H_2O$, and NH_4Cl from Merck, Ph_3P, DCC, DMAP, and Et_3N fom Fluka, DPPA from Aldrich, Pd−C 10% type E 10 N/D from Degussa. All solvents were anhydrous grade and were purchased from Fluka. Brine refers to a saturated aqueous solution of NaCl. Analytical TLC was performed on Merck aluminum sheets (silica gel 60 F_{254}). Compounds that were not visualized by UV light were detected by spraying with Ehrlich's reagent (1 g of *p*-(dimethylamino)-benzaldehyde, 25 mL of HCl concentrated, 75 mL of MeOH) followed by heating. Preparative flash column chromatography was performed using silica gel 60, 40−63 μm, from Merck. Preparative hydrostatic column chromatography was performed using silica gel 60, 63−200 μm, from Merck. Melting points are uncorrected and were obtained on a Mel-Temp II capillary melting point apparatus (Laboratory Devices). Optical rotations were measured on a Perkin-Elmer 241 polarimeter in a thermostated cell. IR spectra were determined in KBr pellets or with NaCl solution cells on a Perkin-Elmer 841 IR spectrophotometer. Mass spectra were measured on a Finnigan MAT 312 (low resolution; EI, 70 eV, 0.8 mA; CI, isobutane or NH_3, 200 eV, 0.5 mA) by Chemisches Laboratorium of the University of Freiburg or on a Voyager-RP biospectrometry workstation by PerSeptive Biosystems, Wiesbaden, Germany (high resolution) (mode, reflector; accelerating voltage, 12 or 15 kV; scans averaged, 15−34; negative ions, off). Elemental analyses were determined on a Perkin-Elmer elemental analyzer 240 by Chemisches Laboratorium of the University of Freiburg. NMR (^1H, ^{13}C) spectra were recorded on a Varian Unity 300 spectrometer (300 and 75.43 MHz, respectively). ^1H NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak at $\delta = 7.26$ with $CDCl_3$ as solvent and to the DMSO peak ($\delta = 2.49$) with DMSO-d_6 as solvent. ^{13}C NMR chemical shifts are reported in ppm relative to the $CHCl_3$ peak ($\delta = 77.00$) with $CDCl_3$ as solvent. All ^1H NMR assignments were supported by homonuclear decoupling experiments or by 2-D COSY experiments. All ^{13}C NMR assignments were supported by 2-D HETCOR experiments. Coupling constants (J) are reported in hertz (Hz).

General Procedures. N-Acylation via Symmetric Anhydrides. N-Protected amino acid (15.5 mmol) was dissolved in 40 mL of dichloromethane at 0 °C; 1.65 g (7.7 mmol) of DCC was added, and the mixture was stirred at 0 °C for 45 min. Insoluble dicyclohexylurea was filtered off, and 7 mmol of aziridine was added in 20 mL of dichloromethane at 0 °C together with a few crystals of DMAP (10 mol %). The reaction was stirred at 0 °C for 1 h and for 3−5 h at room temperature. Dicyclohexylurea was filtered off, and the solvent was removed. The residue was stirred in 10 mL of ethyl acetate for 20 min and again filtered off. The organic layer was washed with saturated $NaHCO_3$ solution (20 mL) and brine (20 mL), dried with $MgSO_4$, and concentrated. The residue was purified for each individual compound by either flash or hydrostatic chromatography as described below.

N-Acylation via Mixed Anhydrides. The coupling reagent EEDQ (426 mg, 1.72 mmol) was added to a solution of N-protected peptide (1.61 mmol) and aziridine (1.61 mmol) in 15 mL of DMF. The mixture was stirred at room temperature for 48 h. The solvent was removed in vacuo; the residue was dissolved in 100 mL of ethyl acetate and extracted with 50 mL of a 5% solution of $NaHCO_3$ and 50 mL of water. The organic layer was dried with $MgSO_4$ and evaporated. The residue was purified for each individual compound by either flash or hydrostatic chromatography as described below.

DPPA-Mediated Peptide Coupling. A stirred solution of carboxylic acid (5 mmol) and C-protected amino acid or peptide (HCl, TFA, or tosylate) (5.2 mmol) in DMF (30 mL) was cooled in an ice−water bath and treated with DPPA (1.2 mL, 5.5 mmol). A solution of Et_3N in 10 mL of DMF (10.5 mmol, 1.43 mL) was added dropwise over a period of 10 min. Stirring and cooling were continued for 10 h. The mixture was diluted with ethyl acetate (150 mL) and washed with 25-mL portions of water (3×), 5% $NaHCO_3$ (1×), and brine (2×). The organic layer was dried with $MgSO_4$ and evaporated in vacuo. The residue was purified for each individual compound by either flash or hydrostatic chromatography as described below.

Catalytic Hydrogenolysis of Benzyl Esters. An evacuated solution of 0.6 mmol of benzyl ester in 30 mL of MeOH and 40 mg of Pd−C (10%) was vigorously stirred at room temperature and atmospheric pressure for 1.5 h (TLC control) under a slow stream of hydrogen. The catalyst was removed by filtration over Celite and washed with methanol (50 mL). The filtrate was evaporated in vacuo, and the residue was recrystallized.

The following compounds were prepared according to literature procedures: **diethyl (2*S*,3*S*)-2,3-epoxysuccinate (8a),**[34] **diethyl (2*R*,3*R*)-2,3-epoxysuccinate (8b),**[34] **diethyl (2*S*,3*S*)-aziridine-2,3-dicarboxylate (10a),**[31] **diethyl (2*R*,3*R*)-aziridine-2,3-dicarboxylate (10b),**[31] **(2*S*,3*S*)-3-(ethoxycarbonyl)aziridine-2-carboxylic acid (11a),**[27] **(2*R*,3*R*)-3-(ethoxycarbonyl)aziridine-2-carboxylic acid (11b),**[27] **benzyl ethyl fumarate (12),**[68] **diethyl (2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (17a) (BOC-Phe-(*S*,*S*)-Azi-(OEt)$_2$),**[31] **diethyl (2*R*,3*R*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (17b) (BOC-Phe-(*R*,*R*)-Azi-(OEt)$_2$),**[31] **diethyl (2*S*,3*S*)-1-[*N*-(benzyloxycarbonyl)-(*S*)-alanyl]aziridine-2,3-dicarboxylate (18a) (Z-Ala-(*S*,*S*)-Azi-(OEt)$_2$),**[31] **and diethyl (2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-alanyl]aziridine-2,3-dicarboxylate (19a) (BOC-Ala-(*S*,*S*)-Azi-(OEt)$_2$).**[31]

(2*S*,3*S*)- and (2*R*,3*R*)-2-Benzyl 3-ethylaziridine-2,3-dicarboxylate (13a+b) (EtO-Azi-OBzl). DPSI[69] (5.2 g, 0.024 mol) and 5.9 g of benzyl ethyl fumarate (**12**) (0.02 mol) were dissolved in 60 mL of toluene and heated at 80 °C for 24 h. The mixture was evaporated in vacuo. Column chromatography (silica gel 60, cyclohexane/ethyl acetate, 4/1, $R_f = 0.2$) yielded 1.2 g (25%) of **13a+b** as a yellowish solid:[70] mp 52 °C. IR (CCl_4): 3282 (br), 1733, 1497 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.30 (t, $J = 7.0$ Hz, 3 H), 1.85 (bs, 1 H, NH), 2.90 (d, $J = 2.3$ Hz, 1 H, CH-Azi), 2.92 (d, $J = 2.3$ Hz, 1 H, CH-Azi), 4.22 (q, $J = 7.0$ Hz, 2 H), 5.15 (d, $J = 12.0$ Hz, 1 H), 5.25 (d, $J = 12.0$ Hz, 1 H), 7.3−7.45 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 13.81, 35.45, 35.60, 61.71, 67.36, 126.60, 128.22, 128.40, 134.73, 169.22

(double peak). MS (CI, C_4H_{10}): *m/z* (%) = 306 (1.1) [M^+ + C_4H_9], 250 (100) [M^+ + 1]. Anal. ($C_{13}H_{15}NO_4$) C,H,N.

***N*-{[(2*S*,3*S*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (14a) (EtO-(*S*,*S*)-Azi-Leu-OBzl).** **11a** was coupled with L-leucine benzyl ester tosylate using the general DPPA procedure. The product was purified by column chromatography on silica gel (ethyl acetate/cyclohexane, 1/1, R_f = 0.44) to give **14a** (760 mg, 42%) as a yellowish viscous liqiud: $[\alpha]_D^{27.3}$ = +47.2° (*c* 1.4, EtOH). IR (CCl_4): 3283 (br), 1739, 1675, 1526 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.9 (d, *J* = 6.1 Hz, 6 H), 1.3 (t, *J* = 7.08 Hz, 3 H), 1.46−1.7 (m, 3 H), 1.8 (bt, *J* = 8.5 Hz, 1 H, NH), 2.55 (dd, *J* = 2.2, 7.8 Hz, 1 H), 2.87 (dd, *J* = 2.2, 9.0 Hz, 1 H), 4.22 (q, *J* = 7.08 Hz, 2 H), 4.55−4.65 (m, 1 H), 5.15 (s, 2 H), 6.78 (bd, *J* = 8.8 Hz, 1 H), 7.28−7.4 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 14.07, 21.73, 22.79, 24.84, 35.44, 37.40, 41.03, 50.36, 62.20, 67.09, 128.20, 128.41, 128.61, 135.29, 167.75, 170.30, 172.22. MS (EI, 70 eV): *m/z* (%) = 363 (14) [M^+ + 1], 227 (100). Anal. ($C_{19}H_{26}N_2O_5$) C,H,N.

***N*-{[(2*R*,3*R*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (14b) (EtO-(*R*,*R*)-Azi-Leu-OBzl).** **11b** was coupled with L-leucine benzyl ester tosylate using the general DPPA method. The product was purified by column chromatography on silica gel (ethyl acetate/cyclohexane, R_f = 0.39) to give **14b** (633 mg, 35%) as a yellowish viscous liqiud: $[\alpha]_D^{25.2}$ = −110.7° (*c* 1.4, EtOH). IR (CCl_4): 3275 (br), 1742, 1670, 1537 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.9 (d, *J* = 6.1 Hz, 6 H), 1.25 (t, *J* = 7.0 Hz, 3 H), 1.45−1.8 (m, 4 H, NH, β-H, γ-H), 2.62 (d, *J* = 2.0 Hz, 1 H), 2.85 (d, *J* = 2.0 Hz, 1 H), 4.2 (q, *J* = 7.0 Hz, 2 H), 4.55−4.65 (m, 1 H), 5.13 (d, *J* = 12.0 Hz, 1 H), 5.18 (d, *J* = 12.0 Hz, 1 H), 6.65 (bd, *J* = 8.5 Hz, 1 H), 7.3−7.4 (m, 5 H).^{13}C NMR ($CDCl_3$): δ 13.83, 21.49, 22.56, 24.58, 35.17, 37.12, 40.72, 50.18, 61.86, 66.80, 127.93, 128.14, 128.35, 135.10, 167.65, 170.02, 171.97. MS (EI, 70 eV): *m/z* (%) = 363 (9) [M^+ + 1], 227 (100). MS (CI, C_4H_{10}): *m/z* (%) = 363 (100) [M^+ + 1]. Anal. ($C_{19}H_{26}N_2O_5$) C,H,N.

***N*-{[(2*R*,3*R*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine (15b) (EtO-(*R*,*R*)-Azi-Leu-OH).** **14b** was hydrogenolyzed using the general hydrogenolysis procedure. Recrystallization from MeOH yielded 95% (155 mg) **15b**: mp 66 °C; $[\alpha]_D^{23}$ = −63.6° (*c* 0.55, EtOH). IR (KBr): 3374 (br), 1737, 1670, 1544 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.94 (d, *J* = 5.9 Hz, 3 H), 0.96 (d, *J* = 6.11 Hz, 3 H), 1.30 (t, *J* = 7.08 Hz, 3 H), 1.5−1.8 (m, 3 H), 2.71 (d, *J* = 2.2 Hz, 1 H), 2.89 (d, *J* = 2.2 Hz, 1 H), 4.23 (q, *J* = 7.3 Hz, 2 H), 4.5−4.6 (m, 1 H), 5.0 (bs, 2 H, NH, COOH), 6.76 (bd, *J* = 8.5 Hz, 1 H, NH). MS (CI, C_4H_{10}): *m/z* (%) = 273 (100) [M^+ + 1]. MS (EI, 70 eV): *m/z* (%) = 273 (12) [M^+ + 1], 227 (100). Anal. ($C_{12}H_{20}N_2O_5 \cdot {}^1/_2$MeOH) C,N; H: calcd, 7.69; found, 7.23.

***N*-{[(2*S*,3*S*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline Benzyl Ester (16a) (EtO-(*S*,*S*)-Azi-Leu-Pro-OBzl).** DPPA-mediated coupling of **11a** with Leu-Pro-OBzl TFA gave, after purification by flash chromatography (cyclohexane/ethyl acetate, 1/2, R_f = 0.19), 51% (1.17 g) of **16a** as a colorless viscous liquid: $[\alpha]_D^{22}$ = −18.3° (*c* 1.45, EtOH). IR (ethyl acetate): 3271, 1740, 1631, 1551, 1453 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.86 (d, *J* = 6.35 Hz, 3 H), 0.91 (d, *J* = 6.35 Hz, 3 H), 1.27 (t, *J* = 7.1 Hz, 3 H), 1.45 (m, 2 H, β-H Leu), 1.5−1.64 (m, 1 H, γ-H Leu), 1.8−2.1 (m, 4 H, β-H Pro, 2 γ-H Pro, NH Azi), 2.1−2.28 (m, 1 H, β-H Pro), 2.58 (d, *J* = 1.95 Hz, 1 H), 2.78 (d, *J* = 2.2 Hz, 1 H), 3.5−3.62 (m, 1 H, δ-H Pro), 3.7−3.8 (m, 1 H, δ-H Pro), 4.2 (q, *J* = 7.3 Hz, 2 H), 4.54 (m, 1 H, α-H Pro), 4.74 (m, 1 H, α-H Leu), 5.06 (d, *J* = 12.2 Hz, 1 H), 5.16 (d, *J* = 12.2 Hz, 1 H), 7.06 (bd, *J* = 8.0 Hz, 1 H, NH Leu), 7.35 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 13.97, 21.63 (Leu), 23.19 (Leu), 24.52 (Leu), 24.76 (Pro), 28.84 (Pro), 35.59 (Azi), 37.35 (Azi), 41.47 (Leu), 46.74 (Pro), 48.55 (Leu), 58.82 (Pro), 61.93 (O-ethyl), 66.79 (O-benzyl), 128.01, 128.18, 128.43, 135.42, 167.68, 170.00, 170.74, 171.49. MS (CI, C_4H_9): *m/z* (%) = 459 (4) [M^+], 368 (100). Anal. ($C_{24}H_{33}N_3O_6$) C,H,N.

***N*-{[(2*R*,3*R*)-3-(Ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline Benzyl Ester (16b) (EtO-(*R*,*R*)-Azi-Leu-Pro-OBzl).** DPPA-mediated coupling of **15b** with Pro-OBzl HCl gave after purification by column chromatography (ethyl acetate, R_f = 0.56) **16b** (1.3 g, 57%) as a colorless viscous liquid: $[\alpha]_D^{21}$ = −125.0° (*c* 0.44, EtOH). IR (ethyl acetate): 3275 (br), 1742, 1656, 1539, 1448 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.88 (d, *J* = 6.6 Hz, 3 H), 0.92 (d, *J* = 6.6 Hz, 3 H), 1.26 (t, *J* = 7.1 Hz, 3 H), 1.4−1.52 (m, 2 H, β-H Leu), 1.58−1.7 (m, 1 H, γ-H Leu), 1.74 (bt, *J* = 8.0 Hz, 1 H, NH Azi), 1.9−2.1 (m, 3 H, β-H Pro, 2 γ-H Pro), 2.2 (m, 1 H, β-H Pro), 2.64 (d, *J* = 7.6 Hz, 1 H, Azi), 2.80 (d, *J* = 8.5 Hz, 1 H, Azi), 3.56 (m, 1 H, δ-H Pro), 3.64 (m, 1 H, δ-H Pro), 4.18 (q, *J* = 7.1 Hz, 2 H), 4.52 (m, 1 H, α-H Pro), 4.70 (m, 1 H, α-H Leu), 5.04 (d, *J* = 12.2 Hz, 1 H), 5.16 (d, *J* = 12.2 Hz, 1 H), 6.86 (bd, *J* = 8.0 Hz, 1 H, NH Leu), 7.2−7.5 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 14.14, 21.78, 23.40, 24.70, 24.99, 20.02, 35.58, 37.49, 41.48, 46.90, 48.40, 58.98, 62.19, 67.03, 128.25, 128.40, 128.63, 135.62, 167.88, 170.50, 170.92, 171.74. MS (EI 70 eV): *m/z* (%) = 459 (27) [M^+], 227 (100). Anal. ($C_{24}H_{33}N_3O_6$) C,H,N.

Diethyl (2*S*,3*S*)-1-[*N*-(Fluoren-9-ylmethoxycarbonyl)-(*S*)-leucyl]aziridine-2,3-dicarboxylate (20a) (Fmoc-Leu-(*S*,*S*)-Azi-(OEt)$_2$). N-Acylation of **10a** via symmetric anhydride using Fmoc-Leu yielded after column chromatography (cyclohexane/ethyl acetate, 5/1, R_f = 0.21) **20a** (1.6 g, 44%): mp 56 °C; $[\alpha]_D^{25.3}$ = −10.52° (*c* 1.065, EtOH). IR (CH_2Cl_2): 3418 (br), 1738, 1527 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.9 (d, *J* = 6.6 Hz, 6 H), 1.23 (t, *J* = 7.1 Hz, 3 H), 1.25 (t, *J* = 7.1 Hz, 3 H), 1.45−1.8 (m, 3 H), 3.5 (s, 2 H, Azi), 4.15−4.3 (m, 3 H, Fmoc), 4.4 (q, *J* = 7.1 Hz, 4 H), 5.15 (m, 1 H, α-H), 5.3 (bd, *J* = 8.0 Hz, 1 H, NH), 7.2−7.4 (m, 4 H), 7.5−7.6 (m, 2 H), 7.7−7.8 (m, 2 H). ^{13}C NMR ($CDCl_3$): δ 13.97, 21.74, 22.73, 24.66, 39.89, 41.44, 47.20, 54.37, 62.62, 66.98, 119.96, 125.11, 127.05, 127.68, 141.29, 143.74, 155.79, 166.06, 180.63. MS (EI, 70 eV): *m/z* (%) = 522 (10) [M^+], 178 (100). MALDI-TOF HRMS: calcd, 545.22637 [M + Na]$^+$; found, 545.2252 ± 0.00174. Anal. ($C_{29}H_{34}N_2O_7$) C,H; N: calcd, 5.36; found, 4.25.

***N*-{(2*S*,3*S*)-1-[[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (21a) (BOC-Phe-(EtO)-(*S*,*S*)-Azi-Leu-OBzl).** N-Acylation of **14a** (1.6 mmol, 579 mg) via the symmetric anhydride procedure using BOC−Phe gave, after column chromatography (cyclohexane/ethyl acetate, 1/1, R_f = 0.7), **21a** (347 mg, 95%): mp 50 °C; $[\alpha]_D^{27.3}$ = −10.6° (*c* 0.25, EtOH). IR (KBr): 3359 (br), 1743, 1710, 1498 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.9 (t, *J* = 6.3 Hz, 6 H), 1.3 (t, *J* = 7.3 Hz, 3 H), 1.4 (s, 9 H), 1.45−1.7 (m, 3 H, Leu), 2.6 (d, *J* = 1.95 Hz, 1 H, Azi), 3.0−3.2 (m, 2 H, β-H Phe), 3.28 (d, *J* = 1.95 Hz, 1 H, Azi), 4.23 (m, 2 H, OCH_2), 4.45 (m, 1 H, α-H Phe), 4.6 (m, 1 H, α-H Leu), 5.18 (s, 2 H), 5.26 (bd, *J* = 8.3 Hz, 1 H, NH Phe), 6.34 (bd, *J* = 8.1 Hz, 1 H, NH Leu), 7.12−7.28 (m, 5 H), 7.28−7.4 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 13.93 (CH_3), 21.85, 22.71 (Leu CH_3), 24.91 (Leu), 28.26 (BOC), 39.58 (Azi), 40.57 (Azi), 41.34 (double peak, β-C Leu, Phe), 50.88 (α-C Leu), 57.0 (α-C Phe), 62.63, 67.25 (OCH_2), 80.01 (BOC), 126.87, 128.22, 128.48, 128.54, 128.65, 129.54, 135.35, 136.73, 154.87, 164.76, 165.76, 171.93, 178.95. HREIMS (70 eV): calcd, 609.3050; found, 609.3051. Anal. ($C_{33}H_{43}N_3O_8$) C,H,N.

***N*-{(2*R*,3*R*)-1-[[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucine Benzyl Ester (21b) (BOC-Phe-(EtO)-(*R*,*R*)-Azi-Leu-OBzl).** N-Acylation of **14b** (1.0 mmol, 362 mg) via the symmetric anhydride procedure using BOC-Phe gave, after column chromatography (cyclohexane/ethyl acetate, 1/1, R_f = 0.7), **21b** (429 mg, 70%) as a colorless viscous liquid: $[\alpha]_D^{24.4}$ = −34.7° (*c* 0.36, EtOH). IR (KBr): 3339 (br), 1742, 1498 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.9 (d, *J* = 6.1 Hz, 6 H), 1.28 (t, *J* = 7.1 Hz, 3 H), 1.38 (s, 9 H), 1.44−1.66 (m, 3 H, Leu), 3.1−3.26 (m, 2 H, β-H Phe), 3.35 (d, *J* = 2.2 Hz, 1 H, Azi), 3.51 (d, *J* = 2.2 Hz, 1 H, Azi), 4.22 (q, *J* = 7.1 Hz, 2 H), 4.44−4.66 (m, 2 H, α-H Leu, Phe), 4.92 (bd, *J* = 9.0 Hz, 1 H, NH Phe), 5.16 (s, 2 H), 6.4 (bd, *J* = 8.0 Hz, 1 H, NH Leu), 7.15−7.4 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.88, 21.75, 22.66, 24.76, 28.15, 38.22, 40.94, 41.19, 41.37, 50.84, 56.16, 62.50, 67.16, 80.09, 126.81, 128.15, 128.44, 128.57, 129.48, 135.14, 136.11, 154.87, 164.93, 165.81, 172.02, 179.26. MS (EI, 70 eV): *m/z* (%) = 609 (31) [M^+], 418 (100). Anal. ($C_{33}H_{43}N_3O_8$) C,H,N.

***N*-{(2*S*,3*S*)-1-[[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucyl-**

(*S*)-proline Benzyl Ester (22a) (BOC-Phe-(EtO)-(*S*,*S*)-Azi-Leu-Pro-OBzl). N-Acylation of **16a** (1.5 mmol, 688 mg) with BOC-Phe via the symmetric anhydride method gave, after purification by flash chromatography (cyclohexane/ethyl acetate, 1/1, $R_f = 0.6$), 560 mg (53%) of **22a**: mp 80 °C; $[\alpha]_D^{25.1} = -46.7°$ (*c* 0.51, EtOH). IR (KBr): 3275 (br), 1744, 1713, 1632, 1498, 1455 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.88 (d, $J = 6.35$ Hz, 3 H, δ-H Leu), 0.92 (d, $J = 6.34$ Hz, 3 H, δ-H Leu), 1.24 (t, $J = 7.1$ Hz, 3 H), 1.35 (s, 9 H, BOC), 1.42−1.8 (m, 3 H, β-H, γ-H Leu), 1.85−2.1 (m, 3 H, 1 β-H, 2 γ-H Pro), 2.1−2.3 (m, 1 H, β-H, Pro), 2.94−3.28 (m, 2 H, β-H Phe), 3.35 (d, $J = 2.0$ Hz, 1 H, Azi), 3.45 (d, $J = 2.0$ Hz, 1 H, Azi), 3.52−364 (m, 1 H, δ-H, Pro), 3.66−3.80 (m, 1 H, δ-H, Pro), 4.12−4.24 (m, 2 H, OCH_2), 4.4 (m, 1 H, α-H Phe), 4.58 (m, 1 H, α-H Pro), 4.76 (m, 1 H, α-H Leu), 5.08 (d, $J = 12.2$ Hz, 1 H), 5.20 (d, $J = 12.2$ Hz, 1 H), 5.36 (bd, $J = 7.81$, 1 H, NH Phe), 6.81 (bd, $J = 8.7$ Hz, 1 H, NH Leu), 7.2−7.4 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.9 (OCH_2CH_3), 21.61, 23.28 (δ-C Leu), 24.58 (γ-C Pro), 24.79 (γ-C Leu), 28.19 (BOC), 28.90 (β-C Pro), 39.67 (β-C Phe), 40.06 (Azi), 40.72 (Azi), 41.57 (β-C Leu), 46.83 (δ-C Pro), 48.96 (α-C Leu), 57.11 (α-C Phe), 58.92 (α-C Pro), 62.51 (OCH_2), 66.91 (OCH_2-Ph), 79.48 (BOC), 126.67, 128.10, 128.30, 128.42, 128.52, 129.65, 135.47 (double peak), 154.71, 164.62, 165.97, 170.57, 171.51, 179.35. MS (CI, C_4H_9): *m*/*z* (%) = 706 (100) [M^+]. Anal. ($C_{38}H_{50}N_4O_9$) C,H; N: calcd, 7.93; found, 7.46.

***N*-{(2*R*,3*R*)-1-[[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridin-2-yl]carbonyl}-(*S*)-leucyl-(*S*)-proline Benzyl Ester (22b) (BOC-Phe-(EtO)-(*R*,*R*)-Azi-Leu-Pro-OBzl).** N-Acylation of **16b** (0.15 mmol, 69 mg) with BOC-Phe via the symmetric anhydride method gave, after purification by flash chromatography (cyclohexane/ethyl acetate, 1/1, $R_f = 0.7$), 60 mg (57%) of **22b**: mp 77 °C; $[\alpha]_D^{24.7} = -61.4°$ (*c* 0.3, EtOH). IR (CH_2Cl_2): 3413 (br), 1742, 1641 cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.9 (d, $J = 6.6$ Hz, 3 H), 0.96 (d, $J = 6.6$ Hz, 3 H), 1.26 (t, $J = 7.1$ Hz, 3 H), 1.36 (s, 9 H), 1.44−1.76 (m, 3 H, Leu), 1.88−2.1 (m, 3 H, Pro), 2.1−2.3 (m, 1 H, Pro), 3.0−3.24 (m, 2 H, Phe), 3.4 (d, $J = 1.96$, 1 H, Azi), 3.47 (d, $J = 1.96$ Hz, Azi), 3.58 (m, 1 H, Pro), 3.76 (m, 1 H, Pro), 4.2 (q, $J = 7.1$ Hz, 2 H), 4.50−4.66 (m, 2 H, α-H Phe, Pro), 4.74 (m, 1 H, α-H Leu), 5.0 (bd, $J = 9.0$ Hz, 1 H, NH Phe), 5.09 (d, $J = 12.2$ Hz, 1 H), 5.2 (d, $J = 12.2$ Hz, 1 H), 6.7 (bd, $J = 8.0$ Hz, 1 H, NH), 7.14−7.4 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.87, 21.68, 23.26, 24.55, 24.83, 28.16, 28.89, 38.42, 41.18, 41.31, 41.42, 46.81, 49.12, 56.25, 58.89, 62.50, 66.90, 79.89, 126.82, 128.10, 128.30, 128.43, 128.52, 129.56, 135.46, 136.13, 154.78, 164.88, 165.92, 170.57, 171.51, 179.52. MS (EI, 70 eV): *m*/*z* (%) = 706 (46) [M^+], 206 (100). Anal. ($C_{38}H_{50}N_4O_9$) C,H; N: calcd, 7.93; found, 7.40.

(2*S*,3*S*)- and (2*R*,3*R*)-2-Benzyl 3-Ethyl 1-[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (23a+b) (BOC-Phe-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OBzl). N-Acylation of **13a+b** (2 mmol, 498 mg) with BOC−Phe via the symmetric anhydride procedure gave, after column chromatography (cyclohexane/ethyl acetate, 2/1, $R_f = 0.65$) **23a+b** (**23a** (*S*,*S*)/**23b** (*R*,*R*) = 5/4) (850 mg, 86%) as a yellowish viscous liquid: $[\alpha]_D^{22.7} = -10.2°$ (*c* 0.71, EtOH). IR (ethyl acetate): 3377 (br), 1743, 1712, 1497, 1455 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.28 (t, $J = 7.1$ Hz, 3 H), 1.36 and 1.38 (s, together 9 H), 3.0−3.3 (m, 2 H, β-H Phe), 3.35 (S,S) and 3.49 (R,R)(d, $J = 2.2$ Hz, together 1 H, CH Azi), 3.40 (S,S) and 3.53 (R,R) (d, $J = 2.2$ Hz, together 1 H, CH Azi), 4.22 (q, $J = 7.1$ Hz, 2 H), 4.5 (S,S) and 4.63 (R,R) (m, together 1 H, α-H Phe), 4.93 (R,R) and 5.1 (S,S) (bd, $J = 9.0$ Hz, together 1 H, NH), 5.2 (m, 2 H), 7.2−7.45 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.75, 28.02, 28.06 (BOC), 38.09, 39.03, 39.61, 39.87, 39.93, 40.21 (β-C Phe, Azi), 55.97, 56.37 (α-C Phe), 62.31, 62.36, 67.91, 67.93 (OCH_2), 79.48, 79.64 (BOC), 126.57, 126.60, 128.14, 128.22, 128.30, 128.40, 128.43, 128.48, 128.52, 128.58, 129.43, 134.38, 134.41, 136.01, 136.11, 154.60, 154.75, 165.67, 165.74, 178.78, 178.99. MS (CI, C_4H_9): *m*/*z* (%) = 496 (75) [M^+], 405 (100). Anal. ($C_{27}H_{32}N_2O_7$) C,H,N.

Diethyl(2*S*,3*S*)-1-[*N*-(Benzyloxycarbonyl)glycylglycyl]-aziridine-2,3-dicarboxylate (24a) (Z-Gly-Gly-(*S*,*S*)-Azi-(OEt)$_2$. N-Acylation of **10a** (1.5 mmol, 280 mg) with Z-Gly-Gly by the general EEDQ procedure gave after column chromatography (ethyl acetate, $R_f = 0.64$) **24a** (320 mg, 49%) as a colorless viscous liquid: $[\alpha]_D^{24.9} = +15.8°$ (*c* 0.92, EtOH). IR (CH_2Cl_2): 3395 (br), 1737, 1532 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.2 (t, $J = 7.1$ Hz, 6 H), 3.38 (s, 2 H, Azi), 3.8−4.08 (m, 4 H, Gly), 4.15 (q, $J = 7.1$ Hz, 4 H), 5.02 (s, 2 H), 5.72 (bt, $J = 5.8$ Hz, 1 H, NH), 6.95 (bs, 1 H, NH), 7.25 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 13.93, 39.72, 43.71, 44.32, 62.77, 67.20, 128.06, 128.17, 128.50, 136.09, 156.52, 166.00, 169.06, 176.54. MS (EI, 70 eV): *m*/*z* (%) = 435 (28) [M^+], 188 (100). MALDI-TOF HRMS: calcd, 458.1539 [M + Na]$^+$; found, 458.16082 ± 0.00637. Anal. ($C_{20}H_{25}N_3O_8$) H,N; C: calcd, 55.17; found, 54.23.

Diethyl (2*S*,3*S*)-1-[*N*-(Benzyloxycarbonyl)-(*S*)-alanyl-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (25a) (Z-Ala-Phe-(*S*,*S*)-Azi-(OEt)$_2$. N-Acylation of **10a** (5 mmol, 935 mg) with Z-Ala-Phe by the general EEDQ procedure gave, after column chromatography (cyclohexane/ethyl acetate, 2/1, $R_f = 0.14$), **25a** (1.17 g, 42.8%) as a yellowish viscous liquid: $[\alpha]_D^{25.1} = +9.2$ (*c* 0.82, EtOH). IR (CH_2Cl_2): 3423 (br), 1738, 1523 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.22 (d, $J = 6.6$ Hz, 3 H, Ala), 1.28 (t, $J = 7.1$ Hz, 6 H), 3.06−3.34 (m, 2 H, Phe), 3.5 (s, 2 H, Azi), 4.18−4.3 (m, 5 H, α-H Ala, 2 OCH_2), 4.9−5.02 (m, 1 H, α-H Phe), 5.08 (d, $J = 12.2$ Hz, 1 H), 5.15 (d, $J = 12.2$ Hz, 1 H), 5.25 (bs, 1 H, NH Ala), 6.44 (bd, $J = 8.7$ Hz, 1 H, NH Phe), 7.16−7.4 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.91, 18.61, 37.77, 40.25, 50.48, 54.39, 62.56, 67.02, 126.98, 128.01, 128.43, 128.48, 129.52, 135.73, 136.12, 155.74, 165.87, 171.70, 178.46. MS (EI, 70 eV): *m*/*z* (%) = 539 (63) [M^+], 91 (100). Anal. ($C_{28}H_{33}N_3O_8$) C,H,N.

Diethyl (2*R*,3*R*)-1-[*N*-(Benzyloxycarbonyl)-(*S*)-alanyl-(*S*)-phenylalanyl]aziridine-2,3-dicarboxylate (25b) (Z-Ala-Phe-(*R*,*R*)-Azi-(OEt)$_2$. N-Acylation of **10b** (3.3 mmol, 617 mg) with Z-Ala-Phe by the general EEDQ procedure gave, after purification by column chromatography (cyclohexane/ethyl acetate, 3/1, $R_f = 0.15$), **25b** (410 mg, 23.1%): mp 57 °C; $[\alpha]_D^{25} = -35.78°$ (*c* 1.034, EtOH). IR (ethyl acetate): 3325 (Br), 1738, 1675, 1520, 1455 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.26 (d, $J = 6.6$ Hz, 3 H, Ala), 1.27 (t, $J = 7.1$ Hz, 6 H), 3.0−3.35 (m, 2 H, Phe), 3.5 (s, 2 H, Azi), 4.16−4.25 (m, 5 H, α-H Ala, 2 OCH_2), 4.90−5.0 (m, 1 H, α-H Phe), 5.05−5.2 (m, 3 H, OCH_2, NH Ala), 6.6 (bd, $J = 8.1$ Hz, 1 H, NH Phe), 7.1−7.4 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.98, 18.16, 37.81, 40.27, 50.56, 54.44, 62.58, 67.19, 127.00, 128.17, 128.28, 128.46, 128.57, 129.54, 135.73, 136.07, 155.80, 165.83, 171.56, 178.32. MS (CI, C_4H_{10}): *m*/*z* (%) = 540 (5) [$M^+ + 1$], 353 (100). Anal. ($C_{28}H_{33}N_3O_8$): C,H,N.

Diethyl (2*S*,3*S*)-1-[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylate (26a) (BOC-Phe-Ala-(*S*,*S*)-Azi-(OEt)$_2$. N-Acylation of **10a** (1.5 mmol, 280 mg) with BOC-Phe-Ala by the general EEDQ method yielded, after purification by column chromatography (cyclohexane/ethyl acetate, 3/1, $R_f = 0.15$), **26a** (580 mg, 77%): mp 99 °C; $[\alpha]_D^{23.8} = -4.7°$ (*c* 0.48, EtOH). IR (ethyl acetate): 3282 (br), 1734, 1446 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.25 (t, $J = 7.1$ Hz, 6 H), 1.35 (s, 9 H, BOC), 1.4 (d, $J = 6.6$ Hz, 3 H, Ala), 2.9−3.1 (m, 2 H, Phe), 3.4 (s, 2 H, Azi), 4.1−4.38 (m, 5 H, α-H Phe, 2 OCH_2), 4.5 (m, 1 H, α-H Ala), 4.96 (bs, 1 H, NH Phe), 6.7 (bd, $J = 7.8$ Hz, 1 H, NH Ala), 7.1−7.3 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 13.98 (OCH_2CH_3), 17.92 (β-C Ala), 28.15 (BOC), 39.75 (β-C Phe), 40.03 (Azi), 49.23 (α-C), 49.94 (α-C), 62.56 (OCH_2), 80.0 (BOC), 126.75, 128.47, 129.35, 136.54, 156.5 (BOC), 165.93, 170.34, 179.96. MS (CI, C_4H_9): *m*/*z* (%) = 505 (100) [M^+]. Anal. ($C_{25}H_{35}N_3O_8$) C,H; N: calcd, 8.31; found, 7.43.

(2*S*,3*S*)- and (2*R*,3*R*)-2-Benzyl 3-Ethyl 1-[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylate (27a+b) (BOC-Phe-Ala-(*S*,*S*+*R*,*R*)-Azi-(OEt)-(OBzl)). N-Acylation of **13a+b** (0.4 mmol, 100 mg) with BOC-Phe-Ala by the general EEDQ procedure gave, after column chromatography (cyclohexane/ethyl acetate, 3/1, $R_f = 0.13$), **27a+b** (150 mg, 66%) (1.3/1): mp 115 °C; $[\alpha]_D^{23.3} = -11.4°$ (*c* 0.37, EtOH). IR (ethyl acetate): 3305 (br), 1741, 1720, 1664, 1524, 1500, 1455 cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.2−1.45 (m, 15 H), 3.0−3.15 (m, 2 H), 3.4−3.55 (m, 2 H), 4.15−4.3 (m, 3 H, α-H Phe, OCH_2), 4.55 und 4.7 (m, together 1 H, α-H Ala), 4.9 (bs, 1 H, NH Phe), 5.2 (s, 2 H), 6.55 and 6.65 (bs, together 1

H, NH Ala), 7.1−7.3 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.9, 18.18, 26.90, 28.21 (BOC), 39.70, 39.99 (β-C Phe), 40.05, 40.23 (Azi), 49.51, 50.01 (α-C Phe), 62.54, 62.72 (OCH_2), 68.16, 68.29 (OCH_2Ph), 80.0 (BOC), 126.97, 128.63, 128.68, 128.72, 128.74, 128.81, 129.14, 129.37, 129.43, 134.46, 134.55, 136.35, 136.44, 156.0, 165.78, 165.82, 165.88, 165.95, 170.36, 170.61, 179.26, 179.96. MS (CI, C_4H_9): *m/z* (%) = 567 (62) [M^+], 476 (100). MALDI-TOF HRMS: calcd, 590.2478 [M + Na]$^+$; found, 590.2464 ± 0.0010. Anal. ($C_{30}H_{37}N_3O_8$) H,N; C: calcd, 63.48; found, 64.15.

(2*S*,3*S*)- and (2*R*,3*R*)-2-Benzyl 3-Ethyl 1-[*N*-(*tert*-Butoxycarbonyl)-(*S*)-leucylglycyl]aziridine-2,3-dicarboxylate (28a+b) (BOC-Leu-Gly-(*S*,*S*+*R*,*R*)-Azi-(OEt)(OBzl)). N-Acylation of **13a+b** (1.6 mmol, 398 mg) with BOC-Leu-Gly via the EEDQ method gave, after flash chromatography (cyclohexane/ethyl acetate, 3/1, R_f = 0.14), **28a+b** (1.5/1) (430 mg, 51.7%): mp 88 °C; $[\alpha]_D^{24.2} = -18.3°$ (*c* 0.59, EtOH). IR (ethyl acetate): 3338 (br), 1741, 1515, 1455 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.9 (d, J = 6.6 Hz, 3 H), 0.92 (d, J = 6.6 Hz, 3 H), 1.25 und 1.28 (t, J = 7.1 Hz, together 3 H), 1.45 (m, 11 H, BOC, β-H Leu), 1.6−1.8 (m, 1 H, γ-H Leu), 3.45−3.55 (m, 2 H, Azi), 3.95−4.10 (m, 1 H, 1 α-H Gly), 4.15−4.35 (m, 4 H, α-H Leu, 1 α-H Gly, OCH_2), 4.9 (bs, 1 H, NH Leu), 5.2 (s, 2 H), 6.7 (bs, 1 H, NH Gly), 7.2−7.4 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 13.94, 14.08 (OCH_2CH_3), 21.74, 22.97 (CH_3 Leu), 24.70 (γ-C Leu), 28.26 (BOC), 39.63, 39.84 (Azi), 41.28 (β-C Leu), 43.77 (α-C Gly), 53.02 (α-C Leu), 61.43, 62.73 (OCH_2), 68.31 (OCH_2-Ph), 80.0 (BOC), 126.98, 128.64, 128.72, 128.83, 129.12, 130.99, 134.36, 156.0 (BOC), 165.87 (double peak), 172.57, 176.45. MS (CI, C_4H_9): *m/z* (%) = 519 [M^+], 428 (100). Anal. ($C_{26}H_{37}N_3O_8$) C,H; N: calcd, 8.09; found, 7.64.

Diethyl (2*S*,3*S*)-1-[*N*-(Benzyloxycarbonyl)glycyl-(*S*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylate (29a) (Z-Gly-Phe-Ala-(*S*,*S*)-Azi-(OEt)$_2$). N-Acylation of **10a** (0.4 mmol, 74 mg) with Z-Gly-Phe-Ala using the general EEDQ procedure gave, after purification by column chromatography (cyclohexan/ethyl acetate, 3/1, R_f = 0.1), **29a** (110 mg, 46%): mp 89 °C; $[\alpha]_D^{24.6} = +6.3°$ (*c* 0.5, EtOH). IR (ethyl acetate): 3305 (br), 1738, 1656, 1455 cm^{-1}. ^{1} H NMR ($CDCl_3$): δ 1.0−1.4 (m, 9 H, β-H Ala, 2 CH_3), 3.08 (m, 2 H, β-H Phe), 3.45 (s, 2 H, Azi), 3.75−4.0 (m, 2 H, Gly), 4.2 (q, J = 7.1 Hz, 4 H, OCH_2), 4.6 (m, 1 H, α-H Ala), 4.8 (m, 1 H, α-H Phe), 5.1 (s, 2 H, OCH_2Ph), 5.7 (bs, 1 H, NH Gly), 6.75 (bd, J = 8.8 Hz, 1 H, NH Phe), 6.88 (bs, 1 H, NH Ala), 7.1−7.5 (m, 10 H). ^{13}C NMR ($CDCl_3$): δ 13.92 (CH_2CH_3), 17.84 (CH_3 Ala), 38.24 (β-C Phe), 40.10 (Azi), 44.66 (Gly), 49.41 (α-C Ala), 54.25 (α-C Phe), 62.56 (OCH_2), 67.22. (OCH_2Phe), 127.02, 128.08, 128.20, 128.50, 128.63, 129.29, 136.11, 136.23, 155.0, 166.08, 169.27, 170.02, 179.36. MS (CI, C_4H_{10}): *m/z* (%) = 596 (93) [M^+], 488 (100). Anal. ($C_{30}H_{36}N_4O_9$) C,H,N.

(2*S*,3*S*)- and (2*R*,3*R*)-1-[*N*-(*tert*-Butoxycarbonyl)-(*S*)-phenylalanyl]-3-(ethoxycarbonyl)aziridine-2-carboxylic Acid (30a+b) (BOC-Phe-(S,S+R,R)-Azi-(OEt)(OH)). Catalytic hydrogenolysis of **23a+b** by the general method described above gave, after recrystallization from acetonitrile, **30a+b** (1/1) (234 mg, 96%): mp 83 °C; $[\alpha]_D^{25.1} = -1.0°$ (*c* 0.49, EtOH). IR (ethyl acetate): 3376 (br), 1742, 1720, 1497, 1455 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.1−1.5 (m, 12 H), 3.1 (m) and 3.3 (m) (together 2 H, β-H Phe), 3.4−3.6 (m, 2 H, Azi), 4.23 (q, J = 7.1 Hz, 2 H), 4.4 (m) and 4.6 (m) (together 1 H, α-H Phe), 5.15 (bd, J = 8.8 Hz) and 5.3 (bd, J = 8.9 Hz) (together 1 H, NH), 7.0−7.4 (m, 5 H), 9.5 (bs, 1 H, COOH).^{13}C NMR ($CDCl_3$): δ 13.81 (CH_3), 27.98, 28.05 (BOC), 38.31 (double peak), 38.83, 39.19, 39.61, 40.66 (β-C Phe, Azi), 56.18 (α-C Phe), 62.17, 62.25 (OCH_2), 80.31 (BOC), 126.64, 126.83, 128.26, 129.09, 129.19, 129.41, 135.79, 135.99, 155.19 (double peak), 166.14 (double peak), 169.00 (double peak), 178.96, 179.60. MS (CI, C_4H_{10}): *m/z* (%) = 463 (4) [$M^+ + C_4H_9$], 407 (46) [$M^+ + 1$], 351 (100). Anal. ($C_{20}H_{26}N_2O_7$) H,N; C: calcd, 59.10; found, 60.30.

Kinetic Measurements. Kinetic measurements were carried out on a Pharmacia LKB Ultrospec III photometer and on a Perkin-Elmer LS-3B fluorescence spectrometer, respectively. The following assay buffers were used: papain/L-BAPA,[53] 50 mM phosphate buffer, pH 6.5, 5 mM EDTA, 5 mM cysteine; papain/Z-Phe-Arg-AMC[54] and cathepsin H/H-Arg-AMC,[55] 50 mM phosphate buffer, pH 6.5, 2.5 mM EDTA, 2.5 mM DTT, 0.005% Brij 35; cathepsin B[55] or cathepsin L/Z-Phe-Arg-AMC,[55−57] 50 mM citrate buffer, pH 6.0, 2.5 mM DTT, 5 mM EDTA, 200 mM NaCl, 0.005% Brij 35; calpain I or II/Suc-Leu-Tyr-AMC,[58] 50 mM TRIS buffer, pH 7.5, 5 mM $CaCl_2$, 2.5 mM EDTA, 2.5 mM DTT; trypsin/L-BAPA[59] or chymotrypsin/Ac-Phe-pNA,[60] 0.1 M phosphate buffer, pH 7.6; elastase/Ac-Ala-Ala-Ala-pNA,[61] 0.1 M TRIS buffer, pH 8.0; thermolysin/FAGLA,[62] 0.1 M TRIS buffer, pH 7.22, 10 mM $CaCl_2$; pepsin/Ac-Phe-3,5-diiodo-TyrOH,[63] 0.22 mM acetic acid. Assay buffers for the inactivation of papain at different pH values were 50 mM citrate for pH 4−6, phosphate for pH 6−8, and borate for pH 8−9. All kinetic experiments were done at 25 °C. Enzyme solutions were prepared fresh by incubating the enzymes in the individual reaction buffers at 25 °C for 30 min. Substrates and inhibitors were dissolved in DMSO, the final concentrations at measurements not being higher than 12% DMSO. Substrate solutions were prepared by diluting a stock solution with buffer, and inhibitor solutions were diluted with water. Substrate hydrolysis in assays using fluorogenic substrates was monitored by the increase of fluorescence at 460 nm (irradiation at 380 nm) and in assays using nitroanilide substrates by absorption increase at 405 nm. Thermolysin was assayed by absorption decrease at 345 nm. Pepsin was assayed by absorption increase at 570 nm. The inactivation rates (k_{obs}) for different inhibitor concentrations in the presence of the substrate were determined according to the continuous method of Tian and Tsou[64] by monitoring the product released from hydrolysis of the substrate in the presence of the inhibitor as a function of time (fluorescence or absorption = $A(1 - \exp(-k_{obs}t)) + B$) until complete inactivation of the enzyme (typically 5−60 min), with steady-state conditions established during inactivation time. This was done at constant enzyme and various (3−7) inhibitor concentrations, respectively. For weak inhibitors where complete inactivation took longer than 60 min, k_{obs} values were determined by the dilution assay of Kitz and Wilson.[65] Thereby enzyme and inhibitor were incubated. After an incubation time of 5−60 min (5−7 values), the incubation mixture was diluted by adding substrate and buffer and the residual enzyme activity [E] was measured. k_{obs} values were then calculated using the equation $[E] = [E_0]\exp(-k_{obs}t)$. These experiments were repeated for 3−7 inhibitor concentrations. Fitting of the k_{obs} values, obtained by either continuous or dilution assays, against the inhibitor concentrations to the hyperbolic equation $k_{obs} = k_i[I]/K_i^{app} + [I]$ gave the individual values of K_i^{app} and k_i. The K_i^{app} values were corrected to zero substrate concentration by the term $1 + [S]/K_m$ in the equation $K_i = K_i^{app}/(1 + [S]/K_m)$. The second-order rate constants $k_{2nd} = k_i/K_i$ were directly calculated from the individual constants. In cases where no saturation kinetics were achieved ($[I] < K_i$) due to limited solubility of the inhibitors or high K_i values, the second-order rate constants were calculated from linear regression to the equation $k_{obs} = k_{app}[I]$ and corrected to zero substrate concentration from the equation $k_{2nd} = k_{app}(1 + [S]/K_m)$. K_i values for the non-time-dependent inhibition of cathepsin H and calpains I and II were obtained by Dixon plots[66] using the equation $v_0/v_i = 1 + [I]/K_i^{app}$ and corrected to zero substrate concentration from $K_i = K_i^{app}/(1 + [S]/K_m)$. The following K_m values were used: papain/Z-Phe-Arg-AMC 0.09 mM, papain/L-BAPA 2.5 mM, cathepsin B/Z-Phe-Arg-AMC 0.15 mM, cathepsin L/Z-Phe-Arg-AMC 6.5 μM, cathepsin H/H-Arg-AMC 0.15 mM, calpain I/Suc-Leu-Tyr-AMC 0.4 mM, calpain II/Suc-Leu-Tyr-AMC 0.22 mM. Enzyme concentrations were: papain 0.48 μg mL^{-1}, cathepsin B 0.015 μg mL^{-1}, cathepsin L 0.05 μg mL^{-1}, cathepsin H 0.082 μg mL^{-1}, calpain I 3.8 μg mL^{-1}, calpain II 0.04 mg mL^{-1}, elastase 7.5 μg mL^{-1}, chymotrypsin 0.15 mg mL^{-1}, trypsin 3 μg mL^{-1}, thermolysin 8.9 μg mL^{-1}, pepsin 0.6 mg mL^{-1}. The kinetic constants were obtained by nonlinear or linear regression analysis using the program GraFit.[67]

Dialysis Experiments. Dialysis experiments were carried out as follows. Papain (0.52 mg mL^{-1}) was inactivated by **17a** (0.37 mM) and **11a** (0.13 mM), respectively (60 min incubation time, each) and then subjected to dialysis (visking dialysis tubing type 27/32, Serva) against reaction buffer (60 min, each). No recovery of enzyme activity (substrate: L-BAPA, 1.18 mM) was detected, whereas control enzymes maintained 70%

and 92% activity, respectively. Cathepsin L (0.07 μg mL^{-1}) was inactivated by **28a**+**b** (50 μM) (60 min incubation time) and then subjected to dialysis against reaction buffer (60 min). No recovery of enzymatic activity was detected, whereas the control enzyme maintained 52% activity.

Stoichiometry of Inactivation of Papain by 11a. A papain solution (2.76 μM, as determined by active site titration with E-64 as described in ref 55) was titrated with **11a** (three values, substrate: L-BAPA, 1.18 mM, pH 6.5) by adding increasing amounts of the inhibitor solution ([I] = 0.528 μM−1.056 mM) to the papain solution. After each aliquot of inhibitor was added, residual enzyme activity [E] was monitored. The intercept with the *x*-axis of a plot of [E] vs [I] gave the active site concentration; 1.11 ± 0.4 equiv of **11a** was required to totally inactivate 1 equiv of papain.

Standard inhibitors were *N*-ethylmaleimide[71] for the dilution assay and E-64[72] for the continuous assay, respectively.

Abbreviations. Amino acids are written in the three-letter codes and are L configured. Others: Azi (aziridine-2,3-dicarboxylic acid), Eps (epoxysuccinic acid), E-64 (1-[*N*-[[(L-3-*trans*-carboxyoxiran-2-yl)carbonyl]-L-leucyl]amino]-4-guanidinobutane), DPSI (diphenyl sulfimide), EEDQ (1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline), DPPA (diphenyl phosphorazidate), DCC (dicyclohexylcarbodiimide), DMAP (4-(dimethylamino)pyridine), PLE (pig liver esterase), CCL (*Candida* Cylindraceae lipase), PPL (porcine pancreatic lipase), LPR (lipase *Penicillium roquefortii*), LRA (lipase *Rhizopus arrhizus*), LAN (lipase *Aspergillus niger*), BPACK ((benzyloxycarbonyl)phenylalanyl-alanine chloromethyl ketone), BAPA (benzoylarginyl *p*-nitroanilide), AMC ((aminomethyl)coumarin), pNA (*p*-nitroanilide), Tris (tris(hydroxymethyl)aminomethane), FAGLA (3-(2-furyl)acryloyl-glycyl-leucine amide), DTT (dithiothreitol), EDTA (ethylenedinitrilotetraacetic acid), Suc (succinyl).

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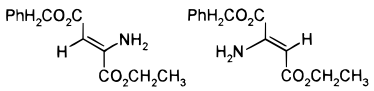
(70) Main product (2.5 g, 50%) of this reaction is a 2/1 mixture of enamines **31** and **32**, isolated by column chromatography (cyclohexane/ethyl acetate, 5/1, $R_f = 0.5$). IR (ethyl acetate): 3485 (br), 3350 (br), 3034 (br), 1729, 1678, 1621, 1556, 1498, 1455 cm^{-1}. ^{1}H NMR ($CDCl_3$, 50 °C): δ 1.3 (t, $J = 7.1$ Hz, **32**) and t (1.35, $J = 7.1$ Hz, **31**) (together 3 H), 4.2 (q, $J = 7.1$ Hz, **32**) and 4.35 (q, $J = 7.1$ Hz, **31**) (together 2 H), 5.2 (s, **31**) and 5.3 (s, **32**) (together 2 H), 5.58 (s, **32**) and 5.61 (s, **31**) (together 1 H), 6.5 (bs, 2 H), 7.2−7.45 (m, 5 H). ^{13}C NMR ($CDCl_3$): δ 14.23 (**31**), 14.63 (**32**) (CH_3), 59.78 (**32**), 62.65 (**31**) (OCH_2), 65.62 (**31**), 68.20 (**32**) (OCH_2Ph), 88.61 (**31**), 89.45 (**32**) (H*C*=C), 128.25, 128.41, 128.60, 128.76, 128.89, 128.93, 135.10, 136.82, 146.38 (**32**), 147.03 (**31**) (HC=*C*), 163.80 (**31**), 163.84 (**32**), 169.86 (**31**), 170.05 (**32**). Anal. ($C_{13}H_{15}NO_4$) C,H,N. Assignment of NMR signals was carried out by INEPT long-range NMR spectroscopy.



31 **32**

(71) A $k_{2nd} = 149 \pm 19$ M^{-1} min^{-1} was found for inhibition of papain by *N*-ethylmaleimide (Anderson, B.; Vasini, E. *Biochemistry* **1970**, *9*, 2248−3352: $k_{2nd} = 162$ M^{-1} min^{-1}).
(72) A $k_{2nd} = 3.4 \pm 0.5 \times 10^7$ M^{-1} min^{-1} was found for inhibition of papain by E-64 (ref 1: 2.2−3.8 × 10^7 M^{-1} min^{-1}).
(73) The following equation was used:[67] $k_{2nd} = (\text{limit} \times 10^{(\text{pH} - pK_{a1})})/(10^{(2\text{pH}-pk_{a1}-pk_{a2})} + 10^{(\text{pH}-pk_{a1})} + 1)$. The following values were found for **11a**: $pK_{a1} = 4.8 \pm 0.2$, $pK_{a2} = 3.8 \pm 0.2$, limit = 6043 M^{-1} s^{-1}.
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(76) Structures have been minimized using the Systematic Search program from Sybyl 6.4 (Tripos Associates Inc., St. Louis, MO, 1997). Energy minimization: Tripos force field, convergence criterion 0.001 kcal mol^{-1}. The superposition has been performed using the program Multifit from Sybyl 6.4.
(77) The docking of **26a** into the active site of papain has been performed using the program FlexiDock from the Sybyl 6.4 Biopolymer Module (Tripos Associates Inc., St. Louis, MO). Energy minimization: Tripos force field, Gasteiger-Hueckel charges on ligand, Kollmann charges on protein. As FlexiDock pocket a region of 0.4 nm around the amino acids Cys25, Tyr67, Pro68, Trp69, Val133, Val157, His159, Ala160, and Phe207 of papain has been defined. The structure of papain has been taken from the Brookhaven Protein Databank (entry 1pe6).

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