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Aziridide-Based Inhibitors of Cathepsin L: Synthesis, Inhibition Activity, and Docking Studies

Radim Vicik,[a] Matthias Busemann,[a, e] Christoph Gelhaus,[d] Nikolaus Stiefl,[a] Josef Scheiber,[a] Werner Schmitz,[b] Franziska Schulz,[a] Milena Mladenovic,[c] Bernd Engels,[c] Matthias Leippe,[d] Knut Baumann,[a] and Tanja Schirmeister*[a]

*A comprehensive screening of N-acylated aziridine (aziridide) based cysteine protease inhibitors containing either Boc-Leu-Caa (Caa = cyclic amino acid), Boc-Gly-Caa, or Boc-Phe-Ala attached to the aziridine nitrogen atom revealed Boc-(S)-Leu-(S)-Azy-(S,S)-Azi(OBn)$_2$ (**18a**) as a highly potent cathepsin L (CL) inhibitor (K_i = 13 nM) (Azy = aziridine-2-carboxylate, Azi = aziridine-2,3-dicarboxylate). Docking studies, which also accounted for the unusual bonding situations (the flexibility and hybridization of the aziridides) predict that the inhibitor adopts a Y shape and spans across the entire active site cleft, binding into both the non-primed and primed sites of CL.*

Introduction

Cysteine proteases are widespread among living organisms. The majority of cysteine proteases belong to the C1 family (papain family) of the clan CA.[1,2] This family consists of a broad variety of proteases such as plant proteases (such as papain), mammalian proteases (such as cathepsins B, H, L, S, C, K, O, F, V, X, and W),[3–5] and proteases of parasitic protozoans (such as rhodesain from *Trypanosoma b. rhodesiense*, cruzain from *Trypanosoma cruzi*, the falcipains of *Plasmodium falciparum*, as well as *Leishmania* proteases, which are referred to as CPB, CPA, and CPC enzymes).[6–9] Based on sequence similarity, CAC1 cysteine proteases have been divided into two subfamilies designated as cathepsin L-like (including mammalian cathepsins L, K, S, protozoan falcipains, rhodesain, cruzain, and *Leishmania* CPA and CPB proteases) and cathepsin B-like (including cathepsin B and *Leishmania* CPC proteases). According to functional aspects these proteases can also be divided into endo- and exopeptidases; the latter class can be further broken down into carboxy- and aminopeptidases. Whereas cathepsin L is a pure endopeptidase, cathepsin B additionally exhibits the activity of a carboxypeptidase, cleaving dipeptides from the C termini of substrate proteins (peptidyl dipeptidase).

From previous studies, two peptidic aziridide (N-acylated aziridine) based inhibitors that exhibit a certain selectivity for cathepsin L over cathepsin B (Table 1, compounds **VII**, **VIII**, and **IX**) were discovered in our research group.[10] These compounds contain either a Leu-Gly (in **VII** and **VIII**)[10] or a Gly-Pro (in **IX**)[11] sequence attached to the aziridine nitrogen atom. Furthermore, it is known that aziridine-2,3-dicarboxylates that contain a Boc-Phe-Ala sequence have superior inhibitory activity than those containing only Boc-Phe attached to the aziridine nitrogen atom (Table 1, compounds **IV** and **V** versus **I**).[12] Furthermore, it was shown that replacement of the ester groups by acids at the three-membered ring may lead to enhanced inhibitory potency (Table 1, compounds **II** and **III** versus **I**, compound **VI** versus **IV** and **V**, and compound **VIII** versus **VII**).[12] Based on these preliminary findings, it was the aim of this study to elucidate the essential features for improved inhibitory activity against cathepsin L.

Thus, we developed a series of aziridide-based cysteine protease inhibitors[13] that consist of either ethyl esters, benzyl esters, or acid groups at the three-membered ring, and an N-protected cyclic amino acid (Caa) or a dipeptide sequence (Boc-Gly-Caa, Boc-Leu-Caa, Boc-Phe-Ala) attached to the aziridine nitrogen atom (Figure 1). The specificity of inhibition with respect to the configurations of the amino acids and the aziri-

[a] *Dr. R. Vicik, Dr. M. Busemann, Dr. N. Stiefl, J. Scheiber, F. Schulz, PD Dr. K. Baumann, Prof. Dr. T. Schirmeister*
Institute of Pharmacy and Food Chemistry
Department of Pharmaceutical/Medicinal Chemistry
University of Würzburg, Am Hubland, 97074 Würzburg (Germany)
Fax: (+49) 931-888-5494
E-mail: schirmei@pzlc.uni-wuerzburg.de

[b] *Dr. W. Schmitz*
Theodor Boveri Institute
Department of Physiological Chemistry II
University of Würzburg, Am Hubland, 97074 Würzburg (Germany)

[c] *M. Mladenovic, Prof. Dr. B. Engels*
Institute of Organic Chemistry
University of Würzburg, Am Hubland, 97074 Würzburg (Germany)

[d] *Dr. C. Gelhaus, Prof. Dr. M. Leippe*
Zoological Institute, Department of Zoophysiology, Olshausenstrasse 40, University of Kiel, 24098 Kiel (Germany)

[e] *Dr. M. Busemann*
4 SC AG, 82152 Martinsried (Germany)

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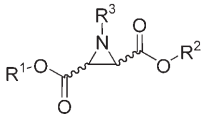
Table 1. Inhibitory activities of aziridinyl peptides containing natural *S*-configured amino acids.



Compd	Aziridine Config.	R^1	R^2	$R^{3[a]}$	Cathepsin L[b]		Cathepsin B[b]	
					K_i [μM]	k_{2nd} [$M^{-1}min^{-1}$]	K_i [μM]	k_{2nd} [$M^{-1}min^{-1}$]
I	*S,S*	Et	Et	Boc-Phe	nd	65	nd	125
II	*S,S*+*R,R*	H	Et	Boc-Phe	nd	188	680	1230
III	*S,S*+*R,R*	H	H	Boc-Phe	27	38081	nd	807
IV	*S,S*	Et	Et	Boc-Phe-Ala	110	469	nd	455
V	*S,S*+*R,R*	Bn	Et	Boc-Phe-Ala	nd	281	nd	443
VI	*S,S*+*R,R*	H	Et	Boc-Phe-Ala	nd	843	nd	676
VII	*S,S*+*R,R*	Bn	Et	Boc-Leu-Gly	9	3237	nd	240
VIII	*S,S*+*R,R*	H	Et	Boc-Leu-Gly	2 nd	13030 (CL *Pt*)[c] 10950 (CL h)[d]	81	766
IX	*S,S*+*R,R*	Bn	Et	Cbz-Gly-Pro	nd nd	10726 (CL *Pt*)[c] 10005 (CL h)[d]	nd	2615

[a] All amino acids are *S*-configured. [b] Values from Refs. [10] and [12]; nd: not determined. [c] CL *Pt*: cathepsin L from *P. tetraurelia*. [d] CL h: human liver cathepsin L.

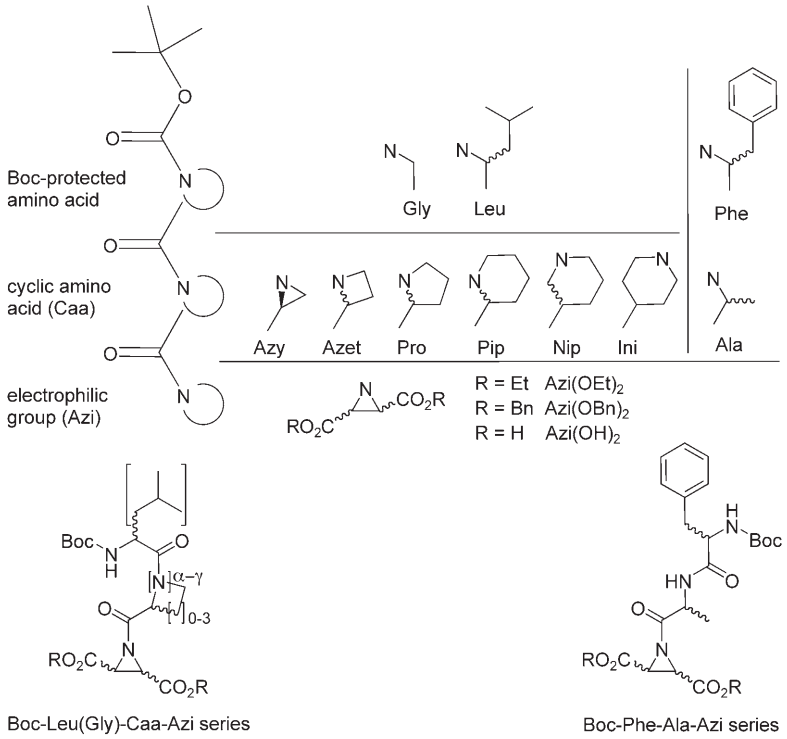


Figure 1. Structures of the Gly-Caa-, Leu-Caa-, and Phe-Ala-containing aziridinyl peptides.

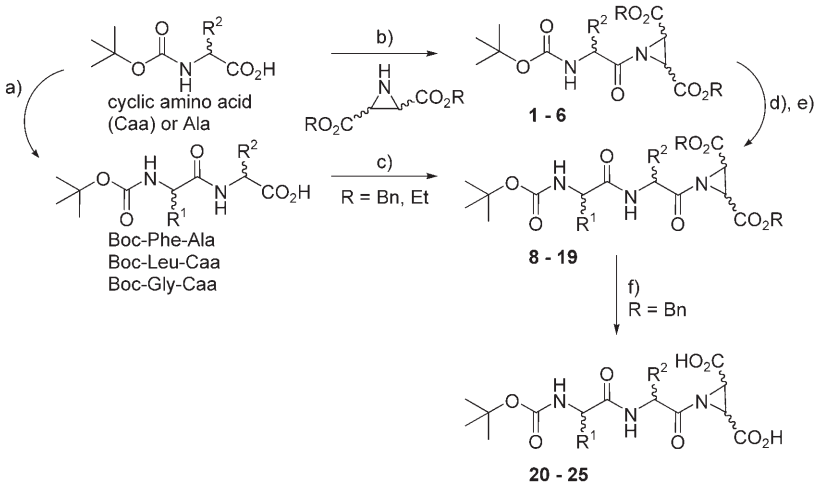
dine ring was investigated by including all possible stereoisomers. Thus, a series of proline-, proline-mimetic-, and Phe-Ala-containing aziridinyl peptides and peptidomimetics were synthesized.

The compounds were tested against cathepsin L (CL) and cathepsin B (CB), the prototypes of cathepsin L-like and cathepsin B-like cysteine proteases. The inhibition mechanism was clarified by using a biotinylated inhibitor. To elucidate the probable binding mode of the most active cathepsin L inhibitor, docking studies were performed with FlexX. As N-acylated aziridines represent nonplanar amides, quantum chemical calculations and various docking settings were used to find the method that best describes this unusual bonding situation.

Results and Discussion

Syntheses

The inhibitors[14] were prepared through successive coupling (Scheme 1: b, d, e) or fragment coupling (Scheme 1: c) of Boc-protected amino acids or dipeptides to the aziridine nitrogen atom of either diethyl or dibenzyl aziridine-2,3-dicarboxylate. The latter two compounds were prepared stereoselectively as published recently.[15] Dipeptides for fragment coupling were synthesized by using standard peptide coupling procedures (Scheme 1: a).[16] These dipeptides were coupled with the aziridine building block with the EEDQ procedure to give aziridinyl tripeptides (Scheme 1: c). Successive coupling was carried out by coupling the aziridine with the symmetric anhydrides of Boc-protected amino acids under DMAP catalysis (Scheme 1: b). In the cases of the β- and γ-amino acids Nip and Ini the DPPA procedure could be applied to acylate the aziridine nitro-



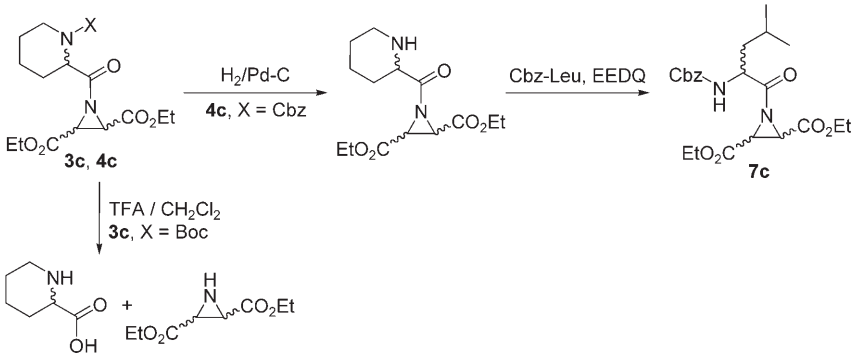
Scheme 1. Synthetic pathways to aziridinyl di- and tripeptides: a) for Boc-Phe-Ala: Boc-Phe-OSuc, Ala, NaOH, H_2O/THF; for Boc-Gly-Caa: Boc-Gly-NCA, CaaOBn, subsequently H_2/Pd-C; for Boc-Leu-Caa: Boc-Leu, DCC, HOBt, subsequently H_2/Pd-C; b) for Boc-Nip and Boc-Ini: DPPA, TEA (Method B); for all other amino acids: DCC, DMAP (Method A); c) EEDQ (Method C); d) TFA, CH_2Cl_2 (Method B); e) Boc-Xaa, DPPA, TEA (Method B); f) H_2/Pd-C (Method D).

gen atom with a Boc-protected amino acid. The resulting aziridinyl dipeptides were then deprotected by TFA (Scheme 1: d). Subsequent coupling with DPPA resulted in aziridinyl tripeptides (Scheme 1: e). The N-acylated aziridine-2,3-dicarboxylic acids were prepared by hydrogenolysis of the corresponding dibenzyl esters (Scheme 1: f).

During the synthesis of pipecolic acid (Pip) containing aziridinyl tripeptides, cleavage of the aziridide bond of the aziridinyl peptides (such as **3c**) was observed if TFA was used for Boc deprotection (Scheme 2). Therefore, the corresponding Cbz-protected aziridinyl peptide **4c** was used to allow the more gentle hydrogenolytic cleavage of the Cbz group. The deprotected aziridinyl dipeptide, which was obtained in 60% yield, was then directly coupled with Cbz-Leu by using the EEDQ method. However, the sole product of this reaction was the aziridine N-acylated with Cbz-Leu (compound **7c**) which suggests that the deprotected Pip-containing aziridinyl peptide is unstable (Scheme 2). Fragment coupling of the aziridine-2,3-dicarboxylate with Boc-Leu-Pip or Boc-Gly-Pip was successful with the glycine-containing dipeptide only, leading to compound **15a**.

(*S*)-Benzyl aziridine-2-carboxylate (AzyOBn) was prepared starting from (*S*)-Ser according to reference [17]. Azetidine-2-carboxylic acid (Azet) was synthesized as a racemate starting from γ-butyrolactone, as described in reference [18]. Cbz-protected Azet was used for the synthesis of aziridinyl dipeptide Cbz-Azet-Azi(OEt)$_2$ **1c+d**; the benzyl ester AzetOBn was used for synthesis of the dipeptide Boc-Leu-AzetOH, which was then coupled to the respective aziridines leading to the aziridinyl tripeptides Boc-Leu-Azet-Azi(OEt)$_2$ **11c+d** and Boc-Leu-Azet-Azi(OBn)$_2$ **12a+b**. Racemic nipecotic acid benzyl ester was prepared by hydrogenation of nicotic acid, Boc protection, esterification with benzyl alcohol, and finally deprotection with TFA. As the cyclic amino acids Azet, Pip, and Nip were used as racemates for coupling with the enantiomerically pure aziridines, the aziridinyl di- or tripeptides were obtained as diastereomeric mixtures. In three cases (Pip-containing aziridinyl dipeptides **3a** and **3b**, **3c** and **3d**, and **4c** and **4d**),[19] the single diastereomers could be separated by preparative-scale column chromatography. Coupling of racemic Boc-Gly-(*R*+*S*)-Pip with (*S*,*S*)-Azi(OBn)$_2$ yielded the homochiral diastereomer (Boc-Gly-(*S*)-Pip-(*S*,*S*)-Azi(OBn)$_2$, **15a**) exclusively.[19]

Table 2 summarizes the synthesized aziridinyl tripeptides containing a cyclic amino acid (Caa) and the aziridine building block as diethyl or dibenzyl dicarboxylate (the structures of aziridinyl dipeptides **1**–**7** are summarized in the Supporting Information). Phe-Ala-containing aziridinyl tripeptides are summarized in Table 3. Aziridinyl tripeptides containing cyclic amino acids and the free aziridine-2,3-dicarboxylic acids are shown in Table 4. The tables also contain the dissociation constants K_i determined with cathepsins L and B.



Scheme 2. Reactions of pipecolic acid (Pip) containing aziridinyl dipeptides. Reaction of Boc-protected dipeptide **3c** with TFA yields Pip aziridine-2,3-dicarboxylate (Azi). Deprotection of the Cbz-protected dipeptide **4c** with H_2/Pd-C yields the deprotected compound as intermediate. This dipeptide, however, is unstable, and coupling with Cbz-Leu results in the Cbz-Leu-containing dipeptide **7c** as sole product.

Enzyme inhibition and elucidation of inhibition mechanism

All inhibitors were tested in fluorimetric microplate assays on the CAC1 proteases cathepsin L and B using the substrate Cbz-Phe-Arg-AMC. Aziridines are electrophilic building blocks susceptible to nucleophilic ring opening. Hence, the enzymes should be inhibited irreversibly, in which case time-dependent inhibition would be expected. However, in most cases only time-

Table 2. Inhibition of CL and CB by aziridinyl tripeptides (Boc-Xaa-Caa-Azi(OR)$_2$) containing cyclic amino acids.

Compd[14]	Xaa	Caa	Aziridine Config.	R	Synthetic Method	CL K_i [μM]	CB K_i [μM]
8a	(*S*)-Leu	(*S*)-Pro	*S,S*	Et	S.C.[a]	7.7 ± 1.3	ni
8c	(*S*)-Leu	(*S*)-Pro	*R,R*	Et	S.C.	ni[c]	ni
9a+b	(*S*)-Leu	(*R*+*S*)-Nip	*S,S*	Et	S.C.	2.4 ± 0.6[d]	24.0 ± 4.1
9c+d	(*S*)-Leu	(*R*+*S*)-Nip	*R,R*	Et	S.C.	5.9 ± 0.8	210 ± 12
10a	(*S*)-Leu	Ini	*S,S*	Et	S.C.	ni	ni
10c	(*S*)-Leu	Ini	*R,R*	Et	S.C.	ni	ni
11c+d	(*S*)-Leu	(*R*+*S*)-Azet	*R,R*	Et	F.C.[b]	4.8 ± 0.3	ni
12a+b	(*S*)-Leu	(*R*+*S*)-Azet	*S,S*	Bn	F.C.	3.8 ± 0.2	ni
13a	(*S*)-Leu	(*S*)-Pro	*S,S*	Bn	F.C.	ni	ni
13b	(*S*)-Leu	(*R*)-Pro	*S,S*	Bn	F.C.	6.0 ± 0.8	ni
13c	(*S*)-Leu	(*S*)-Pro	*R,R*	Bn	F.C.	0.4 ± 0.2[d]	115 ± 18
13e	(*R*)-Leu	(*S*)-Pro	*S,S*	Bn	F.C.	4.0 ± 0.2	ni
13f	(*R*)-Leu	(*R*)-Pro	*S,S*	Bn	F.C.	ni	ni
14a	Gly	(*S*)-Pro	*S,S*	Bn	F.C.	15.8 ± 1.7	43.0 ± 1.9
14b	Gly	(*R*)-Pro	*S,S*	Bn	F.C.	7.1 ± 0.4	32.6 ± 1.4
15a	Gly	(*S*)-Pip	*S,S*	Bn	F.C.	6.4 ± 0.6	ni
16a+b	(*S*)-Leu	(*R*+*S*)-Nip	*S,S*	Bn	F.C.	4.4 ± 0.4	ni
16e+f	(*R*)-Leu	(*R*+*S*)-Nip	*S,S*	Bn	F.C.	4.2 ± 0.4	ni
17a+b	Gly	(*R*+*S*)-Nip	*S,S*	Bn	F.C.	5.8 ± 0.1[d]	ni
18a	(*S*)-Leu	(*S*)-Azy	*S,S*	Bn	F.C.	0.013 ± 0.001	9.4 ± 1.1[d]
18e	(*R*)-Leu	(*S*)-Azy	*S,S*	Bn	F.C.	6.4 ± 0.4	ni

[a] S.C.: successive peptide coupling, Methods A and B described in Experimental Section. [b] F.C.: fragment coupling, Method C described in Experimental Section. [c] ni: No inhibition at [inhibitor] = 140 μM. [d] Time-dependent inhibition, **13c**: $k_i = 0.01 \pm 0.001$ min^{-1}, $k_{2nd} = 26\,115$ M^{-1}min^{-1}; **17a+b**: $k_i = 0.007 \pm 0.001$ min^{-1}, $k_{2nd} = 1\,145$ M^{-1}min^{-1}; **9a+b**: $k_i = 0.02 \pm 0.001$ min^{-1}, $k_{2nd} = 833$ M^{-1}min^{-1}; **18a**: $k_i = 0.02 \pm 0.001$ min^{-1}, $k_{2nd} = 1\,915$ M^{-1}min^{-1}.

showed time-independent inhibition of cathepsin L and the related CAC1 cysteine protease falcipain 2 of the malaria parasite *Plasmodium falciparum*.[20,24] However, it was shown by 1) dialysis experiments, 2) detection of plasmodial cysteine proteases by affinity labeling after 2D gel electrophoresis and Western blotting, and 3) identification of labeled plasmodial cysteine proteases by mass spectrometry that the inhibition of the cysteine proteases was irreversible.[20,24] Owing to the fact that the biotin group is easily detectable, compound **26** is an optimal model compound for affinity labeling, representing N-acylated aziridine-2,3-dicarboxylates. To evaluate whether the inhibition by aziridine-2,3-dicarboxylates is active-site directed, affinity labeling assays and competition experiments on cathepsin L were performed using this model compound

Table 3. Inhibition of CL and CB by Boc-Phe-Ala-containing aziridinyl tripeptides (Boc-Phe-Ala-(*S*,*S*)-Azi(OR)$_2$).

Compd[14]	Phe Config.	Ala Config.	R	CL K_i [μM]	CB K_i [μM]
19a	*S*	*S*	Bn	16.6 ± 3.9	170 ± 19
19b	*S*	*R*	Bn	15.2 ± 2.1	190 ± 13
19e	*R*	*S*	Bn	14.7 ± 1.1	ni
19f	*R*	*R*	Bn	21.6 ± 4.1	250 ± 12
20a	*S*	*S*	H	15.3 ± 1.0	18.3 ± 0.7[b]
20b	*S*	*R*	H	17.8 ± 0.9	106 ± 10
20e	*R*	*S*	H	ni[a]	160 ± 12
20f	*R*	*R*	H	26.0 ± 1.9	174 ± 10

[a] ni: No inhibition at [I] = 140 μM. [b] Time-dependent inhibition, **20a**: $k_i = 0.027 \pm 0.001$ min^{-1}, $k_{2nd} = 1\,475$ M^{-1}min^{-1}.

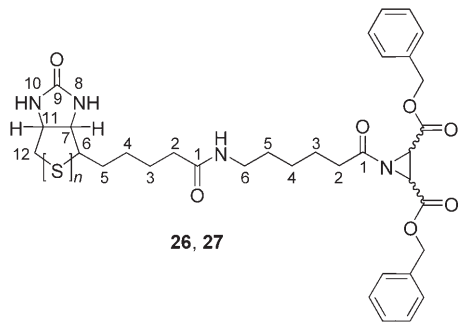
Table 4. Inhibition of CL and CB by tripeptides containing aziridine-2,3-dicarboxylic acid (Boc-Xaa-Caa-(*S*,*S*)-Azi(OH)$_2$).

Compd[14]	Xaa	Caa	CL K_i [μM]	CB K_i [μM]
21a	(*S*)-Leu	(*S*)-Pro	18.7 ± 1.2	139 ± 12
22a	Gly	(*S*)-Pro	15.9 ± 1.9	88.3 ± 6.5[a]
22b	Gly	(*R*)-Pro	ni	ni
23a+b	(*S*)-Leu	(*R*+*S*)-Nip	10.1 ± 0.6	128 ± 8
23e+f	(*R*)-Leu	(*R*+*S*)-Nip	52.9 ± 4.3	134 ± 16[a]
24a+b	Gly	(*R*+*S*)-Nip	ni	ni
25a	(*S*)-Leu	Ini	17.8 ± 0.8	ni

[a] Time-dependent inhibition, **21a**: $k_i = 0.058$ min^{-1}, $k_{2nd} = 417$ M^{-1}min^{-1}; **22a**: $k_i = 0.028$ min^{-1}; $k_{2nd} = 317$ M^{-1}min^{-1}; **23e+f**: $k_i = 0.054$ min^{-1}, $k_{2nd} = 408$ M^{-1}min^{-1}. [b] ni: No inhibition at [inhibitor] = 140 μM.

independent inhibition was observed, which is consistent with previously published work with aziridine-containing inhibitors.[20,13] In these cases only the dissociation constants K_i (Tables 2–4) could be determined instead of the second-order rate constants $k_{2nd} = k_i/K_i$. From previous experimental[21] and theoretical[22,23] studies, it is well known that aziridines are far less reactive toward ring opening by thiolates than the corresponding epoxides. As a consequence, the k_i values are very low. This fact is also corroborated by the present results which revealed k_i values between 0.007 and 0.058 min^{-1}.

Previous studies with the biotinylated aziridine-2,3-dicarboxylate (**26**, termed bADA in reference [24], Figure 2, Table 5) also



26, **27**

Figure 2. Biotinylated **26** ($n = 1$) and desthiobiotinylated **27** ($n = 0$) aziridine-2,3-dicarboxylates as affinity labels for cysteine proteases. Numbers are referred to in the NMR data.

Table 5. Inhibition of cysteine proteases by affinity labeling aziridine-2,3-dicarboxylates.

Compd[a]	*n*	Aziridine Config.	CL K_i [μM]	CB K_i [μM]	Papain K_i [μM]
26	1	*S,S*	1.4 ± 0.1[20]	50.5 ± 6.0[b]	5.8 ± 0.9
27	0	*S,S* + *R,R*	7.9 ± 2.7	nd[c]	3.8 ± 0.2

[a] For structural details of the compounds see Figure 2. [b] Time-dependent inhibition, $k_i = 0.036 \pm 0.002\ min^{-1}$, $k_{2nd} = 720\ M^{-1}\ min^{-1}$. [c] nd: Not determined.

and E-64, a commonly used irreversible inhibitor that is specific for the active site of cysteine proteases.[25] In addition, the corresponding desthiobiotinylated inhibitor **27** was tested (Figure 2, Table 5) which is supposed to exhibit a weaker binding affinity to streptavidin.[26] The results of these assays are shown in Figures 3 and 4.

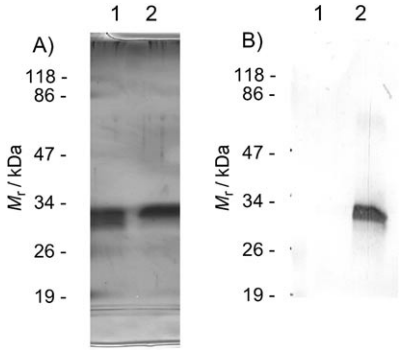


Figure 3. Affinity labeling of cathepsin L by the biotinylated model compound **26**. Cathepsin L was incubated with compound **26** (lanes 2). Untreated cathepsin L served as a control (lanes 1). Samples were subjected to SDS-PAGE and stained with A) silver nitrate or B) electrotransferred to a membrane for affinity labeling using a streptavidin–alkaline phosphatase conjugate. Standards M_r values are indicated at left.

Figure 3 shows that compound **26** binds to cathepsin L irreversibly. The resulting complex was detectable on Western blots after SDS-PAGE. The biotin group of compound **26** is accessible for alkaline-phosphatase-conjugated streptavidin, leading to a strong labeling of enzyme–inhibitor complexes on the blotting membrane. Accordingly, untreated cathepsin L does not result in any label on the membrane. As shown in the competition experiment (Figure 4), the binding of compounds **26** and **27** occurs specifically at the active site of the enzyme. Cathepsin L was only detected by affinity labeling when incubated with compound **26** or **27** prior to adding the inhibitor E-64 (Figure 4 A). These results clearly show that cathepsin L is inactivated irreversibly despite the observed time-independent inhibition. The inhibitors are directed against the active site of the enzyme as they do compete with E-64.

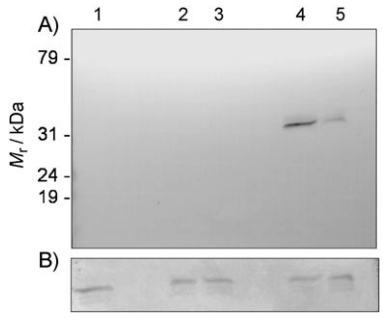


Figure 4. Competitive binding of compounds **26** and **27** and E-64 to cathepsin L. Cathepsin L (CL) was incubated with E-64 alone (lane 1) at 22 °C for 10 min and subsequently incubated with compound **26** (lane 2) or compound **27** (lane 3) at 22 °C for 30 min. In addition, cathepsin L was incubated first with the compounds **26** (lane 4) or **27** (lane 5) and then incubated with E-64. Samples were subjected to SDS-PAGE and A) electrotransferred on a PVDF membrane for affinity labeling using streptavidin–alkaline phosphatase conjugate or B) stained with silver nitrate. Note that the enzyme was labeled only when the compounds **26** or **27** were present prior to E-64 treatment (lanes 4 and 5).

Inhibition activity

Strikingly, most aziridinyl dipeptides are inactive or only weakly active on both CL and CB (see Supporting Information). The only exception is the Cbz-Leu-containing compound **7c**, which exhibits a K_i value of 0.3 μM for CL. With the exception of tripeptide **20a**, which is equipotent toward both enzymes (K_i = 15.3 μM (CL), 18.3 μM (CB); Table 3), all active compounds are selective CL inhibitors. Several compounds that are inactive toward CB up to concentrations of 140 μM possess affinities for CL in the low micromolar range ($K_i < 10$ μM). These include the Cbz-Leu-containing dipeptide **7c** ($K_i = 0.3$ μM) and some of the Leu(Gly)-Caa-containing tripeptides shown in Table 2 (**8a**, **11c+d**, **12a+b**, **13b**, **15a**, **16a+b**, **16e+f**, **17a+b**, and **18e**). Interestingly, the Ini-containing diesters **10a** and **10c** are inactive, and the corresponding diacid **25a** is only moderately active toward CL ($K_i = 17.8$ μM). Most of the Phe-Ala-containing inhibitors also exhibit some activity toward CB which means that these compounds are not selective (Table 3). This behavior is in agreement with previous findings.[10] Within this series, the dibenzyl esters (compounds **19**) show an approximate tenfold selectivity in favor of CL. A slight improvement in inhibitory potency against CB is found when the diesters **19** are converted into diacids **20**; compound **20a** is the most active CB inhibitor ($K_i = 18.3$ μM), as it is tenfold more potent than its dibenzyl ester counterpart **19a**. Within the other series, only the diacids Boc-(*S*)-Leu-(*S*)-Pro-(*S,S*)-Azi(OH)$_2$ (**21a**) and (*R*)-Leu-(*R*+*S*)-Nip-(*S,S*)-Azi(OH)$_2$ (**23e+f**) are better inhibitors than their corresponding diesters (**8a**, **13a**; **16e+f**). This again holds true only for CB, and not for CL. In all other cases, the acid groups do not enhance inhibitory activity (**17a+b** versus **24a+b**, **14a** versus **22a**, **14b** versus **22b**, **9a+b** and **16a+b** versus **23a+b**). No general trend can be found for CL with respect to the different ester moieties (ethyl versus benzyl: **8a** versus **13a**, **8c** versus **13c**, **9a+b** versus **16a+b**). For CB the differences between the diethyl esters **9a+b** and their corresponding dibenzyl esters **16a+b** (Table 2) are remarkable.

Whereas the diethyl esters are moderately active, the dibenzyl esters no longer exhibit inhibitory potency against CB. On the other hand, both esters are equipotent against CL.

Three compound pairs differ only in the configuration of the aziridine ring: **8a** versus **8c**, **9a+b** versus **9c+d**, and **13a** versus **13c**). In accordance with previous results,[10] the *S*,*S*-configured diethyl esters **8a** and **9a+b** are superior to their *R*,*R*-configured diastereomeric counterparts. Unexpectedly, and in contrast to this, the dibenzyl ester **13c**, with an *R*,*R*-configured aziridine moiety is far more active than its diastereomer **13a**. This dibenzyl ester (**13c**, Boc-(*S*)-Leu-(*S*)-Pro-(*R*,*R*)-Azi(OBn)$_2$, K_i = 0.4 μM) is the most active compound within the Leu-Pro and Gly-Pro series which is in agreement with the results obtained for the cathepsin L-like protease rhodesain (K_i = 0.5 μM).[13]

All Nip and Azet-containing inhibitors were tested as diastereomeric mixtures. Most of these compounds (**9a+b**, **9c+d**, **16a+b**, **16e+f**, **23a+b**, **23e+f**, **11c+d**, **12a+b**, **17a+b**, and **23a+b**) have dissociation constants in the low micromolar range, possibly because of a relatively high activity of one of the diastereomers.

The striking aspect of these results is the fact that most of the X-Leu(Gly)-Caa-containing aziridinyl tripeptides are highly CL-selective, whereas the Boc-Phe-Ala-containing derivatives are not. In contrast to cathepsin L, inhibition of cathepsin B is improved when diesters are replaced by diacids.

The most potent compound is the Azy-containing inhibitor **18a**, with K_i = 13 nM toward CL and 9.4 μM toward CB which gives a CL/CB selectivity of > 700. This compound is also one of the most potent inhibitors of the CL-like parasite proteases rhodesain (K_i = 0.3 μM),[13] and falcipains 2 and 3.[27] With the aziridine-2-carboxylate moiety, (Azy) this compound not only possesses the smallest heterocycle of the Caa series leading to a particular conformation, but also possesses a second electrophilic center which can be attacked by the active site Cys residue. The diastereomeric inhibitor **18e**, which contains (*R*)-Leu, is about 500-fold less potent against cathepsin L and does not inhibit cathepsin B.

Theoretical studies, investigation of binding modes

Because a covalent and irreversible inhibition mechanism was elucidated, theoretical studies to investigate enzyme–inhibitor interactions have to take into account both noncovalent complex formation between enzyme and inhibitor (characterized by K_i) and nucleophilic ring opening by the active site cysteine residue (characterized by k_i). The latter step can only be treated accurately by using quantum chemical methods. However, the first step, that is, the formation of the noncovalent complex, can be addressed by docking methods. Hence, the docking tool FlexX[28,29] was used to investigate the binding mode of the most active CL inhibitor **18a**. In addition, the probable binding mode of the less active diastereomer **18e** was investigated.

The structure of CL (PDB code: 1MHW[30]) was taken from published X-ray crystallographic structures. As no 3D structure is published yet for the commercially available CL from *Paramecium tetraurelia*,[31] the X-ray crystallographic structure of human CL[30] was used for docking. This was done because previous studies showed that the inhibitors are equipotent toward both enzymes (see also Table 1).[12] Moreover, a sequence alignment between human and *Paramecium* CL forms showed high sequence homology with a difference of five residues in the binding site (see Supporting Information).[32]

It is reasonable to assume that the initial complex formed between enzyme and inhibitor is driven mainly by noncovalent interactions. Ring opening and the formation of the covalent bond can only occur if the aziridine ring of the inhibitor is directed close to the sulfur atom of the active site cysteine residue by the remainder of the molecule. Hence, to check whether the inhibitor is placed in the active site in a way that allows reaction with the enzyme, the ring-closed compounds were docked into the binding site. Furthermore, the influence of the Cys 25 sulfur atom was removed to check whether the aziridine ring could approach this atom reasonably close (a validation of this procedure can be found in the Supporting Information). In this way, a simulation of the pre-transition state was attempted. With standard FlexX settings, the bond between the aziridine nitrogen atom and the carbonyl carbon atom in the N-acylated aziridine is rebuilt with a dihedral angle of 180°. However, it is known that N-acylated aziridines (aziridides) do not possess a planar amide structure, but a tilted one[33] that mirrors the partial sp^3 character of the aziridine nitrogen atom. For estimating the dihedral and the out-of-plane angles, quantum chemical calculations on several N-acylated model aziridines were carried out (see Supporting Information). To gain insight into the barriers between the particular structures (planar versus tilted), calculations of the flexibility of the aziridide bond were performed.

Independent of the substituents at the carbonyl group, a dihedral angle of about 115° and an out-of-plane angle of about 50° were found. The barrier between the tilted (dihedral $\measuredangle(C^1–C^2–N–C) = 115°$, for which C^1 and C^2 denote the carbon atoms of the aziridine ring) and planar (dihedral $\measuredangle(C–C–N–C) = 180°$) conformation was predicted to be only 4 kcal mol^{-1}. This energy could easily be compensated for by binding of the inhibitor to the enzyme through the formation of two hydrogen bonds, for example. In contrast to this, the barrier in an N-alkylated model aziridine (*N*-methyl, $\measuredangle(C–C–N–C) = 107°$) was predicted to be 17 kcal mol^{-1}. Both the magnitude of the dihedral angles and the low barriers between tilted and planar conformation in aziridides reflects the hybridization of the aziridine nitrogen atom as partially sp^2 and partially sp^3. This shows that the docking results determined with standard settings, where the aziridide bonds are planar, are not necessarily wrong but could as well reflect reasonable binding conformations. Hence, both geometries (tilted and planar) were docked. In addition to that, sp^2 and sp^3 hybridization parameter settings for the aziridine nitrogen atoms were examined, as it is not a priori clear which combination would yield the most sensible results.[34] The necessary changes to the docking methods are given in the Experimental Section. Because **18a** and **18e** contain a second N-acylated aziridine (Azy) which is substituted only on one ring carbon atom, two diastereomeric structures are possible (**18a**$_R$ and **18a**$_S$ designated according to the con-

figuration of the Azy nitrogen atom). Again, both diastereomers were docked to take into account the flexibility of the Azy aziridide as shown by the quantum chemical calculations.

Results of the docking studies are shown in Figure 5. The results of the docking for the planar aziridide structure of **18a** (**18a**$_{planar}$) (obtained by using sp^2 hybridization parameter settings at both aziridine nitrogen atoms) and for the tilted aziridide structure of **18e** (obtained by using sp^3 hybridization parameter settings for both aziridine nitrogen atoms) can be found in the Supporting Information. The interaction pattern between inhibitor and protein, as well as the distance between the aziridine carbon atoms and the active site sulfur atom (the position of the Cys S atom was taken from the X-ray crystallographic structure PDB code: 1MHW) were determined for the docked compounds (for a detailed description, see Supporting Information).

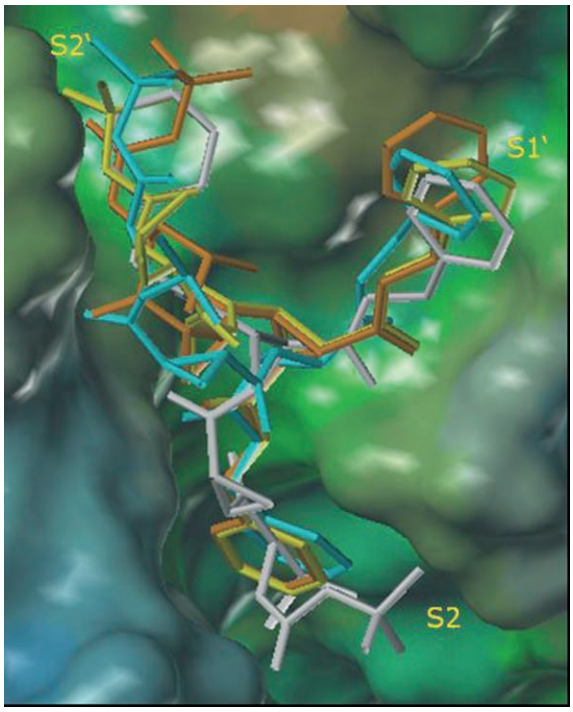


Figure 5. Overlay of docking results for the noncovalent pre-transition state of the binding of inhibitor **18a** to CL. yellow: **18a**$_R$ (N: sp^3), orange: **18a**$_S$ (N: sp^3), cyan: **18a**$_R$ (N: sp^2), white: **18a**$_S$ (N: sp^2). The surface of the protein is colored according to its molecular lipophilic potential (blue: hydrophilic, green: intermediate, brown: lipophilic). N: sp^3 means that sp^3 parameter settings were applied in FlexX; N: sp^2 means that sp^2 parameter settings were applied in FlexX.

Independent of the configuration of the inhibitor (**18a**$_{R/S/planar}$) and independent of the parameterization of the aziridine nitrogen atom with respect to the hybridization state, a binding mode in which the inhibitor is predicted to bind into both the primed and nonprimed substrate binding sites is found for the interaction of **18a** with CL (Figure 5). Either one benzyl ester or the isobutyl group of the N-terminal Boc-Leu residue is located in the hydrophobic S2 pocket, which consists of the amino acids Met 70, Ala 135, Ala 214, and Met 161. The second benzyl ester binds into the S1′ pocket (Ala 138, Asp 162, His 163, and Trp 189). If the planar structure is docked, the Boc group binds into the S1′ pocket. The Boc-Leu-Azy sequence interacts with the S2′ pocket (Gln 19, Gly 20, Gln 21, Cys 22, and Gly 23) through H bonds to Gln 19 (side chain NH_2 group), Gly 20 (backbone C=O), or Cys 22 (backbone NH). Among the different configurations of **18a** (**18a**$_{S/R/planar}$), **18a**$_S$ seems to be most plausible. This binding mode is stabilized by a large number of H bonds between enzyme and ligand (seven H bonds, mainly to residues in the S2′ pocket, namely Gln 19, Gly 20, and Cys 22) and hydrophobic interactions between the ligand OBn moieties and Met 70 (S2), Trp 189, and Ala 138 (S1′). According to the different docking results, it is not possible to predict which aziridine ring carbon atom will most likely be attacked by the active site Cys sulfur atom. Based on the results obtained for **18a**$_S$ (N: sp^3) it is most likely to be one of the Azi carbon atoms (the distances between the Cys S atom and the aziridine ring carbon atoms are: Azi–C^2, 3.38 Å; Azi–C^3, 4.85 Å; Azy–C^2, 5.13 Å; Azy–C^3, 4.88 Å; for a detailed analysis of all docking poses obtained, see Supporting Information). However, according to the results obtained with the sp^2 hybridization parameter setting (N: sp^2) it is most likely the Azy–C^2 carbon atom (distance from Cys S: 2.44 Å).[35]

The docking results obtained with **18e**, which only differs from **18a** in the configuration of the N-terminal Leu residue, also predict binding into primed and nonprimed sites of CL (benzyl into S1′, second benzyl into S2, Boc-Leu-Azy into S2′, see Supporting Information). However, a comparison of the binding mode of **18e** with **18a** shows that as a result of the inverted configuration of the Leu residue, the isobutyl side chain of the Leu residue of **18e** does not make contact to the surface of the S2′ pocket, but points into the space between S2′ and S1′ without any contacts to an amino acid side chain of the protein. This probably explains the decreased inhibition potency.

Summary

A comprehensive series of aziridine-type cysteine protease inhibitors with the aziridine nitrogen atom acylated either with Boc-Leu-Caa, Boc-Gly-Caa, or Boc-Phe-Ala were synthesized. Cyclic amino acids (Caa) of varying ring size and position of the nitrogen atom were used (Azy, Azet, Pro, Pip, Nip, Ini). The compounds also differ in the configurations at all chiral centers and in the substituents at the aziridine-2,3-dicarboxylic acid moiety (acid, diethyl ester, dibenzyl ester).

The compounds were tested against the CAC1 proteases CL and CB. Affinity labeling studies of CL with biotinylated model compounds were performed to confirm the irreversible and active-site-directed inhibition mechanism. Most of the tripeptides containing a cyclic amino acid in combination with a dibenzyl ester moiety are highly selective for CL, whereas the Phe-Ala-containing inhibitors are not.

The most potent inhibitor is Boc-(*S*)-Leu-(*S*)-Azy-(*S*,*S*)-Azi-$(OBn)_2$ **18a** with a K_i value of 13 nM for CL. To elucidate the possible binding mode of **18a** and its less active diastereomer **18e**, these compounds were docked with FlexX into CL. The

docking results predict a near perfect fit of **18a** into the S2, S1′, and S2′ subsites of CL which explains the high-affinity interaction. As **18a** is also one of the most potent rhodesain[13] and falcipain inhibitors[27] within the presented series, a similar prediction of the binding mode for these CL-like enzymes is expected.

Abbreviations

AMC	7-amino-4-methylcoumarin
Azet	azetidine-2-carboxylic acid
Azi	aziridine-2,3-dicarboxylic acid
Azy	aziridine-2-carboxylic acid
Bio	biotin
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
Caa	cyclic amino acid
CAC1	clan CA family C1 cysteine proteases
CB	cathepsin B
Cbz	benzyloxycarbonyl
cc	column chromatography
CL	cathepsin L
config.	configuration
DCC	*N*,*N*-dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DPPA	diphenylphosphoryl azide
DsBio	desthiobiotinyl
E-64	(((2*S*,3*S*)-epoxysuccinyl-(*S*)-leucyl)amino)-4-guanidinobutane
E-64c	(2*S*,3*S*)-3-(1-(*N*-(3-methylbutyl)amino)-(*S*)-leucylcarbonyl)oxirane-2-carboxylic acid
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
F	fluorescence units
HOBt	1-hydroxybenzotriazole
HOSuc	*N*-hydroxysuccinimide
Hxa	6-aminohexanoic acid
Ini	isonipecotic acid
Nip	nipecotic acid
NCA	*N*-carboxyanhydride
Pip	pipecolic acid
TEA	triethylamine
TFA	trifluoroacetic acid
Xaa	any amino acid

Supporting Information

Structures and analytical data of aziridinyl dipeptides **1**–**7**, purity of the inhibitors, inhibition data for compounds **1**–**7**, results and discussion of the homology modeling, results of the quantum chemical calculations, detailed analysis of the binding modes obtained with the different parameters, validation of the docking procedure, and further docking results are presented in the Supporting Information.

Experimental Section

General information: HR ESI mass spectra were recorded on a FTICR mass spectrometer APEX II, Bruker. NMR spectra were recorded on an AVANCE 400 MHz spectrometer from Bruker Biospin GmbH, Germany (solvent: $CDCl_3$, ^1H NMR: 400.13 MHz; ^{13}C NMR: 100.61 MHz). IR spectra were recorded on a PharmalyzIR FTIR spectrometer from BioRad, USA. α Values were determined on a 241 polarimeter from PerkinElmer, USA. CHN analyses were determined with a CHNS-932 instrument, Leco, USA. Column chromatography was performed with silica gel 60 from Merck (0.063–0.2 mm or 70–230 mesh). For TLC, alumina sheets from Merck coated with silica gel 60 F_{254} were used. All solvents were purified and dried prior to use according to standard literature procedures. All reactions were performed in a N_2 atmosphere under strict exclusion of humidity. Cathepsin L (*P. tetraurelia*) and cathepsin B (recombinant, human liver) were purchased from Calbiochem. Cbz-Phe-Arg-AMC was purchased from Bachem.

General synthetic methods: *Method A: N-acylation of aziridine-2,3-dicarboxylates with symmetric anhydrides*. 290 mg (1.1 mmol) DCC are added to an ice-cooled solution (0 °C) of 2.2 mmol N-protected amino acid in 10 mL CH_2Cl_2. After stirring for 60 min, dicyclohexylurea is filtered off, and 1 mmol aziridine-2,3-dicarboxylate is added together with a catalytic amount of DMAP (0.1 equiv). The reaction mixture is allowed to warm at room temperature. After 3–12 h stirring (reaction control by TLC) the solvent is removed in vacuo, 50 mL EtOAc are added, and the mixture is stirred for 1 h. Precipitated dicyclohexylurea is filtered off, the filtrate is washed with 2% Na_2CO_3, water, and brine. The residue remaining after removal of the solvent is purified by cc.

Method B: N-acylation of aziridine-2,3-dicarboxylates or aziridinyl dipeptides with DPPA. 303 mg (1.1 mmol) DPPA are added at 0 °C to a solution of 1 mmol carboxylic acid and 1 mmol aziridine-2,3-dicarboxylate or N-deprotected aziridinyl dipeptide in 5 mL DMF. 2 equiv TEA dissolved in 3 mL DMF are added dropwise over a period of 30 min. The mixture is stirred for 12 h at 0 °C, then 50 mL EtOAc are added, and the solution is washed with water, saturated $NaHCO_3$, and brine. The residue remaining after removal of the solvent is purified by cc.

For the preceding deprotection of aziridinyl dipeptides, 1 mmol Boc-protected aziridinyl dipeptide is dissolved in CH_2Cl_2/TFA (5:1). After stirring for 30 min, the solvents are removed in vacuo, and the residue is dissolved in CH_2Cl_2. The solvent is removed again, and this procedure is repeated twice to remove residual TFA. The resulting TFA salt is used for coupling with an N-protected amino acid using DPPA as coupling reagent as described above.

Method C: coupling with EEDQ. 248 mg (1 mmol) EEDQ are added to a solution of 0.5 mmol aziridine-2,3-dicarboxylate and 1 mmol N-protected dipeptide in 2 mL DMF or CH_2Cl_2. After 5 days stirring at room temperature, the mixture is diluted with 50 mL EtOAc and washed with 2% Na_2CO_3, water, and brine. The residue remaining after removal of the solvent is purified via cc.

Method D: deprotection of dibenzyl esters. 10 μmol of the respective dibenzyl ester are dissolved in EtOAc and 20 mg Pd-C are added. The mixture is stirred, evacuated, and put under a stream of H_2. The reaction is monitored by TLC. After completion of the reaction Pd-C is filtered off over celite, the solvent is removed in vacuo, the remaining residue is recrystallized, and is finally dissolved in DMSO for enzyme assays.

Synthesized compounds: Aziridine-2,3-dicarboxylates were prepared as published previously.[15] Dipeptides (Boc-Phe-Ala, Boc-Leu-

Caa, Boc-Gly-Caa) were synthesized and deprotected prior to coupling with the aziridine according to standard peptide-coupling procedures (for details, see Scheme 1).[16] All ^{1}H NMR assignments were supported by 2D COSY experiments. All ^{13}C NMR assignments were supported by 2D HMBC and HMQC experiments.

Aziridinyl tripeptides: (2*S*,3*S*)+(2*R*,3*R*)-2-Benzyl-3-ethyl-1-[*N*-benzyloxycarbonyl)-glycyl-(*S*)-prolyl]aziridine-2,3-dicarboxylate (IX): Method C starting with 1.61 mmol racemic *trans*-2-benzyl-3-ethylaziridine-2,3-dicarboxylate[10] yielded 358 mg (41%) of a 1:1 mixture of diastereomers **IX** as a colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, R_f=0.05; $[\alpha]_D^{20}$=−41.5 (c=0.27, EtOH); IR (EtOAc): $\tilde{\nu}$=3372, 2953, 1729, 1658, 1441, 1374 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ=1.30 (t, J=7.1 Hz, 3H), 1.8–2.2 (m, 3H, 1×β*H* Pro, 2×γ*H* Pro), 2.2–2.35 (m, 1H, β*H* Pro), 3.48, 3.52 (2× d, J=1.95 Hz) and 3.58, 3.61 (2×d, J=1.95 Hz) (together 2H, ring-*H*), 3.30–3.60 (m, 2H, δ*H* Pro), 3.80–4.10 (m, 2H, Gly), 4.20 (d, J=7.0 Hz, 2H, ester), 4.68 (m, 1H, α*H* Pro), 5.10 (s, 2H), 5.21 (s, 2H), 5.71 (bs, 1H, N*H*), 7.2–7.4 ppm (m, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ=13.88 (*C*H$_3$), 24.26, 24.37 (γ*C* Pro), 28.98, 20.96 (β*C* Pro), 39.41, 30.49, 39.94, 40.08 (ring-*C* Azi), 43.26 (α*C* Gly), 45.73, 45.85 (δ*C* Pro), 60.64, 60.65 (α*C* Pro), 62.26, 62.37 (O*C*H$_2$ ethyl), 66.72, 67.82, 67.93 (O*C*H$_2$ benzyl), 127.85, 127.93, 128.35, 128.44, 128.54, 128.57, 128.62, 134.58, 134.65, 136.37, 156.10, 165.54, 165.67, 165.81, 167.00, 167.24, 177.64, 178.50 ppm; EIMS (70 eV): m/z (%)=537.2 (2.8) [M^+], 91.1 (100); CHN: calcd for $C_{28}H_{31}N_3O_8$: C 62.56, H 5.81, N 7.82, found: C 63.01, H 5.99, N 7.53.

(2*S*,3*S*)-Diethyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*S*)-prolyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*S*)-Pro-(*S*,*S*)-Azi(OEt)$_2$ (8a): Method B starting with 1 mmol N-deprotected aziridinyl dipeptide yielded 286 mg (57%) **8a** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, R_f=0.16; $[\alpha]_D^{20}$=−45.9 (c=1.01, $CHCl_3$); IR (neat):$\tilde{\nu}$=3325, 1735, 1704, 1645, 1441, 1368, 1253, 1192, 1165, 1125, 1026, 735 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ=0.72 (d, J=6.7 Hz, Leu-δ′ C*H*$_3$, 3H), 0.77 (d, J=6.5 Hz, Leu-δ C*H*$_3$, 3H), 1.09 (t, J=7.2 Hz, 2×C*H*$_3$, 6H), 1.14–1.32 (m, Boc+Leu-β C*H*$_2$, 11H), 1.55 (sep., J=6.7 Hz, Leu-γ C*H*, 1H), 1.73–2.07 (m, Pro-β+β′+Pro-γ+γ′ C*H*$_2$, 4H), 3.24 (s, 2×Azi C*H*, 2H), 3.33–3.44 (m, Pro-δ′ C*H*$_2$, 1H), 3.50–3.61 (m, Pro-δ C*H*$_2$, 1H), 3.99–4.11 (m, 2×OC*H*$_2$, 4H), 4.48 (dt, J=4.5, 7.5 Hz, Leu-α C*H*, 1H), 4.68 (dd, J=4.5 Hz, 7.5 Hz, Pro-α C*H*, 1H), 5.13 ppm (bd, J=9.1 Hz, N*H* Leu, 1H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ=13.99 (OCH$_2$-*C*H$_3$), 21.71 (Leu-δ′ CH$_3$), 23.37 (Leu-δ *C*H$_3$), 24.52 (Leu-γ *C*H), 24.85 (Pro-γ *C*H$_2$), 28.32 (Boc), 28.55 (Pro-β *C*H$_2$), 39.60 (Azi *C*H), 42.11 (Leu-β *C*H$_2$), 46.70 (Pro-δ *C*H$_2$), 50.23 (Leu-α *C*H), 60.17 (Pro-α *C*H), 62.44 (OCH$_2$-CH$_3$), 79.40 (Boc q. *C*), 155.63 (*C*=O Boc), 165.78 (*C*=OEt), 171.83 (N-*C*=O Leu), 177.70 ppm (N-*C*=O Pro); HR ESIMS: calcd for $C_{24}H_{39}N_3O_8$, [M+H$^+$]: 498.2815, found: 498.2813.

(2*R*,3*R*)-Diethyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*S*)-prolyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*S*)-Pro-(*R*,*R*)-Azi(OEt)$_2$ (8c): Method B starting with 1 mmol N-deprotected aziridinyl dipeptide yielded 207 mg (42%) **8c** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, R_f=0.23; $[\alpha]_D^{20}$=−39.8 (c=1.52, $CHCl_3$); IR (neat):$\tilde{\nu}$=1740, 1709, 1646, 1503, 1429, 1368, 1252, 1166, 1028, 915, 644 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ=0.88 (d, J=6.7 Hz, Leu-δ′ C*H*$_3$, 3H), 0.93 (d, J=6.4 Hz, Leu-δ C*H*$_3$, 3H), 1.24 (t, J=7.1 Hz, 2×C*H*$_3$, 6H), 1.27–1.45 (m, Boc+Leu-β C*H*$_2$, 11H), 1.7 (sep., J=6.8 Hz, Leu-γ C*H*, 1H), 1.84–2.30 (m, Pro-β+β′+Pro-γ+γ′ C*H*$_2$, 4H), 3.50 (s, 2×Azi C*H*, 2H), 3.51–3.59 (m, Pro-δ′ C*H*$_2$, 1H), 3.65–3.77 (m, Pro-δ C*H*$_2$, 1H), 4.18 (q, 4H, J=7.2 Hz, 2×OC*H*$_2$), 4.38–4.49 (m, Leu-α C*H*, 1H), 4.67 (dd, J=2.6 Hz, 7.4 Hz, Pro-α C*H*, 1H), 5.15 ppm (bd, J=9.0 Hz, N*H* Leu, 1H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ=13.88 (OCH$_2$-*C*H$_3$), 21.62 (Leu-δ′ *C*H$_3$), 23.22 (Leu-δ *C*H$_3$), 24.52 (Leu-γ *C*H), 24.73 (Pro-γ *C*H$_2$), 28.19 (Boc), 28.58 (Pro-β *C*H$_2$), 40.19 (Azi *C*H), 42.10 (Leu-β *C*H$_2$), 46.57 (Pro-δ *C*H$_2$), 50.04 (Leu-α *C*H), 60.00 (Pro-α *C*H), 62.02 (OCH$_2$-CH$_3$), 79.28 (Boc q. *C*), 155.47 (*C*=O Boc), 165.92 (EtO-*C*=O), 171.93 (N-*C*=O Leu), 179.12 ppm (N-*C*=O Pro); HR ESIMS: calcd for $C_{24}H_{39}N_3O_8$, [M+H$^+$]: 498.2815, found: 498.2808.

(2*S*,3*S*)-Diethyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*R*+*S*)-nipecotyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*R*+*S*)-Nip-(*S*,*S*)-Azi(OEt)$_2$ (9a+b): Method B starting with 0.25 mmol N-deprotected aziridinyl dipeptide yielded 61 mg (48%) of a 1:1 mixture of diastereomers **9a** and **9b** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, R_f=0.21; $[\alpha]_D^{20}$=+10.4 (c=0.68, $CHCl_3$); IR (neat): $\tilde{\nu}$=3385, 1731, 1698, 1661, 1518, 1441, 1367, 1327, 1219, 1192, 1167, 1024, 856 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ=0.78–1.05 (m, δ-Leu C*H*$_3$, 6H), 1.17–1.49 (m, Boc+5′-Nip+β-Leu C*H*$_2$+OCH$_2$*C*H$_3$, 18H), 1.58–1.86 (m, 4′-Nip+5-Nip C*H*$_2$+γ-Leu C*H*, 3H), 2.00–2.21 (m, 4-Nip C*H*$_2$, 1H), 2.25–2.71 (m, 3-Nip C*H*, 1H), 2.75–3.34 (m, 2′-Nip+6′-Nip C*H*$_2$, 2H), 3.35–3.51 (m, C*H* Azi, 2H), 3.79–4.10 (m, 6-Nip C*H*$_2$, 1H), 4.11–4.34 (m, OC*H*$_2$CH$_3$+2-Nip C*H*$_2$, 5H), 4.50–4.69 (m, α-Leu C*H*, 1H), 5.11–5.39 ppm (m, N*H* Leu, 1H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ=14.40, 14.34 (OCH$_2$*C*H$_3$), 23.82, 21.87 (Leu-δ *C*H$_3$), 24.88 (Leu-γ *C*H), 25.06, 25.00 (5- *C*H$_2$ Nip), 26.94, 26.56 (4-*C*H$_2$ Nip), 28.73, 28.69 (Boc), 40.44, 40.39 (*C*H Azi), 42.86, 42.61 (Leu-β *C*H$_2$), 43.68, 43.33 (6-*C*H$_2$ Nip), 44.35, 44.17 (3-*C*H Nip), 47.29, 46.41 (2-*C*H$_2$ Nip), 49.10, 49.00 (α-Leu *C*H), 63.15, 62.88 (OCH$_2$CH$_3$), 79.57, 79.51 (q. *C* Boc), 155.65, 155.57 (*C*=O Boc), 166.78, 166.56 (EtO-C=O), 171.64, 171.39 (N-*C*=O Leu), 179.78, 179.52 ppm (N-*C*=O Nip); HR ESIMS: calcd for $C_{25}H_{41}N_3O_8$, [M+H$^+$]: 512.2972, found: 512.2976.

(2*R*,3*R*)-Diethyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*R*+*S*)-nipecotyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*R*+*S*)-Nip-(*R*,*R*)-Azi(OEt)$_2$ (9c+d): Method B starting with 0.25 mmol N-deprotected aziridinyl dipeptide yielded 76 mg (60%) of a 1:1 mixture of diastereomers **9c** and **9d** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, R_f=0.22; $[\alpha]_D^{20}$=−9.3 (c=1.17, $CHCl_3$); IR (neat): $\tilde{\nu}$=1737, 1707, 1640, 1504, 1445, 1368, 1327, 1270, 1195, 1166, 1129, 1026, 857 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ=0.78–0.99 (m, δ-Leu C*H*$_3$, 6H), 1.19–1.52 (m, Boc+5′-Nip+β-Leu C*H*$_2$+OCH$_2$*C*H$_3$, 18H), 1.57–1.87 (m, 4′-Nip+5-Nip C*H*$_2$+γ-Leu C*H*, 3H), 1.95–2.31 (m, 4-Nip C*H*$_2$, 1H), 2.30–2.65 (m, 3-Nip C*H*, 1H), 2.70–3.29 (m, 2′-Nip+6′-Nip C*H*$_2$, 2H), 3.38–3.47 (m, C*H* Azi, 2H), 3.76–3.90 (m, 6-Nip C*H*$_2$, 1H), 4.02–4.33 (m, OC*H*$_2$CH$_3$+2-Nip C*H*, 5H), 4.42–4.72 (m, α-C*H* Leu, 1H), 5.13–5.35 ppm (m, N*H* Leu, 1H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ=13.93 (OCH$_2$*C*H$_3$), 23.28, 21.80 (Leu-δ′ CH$_3$), 24.53 (Leu-γ *C*H), 25.26 (5-*C*H$_2$ Nip), 26.76, 26.56 (4-*C*H$_2$ Nip), 28.29 (Boc), 39.98, 39.88 (CH Azi), 42.85, 42.67 (Leu-β *C*H$_2$), 43.59, 43.20 (6-CH$_2$ Nip), 43.90, 43.64 (3-*C*H Nip), 46.49, 45.87 (2-*C*H$_2$ Nip), 48.85, 48.53 (α-Leu *C*H), 62.59, 62.43 (OCH$_2$CH$_3$), 79.57, 79.51 (q. C Boc), 155.63, 155.55 (*C*=O Boc), 166.66, 166.57 (EtO-*C*=O), 171.44, 171.29 (N-C=O Leu), 179.80, 179.62 ppm (N-*C*=O Nip); HR ESIMS: calcd for $C_{25}H_{41}N_3O_8$, [M+H$^+$]: 512.2972, found: 512.2970.

(2*S*,3*S*)-Diethyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-isonipecotyl]-aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-Ini-(*S*,*S*)-Azi(OEt)$_2$ (10a): Method B starting with 0.25 mmol N-deprotected aziridinyl dipeptide yielded 72 mg (56%) **10a** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, R_f=0.15; $[\alpha]_D^{20}$=+17.7, (c=1.02, $CHCl_3$); IR (neat): $\tilde{\nu}$=1737, 1700, 1640, 1447, 1369, 1327, 1269, 1194, 1167, 1140, 1024, 857 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ=0.86–1.07 (m, 2×C*H*$_3$ δ-Leu, 6H), 1.31 (t, J=7.1 Hz, OCH$_2$*C*H$_3$, 6H), 1.35–1.53 (m, 3×C*H*$_3$ Boc+β-Leu C*H*$_2$, 11H), 1.59–1.85 (m, 3′-Ini+5′-Ini C*H*$_2$+γ-Leu C*H*, 3H), 1.89 (bt, J=13.6 Hz, 5-Ini C*H*$_2$, 1H), 2.01 (bd, J=13.3 Hz, 3-Ini C*H*$_2$, 1H), 2.39–2.53 (m, 4-Ini C*H*$_2$, 1H), 2.76–

2.89 (dt, $J=11.1$ Hz, 2.8 Hz, 2′-Ini CH_2, 1H), 3.13 (dt, $J=10.9$ Hz, 2.2 Hz, 6′-Ini CH_2, 1H), 3.49 (s, 2×*CH* Azi, 2H), 3.88–3.91 (bd, $J=$ 13.6 Hz, 6-Ini CH_2, 1H), 4.16–4.29 (m, OCH_2CH_3, 4H), 4.35–4.44 (bd, $J=13.5$ Hz, 2-Ini CH_2, 1H), 4.64 (t, $J=7.2$ Hz, α-Leu *CH*, 1H), 5.21 ppm (s, N*H*-Leu, 1H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=14.06$ (OCH_2CH_3), 21.87 (Leu-δ′ CH_3), 23.49 (Leu-δ CH_3), 24.70 (γ-CH Leu), 27.61 (5-CH_2 Ini), 28.37 (3×CH_3 Boc), 28.73 (3-CH_2 Ini), 39.97 (2×CH Azi), 41.36 (6-CH_2 Ini), 42.90 (β-CH_2 Leu), 43.31 (4-*CH* Ini), 44.32 (2-CH_2 Ini), 48.63 (α-CH Leu), 62.61 (OCH_2CH_3), 79.44 (q. *C* Boc), 155.68 (*C*=O Boc), 166.47 (2×EtO-*C*=O), 171.33 (N-*C*=O Leu), 181.50 ppm (N-*C*=O Ini); HR ESIMS: calcd for $C_{25}H_{41}N_3O_8$, [*M*+H$^+$]: 512.2972, found: 512.2969.

(2*R*,3*R*)-Diethyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-isonipecotyl]-aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-Ini-(*R*,*R*)-Azi(OEt)$_2$ (10c): Method B starting with 0.5 mmol N-deprotected aziridinyl dipeptide yielded 167 mg (65%) **10c** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.15$; $[\alpha]_D^{20}=-14.6$, ($c=1.32$, $CHCl_3$); IR (neat): $\tilde{\nu}=3300$, 1737, 1706, 1638, 1504, 1448, 1368, 1327, 1269, 1194, 1165, 1126, 1014 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): $\delta=0.86$–1.01 (m, 2×CH_3 δ-Leu, 6H), 1.31 (t, $J=$ 7.1 Hz, OCH_2CH_3, 6H), 1.35–1.53 (m, 3×CH_3 Boc+β-Leu CH_2, 11H), 1.60–1.84 (m, 3′-Ini+5′-Ini CH_2+γ-Leu *CH*, 3H), 1.90 (bt, $J=$ 13.6 Hz, 5-Ini, 1H), 2.02 (bd, $J=13.3$ Hz, 3-Ini CH_2, 1H), 2.40–2.54 (m, 4-Ini *CH*, 1H), 2.78–2.90 (dt, $J=11.1$ Hz, 2.8 Hz, 2′-Ini CH_2, 1H), 3.14 (dt, $J=10.9$ Hz, 2.2 Hz, 6′-Ini CH_2, 1H), 3.49 (s, 2×*CH* Azi, 2H), 3.89–3.96 (bd, $J=13.6$ Hz, 6-Ini CH_2, 1H), 4.17–4.31 (m, OCH_2CH_3, 4H), 4.36–4.46 (bd, $J=13.5$ Hz, 2-Ini CH_2, 1H), 4.64 (t, $J=7.2$ Hz, α-Leu *CH*, 1H), 5.25 ppm (s, N*H*-Leu, 1H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=14.06$ (OCH_2CH_3), 23.39, 21.84 (Leu-δ CH_3), 24.69 (γ-CH Leu), 27.61 (5-CH_2 Ini), 28.37 (3×CH_3 Boc), 28.73 (3-CH_2 Ini), 39.97 (2×CH Azi), 41.26 (6-CH_2 Ini), 42.90 (β-CH_2 Leu), 43.31 (4-*CH* Ini), 44.32 (2-CH_2 Ini), 48.53 (α-CH Leu), 62.61 (OCH_2CH_3), 79.44 (q. *C* Boc), 155.68 (*C*=O Boc), 166.47 (2×EtO-*C*=O), 171.43 (N-*C*=O Leu), 181.60 ppm (N-*C*=O Ini); HR ESIMS: calcd for $C_{25}H_{41}N_3O_8$, [*M*+H$^+$]: 512.2972, found: 512.2970.

(2*R*,3*R*)-Diethyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*R*+*S*)-azetidine-2-carbonyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*R*+*S*)-Azet-(*R*,*R*)-Azi(OEt)$_2$ (11c+d): Method C starting with 0.25 mmol aziridine yielded 40 mg (33%) of a 1:1 mixture of diastereomers **11c** and **11d** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.27$; $[\alpha]_D^{20}=-22.3$ ($c=1.35$, $CHCl_3$); IR (neat): $\tilde{\nu}=1738$, 1706, 156, 1497, 1449, 1324, 1250, 1170, 1021, 736 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): $\delta=0.91$ (d, $J=6.4$ Hz, Leu-δ′ CH_3, 3H), 0.92 (d, $J=6.3$ Hz, Leu-δ CH_3, 3H), 1.28 (t, $J=7.1$ Hz, OCH_2CH_3, 6H), 1.39–1.49 (m, Boc+Leu-β CH_2, 11H), 1.64–1.74 (m, Leu-γ *CH*, 1H), 2.52–2.68 (m, Azet-β CH_2, 2H), 3.52 (s, 2×Azi *CH*, 2H), 4.15–4.30 (m, Leu-α *CH*, Azet-γ CH_2, 3H), 4.22 (q, $J=7.1$ Hz, OCH_2CH_3, 4H), 4.78 (dd, $J=6.1$, 8.7 Hz, Azet-α *CH*, 1H), 4.98 ppm (bd, $J=8.7$ Hz, Leu-N*H*, 1H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=14.19$, 14.06 (OCH_2-CH_3), 21.18, 21.06 (Azet-β CH_2), 23.12, 21.09 (Leu-δ CH_3), 24.52 (Leu-γ *CH*), 28.29, 28.25 (Boc), 40.23, 40.17 (Azi *CH*), 42.36, 42.10 (Leu-β CH_2), 48.07, 47.96 (Leu-α *CH*), 49.11, 48.93 (Azet-γ CH_2), 61.76, 61.65 (Azet-α *CH*), 62.26, 62.03 (OCH_2-CH_3), 79.63, 79.56 (Boc q. *C*), 155.53, 155.45 (*C*=O Boc), 165.91, 165.78 (EtO-*C*=O), 173.65, 173.44 (N-*C*=O Leu), 178.92, 178.77 ppm (N-*C*=O Azet); HR ESIMS: calcd for $C_{23}H_{37}N_3O_8$, [*M*+H$^+$]: 484.2659, found: 484.2653.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*R*+*S*)-azetidine-2-carbonyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*R*+*S*)-Azet-(*S*,*S*)-Azi(OBn)$_2$ (12a+b): Method C starting with 0.25 mmol aziridine yielded 52 mg (34%) of a 1:1 mixture of diastereomers **12a** and **12b** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.26$; $[\alpha]_D^{20}=+36.5$, ($c=1.06$, $CHCl_3$); IR (neat): $\tilde{\nu}=1740$, 1708, 1657, 1499, 1451, 1427, 1367, 1326, 1249, 1171, 1120, 1022, 698 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): $\delta=0.91$ (d, $J=$ 6.4 Hz,Leu-δ′ CH_3, 3H), 0.92 (d, $J=6.2$ Hz, Leu-δ CH_3, 3H), 1.39–1.49 (m, Boc+Leu-β CH_2, 11H), 1.64–1.74 (m, Leu-γ *CH*, 1H), 2.50–2.59 (m, Azet-β CH_2, 2H), 3.56 (s, 2×Azi *CH*, 2H), 4.13–4.34 (m, Leu-α *CH*, Azet-γ CH_2, 3H), 4.78 (dd, $J=6.2$, 8.8 Hz, Azet-α *CH*, 1H), 4.98 (d, $J=8.7$ Hz, Leu-N*H*, 1H), 5.14–5.26 (m, 2×OCH_2Ph, 4H), 7.30–7.44 ppm (m, arom., 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=21.03$ (Azet-β CH_2), 23.08, 21.99 (Leu-δ CH_3), 24.52 (Leu-γ *CH*), 28.35 (Boc), 40.63 (Azi *CH*), 42.12 (Leu-β CH_2), 47.92 (Leu-α *CH*), 49.02 (Azet-γ CH_2), 61.73 (Azet-α *CH*), 67.97 (OCH_2Ph), 79.63 (Boc q. *C*), 128.56, 128.50 (arom. *CH*), 134.69 (q. arom. *C*), 155.49 (*C*=O Boc), 166.18 (BnO-*C*=O), 173.75 (N-*C*=O Leu), 178.66 ppm (N-*C*=O Azet); HR ESIMS: calcd for $C_{33}H_{41}N_3O_8$, [*M*+H$^+$]: 608.2972, found: 608.2957.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*S*)-prolyl]-aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*S*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (13a): Method C starting with 0.5 mmol aziridine yielded 188 mg (60%) **13a** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.34$; $[\alpha]_D^{20}=-37.8$ ($c=1.22$, $CHCl_3$); IR (neat): $\tilde{\nu}=$ 3403, 2959, 1742, 1708, 1647, 1500, 1428, 1367, 1327, 1249, 1169, 1122, 1022, 698 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): $\delta=0.91$ (d, 1H, $J=6.7$ Hz, Leu-δ′ CH_3, 3H), 0.97 (d, $J=6.2$ Hz, Leu-δ CH_3, 3H), 1.36–1.52 (m, Boc+Leu-β CH_2, 11H), 1.65–1.83 (m, Leu-γ *CH*, 1H), 1.91–2.06 (m, Pro-β′, Pro-γ′ CH_2, 2H), 2.07–2.17 (m, Pro-γ CH_2, 1H), 2.18–2.31 (m, Pro-β CH_2, 1H), 3.55–3.62 (m, Pro-δ′ CH_2, 1H), 3.63 (s, 2×Azi *CH*, 2H), 3.69–3.81 (m, Pro-δ CH_2, 1H), 4.43–4.54 (m, Leu-α *CH*, 1H), 4.71 (dd, 1H, $J=3.3$ Hz, 8.3 Hz, Pro-α *CH*, 1H), 5.13 (d, 1H, $J=$ 9.0 Hz, Leu N*H*, 1H), 5.15–5.25 (m, 2×OCH_2Ph, 4H), 7.27–7.45 ppm (m, arom. *CH*, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=23.35$, 21.73 (Leu-δ CH_3), 24.51 (Leu-γ *CH*), 24.68 (Pro-γ CH_2), 28.34 (Boc), 28.64 (Pro-β CH_2), 40.39 (2×Azi *CH*), 42.19 (Leu-β CH_2), 46.67 (Pro-δ CH_2), 50.18 (Leu-α *CH*), 60.23 (Pro-α *CH*), 67.81 (OCH_2Ph), 79.42 (Boc q. *C*), 128.59, 128.49 (arom. *CH*), 134.80 (q. arom. *C*), 155.60 (*C*=O Boc), 165.87 (BnO-*C*=O), 172.08 (N-*C*=O Leu), 179.19 ppm (N-*C*=O Pro); HR ESIMS: calcd for $C_{34}H_{43}N_3O_8$, [*M*+H$^+$]; 622.3128, found: 622.3113.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*R*)-prolyl]-aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*R*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (13b): Method C starting with 0.5 mmol aziridine yielded 95 mg (31%) **13b** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.29$; $[\alpha]_D^{20}=+7.3$ ($c=1.13$, $CHCl_3$); IR (neat): $\tilde{\nu}=$ 3396, 2959, 1742, 1707, 1646, 1500, 1429, 1367, 1326, 1249, 1167, 1126, 1022, 698 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400.13 MHz): $\delta=0.93$ (d, $J=$ 6.7 Hz, Leu-δ′ CH_3, 3H), 0.99 (d, $J=6.3$ Hz, Leu-δ CH_3, 3H), 1.35–1.52 (m, Boc+Leu-β CH_2, 11H), 1.66–1.77 (m, Leu-γ *CH*, 1H), 1.92–2.04 (m, Pro-β′, Pro-γ′ CH_2, 2H), 2.05–2.19 (m, Pro-γ CH_2, 1H), 2.20–2.30 (m, Pro-β CH_2, 1H), 3.55–3.61 (m, Pro-δ′ CH_2, 1H), 3.64 (s, 2×Azi *CH*, 2H), 3.76–3.90 (m, Pro-δ CH_2, 1H), 4.45–4.57 (m, Leu-α *CH*, 1H), 4.62 (dd, $J=2.5$, 8.0 Hz, Pro-α *CH*, 1H), 5.13–5.24 (m, 2×OCH_2Ph+Leu NH, 5H), 7.31–7.39 ppm (m, arom. *CH*, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=23.49$, 22.06 (Leu-δ CH_3), 24.49 (Leu-γ *CH*), 24.75 (Pro-γ CH_2), 28.35 (Boc), 29.43 (Pro-β CH_2), 40.15 (Azi *CH*), 42.56 (Leu-β CH_2), 46.62 (Pro-δ CH_2), 50.37 (Leu-α *CH*), 60.70 (Pro-α *CH*), 67.89 (OCH_2Ph), 79.36 (Boc q. *C*), 128.63, 128.63 (arom. *CH*), 134.66 (q. arom. *C*), 155.50 (*C*=O Boc), 165.69 (BnO-*C*=O), 171.66 (N-*C*=O Leu), 178.53 ppm (N-*C*=O Pro); HR ESIMS: calcd for $C_{34}H_{43}N_3O_8$, [*M*+H$^+$]: 622.3128, found: 622.3138.

(2*R*,3*R*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*S*)-prolyl]-aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*S*)-Pro-(*R*,*R*)-Azi(OBn)$_2$ (13c): Method C starting with 0.5 mmol aziridine yielded 152 mg (49%) **13c** colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.32$; $[\alpha]_D^{20}=-26.0$ ($c=1.19$, $CHCl_3$); IR (neat): $\tilde{\nu}=$

3355, 2959, 1746, 1699, 1500, 1456, 1367, 1250, 1167, 1104, 1022, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 0.93$ (d, $J = 6.8$ Hz, Leu-δ′ CH_3, 3H), 0.98 (d, $J = 6.6$ Hz, Leu-δ CH_3, 3H), 1.38–1.52 (m, Boc + Leu-β CH_2, 11H), 1.74 (sep, $J = 6.7$ Hz, Leu-γ CH, 1H), 1.94–2.08 (m, Pro-β′, Pro-γ′ CH_2, 2H), 2.08–2.20 (m, Pro-γ CH_2, 1H), 2.22–2.33 (m, Pro-β CH_2, 1H), 3.56–3.62 (m, Pro-δ′ CH_2, 1H), 3.64 (s, 2×Azi CH, 2H), 3.71–3.78 (m, Pro-δ CH_2, 1H), 4.48 (dt, $J = 5.9$, 8.8 Hz, Leu-α CH, 1H), 4.72 (dd, $J = 2.9$, 7.7 Hz, Pro-α CH, 1H), 5.14 (d, $J = 9.3$ Hz, Leu NH, 1H); 5.17–5.27 (m, 2×OCH_2Ph, 4H); 7.34–7.42 ppm (m, arom. CH, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 21.75$ (Leu-δ′ CH_3), 23.38 (Leu-δ CH_3), 24.55 (Leu-γ CH), 24.72 (Pro-γ CH_2), 28.37 (CH_3 Boc), 28.68 (Pro-β CH_2), 40.42 (Azi CH), 42.20 (Leu-β CH_2), 46.70 (Pro-δ CH_2), 50.22 (Leu-α CH), 60.27 (Pro-α CH), 67.86 (OCH_2Ph), 79.48 (Boc q. C), 128.63, 128.53 (arom. CH), 134.82 (q. arom. C), 155.64 (C=O Boc), 165.91 (BnO-C=O), 172.15 (N-C=O Leu), 179.22 ppm (N-C=O Pro); HR ESIMS: calcd for $C_{34}H_{43}N_3O_8$, $[M+H^+]$: 622.3128, found: 622.3209.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-leucyl-(*S*)-prolyl]-aziridine-2,3-dicarboxylate, Boc-(*R*)-Leu-(*S*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (13 e): Method C starting with 0.25 mmol aziridine yielded 36 mg (23%) **13 e** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.23$; $[\alpha]_D^{20} = -19.5$ ($c = 1.05$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3300$, 2959, 1739, 1708, 1647, 1497, 1427, 1367, 1245, 1165, 1121, 1026 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 0.77$ (d, $J = 6.7$ Hz, Leu-δ′ CH_3, 3H), 0.84 (d, $J = 6.5$ Hz, Leu-δ CH_3, 3H), 1.21–1.35 (m, Boc + Leu-β CH_2, 11H), 1.48–1.61 (m, Leu-γ CH, 1H), 1.70–1.80 (m, Pro-γ′ CH_2, 1H), 1.86–1.96 (m, Pro-β′, Pro-γ CH_2, 2H), 1.97–2.08 (m, Pro-β CH_2, 1H), 3.24–3.34 (m, Pro-δ′ CH_2, 1H), 3.37 (s, 2× Azi CH, 2H), 3.47–3.56 (m, Pro-δ CH_2, 1H), 4.35 (dt, $J = 4.0$, 9.3 Hz, Leu-α CH, 1H), 4.44 (dd, $J = 2.9$, 7.7 Hz, Pro-α CH, 1H), 5.02–5.09 (m, 2×OCH_2Ph, 4H), 5.12 (d, $J = 9.0$ Hz, Leu NH, 1H), 7.14–7.28 ppm (m, arom. CH, 10H);^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta =$ 21.96 (Leu-δ′ CH_3), 23.43 (Leu-δ CH_3), 24.52 (Leu-γ CH), 24.60 (Pro-γ CH_2), 28.31 (Boc), 28.80 (Pro-β CH_2), 39.60 (Azi CH), 42.89 (Leu-β CH_2), 46.61 (Pro-δ CH_2), 50.35 (Leu-α CH), 60.62 (Pro-α CH), 68.02 (OCH_2Ph), 79.36 (Boc q. C), 134.66 (q. arom. C), 155.40 (C=O Boc), 165.59 (BnO-C=O), 171.56 (N-C=O Leu), 128.78, 128.75, 128.63 (arom. CH), 177.53 ppm (N-C=O Pro); HR ESIMS: calcd for $C_{34}H_{43}N_3O_8$, $[M+H^+]$: 622.3128, found: 622.3141.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-leucyl-(*R*)-prolyl]-aziridine-2,3-dicarboxylate, Boc-(*R*)-Leu-(*R*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (13 f): Method C starting with 0.5 mmol aziridine yielded 73 mg (24%) **13 f** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.21$; $[\alpha]_D^{20} = +40.0$ ($c = 1.15$, $CHCl_3$); IR (neat): $\tilde{\nu} =$ 3385, 2958, 1743, 1708, 1645, 1500, 1428, 1367, 1333, 1269, 1250, 1166, 1122, 1024, 699 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 0.92$ (d, $J = 6.7$ Hz, Leu-δ′ CH_3, 3H), 0.97 (d, $J = 6.5$ Hz, Leu-δ CH_3, 3H), 1.37–1.50 (m, Boc + Leu-β CH_2, 11H), 1.73 (h, $J = 6.7$ Hz, Leu-γ CH, 1H), 1.93–2.06 (m, Pro-β′, Pro-γ′ CH_2, 2H), 2.07–2.18 (m, Pro-γ CH_2, 1H), 2.21–2.31 (m, Pro-β CH_2, 1H), 3.63 (s, 2×Azi CH, 2H), 3.70–3.77 (m, Pro-δ CH_2, 1H), 3.55–3.61 (m, Pro-δ′ CH_2, 1H), 4.47 (dt, $J = 6.0$, 8.8 Hz, Leu-α CH, 1H), 4.71 (dd, $J = 2.8$, 7.7 Hz, Pro-α CH, 1H), 5.13 (d, $J = 9.2$ Hz, Leu NH, 1H), 5.16–5.25 (m, 2×OCH_2Ph, 4H), 7.33–7.40 ppm (m, arom. CH, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta =$ 23.37, 21.74 (Leu-δ CH_3), 24.54 (Leu-γ CH), 24.71 (Pro-γ CH_2), 28.36 (Boc), 28.67 (Pro-β CH_2), 40.41 (Azi CH), 42.19 (Leu-β CH_2), 46.69 (Pro-δ CH_2), 50.21 (Leu-α CH), 60.26 (Pro-α CH), 67.85 (OCH_2Ph), 79.47 (Boc q. C), 134.81 (q. arom. C), 155.63 (C=O Boc), 165.90 (BnO-C=O), 172.14 (N-C=O Leu), 179.21 (N-C=O Pro), 128.62, 128.52 ppm (arom. CH); HR ESIMS: calcd for $C_{34}H_{43}N_3O_8$, $[M+H^+]$: 622.3128, found: 622.3133.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-glycyl-(*S*)-prolyl]aziridine-2,3-dicarboxylate, Boc-Gly-(*S*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (14 a): Method C starting with 1 mmol aziridine yielded 301 mg (53%) **14 a** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.10$; $[\alpha]_D^{20} = -18.3$ ($c = 1.05$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3401$, 2978, 1739, 1713, 1649, 1451, 1368, 1281, 1248, 1164, 699 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 1.97$–2.06 (m, Pro-β′ + Pro-γ CH_2, 3H), 1.37–1.48 (s, Boc, 9H), 2.17–2.22 (m, Pro-β CH_2, 1H), 3.44–3.47 (m, Pro-δ′ CH_2, 1H), 3.53 (s, 2×CH Azi, 2H), 3.54–3.57 (m, Pro-δ CH_2, 1H), 3.85–4.04 (m, Gly CH_2, 2H), 4.49 (dd, $J = 3.3$, 8.5 Hz, Pro-α CH, 1H), 5.17–5.21 (m, 2×OCH_2Ph, 4H), 5.25–5.30 (bs, Gly NH, 1H), 7.27–7.42 ppm (m, arom. CH, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 24.56$ (Pro-γ CH_2), 28.28 (Boc), 28.96 (Pro-β CH_2), 39.51 (Azi CH), 42.94 (CH_2 Gly), 45.87 (Pro-δ CH_2), 59.00 (Pro-α CH), 68.06 (OCH_2Ph), 79.64 (Boc q. C), 128.69, 128.64 (arom. CH), 134.57 (q. arom. C), 155.93 (C=O Boc), 165.54 (BnO-C=O), 167.48 (N-C=O Gly), 177.79 ppm (N-C=O Pro); HR ESIMS: calcd for $C_{30}H_{35}N_3O_8$, $[M+H^+]$: 566.2502, found: 566.2491.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-glycyl-(*R*)-prolyl]aziridine-2,3-dicarboxylate, Boc-Gly-(*R*)-Pro-(*S*,*S*)-Azi(OBn)$_2$ (14 b): Method C starting with 0.5 mmol aziridine yielded 102 mg (36%) **14 b** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.14$; $[\alpha]_D^{20} = +29.6$ ($c = 1.13$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3418$, 2977, 1739, 1708, 1654, 1499, 1432, 1326, 1271, 1248, 1167, 1024, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 1.34$–1.50 (s, Boc, 9H), 1.91–2.09 (m, Pro-β′ + Pro-γ′ CH_2, 3H), 2.05–2.19 (m, Pro-γ CH_2, 1H), 2.17–2.31 (m, Pro-β CH_2, 1H), 3.35–3.47 (m, Pro-δ′ CH_2, 1H), 3.50–3.59 (m, Pro-δ CH_2, 1H), 3.64 (s, 2×CH Azi, 2H), 3.74–4.06 (m, Gly CH_2, 2H), 4.59 (dd, $J = 3.2$, 8.6 Hz, Pro-α CH, 1H), 5.12–5.25 (m, 2× OCH_2Ph, 4H), 5.32 (bs, Gly NH, 1H), 7.28–7.43 ppm (m, arom. CH, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 24.49$ (Pro-γ CH_2), 28.30 (Boc), 29.07 (Pro-β CH_2), 40.09 (Azi CH), 42.9 (CH_2 Gly), 45.89 (Pro-δ CH_2), 60.57 (Pro-α CH), 67.95 (OCH_2Ph), 79.56 (Boc q. C), 128.77, 128.61 (arom. CH), 134.66 (q. arom. C), 155.66 (C=O Boc), 165.80 (BnO-C=O), 167.46 (N-C=O Gly), 178.66 ppm (N-C=O Pro); HR ESIMS: calcd for $C_{30}H_{35}N_3O_8$, $[M+H^+]$: 566.2502, found: 566.2510.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-glycyl-(*S*)-pipecolyl]-aziridine-2,3-dicarboxylate, Boc-Gly-(*S*)-Pip-(*S*,*S*)-Azi(OBn)$_2$ (15 a): Method C starting with 0.5 mmol aziridine yielded 100 mg (34%) of a 2:1 mixture of rotamers of **15 a** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.28$; $[\alpha]_D^{20} = +31.1$ ($c =$ 1.31, $CHCl_3$); IR (neat): $\tilde{\nu} = 3422$, 2941, 1735, 1710, 1651, 1497, 1451, 1436, 1367, 1325, 1244, 1166, 1014, 738, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 1.40$–1.50 (br. m, Pip-δ′ + Pip-γ CH_2 + Boc, 12H), 1.66–1.76 (m, Pip-δ + Pip-β′ CH_2, 2H), 2.32 (bd, $J = 13.3$ Hz, Pip-β CH_2, 1H), 3.27 (bdt, $J = 2.5$, 13.5, 13.6 Hz, Pip-ε′ CH_2, 1H), 3.42 (s, 2× Azi-CH, 2H), 3.61 (bd, $J = 12.8$ Hz, Pip-ε CH_2, 1H), 3.88–4.13 (m, Gly CH_2, 2H), 5.27 (bd, $J = 5.1$ Hz, Pip-α CH, 1H), 5.28 (bs, 2×CH_2OBn, 4H), 5.44 (bd, Gly-NH, 1H), 7.27–7.38 ppm (m, arom. CH, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 20.81$, 20.62 (Pip-γ CH_2), 25.25, 24.99 (Pip-δ CH_2), 26.55, 25.85 (Pip-β CH_2), 28.40, 27.79 (Boc), 40.03 (Azi-2×CH), 42.33, 42.25 (Pip-ε CH_2), 42.99 (Gly-CH_2), 54.33, 52.43 (Pip-α CH), 68.14 (2×CH_2OBn), 79.67 (Boc q. C), 128.69, 128.65, 128.71 (arom. CH), 134.63 (C=O Boc), 166.06 (BnO-C=O), 167.75 (N-C=O Gly), 178.23 ppm (N-C=O Pip); HR ESIMS: calcd for $C_{31}H_{37}N_3O_8$, $[M+H^+]$: 580.2659, found: 580.2670.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*R*+*S*)-nipecotyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(R+S)-Nip-(*S*,*S*)-Azi(OBn)$_2$ (16 a+b): Method C starting with 0.5 mmol aziridine yielded 140 mg (44%) of a 1:1 mixture of diastereomers **16 a** and **16 b** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.28$; $[\alpha]_D^{20} = +21.6$ ($c = 1.16$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3398$,

2955, 1737, 1705, 1640, 1499, 1446, 1367, 1325, 1265, 1246, 1173, 1126, 1022, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 0.83$–1.08 (m, 2×δ-Leu CH_3, 6H), 1.19–1.51 (m, Boc + 5′-Nip CH_2 + β-Leu CH_2, 12H), 1.52–1.82 (m, 4′-Nip + 5-Nip CH_2 + γ-Leu C*H*, 3H), 2.04–2.41 (m, 4-Nip CH_2, 1H), 2.45–2.66 (m, 3-Nip C*H*, 1H), 2.92–3.07 (m, 6′-Nip CH_2, 1H), 3.08–3.34 (m, 2′-Nip CH_2, 1H), 3.43–3.65 (m, C*H* Azi, 2H), 3.74–3.94 (m, 6-Nip CH_2, 1H), 4.04–4.23 (m, 2-Nip CH_2, 1H), 4.50–4.76 (m, α-Leu C*H*, 1H), 5.08–5.37 (m, OCH_2Ph + N*H* Leu, 5H), 7.29–7.49 ppm (m, arom. C*H*, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 21.89$, 21.69 (Leu-δ′ CH_3), 23.44, 23.34 (Leu-δ CH_3), 24.69, 24.62 (5-CH_2 Nip), 26.67, 26.43 (4-CH_2 Nip), 28.33, 28.28 (Boc), 40.05, 39.97 (Azi C*H*), 42.82, 42.45 (Leu-β CH_2), 43.67, 43.33 (3-C*H* Nip), 45.54, 45.22 (6-CH_2 Nip), 46.79, 45.91 (2-CH_2 Nip), 48.74, 48.58 (α-Leu C*H*), 68.31, 68.14 (OCH_2Ph), 79.44, 79.39 (q. *C* Boc), 128.78, 128.69, 128.58 (arom. C*H*), 134.51 (q. arom. *C*), 155.61, 155.53 (*C*=O Boc), 166.15, 166.10 (BnO-*C*=O), 171.42, 171.27 (N-*C*=O Leu), 179.78, 179.60 ppm (N-*C*=O Nip); HR ESIMS: calcd for $C_{35}H_{45}N_3O_8$, [*M*+H$^+$]: 636.3285, found: 636.3296.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-leucyl-(*R*+*S*)-nipecotyl]aziridine-2,3-dicarboxylate, Boc-(*R*)-Leu-(*R*+*S*)-Nip-(*S*,*S*)-Azi(OBn)$_2$ (16e+f): Method C starting with 0.5 mmol aziridine yielded 136 mg (43%) of a 1:1 mixture of diastereomers **16e** and **16f** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.28$; $[\alpha]_D^{20} = -1.8$ ($c = 1.08$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3409$, 2955, 1738, 1705, 1640, 1499, 1446, 1367, 1325, 1246, 1173, 1126, 1022, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 0.84$–1.10 (m, δ-Leu CH_3, 6H), 1.21–1.53 (m, Boc + 5′-Nip + β-Leu CH_2, 12H), 1.54–1.84 (m, 4′-Nip + 5-Nip CH_2 + γ-Leu C*H*, 3H), 2.06–2.43 (m, 4-Nip CH_2, 1H), 2.46–2.68 (m, 3-Nip C*H*, 1H), 2.94–3.08 (m, 6′-Nip CH_2, 1H), 3.10–3.35 (m, 2′-Nip CH_2, 1H), 3.45–3.67 (m, C*H* Azi, 2H), 3.75–3.96 (m, 6-Nip CH_2, 1H), 4.51–4.78 (m, α-C*H* Leu, 1H), 5.09–5.39 (m, OCH_2Ph + N*H* Leu, 5H), 7.50–7.30 ppm (m, arom. C*H*, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 21.91$, 21.70 (Leu-δ′ CH_3), 23.45, 23.35 (Leu-δ CH_3), 24.51 (Leu-γ CH), 24.70, 24.63 (5-CH_2 Nip), 46.80, 45.93 (2-CH_2 Nip), 45.56, 45.24 (6-CH_2 Nip), 43.69, 43.35 (3-C*H* Nip), 26.69, 26.44 (4-CH_2 Nip), 28.34, 28.29 (Boc), 40.06, 39.99 (Azi C*H*), 42.83, 42.47 (Leu-β CH_2), 48.74, 48.58 (α-Leu C*H*), 68.32, 68.15 (OCH_2Ph), 79.45, 79.40 (q. *C* Boc), 128.80, 128.71, 128.60 (arom. C*H*), 134.53 (q. arom. *C*), 155.63, 155.55 (*C*=O Boc), 166.17, 166.12 (BnO-*C*=O), 171.44, 171.29 (N-*C*=O Leu), 179.80, 179.62 ppm (N-*C*=O Nip); HR ESIMS: calcd for $C_{35}H_{45}N_3O_8$, [*M*+H$^+$]: 636.3285, found: 636.3295.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-glycyl-(*R*+*S*)-nipecotyl]aziridine-2,3-dicarboxylate, Boc-Gly-(*R*+*S*)-Nip-(*S*,*S*)-Azi(OBn)$_2$ (17a+b): Method C starting with 1 mmol aziridine yielded 361 mg (62%) of a 1:1 mixture of diastereomers **17a** and **17b** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.15$; $[\alpha]_D^{20} = +12.5$ ($c = 1.19$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3417$, 2975, 1710, 1650, 1497, 1459, 1442, 1366, 1250, 1166, 1049, 1028, 699 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 1.38$–1.54–1.38 (m, Boc + 5′-Nip CH_2, 10H), 1.61–1.88 (m, 4′-Nip + 5-Nip CH_2, 2H), 2.04–2.14 (m, 4-Nip CH_2, 1H), 2.44–2.61 (m, 3-Nip C*H*, 1H), 2.97–3.09 and 3.10–3.23 (m each, together 1H, 6′-Nip CH_2), 3.51–3.58 and 3.85–4.04 (m each, together 3H, 6-Nip CH_2 + Gly CH_2), 3.61–3.71 and 4.46–4.56 (m each, together 1H, 2-Nip CH_2), 2.97–3.09 and 3.33–3.45 (m each, together 1H, 2′-Nip CH_2), 3.60 (s, 2×Azi C*H*, 2H), 5.12 (bd, $J = 6.1$ Hz, 1H, N*H* Gly), 5.15–5.24 (m, 2×OCH_2Ph, 4H), 7.26–7.44 ppm (m, arom. C*H*, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 24.66$, 24.61 (5-CH_2 Nip), 26.61, 26.42 (4-CH_2 Nip), 28.35, 28.30 (Boc), 40.71, 40.39 (2×Azi C*H*), 42.27, 42.02 (Gly-CH_2), 43.65, 43.35 (3-C*H* Nip), 45.43, 45.26 (6-CH_2 Nip), 46.75, 45.95 (2-CH_2 Nip), 67.81, 67.70 (OCH_2Ph), 79.33, 79.29 (q. *C* Boc), 128.59, 128.49, 128.47 (arom. C*H*), 134.79 (q. arom. *C*), 155.48, 155.42 (*C*=O Boc), 165.87, 165.81 (BnO-*C*=O), 171.40, 171.33 (N-*C*=O Gly), 179.80, 179.64 ppm (N-*C*=O Nip); HR ESIMS: calcd for $C_{31}H_{37}N_3O_8$, [*M*+H$^+$]: 580.2659, found: 580.2669.

(2*S*,3*S*)-Dibenzyl-1-{1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl]-(*S*)-aziridine-2-carbonyl}aziridine-2,3-dicarboxylate, Boc-(*S*)-Leu-(*S*)-Azy-(*S*,*S*)-Azi(OBn)$_2$ (18a): Method C starting with 1 mmol aziridine-2,3-dicarboxylate yielded 123 mg (23%) **18a** as colorless viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.50$; $[\alpha]_D^{20} = +31.1$ ($c = 1.06$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3352$, 2959, 1742, 1695, 1500, 1454, 1367, 1250, 1167, 1105, 1022, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 0.87$ (d, $J = 4.2$ Hz, Leu-δ′ CH_3, 3H), 0.89 (d, $J = 4.3$ Hz, Leu-δ CH_3, 3H), 1.40 (bs, Boc, 9H), 1.45–1.58 (m, β′-CH_2 Leu, 1H), 1.62–1.82 (m, β-CH_2 Leu + γ-Leu C*H*, 2H), 2.55 (d, $J = 1.5$ Hz, 3′-CH_2 Azy, 1H), 2.58 (d, $J = 5.7$ Hz, 3-CH_2 Azy, 1H), 3.40 (dd, $J = 2.9$, 5.4 Hz, 2-C*H* Azy, 1H), 3.61 (s, 2×Azi C*H*, 2H), 4.25 (dt, $J = 4.6$, 8.7 Hz, Leu-α C*H*, 1H), 4.96 (d, 1H, $J = 8.0$ Hz, Leu N*H*), 5.14–5.24 (m, 2×OCH_2Ph, 4H), 7.25–7.44 ppm (m, arom. C*H*, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 22.83$, 21.84 (Leu-δ CH_3), 24.75 (Leu-γ C*H*), 28.29 (Boc), 30.46 (3-CH_2 Azy), 34.36 (2-C*H* Azy), 41.38 (2×Azi C*H*), 41.88 (Leu-β CH_2), 53.96 (Leu-α C*H*), 67.80 (OCH_2Ph), 79.87 (Boc q. *C*), 128.62, 128.54, 128.49 (arom. C*H*), 134.78 (q. arom. *C*), 155.55 (*C*=O Boc), 165.86 (BnO-*C*=O), 181.31 (N-*C*=O Azy), 184.26 ppm (N-*C*=O Leu); HR ESIMS: calcd for $C_{32}H_{39}N_3O_8$, [*M*+H$^+$]: 593.2737, found: 593.2734.

(2*S*,3*S*)-Dibenzyl-1-{1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-leucyl]-(*S*)-aziridine-2-carbonyl}aziridine-2,3-dicarboxylate, Boc-(*R*)-Leu-(*S*)-Azy-(*S*,*S*)-Azi(OBn)$_2$ (18e): Method C starting with 1 mmol aziridine-2,3-dicarboxylate yielded 318 mg (59%) **18e** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.48$; $[\alpha]_D^{20} = +64.4$ ($c = 1.26$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3350$, 2959, 1742, 1699, 1500, 1454, 1367, 1250, 1166, 1020, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 0.88$ (d, $J = 4.1$ Hz, Leu-δ′ CH_3, 3H), 0.89 (d, $J = 4.2$ Hz, Leu-δ CH_3, 3H), 1.40 (bs, Boc, 9H), 1.46–1.58 (m, β′-Leu, 1H), 1.64–1.78 (m, β-Leu CH_2 + γ-Leu C*H*, 2H), 2.55 (d, $J = 1.5$ Hz, 3′-CH_2 Azy, 1H), 2.59 (dd, $J = 5.8$, 1.5 Hz, 3-CH_2 Azy, 1H), 3.40 (dd, $J = 2.9$, 5.5 Hz, 2-C*H* Azy, 1H), 3.63 (s, 2×Azi C*H*, 2H), 4.25 (dt, $J = 4.7$, 8.6 Hz, Leu-α C*H*, 1H), 4.96 (d, $J = 8.2$ Hz, Leu N*H*, 1H), 5.12–5.25 (m, OCH_2Ph, 4H), 7.27–7.45 ppm (m, arom. C*H*, 10H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 22.37$, 21.38 (δ-CH_3 Leu), 24.29 (γ Leu C*H*), 27.83 (Boc), 30.00 (3-CH_2 Azy), 33.90 (2-C*H* Azy), 40.38 (2×Azi C*H*), 41.43 (β-CH_2 Leu), 53.50 (α-C*H* Leu), 67.80 (OCH_2Ph), 79.41 (q. *C* Boc), 128.16, 128.08, 128.03 (arom. C*H*), 134.78 (q. arom. *C*), 155.10 (*C*=O Boc), 165.86 (BnO-*C*=O), 180.22 (N-*C*=O Azy), 183.81 ppm (N-*C*=O Leu); HR ESIMS: calcd for $C_{32}H_{39}N_3O_8$, [*M*+H$^+$]: 593.2737, found: 593.2731.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Phe-(*S*)-Ala-(*S*,*S*)-Azi-(OBn)$_2$ (19a): Method C starting with 0.25 mmol aziridine yielded 65 mg (41%) **19a** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f = 0.22$; $[\alpha]_D^{20} = +27.4$ ($c = 1.96$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3485$, 2924, 1741, 1656, 1593, 1499, 1450, 1426, 1366, 1325, 1248, 1170, 1119, 1021, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta = 1.37$ (d, $J = 7.2$ Hz, 3H, β-Ala CH_3), 1.40 (bs, 9H, Boc), 2.98–3.09 (m, 2H, β-Phe CH_2), 3.69 (s, 2H, 2×Azi C*H*), 4.31–4.40 (bs, 1H, α-Ala C*H*), 4.43 (p, $J = 7.0$ Hz, 1H, α-Phe C*H*), 5.05–5.11 (bs, 1H, N*H*-Phe), 5.15–5.25 (m, 2×OCH_2Ph, 4H), 6.63 (bs, 1H, N*H*-Ala), 7.15–7.45 ppm (m, 15H, arom. C*H*); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta = 18.26$ (β-CH_3 Ala), 28.26 (CH_3 Boc), 38.43 (β-CH_2 Phe), 40.07 (2×Azi C*H*), 48.21 (α-C*H* Phe), 55.60 (α-C*H* Ala), 67.85 (OCH_2Ph), 80.15 (q. *C* Boc), 129.43, 128.69, 128.65, 128.44, 126.89 (arom. C*H*), 134.85 (q. arom. *C*), 136.66 (q. arom *C*, Phe), 155.46 (O-*C*=O Boc), 165.92 (BnO-*C*=O), 171.11 (N-*C*=O Phe), 180.15 ppm (N-

C=O Ala); HR ESIMS: calcd for $C_{35}H_{39}N_3O_8$, $[M+H^+]$: 630.2815, found: 630.2809.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl-(*R*)-alanyl]aziridine-2,3-dicarboxylate, Boc-(*S*)-Phe-(*R*)-Ala-(*S*,*S*)-Azi-(OBn)$_2$ (19 b): Method C starting with 0.25 mmol aziridine yielded 19 mg (12%) **19 b** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.24$; $[\alpha]_D^{20}=+31.5$ ($c=2.54$, $CHCl_3$); IR (neat): $\tilde{\nu}=3496$, 2928, 1740, 1658, 1596, 1498, 1451, 1426, 1367, 1326, 1248, 1170, 1120, 1021, 699 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta=1.31$ (d, 3H, $J=6.9$ Hz, β-CH_3 Ala), 1.45 (bs, 9H, Boc), 3.10 (d, $J=6.4$ Hz, 2H, β-CH_2 Phe), 3.69 (s, 2×Azi C*H*, 2H), 4.40 (bs, 1H, α-C*H* Ala), 4.55–4.70 (m, 1H, α-C*H* Phe), 5.17 (bs, 1H, N*H*-Phe), 5.21–5.33 (m, 2×OCH_2Ph, 4H), 6.46 (bd, $J=7.3$ Hz, 1H, N*H*-Ala), 7.22–7.53 ppm (m, 15H, arom. C*H*); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=18.20$ (β-CH_3 Ala), 28.38 (Boc), 38.89 (β-CH_2 Phe), 40.59 (2×Azi *C*H), 48.13 (α-*C*H Phe), 55.83 (α-*C*H Ala), 67.98 (O*C*H_2Ph), 80.27 (q. *C* Boc), 129.43, 128.73, 128.53, 128.20, 127.02 (arom. *C*H), 134.98 (q. arom. *C*), 136.79 (q. arom *C*, Phe), 155.47 (*C*=O Boc), 166.05 (BnO-*C*=O), 172.49 (N-*C*=O Phe), 180.18 ppm (N-*C*=O Phe); HR ESIMS: calcd for $C_{35}H_{39}N_3O_8$, $[M+H^+]$: 630.2815, found: 630.2813.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylate, Boc-(*R*)-Phe-(*S*)-Ala-(*S*,*S*)-Azi-(OBn)$_2$ (19 e): Method C starting with 0.25 mmol aziridine yielded 25 mg (16%) **19 e** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.35$; $[\alpha]_D^{20}=-52.1$ ($c=1.91$, $CHCl_3$); IR (neat): $\tilde{\nu}=3486$, 2934, 1739, 1659, 1594, 1451, 1426, 1368, 1322, 1248, 1169, 1120, 1022, 698 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta=$ 1.26 (d, 3H, $J=6.9$ Hz, β-CH_3 Ala), 1.40 (bs, 9H, Boc), 3.05 (d, $J=$ 6.4 Hz, 2H, β-Phe CH_2), 3.64 (s, 2H, 2×Azi C*H*), 4.35–4.47 (bs, 1H, α-Ala C*H*), 4.50–4.63 (m, 1H, α-Phe C*H*), 5.11 (bs, 1H, N*H*-Phe), 5.16–5.26 (m, 2×OCH_2Ph, 4H), 6.41 (bd, $J=7.4$ Hz, 1H, N*H*-Ala), 7.16–7.46 ppm (m, 15H, arom. C*H*); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=18.02$ (β-CH_3 Ala), 28.20 (Boc), 38.71 (β-CH_2 Phe), 40.41 (2×Azi *C*H), 47.95 (α-*C*H Phe), 55.65 (α-*C*H Ala), 67.80 (OCH_2Ph), 80.11 (q. *C* Boc), 129.27, 128.57, 128.37, 128.04, 126.86 (arom. *C*H), 134.82 (q. arom. *C*), 136.63 (q. arom *C*, Phe), 155.31 (*C*=O Boc), 165.88 (BnO-*C*=O), 172.33 (N-*C*=O Phe), 180.02 ppm (N-*C*=O Phe); HR ESIMS: calcd for $C_{35}H_{39}N_3O_8$, $[M+H^+]$: 630.2815, found: 630.2822.

(2*S*,3*S*)-Dibenzyl-1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-phenylalanyl-(*R*)-alanyl]aziridine-2,3-dicarboxylate, Boc-(*R*)-Phe-(*R*)-Ala-(*S*,*S*)-Azi-(OBn)$_2$ (19 f): Method C starting with 0.25 mmol aziridine yielded 46 mg (29%) **19 f** as yellowish viscous liquid; cc silica gel 60, cyclohexane/EtOAc 2:1, $R_f=0.27$; $[\alpha]_D^{20}=+14.3$ ($c=2.03$, $CHCl_3$); IR (neat): $\tilde{\nu}=3495$, 2918, 1739, 1651, 1596, 1499, 1449, 1425, 1365, 1324, 1246, 1168, 1119, 1022, 699 cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): $\delta=1.35$ (d, 3H, $J=7.2$ Hz, β-CH_3 Ala), 1.37 (bs, 9H, Boc), 2.96–3.06 (m, 2H, β-CH_2 Phe), 3.67 (s, 2H, 2×Azi C*H*), 4.29–4.37 (bs, 1H, α-C*H* Ala), 4.41 (p, $J=7.0$ Hz, 1H, α-C*H* Phe), 5.03–5.08 (bs, 1H, N*H*-Phe), 5.13–5.22 (m, 4H, 2×OCH_2Ph), 6.62 (bs, 1H, N*H*-Ala), 7.12–7.42 ppm (m, 15H, arom. H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): $\delta=17.84$ (β-CH_3 Ala), 27.84 (*C*H$_3$ Boc), 38.01 (β-CH_2 Phe), 39.65 (2×Azi *C*H), 47.79 (α-*C*H Phe), 55.18 (α-*C*H Ala), 67.80 (OCH_2Ph), 80.10 (q. *C* Boc), 129.37, 128.63, 128.59, 128.39, 126.83 (arom. *C*H), 134.79 (q. arom. *C*), 136.61 (q. arom *C*, Phe), 155.40 (*C*=O Boc), 165.87 (BnO-*C*=O), 171.05 (N-*C*=O Phe), 180.10 ppm (N-*C*=O Ala); HR ESIMS: calcd for $C_{35}H_{39}N_3O_8$, $[M+H^+]$: 630.2815, found: 630.2807.

(2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarbocylic acid, Boc-(*S*)-Phe-(*S*)-Ala-(*S*,*S*)-Azi(OH)$_2$ (20 a): Method D starting with 10 μmol **19 a** yielded 4.3 mg (96%) **20 a** as colorless crystals; ESIMS: $C_{21}H_{27}N_3O_8$ $[M-H^+]$: 448.1.

(2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-phenylalanyl-(*R*)-alanyl]aziridine-2,3-dicarboxylic acid, Boc-(*S*)-Phe-(*R*)-Ala-(*S*,*S*)-Azi(OH)$_2$ (20 b): Method D starting with 10 μmol **19 b** yielded 4.2 mg (94%) **20 b** as colorless crystals; ESIMS: $C_{21}H_{27}N_3O_8$ $[M-H^+]$: 448.2.

(2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-phenylalanyl-(*S*)-alanyl]aziridine-2,3-dicarboxylic acid, Boc-(*R*)-Phe-(*S*)-Ala-(*S*,*S*)-Azi(OH)$_2$ (20 e): Method D starting with 10 μmol **19 e** yielded 4.4 mg (98%) **20 e** as colorless crystals; ESIMS: $C_{21}H_{27}N_3O_8$ $[M-H^+]$: 448.2.

(2*S*,3*S*)-1-[N-(tert-butoxycarbonyl)-(*R*)-phenylalanyl-(*R*)-alanyl]aziridine-2,3-dicarboxylic acid, Boc-(*R*)-Phe-(*R*)-Ala-(*S*,*S*)-Azi(OH)$_2$ (20 f): Method D starting with 10 μmol **19 f** yielded 4.3 mg (96%) **20 f** as colorless crystals; ESIMS: $C_{21}H_{27}N_3O_8$ $[M-H^+]$: 448.3.

(2*S*,3*S*)-1-[N-(tert-butoxycarbonyl)-(*S*)-leucyl-(*S*)-prolyl]aziridine-2,3-dicarboxylic acid, Boc-(*S*)-Leu-(*S*)-Pro-(*S*,*S*)-Azi(OH)$_2$ (21 a): Method D starting with 10 μmol of the respective dibenzyl ester **13 a** yielded 4.3 mg (97%) **21 a** as colorless crystals; ESIMS: $C_{20}H_{31}N_3O_8$ $[M+OH^-]$: 458.1.

(2*S*,3*S*)-1-[N-(tert-butoxycarbonyl)-glycyl-(*S*)-prolyl]aziridine-2,3-dicarboxylic acid, Boc-Gly-(*S*)-Pro-(*S*,*S*)-Azi(OH)$_2$ (22 a): Method D starting with 10 μmol of the respective dibenzyl ester **14 a** yielded 3.6 mg (94%) **22 a** as colorless crystals; ESIMS: C_{16} H_{23} N_3 O_8 $[M-H^+]$: 384.1.

(2*S*,3*S*)-1-[N-(tert-butoxycarbonyl)-glycyl-(*R*)-prolyl]aziridine-2,3-dicarboxylic acid, Boc-Gly-(*R*)-Pro-(*S*,*S*)-Azi(OH)$_2$ (22 b): Method D starting with 10 μmol of the respective dibenzyl ester **14 b** yielded 3.5 mg (92%) **22 b** as colorless crystals; ESIMS: C_{16} H_{23} N_3 O_8 $[M-H^+]$: 384.3.

(2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-(*R*+*S*)-nipecotyl]aziridine-2,3-dicarboxylic acid, Boc-(*S*)-Leu-(*R*+*S*)-Nip-(*S*,*S*)-Azi(OH)$_2$ (23 a+b): Method D starting with 10 μmol of a mixture of the respective dibenzyl esters **16 a+b** yielded 4.4 mg (98%) of a 1:1 mixture of diastereomers **23 a** and **23 b** as colorless crystals; ESIMS: $C_{21}H_{33}N_3O_8$ $[M-H^+]$: 454.1.

(2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*R*)-leucyl-(R+S)-nipecotyl]aziridine-2,3-dicarboxylic acid, Boc-(*R*)-Leu-(*R*+*S*)-Nip-(*S*,*S*)-Azi(OH)$_2$ (23 e+f): Method D starting with 10 μmol of a mixture of the respective dibenzyl esters **16 e+f** yielded 4.2 mg (93%) of a 1:1 mixture of diastereomers **23 e** and **23 f** as colorless crystals; ESIMS: $C_{21}H_{33}N_3O_8$ $[M-H^+]$: 454.4.

(2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-glycyl-(*R*+*S*)-nipecotyl]aziridine-2,3-dicarboxylic acid, Boc-Gly-(*R*+*S*)-Nip-(*S*,*S*)-Azi(OH)$_2$ (24 a+b): Method D starting with 10 μmol of a mixture of the respective dibenzyl esters **17 a+b** yielded 3.6 mg (90%) of a 1:1 mixture of diastereomers **24 a** and **24 b** as colorless crystals; ESIMS: $C_{17}H_{25}N_3O_8$ $[M-H^+]$: 398.1.

(2*S*,3*S*)-1-[*N*-(*tert*-butoxycarbonyl)-(*S*)-leucyl-isonipecotyl]aziridine-2,3-dicarboxylic acid, Boc-(*S*)-Leu-Ini-(*S*,*S*)-Azi(OH)$_2$ (25 a): Method D starting with 10 μmol of the respective dibenzyl ester yielded 4.1 mg (90%) **25 a** as colorless crystals; ESIMS: $C_{21}H_{33}N_3O_8$ $[M-H^+]$: 454.3.

(2*S*,3*S*)-Dibenzyl-1-[biotinyl-6-aminohexanoyl]-aziridine-2,3-dicarboxylate, Bio-Hxa-(*S*,*S*)-Azi(OBn)$_2$ (26): 508 mg (2.2 mmol) Boc-6-amino hexanoic acid (Boc-Hxa) and 227 mg (1.1 mmol) DCC are stirred in 15 mL CH_2Cl_2 at 0 °C for 30 min. Insoluble dicyclohexylurea is filtered off, and 311 mg (1 mmol) aziridine-2,3-dicarboxylate dissolved in 1 mL CH_2Cl_2 are added, followed by addition of 60 mg

(0.5 mmol) DMAP. The mixture is stirred for 12 h at room temperature. The solvent is removed in vacuo, and the residue is dissolved in 100 mL diethyl ether. The organic phase is washed twice with 10% Na_2CO_3 (30 mL) and 10% citric acid (30 mL). After removal of the solvent, the intermediate Boc-Hxa-(*S*,*S*)-Azi-$(OBn)_2$ is dissolved in 5 mL TFA/CH_2Cl_2 (1:5) at 0 °C and stirred for 1 h. The residue remaining after evaporation of the solvent is dissolved in 5 mL DMF. 2 μL (2 mmol) Et_3N and 375 mg (1.1 mmol) biotin-*N*-hydroxysuccinimidate[36] are added at room temperature, and the solution is stirred for 5 days. After removal of the solvent Bio-Hxa-(*S*,*S*)-Azi-$(OBn)_2$ (**26**) is purified by flash chromatography on silica gel 60 (methanol/$CHCl_3$ gradient); overall yield: 253 mg (39%) **26** as yellowish solid; TLC $R_f = 0.45$ with silica gel 60, methanol/$CHCl_3$ 1:10; $[\alpha]_D^{20} = -30.4$ ($c = 1.01$, MeOH); IR (neat): $\tilde{\nu} = 3250$ (m, br.), 2930 (m), 2862 (m), 2360 (s), 1735 (s), 1697 (s, C=O), 1637 (s), 1379 (s), 1324 (s), 1182 (s), 1074 (s), 954 (m), 730 (s), 649 (m) cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): aziridine: $\delta = 3.49$ (s, 2H, 2×C*H* ring), 5.19 (s, 4H, 2×CH_2), 7.30–7.37 (m, 10H, 2C_6H_5); amino hexanoic acid: $\delta = 1.15$–1.30 (m, 2H, 4-CH_2), 1.33–1.48 (m, 2H, 5-CH_2), 1.58–1.75 (m, 2H, 3-CH_2), 2.10–2.25 (m, 1H, 2-CH_a), 2.28–2.40 (m, 1H, 2-CH_b), 3.07–3.15 (m, 2H, 6-CH_2), 6.76 (bt, 1H, N*H*); biotin: 1.33–1.48 (m, 2H, 4-CH_2), 1.48–1.75 (m, 4H, 3+5-CH_2), 2.20–2.25 (m, 2H, 2-CH_2), 2.73 (d, 1H, $J = 12.1$ Hz, *cis* 12-CH_2), 2.87 (dd, 1H, $J = 12.1$, 5.0 Hz, *trans* 12-CH_2), 4.30–4.35 (m, 1H, 7-C*H*), 4.50–4.55 (m, 1H, 11-C*H*), 5.7 (bs, 1H, 10-N*H*), 6.32 ppm (bs, 1H, 8-N*H*); ^{13}C NMR ($CDCl_3$, 100.62 MHz): aziridine: $\delta = 40.0$ (2×*C*H ring), 68.2 (2×CH_2), 128.6, 128.7, 128.8, 134.5 (arom. *C*), 168.4 ppm (2×*C*=O); amino hexanoic acid: $\delta = 23.8$ (3-CH_2), 25.6 (4-CH_2), 28.9 (5-CH_2), 36.5 (2-CH_2), 39.4 (6-CH_2), 173.2 (1-*C*=O); biotin: 26.2 (3-CH_2), 27.8 (5-CH_2), 27.9 (4-CH_2), 35.7 (2-CH_2), 40.5 (12-CH_2), 55.4 (6-CH), 60.5 (11-*C*H), 62.2 (7-*C*H), 169.4 (1-*C*=O), 180.5 ppm (9-*C*=O); ESIMS *m/z*: 651.3 [*M*+H^+] (100%), 673.3 [*M*+Na^+] (25%); HR ESIMS: calcd for $C_{34}H_{42}N_4O_7S$, [*M*+H^+]: 651.2852, found: 651.2855.

(2*S*,3*S*+2*R*,3*R*)-Dibenzyl-1-[desthiobiotinyl-6-aminohexanoyl]-aziridine-2,3-dicarboxylate, DsBio-Hxa-(*S*,*S*+*R*,*R*)-Azi$(OBn)_2$ (27): 522 mg (2.26 mmol) Boc-6-amino hexanoic acid (Boc-Hxa) and 256 mg (1.24 mmol) DCC are stirred in 20 mL CH_2Cl_2 at 0 °C for 30 min. Insoluble dicyclohexylurea is filtered off and 352.3 mg (1.13 mmol) racemic dibenzyl aziridine-2,3-dicarboxylate[12] dissolved in 2 mL CH_2Cl_2 are added, followed by addition of 67.8 mg (0.565 mmol) DMAP. The mixture is stirred for 12 h at room temperature. The solvent is removed in vacuo and the residue is dissolved in 100 mL diethyl ether. The organic phase is washed twice with 10% Na_2CO_3 (30 mL) and 10% citric acid (30 mL). After removal of the solvent the intermediate Boc-Hxa-(*S*,*S*+*R*,*R*)-Azi-$(OBn)_2$ is dissolved in 5 mL TFA/CH_2Cl_2 (1:5) at 0 °C and stirred for 1 h. The residue remaining after removal of the solvent is dissolved in 25 mL DMF, and 356.4 mg (1.44 mmol) EEDQ, 308.59 mg (1.44 mmol) desthiobiotin and 307 μL (1.44 mmol) TEA are added. After 48 h stirring at room temperature the solvent is removed in vacuo and the crude product is purified by flash chromatography on silica gel 60 (1. $CHCl_3$/MeOH 5:1; 2. 5% MeOH in $CHCl_3$); overall yield: 73.4 mg (16%) **27** as yellowish viscous liquid; TLC $R_f = 0.63$ with silica gel 60, methanol/$CHCl_3$ 1:5; $[\alpha]_D^{20} = +12$ ($c = 0.8$, $CHCl_3$); IR (neat): $\tilde{\nu} = 3276.5$ (m, br.), 2961 (m), 2929 (m), 2858 (m), 2361 (s), 1734 (s), 1694 (s, C=O), 1645 (s), 1259 (s), 1015 (s), 795 (m), 679 (m) cm^{-1}; ^1H NMR ($CDCl_3$, 400.13 MHz): aziridine: $\delta = 3.41$ (s, 2H, 2×C*H* ring), 5.13 (s, 4H, 2×CH_2), 7.28–7.31 ppm (m, 10H, 2×C_6H_5); amino hexanoic acid: $\delta = 1.17$–1.27 (m, 2H, 4-CH_2), 1.52–1.62 (m, 4H, 5-CH_2 and 3-CH_2), 2.07–2.15 (m, 1H, 2-CH_a), 2.23–2.33 (m, 1H, 2-CH_b), 3.12–3.17 (m, 2H, 6-CH_2), 4.66 ppm (bs, 1H, N*H*); desthiobiotin: $\delta = 1.05$ (d, 3H, 12-CH_3, $^3J = 6.3$ Hz), 1.32–1.46 (m, 4H, 4-CH_2 and 5-CH_2), 1.52–1.63 (m, 4H, 3-CH_2 and 6-CH_2), 2.23–2.33 (m, 2H, 2-CH_2), 3.59–3.65 (m, 1H, 7-C*H*), 3.76–3.81 (m, 1H, 11-C*H*), 5.69 (bs, 1H, N*H*), 5.75 ppm (bs, 1H, N*H*); ^{13}C NMR ($CDCl_3$, 100.62 MHz): aziridine: $\delta = 39.01$ (*C*H ring), 67.17 (CH_2), 127.64, 127.74, 127.84, 133.49 (aromatic), 171.93 ppm (*C*=O); amino hexanoic acid: $\delta = 23.47$ (3-CH_2), 25.02 (4-CH_2), 28.47 (5-CH_2), 35.31 (2-CH_2), 38.19 (6-CH_2), 172.46 ppm (1-*C*=O); desthiobiotin: $\delta = 14.74$ (12-CH_3), 25.35 (5-CH_2), 27.87 (6-CH_2), 28.15 (4-CH_2), 35.46 (3-CH_2), 38.10 (2-CH_2), 50.42 (11-*C*H), 55.01 (7-*C*H), 172.52 (1-*C*=O), 179.23 ppm (9-*C*=O); ESIMS: $C_{34}H_{44}N_4O_7$ [*M*]$^+$ 620.75.

Enzyme assays: The fluorimetric enzyme assays were performed on a Cary Eclipse fluorescence spectrophotometer from Varian, Darmstadt, Germany, using a microplate reader ($\lambda_{excitation} = 365$ nm, $\lambda_{emission} = 460$ nm). For the inhibition assays, 96-well microplates from Nunc GmbH, Wiesbaden, Germany, were used. Assays were performed at 25 °C in a 20 mM Tris-HCl buffer, pH 6.0, containing 5 mM EDTA, 2.5 mM DTT, 200 mM NaCl, 0.005% Brij 35 in a total volume of 285 μL. Substrate (Cbz-Phe-Arg-AMC for both enzymes) and inhibitor stock solutions were prepared in DMSO (10% final concentration) and were diluted with assay buffer; enzymes were held in buffer. The final substrate concentration for inhibition assays was between 10.0 and 81.0 μM; the final enzyme concentration was 53 ng mL^{-1} for CL (*P. tetraurelia*) and 58 pg mL^{-1} for CB (recombinant, human liver). Inhibitors were used at concentrations between 0.35 μM and 140 μM.

K_i values for time-independent inhibition were obtained by Dixon plots[37] using the equation $v_0/v_i = 1 + [I]/K_i^{app}$ and correction to zero substrate concentration from $K_i = K_i^{app}/(1+[S]/K_M)$. In cases of time-dependent inhibition, 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[38] by monitoring the product released from hydrolysis of the substrate in the presence of the inhibitor as a function of time (fluorescence = $A(1-\exp(-k_{obs}t)) + B$). Fitting of the k_{obs} values 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. K_i and k_i values were calculated by nonlinear regression analyses using the program GraFit.[39] All values are mean values from at least two independent assays. The K_M values used to correct K_i^{app} values were 6.5 μM for CL and 150.0 μM for CB.

Affinity labeling: Cathepsin L from *P. tetraurelia* was incubated in 50 mM sodium acetate, 2 mM dithiothreitol pH 5.5, supplemented with 10 μM of the biotinylated compound at 25 °C for 1 h. Excess of the compound was removed from the sample by use of a PD10 column (Amersham Biosciences) equilibrated in the same buffer. Protein was subjected to reversed-phase chromatography using a PRP-3 column (Hamilton, Reno, USA). Eluted protein was collected and subjected to SDS-polyacrylamide 12.5% separating gels. Proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were electrotransferred to a polyvinylidene difluoride (PVDF) membrane or directly stained with silver nitrate.[40] As a control, untreated cathepsin L was used. Enzyme–inhibitor complexes were detected on the membrane by alkaline phosphatase conjugated streptavidin (Promega). BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitro blue tetrazolium) were used as substrates for the colorimetric detection of alkaline phosphatase activity (both obtained from Promega).

Docking procedures: *Ligands*: The ligand input structures were sketched and energy-minimized using Sybyl (Tripos force field, 2000 iterations, Powell minimizer). The tilted angle at the aziridine

nitrogen atom obtained from quantum chemical calculations was kept rigid during minimization. Within all ligand mol2 files, the bond type "UN" was assigned to the carbon–carbon bond of the aziridine ring. This prevented CORINA, which is automatically executed during FlexX docking for the generation of ring conformations, from processing the aziridine ring and altering the tilted bond angle.

Docking with FlexX was performed with default settings except the parameters SOLUTIONS_PER_IT 800 and SOLUTIONS_PER_FRAG 400. Although this results in longer computation times, more solutions are generated and evaluated during the docking process, leading to a larger probability of finding the correct binding mode.

Additionally, the geometry of the spherical cap representing the hydrogen bonding interaction of the aziridine nitrogen atom had to be slightly adjusted (sp^3 hybridization model only). In the geometry.dat parameter file, the respective interaction center was defined with the entry "cone e −1 −0.55 −0.55 0 40". Furthermore, in the contact.dat file, a "@subgraph" entry matching the sp^3 aziridine moiety was defined (using the respective amino subgraph as a template).

Receptors: The X-ray crystal structure with the PDB code 1MHW was taken as target structure cathepsin L. The active site was defined with Sybyl as a region about 12 Å around the cysteine residue responsible for covalent ligand binding (Cys 25). The sulfur atom of this active site cysteine was deleted to deliberately remove its influence. Otherwise, the noncovalent pre-transition state could not be reliably simulated. Applying this procedure to 10 cysteine protease–inhibitor complexes taken from the Brookhaven protein data bank showed that the crystal structure binding mode could be reproduced with a median RMSD of about 2 Å for the best docking pose (see Supporting Information). Given the high flexibility of the investigated compounds (the compound with the fewest rotatable bonds has 17 of them), the prediction was found sufficient. The FlexX his+ template was assigned to the histidine residue in the active site, leading to a positively charged histidinium ion. Water molecules were removed from the protein structure.

The parameters to obtain the two different aziridide geometries and to simulate the two different hybridization states are explained in the following. The tilted angles were conserved by preventing CORINA from processing the aziridine rings. Thus, for the latter fragment FlexX used the given structure obtained from the quantum chemical calculations. The docking models for sp^2 and sp^3 hybridization differ only in the atom type of the aziridine nitrogen atom within the Sybyl mol2 file. For the sp^2 and sp^3 model, we assigned to it the N.am and N.3 atom type, respectively.

For the sp^3 hybridization model, minor changes in the hydrogen bond interaction geometry of the aziridine nitrogen atom were required. The special situation of a tilted "amide" bond with the nitrogen atom having partial sp^3 character is not defined in the FlexX standard parameterization. Therefore, we had to slightly adjust the direction of the hydrogen bonding interaction of the aziridine nitrogen atom.

Quantum chemical calculations: All the structures were first optimized using the DFT method combined with the B[41]-LYP[42] functional and the TZVP[43] basis set. The RI approach[44,45] was employed. In one set of calculations all internal coordinates were allowed to change, while in the other set the geometry was kept planar ($\measuredangle$(C–C–N–C) = 180°). The final energies were obtained in the single-point calculations on the optimized structures using the B3[46]-LYP functional and TZVP basis set. All the calculations were done in the TURBOMOLE[47] program package.

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Keywords: aziridines · cathepsins · cysteine proteases · docking · inhibitors

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[14] The following list of descriptors are used for the different stereoisomers of aziridinyl peptides.

X-Xaa-Caa-Azi[a]			
Xaa	Caa or Ala	Azi	Descriptor
	S	*S*,*S*	**a**
	R	*S*,*S*	**b**
	S	*R*,*R*	**c**
	R	*R*,*R*	**d**
S	*S*	*S*,*S*	**a**
S	*R*	*S*,*S*	**b**
S	*S*	*R*,*R*	**c**
S	*R*	*R*,*R*	**d**
R	*S*	*S*,*S*	**e**
R	*R*	*S*,*S*	**f**

[a] X = protecting group; Xaa = Leu, Gly, Phe; Caa = cyclic amino acid: Azy, Azet, Pro, Pip, Nip, Ini; Azi = aziridine-2,3-dicarboxylate.

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[19] The diastereomers were separated by cc on silica gel 60, petrolether/diethyl ether 3:1. Assignment of configuration to pipecolic acid in the aziridinyl dipeptides **3a** and **3b**, **3c** and **3d**, and **4c** and **4d**, and in the aziridinyl tripeptide **15a** was done by comparison with samples prepared with enantiomerically enriched Boc-protected pipecolic acids. These are commercially available as Boc-(*S*)-Pip and Boc-(*R*)-Pip. However, chromatographic analysis on a chiral HPLC column (Chirobiotic T, 250×4.6 mm, 5 μm, isopropanol/water 60:40, flow 0.5 mL min^{-1}) showed the enantiomeric purity of the *R* enantiomer to be only 76% *ee* and of the *S* enantiomer, 92% *ee* (see Supporting Information).

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