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Novel 3-amino-2-hydroxy acids containing protease inhibitors. Part 1: Synthesis and kinetic characterization as aminopeptidase P inhibitors

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Abstract—Novel, potent inhibitors of aminopeptidase P, containing a 3-amino-2-hydroxy acid and a proline or a proline analogues, have been prepared. One part of the bestatin-derived inhibitors was found to inhibit APP from *Escherichia coli* and from rat intestine according to a mixed-type mechanism, with K_i values up to 1.26 μM. The other compounds, 3-amino-2-hydroxy acyl prolines of a different configuration, inhibit APP competitively, according to a slow-binding mechanism, with K_i values in the nanomolar up to the micromolar range.

1. Introduction

Aminopeptidase P (APP, EC 3.4.11.9) is a high specific exopeptidase cleaving N-terminal Xaa-Pro peptide bonds. Originally, APP was isolated from *Escherichia coli*.[1] Later it was found in other bacteria and mammals.[2] The enzyme is multimer. In mammals, APP occurs membrane bound by a glycosylinositol anchor or in soluble form.[2c,3] It was shown that recombinant APP from *E. coli* contains a dinuclear manganese center.[4] The enzyme is a member of the methionyl aminopeptidase family (M24). This peptidase family represents a set of aminopeptidases (APP, methionine aminopeptidase (MetAP), and prolidase). All these enzymes contain cocatalytic metal atoms and are so-called 'pita-bread'-fold enzymes.[5]

On account of its unique substrate specificity, APP is involved in protein turnover of collagen and regulation of biological active peptides, such as substance P and bradykinin.[6] Kitamura et al.[7] and Prechel et al.[8] have shown that APP together with angiotensin-converting enzyme inactivates bradykinin.

3-Amino-2-hydroxy acids are the characteristic structural element of several important natural products and therapeutics, such as taxol, bestatin, and HIV-protease inhibitors.[9] The natural occurring aminopeptidase inhibitors bestatin, amastatin, probestin, and leuhistin contain the 3-amino-2-hydroxy acid in the configuration 2*S*,3*R*.[9c,10] The 3-amino-2-hydroxy acyl residue takes part in the chelation of the Zn^{2+} in the active site of metal-dependent enzymes.[11] Bestatin and amastatin are described as very potent inhibitors of leucine aminopeptidase (LAP, EC 3.4.11.1) and aminopeptidase M (APM, EC 3.4.11.2).[10a,11,12] However, only millimolar concentrations of both compounds are able to inhibit APP.[2b,c] The reason seems to be the different substrate specificities of these aminopeptidases. Bestatin and amastatin contain Leu and Val, respectively, at the P_1'-position, while APP has an absolute requirement for Pro or proline homologous structures in this position.[13] Therefore, we synthesized different stereoisomers of 3-amino-2-hydroxyacyl-prolines, 3-amino-2-hydroxy acid pyrrolidides, and thiazolidides as potential inhibitors of APP

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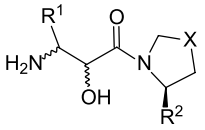


No	configuration	compound	R^1	R^2	X
13	2*S*,3*R*	AHPB-Pro-OMe	benzyl	$COOCH_3$	CH_2
14	2*S*,3*R*	AHPB-Pro-OH	benzyl	COOH	CH_2
16	2*S*,3*R*	AHPB-Pro-Phe-OMe	benzyl	CO-Phe-OCH_3	CH_2
17	2*S*,3*R*	AHPB-Pyrr	benzyl	H	CH_2
18	2*S*,3*R*	AHPB-Thia	benzyl	H	S
19	2*R*,3*R*	AHPB-Pro-OH	benzyl	COOH	CH_2
20	2*R*,3*R*	AHPB-Thia	benzyl	H	S
21	2*R*,3*S*	AHPB-Pro-OH	benzyl	COOH	CH_2
23	2*R*,3*S*	AHPB-Thia	benzyl	H	S
24	2*S*,3*S*	AHPB-Pro-OMe	benzyl	$COOCH_3$	CH_2
25	2*S*,3*R*	AHMH-Pro-OH	isobutyl	COOH	CH_2
26	2*S*,3*R*	AHMH-Thia	isobutyl	H	S

Scheme 1. General structure of APP inhibitors.

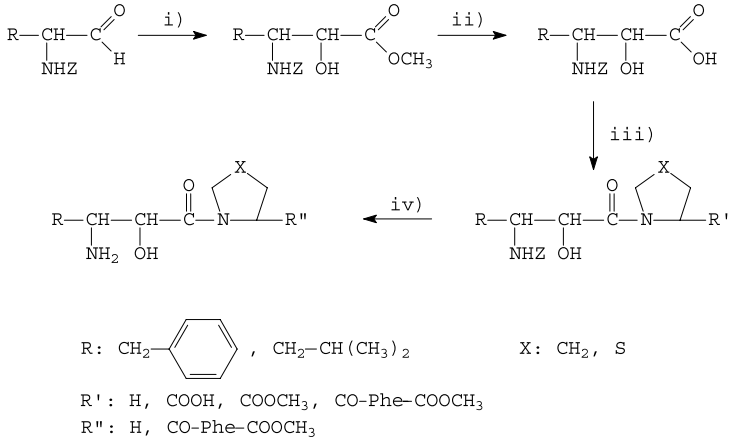
(Scheme 1). Our investigations show that the compounds exhibit different inhibition mechanisms. For the first time, slow-binding inhibitors of APP are described.

2. Results and discussion

2.1. Chemistry

3-Amino-2-hydroxy acids containing peptides and amides were designed as potential APP inhibitors based on the fact that bestatin and amastatin are very potent inhibitors of APM, LAP, and aminopeptidase B.[11–13] Peptides (AHPB/AHMH-Pro; AHPB, 3-amino-2-hydroxy-4-phenylbutanoic acid; AHMH, 3-amino-2-hydroxy-5-methylhexanoic acid), as well as amino acid pyrrolidides (AHPB-Pyrr; Pyrr, pyrrolidide) and amino acid thiazolidides (AHPB/AHMH-Thia; Thia, thiazolidides) were synthesized and investigated for their inhibitory efficiency toward APP of a different origin (Scheme 1).

Z-protected (Z, benzyloxycarbonyl) 3-amino-2-hydroxy acids were synthesized using methods described by Fehrentz and Castro[14], and Herranz et al.[15] (Scheme 2). Thus, Z-protected (*R*)- or (*S*)-amino acid *N*-methyl-*O*-methylcarboxamides were reduced to the corresponding α-amino aldehydes by $LiAlH_4$.[15] These aldehydes were converted to a mixture of two diastereomers of Z-protected 3-amino-2-hydroxy acid methyl esters via *O*-(trimethylsilyl)cyanhydrin. As described by Herranz et al.[15] this synthesis yielded favored the threo isomer of the Z-protected 3-amino-2-hydroxy acid methyl ester with the configuration (2*S*,3*R*) or (2*R*,3*S*) starting from the (*R*)- or (*S*)-amino acid, respectively. The two diastereomers were separated by flash chromatography and saponified, subsequently. Pure diastereomers of Z-protected 3-amino-2-hydroxy acids were coupled with (*S*)-Pro-OMe, (*S*)-Pro-(*S*)-Phe-OMe, pyrrolidine, or thiazolidine using the carbodiimide method. Acidolytic or hydrogenolytic deprotection of Z-protecting group provided the expected final compounds in the following cases: (2*S*,3*R*)-AHPB-(*S*)-Pro-(*S*)-Phe-OMe (**16**), (2*S*,3*R*)-AHPB-Pyrr (**17**), (2*S*,3*R*)-AHPB-Thia (**18**), (2*R*,3*R*)-AHPB-Thia (**20**), (2*R*,3*S*)-AHPB-Thia (**23**), and (2*S*,3*R*)-AHMH-Thia (**26**).



Scheme 2. Synthesis of bestatin-derived inhibitors of APP. (i) 1. TMSCN, 2. HCl/Ether/MeOH, and 3. H_2O; (ii) NaOH/dioxane; (iii) EDC/HOBT/amino compound; and (iv) HBr/acetic acid or H_2/Pd/C.

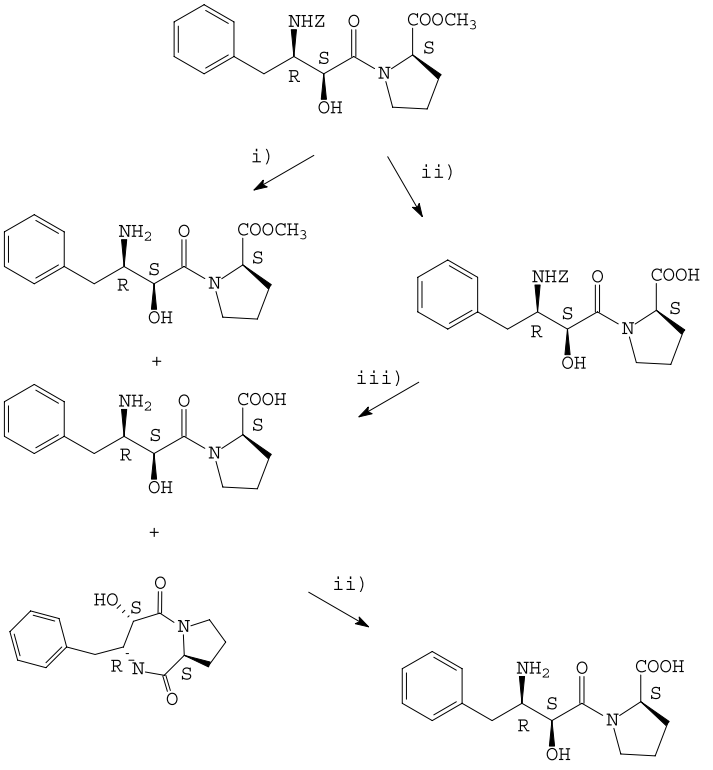
In contrast, the deprotection of the AHPB- and AHMH-containing dipeptide methyl esters (**1**, **5**, **7**, **9**, and **10**) resulted in nonhomogeneous products. Depending on the configuration of the 3-amino-2-hydroxy acid, up to three reaction products were obtained. As an example, the deprotection of compound **1** is shown in Scheme 3, yielding the methyl ester **13**, the acid **14**, and the diketodiazepine **15**. After hydrogenation of Z-(2*S*,3*R*)-AHPB-(*S*)-Pro-OMe (**1**), the mass spectrum showed three peaks representing three reaction products. The expected (2*S*,3*R*)-AHPB-(*S*)-Pro-OMe (**13**) was detected only by mass spectrometry (*m*/*z* $[M+H]^+$ 307.21). It was possible to isolate the unexpected diketodiazepine **15** (Scheme 3), which was identified by mass spectrometry (*m*/*z* $[M+H]^+$ 275.13), and ^1H and ^{13}C NMR spectroscopy. In the ^1H NMR spectrum of **15**, a singlet peak for the protons of a methyl ester group, which was expected for (2*S*,3*R*)-AHPB-(*S*)-Pro-OMe (**13**), was not found. A doublet for the proton of the hydroxyl group was detected at 6.33 ppm ($^3J(H,H) = 4.3$ Hz). Furthermore, the intensity of the signal of the nitrogen-bound hydrogen (NH) represented only one proton. The second isolated product was (2*S*,3*R*)-AHPB-(*S*)-Pro-OH (**14**), characterized by mass spectrometry (*m*/*z* $[M+H]^+$ 293.15) and ^1H NMR spectroscopy. The acidolytic deprotection (HBr/AcOH) of the dipeptide Z-(2*S*,3*R*)-AHPB-(*S*)-Pro-OMe (**1**) also yielded a mixture of products and crystallization provided the desired dipeptide methylester **13**.

Crystallization, after hydrogenolytic deprotection of the dipeptide Z-(2*R*,3*S*)-AHPB-(*S*)-Pro-OMe (**7**), gave the diketodiazepine **22**, which was also identified by mass spectrometry (*m*/*z* $[M+H]^+$ 275.13) and NMR spectroscopy. The remaining residue was saponified and purification by HPLC yielded the N- and C-terminal unprotected dipeptide (2*S*,3*R*)-AHPB-(*S*)-Pro-OH (**21**).

In the case of deprotection of the dipeptide Z-(2*S*,3*S*)-AHPB-(*S*)-Pro-OMe (**9**), the reaction was started with saponification. Subsequent hydrogenation in methanol yielded the dipeptide methyl ester (2*S*,3*S*)-AHPB-(*S*)-Pro-OMe (**24**), characterized by mass spectrometry (*m*/*z* $[M+H]^+$ 307.21) and NMR spectroscopy. The ^1H NMR spectrum exhibits a typical singlet for the protons of the methyl ester group at 3.67 ppm.

The dipeptides, (2*R*,3*R*)-AHPB-(*S*)-Pro-OH (**19**) and (2*S*,3*R*)-AHMH-(*S*)-Pro-OH (**25**), were isolated as homologous products after hydrogenation, immediately following saponification and purification by HPLC of the protected dipeptides Z-(2*R*,3*R*)-AHPB-(*S*)-Pro-OMe (**5**) and Z-(2*S*,3*R*)-AHMH-(*S*)-Pro-OMe (**10**), respectively.

At first, formation of seven-membered rings of the diketodiazepines (3*S*,4*R*,7*S*)-4-benzyl-3-hydroxy-1,5-diazabicyclo-[5.3.0]-decan-2,6-dion (**15**) and (3*R*,4*S*,7*S*)-4-benzyl-3-hydroxy-1,5-diazabicyclo-[5.3.0]-decan-2,6-dion (**22**), during the deprotection of the Z-protected dipeptide methyl esters, was a surprising result, but such ring formations are not unknown in peptide chemistry. Xaa-Pro dipeptides form 2,5-diketopiperazines, as they contain a higher amount of cis peptide bonds.[16] A



Scheme 3. Deprotection of Z-(2*S*,3*R*)-AHPB-(*S*)-Pro-OMe (**1**) as an example for the synthesis of 3-amino-2-hydroxy acyl-(*S*)-proline, 3-amino-2-hydroxy acyl-(*S*)-proline methyl ester, and 4-benzyl-3-hydroxy-1,5-diazabicyclo-[5.3.0]-decan-2,6-dion (diketodiazepin **15** and **22**). (i) HBr/acetic acid or H_2/Pd/C; (ii) aqueous NaOH (0.1 N) in dioxane; and (iii) HBr/acetic acid or H_2/Pd/C.

similar reaction was observed by Sakurai et al.[17] during the synthesis of HIV-protease inhibitors. The tripeptide, (2*S*,3*S*)-3-(*N*-benzyloxycarbonyl-L-asparaginyl)amino-2-hydroxy-4-phenylbutyryl-L-proline *tert*-butyl ester, was converted into the diketooxazine (3*S*,6*S*,1′*S*)-3-{1′-(*N*-benzyloxycarbonyl-L-asparaginyl)-amino-2′-phenylethyl}-1-aza-4-oxabicyclo[4.3.0]-nonan-2.5-dion by intramolecular cyclization.

2.2. Mixed-type inhibition of APP

One part of the investigated compounds inhibited rat APP and recombinant *E. coli* APP, according to a linear mixed-type mechanism, with inhibition constants in the micromolar range (Table 1, Scheme 1). In proof of concept, the Dixon plot of the inhibition of rat APP by (2*S*,3*R*)-AHPB-Thia **18** is shown in Figure 1. All lines are linear and have a common point of intersection in the second quadrant (Fig. 1A). Linearity of the lines means the EIS-complex is catalytically inactive. Generally, APP is inhibited according to a competitive or a linear mixed-type mechanism. The replot of slopes versus 1/[*S*] allows us to distinguish between pure competitive and linear mixed-type inhibition ($\alpha \neq 1$). The inhibition of APP is a linear mixed-type one, because the line in the replot of the slopes does not pass through the origin, as shown in Fig. 1B.[18] Furthermore, replots of slopes versus 1/[*S*], as well as intercepts versus 1/[*S*], were used to calculate the kinetic constants K_i and α (Eqs. 3 and 4).

Like thiazolidide **18**, dipeptide methyl esters (**13**, **24**), tripeptide methyl ester (**16**), pyrrolidide (**17**), and other thiazolidides (**20**, **23**, and **26**) inhibit APP from both sources according to the linear mixed-type mechanism (Scheme 4). In all cases, the inhibition constants K_i are in the micromolar range (Table 1). Nevertheless, configuration of a 3-amino-2-hydroxy acyl residue exerts an influence on the K_i value. A comparison of the K_i values of the dipeptide methyl esters (2*S*,3*R*)-AHPB-(*S*)-Pro-OMe **13** and (2*S*,3*S*)-AHPB-(*S*)-Pro-OMe **24** demonstrates a great influence of the configuration of the carbon atom C-3. The K_i values differ around 60 times and 40 times for *E. coli* APP and rat APP, respectively. Both enzymes prefer the 3*R* configuration. Furthermore, it is known that the IC_{50} values differ around 180 times for the inhibition of rat APP by the apstatin analogous compounds (2*S*,3*R*)- and (2*S*,3*S*)-AHMH-L-Pro-L-Ala-NH_2.[19] In contrast to the dipeptides, the K_i values for the inhibition of bacterial APP by the thiazolidides **18**, **20**, and **23** are in the same range, irrespective of configuration. In the case of rat APP, there are some differen-

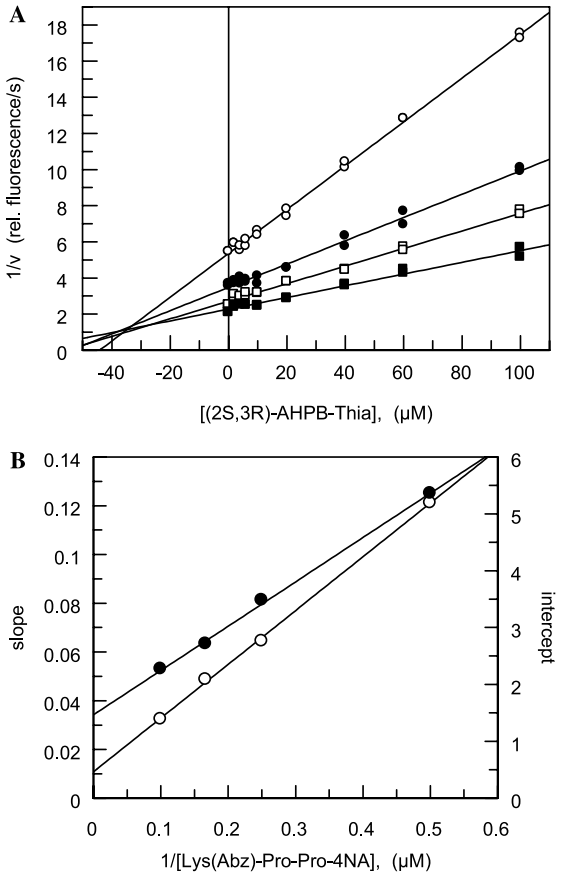


Figure 1. Inhibition of rat APP by (2*S*,3*R*)-AHPB-Thia **18**. The data were obtained by the hydrolysis of Lys(Abz)-Pro-Pro-4NA (2–10 μM) in 40 mM Tricine buffer, pH 7.4, containing 2.4 mM $MnCl_2$ and different concentrations of **18** at 30 °C. Final enzyme concentration was 0.24 nM. (A) Dixon plot 1/*v* vs. [*I*]. (B) Replot of slopes (○) and intercepts (●) of the Dixon plot vs. 1/[*S*].

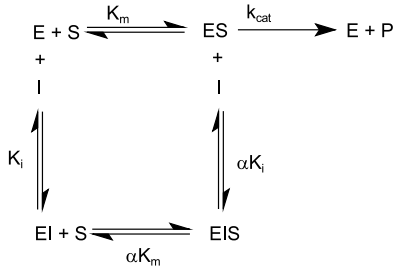
Table 1. Linear mixed-type inhibition of APP

	Inhibitor	*E. coli* APP[a]		Rat APP[b]	
		K_i[c] (μM)	α	K_i[c] (μM)	α
13	(2*S*,3*R*)-AHPB-Pro-OMe	2.60	1.5	24.0	0.83
16	(2*S*,3*R*)-AHPB-Pro-Phe-OMe	1.26	1.7	57.0	0.84
17	(2*S*,3*R*)-AHPB-Pyrr	19.8	2.2	37.6	1.1
18	(2*S*,3*R*)-AHPB-Thia	30.7	0.44	27.4	5.2
20	(2*R*,3*R*)-AHPB-Thia	60.3	2.3	269	3.8
23	(2*R*,3*S*)-AHPB-Thia	28.2	0.38	78.9	2.9
24	(2*S*,3*S*)-AHPB-Pro-OMe	158	0.62	854	2.4
26	(2*S*,3*R*)-AHMH-Thia	22.9	2.1	4.32	6.6

[a] Inhibition of *E. coli* APP-catalyzed hydrolysis of Lys(Abz)-Pro-Pro-4NA (4–15 μM) was monitored in 40 mM Tris buffer, pH 7.4, containing 0.75 mM $MnCl_2$ and 24 nM APP at 30 °C, as well as different concentrations of inhibitor.

[b] lRat APP was assayed using Lys(Abz)-Pro-Pro-4NA (2–20 μM) in 40 mM Tricine buffer, pH 7.4, containing 2.4 mM $MnCl_2$, different concentrations of inhibitor, and 0.24 nM APP at 30 °C.

[c] Calculations were performed, as indicated in the experimental section.



Scheme 4. Kinetic model of linear mixed-type inhibition.

ces between the K_i values of diastereomeric thiazolidides (**18**, **20**, and **23**). In addition, the IC_{50} values of (2*S*,3*R*)- and (2*R*,3*S*)-AHMH-L-Pro-L-Ala-NH_2 are also in the same range for the inhibition of different mammalian APPs.[19]

In general, our results and data from the literature indicate that amides and peptides containing a 3-amino-2-hydroxy acid are able to inhibit APP from different sources according to the linear mixed-type mechanism.[8,19] Thus, the mixed-type inhibitor apstatin ((2*S*,3*R*)-AHPB-Pro-Pro-Ala-NH_2), described by Prechel et al.[8], has K_i values of 2.6 and 0.64 μM for APP from rat lung and human lung, respectively. That means, apstatin is a slightly more potent inhibitor of mammalian APP than even the best mixed-type inhibitor (**26**) described here. This could have been caused by substrate specificity of the enzyme. The new mixed-type inhibitors correspond only partially to the substrate specificity of rat APP. Previous studies from Yoshimoto et al.[13] showed that mammalian APP does not hydrolyze dipeptides. Furthermore, an aromatic residue at the P'_2-position is disadvantageous. Apstatin corresponds to these requirements better than our compounds. *E. coli* APP does not have such restrictions with regard to substrate specificity. Therefore, the K_i values of the investigated compounds are in the same range as apstatin ($APP_{E.\ coli}$ K_i = 14 μM) or even up to 10 times better, for example, the tripeptide **16** ($APP_{E.\ coli}$ K_i = 1.26 μM).[8]

Like Maggiora et al.,[19] we found a more effective inhibition of rat APP by compounds containing AHMH, instead of AHPB, suggesting a more preferential interaction of the isobutyl side chain with the mammalian enzyme. These results have been corroborated by substrate specificity studies.[13]

2.3. Slow-binding inhibition of APP

The mechanism of inhibition of APP by 3-amino-2-hydroxy acid-derived dipeptides with a free carboxyl group at the C-terminus ((2*S*,3*R*)-AHPB-(*S*)-Pro-OH **14**, (2*R*,3*R*)-AHPB-(*S*)-Pro-OH **19**, (2*R*,3*S*)-AHPB-(*S*)-Pro-OH **21**, (2*S*,3*R*)-AHMH-(*S*)-Pro-OH **25**), and the diketodiazepine **15** differs from that discussed above. The single progress curves of APP inhibition have a characteristic shape (Fig. 2). The nonlinear nature of these progress curves demonstrates that these compounds are time-dependent inhibitors of APP. This time-dependent reaction is reversible. Figure 2 also shows that after preincubation of enzyme and inhibitor

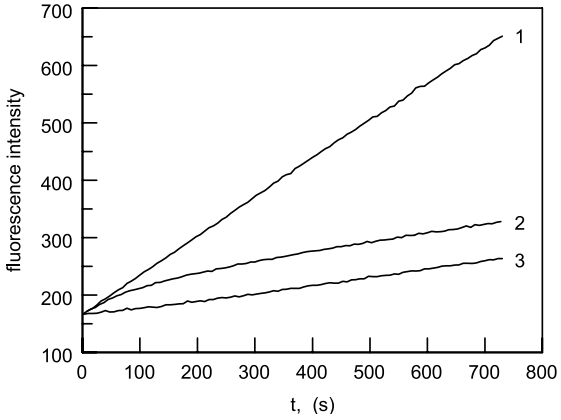
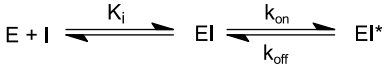


Figure 2. Progress curves of the inhibition of rat APP by (2*S*,3*R*)-AHPB-(*S*)-Pro-OH **14**. APP (0.12 nM) was incubated with Lys(Abz)-Pro-Pro-4NA (7 μM) in the presence and absence of **14** (20 μM) in Tricine buffer, pH 7.4, with 2.4 mM $MnCl_2$ at 30 °C. (1) Without inhibitor, (2) start of the reaction by the addition of APP, (3) preincubation of APP and **14** and start of the reaction by addition of S.

the enzyme-catalyzed reaction reaches the same steady-state velocity v_s as in the case when the reaction is triggered by addition of the enzyme.

For calculation of the kinetic constants of all inhibitors exhibiting a slow-binding behavior, each progress curve was fitted to Eq. 5 to yield values for the first-order rate constant k_{obs}, the initial velocity v_i, and the steady-state velocity v_s. The velocities v_i and v_s were fitted into Eq. 6 to calculate the inhibition constant K_i and the overall inhibition constants K_i^*, respectively. The constants k_{on} and k_{off} representing the forward and reverse rate constants, respectively, for a slow conversion of the initial EI-complex into a tight-binding complex EI* were determined according to Eqs. 7 and 8 (Scheme 5). A plot of k_{obs} versus [*I*] representative for all the investigated slow-binding inhibitors is shown in Figure 3. The shape



Scheme 5. Kinetic model of slow-binding inhibition.

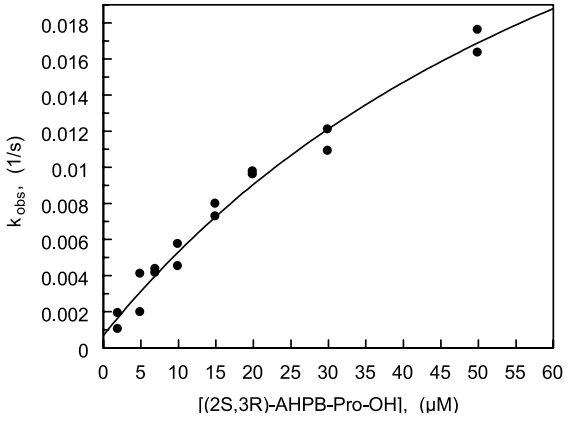


Figure 3. Replot of k_{obs} vs. [*I*] for the inhibition of APP by (2*S*,3*R*)-AHPB-(*S*)-Pro-OH **14**. The solid line is the fit to Eq. 7.

of this curve corresponds to the slow-binding mechanism B, according to Morrison and Walsh (Scheme 5).[20]

In all cases, the Dixon plots $1/v_i$ versus $[I]$ and $1/v_s$ versus $[I]$ show that all lines have a common point of intersection in the second quadrant (for example, Fig. 4A). That means, slow-binding inhibition takes place, according to a competitive or a linear mixed-type mechanism. Distinction between competitive and linear mixed-type inhibition was made by replotting the slopes versus $1/[S]$ (Fig. 4B). In all cases, the straight line in the replot of slopes from Dixon plot versus $1/[S]$ passes through the origin. That means, N- and C-terminal unprotected dipeptides **14**, **19**, **21**, and **25**, as well as the diketodiazepine **15**, inhibit APP competitively, according to a slow-binding mechanism. The kinetic constants are given in Table 2. (2*S*,3*R*)-AHPB-(*S*)-Pro-OH (**14**) is the most efficient inhibitor with an overall inhibition constant K_i^* of 170 nM and a second-order rate constant k_{on}/K_i^* of 588,000 s^{-1} M^{-1}, the most potent of all known inhibitors of bacterial APP and is 100-fold more potent than apstatin (K_i = 14 μM).[8] (2*S*,3*R*)-AHMH-(*S*)-Pro-OH (**25**) is the most efficient inhibitor for rat APP with an overall inhibition constant K_i^* of 1.1 μM and a second-order rate constant k_{on}/K_i^* of 58,200 s^{-1} M^{-1}. Thus, compound **25** is a slightly better inhibitor than apstatin (K_i = 2.6 μM).[8]

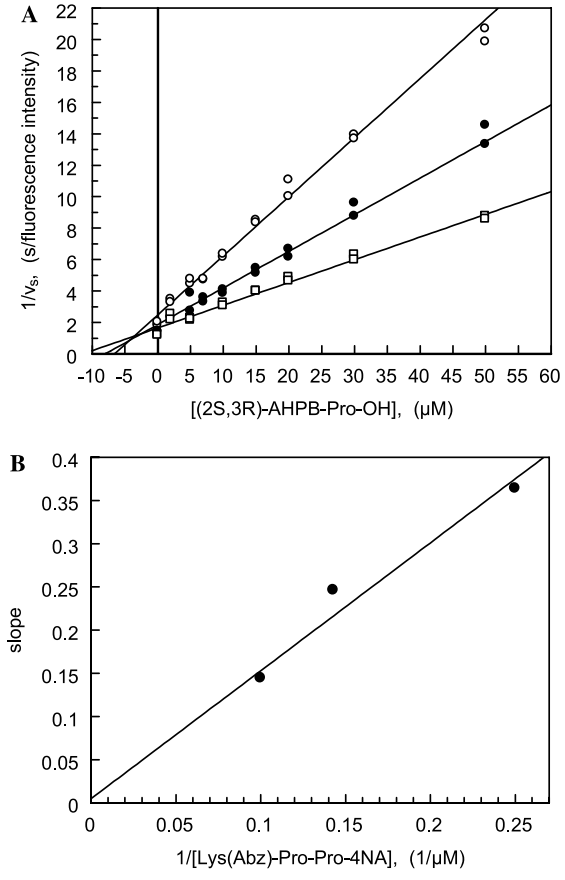


Figure 4. (A) Estimation of the inhibition mechanism and the overall inhibition constant K_i^* by the Dixon plot ($1/v$ vs. $[I]$) for the inhibition of rat APP by (2*S*,3*R*)-AHPB-(*S*)-Pro-OH **14**. v_s was determined from the progress curves according to Eq. 5. (B) Replot of the slopes from panel A vs. $1/[S]$.

A comparison of the above-mentioned N- and C-terminal unprotected dipeptides (**14**, **19**, **21**, and **25**) with mixed-type inhibitors discussed above shows that the slow-binding inhibitors are more potent for *E. coli*, as well as for rat APP. It is presumed that free carboxyl group of the C-terminal unprotected dipeptides (**14**, **19**, **21**, and **25**) is relevant to the occurrence of an initial EI-complex entailing efficient inhibition.

Surprisingly, the diketodiazepine **15** inhibits bacterial APP and rat APP with overall inhibition constants K_i^* of 970 nM and 5.1 μM, respectively, nearly to the same extent as the corresponding aliphatic dipeptide (2*S*,3*R*)-AHPB-(*S*)-Pro-OH **14** ($APP_{E.\ coli}$ K_i = 170 nM, APP_{rat} K_i = 2.1 μM).

The slow-binding inhibitors confirm the preference of both APPs for compounds containing a 3-amino-2-hydroxy acid in the configuration (2*S*,3*R*). (2*S*,3*R*)-AHPB-(*S*)-Pro-OH (**14**) has nearly 5-fold and 100-fold lower K_i^* values for both enzymes than (2*R*,3*S*)-AHPB-(*S*)-Pro-OH (**21**) and (2*R*,3*R*)-AHPB-(*S*)-Pro-OH (**19**), respectively. Nevertheless, the influence of stereochemistry of the AHPB residue in bestatin ((2*S*,3*R*)-AHPB-(*S*)-Leu-OH) on the inhibition of LAP and aminopeptidase B is higher than in case of the inhibition of APP by bestatin-derived compounds. Aoyagi et al.[21] found differences up to 1000-fold in the IC_{50} values for the inhibition of LAP and aminopeptidase B by the four stereoisomers of bestatin. Both enzymes have an absolute requirement for the 2*S*-configuration of the 3-amino-2-hydroxy acid, which was confirmed by Rich et al.[13]

Both APPs do not show a great preference for the AHPB (**14**) or AHMH (**25**) containing dipeptide in regard of the K_i^* value but the second-order rate constant k_{on}/K_i^* differs for both compounds. *E. coli* APP prefers the AHPB-containing dipeptide (**14**), while the rat APP is inhibited more efficiently by an AHMH-containing dipeptide (**25**). These preferences were observed in the case of mixed-type inhibitors discussed above, too.

2.4. A comparison of the inhibition of APP with prolidase and methionine aminopeptidase

In general, 3-amino-2-hydroxy acid pyrrolidides and thiazolidides are potent inhibitors of APP. On the other hand, common amino acid pyrrolidides and thiazolidides, for example, Phe-Thia and Ile-Thia, are not able to inhibit APP efficiently.[22] For this reason, the question arose whether 2-amino-3-hydroxy acid pyrrolidides and thiazolidides are also inhibitors of APP or not. Inhibition studies with Ser-Thia and Thr-Thia as 2-amino-3-hydroxy acid thiazolidides revealed that both compounds (5 mM) inhibit APP from both sources only up to around 25%. These results demonstrate that the 3-amino-2-hydroxy acid represents an essential structural element of the inhibitors described in this paper.

This result corresponds to X-ray crystallographic investigations of APP and the related enzymes MetAP and prolidase.[23] All these enzymes contain a dinuclear metal center at the active site. They are characterized by a relatively nar-

Table 2. Slow-binding inhibition of APP

	Inhibitor	K_i[a] (μM)	K_i^* (μM)	k_{on} (s^{-1})	k_{on}/K_i^* ($s^{-1}\,M^{-1}$)
E. coli APP[b]					
14	(2*S*,3*R*)-AHPB-Pro	14 ± 4	0.17 ± 0.04	0.10	588,000
15	Diketodiazepine	130 ± 10	0.97 ± 0.15	0.037	38,100
19	(2*R*,3*R*)-AHPB-Pro	190 ± 30	12 ± 2	0.015	1250
21	(2*R*,3*S*)-AHPB-Pro	16 ± 3	0.74 ± 0.08	0.023	31,100
25	(2*S*,3*R*)-AHMH-Pro	14 ± 5	0.37 ± 0.07	0.060	162,000
Rat APP[c]					
14	(2*S*,3*R*)-AHPB-Pro	32 ± 5	2.1 ± 0.4	0.026	12,400
15	Diketodiazepine	109 ± 34	5.1 ± 0.8	0.0081	1590
19	(2*R*,3*R*)-AHPB-Pro	2100 ± 400	170 ± 20	0.014	82
21	(2*R*,3*S*)-AHPB-Pro	120 ± 1	11 ± 1	0.017	1550
25	(2*S*,3*R*)-AHMH-Pro	110 ± 3	1.1 ± 0.1	0.064	58,200

K_i and K_i^* are the dissociation constant of the initial complex and the overall inhibition constant, respectively. k_{on} is the forward constant for the conversion to the EI*-complex.

[a] Progress curves were calculated, as described in the experimental section.

[b] Inhibition of *E. coli* APP-catalyzed hydrolysis of Lys(Abz)-Pro-Pro-4NA (4–15 μM) was determined in 40 mM Tris buffer, pH 7.4, containing 0.75 mM $MnCl_2$, different concentrations of inhibitor, and 5 nM APP at 30 °C.

[c] Rat APP was assayed by use of Lys(Abz)-Pro-Pro-4NA (4–10 μM) in Tricine buffer containing 2.4 mM $MnCl_2$, different concentrations of inhibitor, and 0.12 nM APP.

row substrate specificity in comparison to other metal-dependent aminopeptidases.[23b] All three enzymes show a close agreement of the active site on the coordination of the two metal atoms and their bridging water molecule, as well as the distance between the metal atoms.[24] All the above-mentioned aminopeptidases, as well as LAP, contain a metal-bridging water molecule or hydroxide ion, which should act as a nucleophile during catalysis.[23b] It was shown for the inhibition of MetAP by (2*S*,3*R*)-3-amino-2-hydroxy heptanoic acid, for the inhibition of prolidase by (2*S*,3*R*)-AHMH-(*S*)-Pro-OH, as well as for the inhibition of LAP by bestatin that the 2-hydroxy group of the inhibitors substitutes this important water molecule or hydroxide ion and bridges the two metal atoms.[24,25]

In the meantime, Graham et al.[26] solved the crystal structure of *E. coli* APP, which was in complex with the inhibitor apstatin. As postulated from MetAP and prolidase, they found the replacement of the bridging hydroxyl group between the two metal atoms by the hydroxy group at the carbon atom C2 of the inhibitor. These studies explain the competitive binding of an inhibitor to APP because the mixed-type they show only an EI-complex. For confirmation of the mixed-type inhibition mechanism, addition of a substrate is necessary. Unfortunately, the authors did not mention the mixed-type inhibition pattern of apstatin.

Nevertheless, (2*S*,3*R*)-3-amino-2-hydroxy heptanoic acid showed a competitive inhibition pattern of the MetAP, whereas our investigated compounds inhibited APP, according to the mixed-type mechanism or competitive slow-binding mechanism.[27] Slow-binding inhibition was also described for the inhibition of LAP by bestatin.[12]

3. Conclusion

In conclusion, we have developed very potent inhibitors of APP starting from the structure of the well-known aminopeptidase inhibitor bestatin. This approach has led to compounds inhibiting APP according to a classical linear mixed-type mechanism or a competitive slow-binding mechanism with inhibition constants in the nanomolar up to the low micromolar range. We have shown that the 3-amino-2-hydroxy acid is the decisive structural element of this new inhibitor. A further paper will describe the inhibition of other metallopeptidases and serine proteases by these compounds.

4. Experimental section

4.1. General procedures

Commercially available reagents were purchased from Fluka, Merck, Sigma, Aldrich, and BACHEM (Germany). Solvents were dried by standard procedures and distilled before use. Purity of compounds was checked by thin-layer chromatography (TLC), reversed-phase HPLC (Merck, Germany) and by capillary electrophoresis (BioFocus™, Bio-Rad, Germany), and ^{1}H NMR and/or ^{13}C NMR spectroscopy. TLC was performed with Kieselgel 60 F_{254} plates (Merck, Germany) using chloroform/MeOH (9:1) or *n*-BuOH/EtOAc/AcOH/water (1:1:1:1) mixtures as eluent. Melting points were determined with a BOETIUS melting point apparatus and are uncorrected. Optical rotations were measured on a Polamat A (Carl Zeiss Jena, Germany) or a chiral detector (Knauer, Germany) at 25 °C. Electrospray mass spectra were performed in positive mode on a VG-BIO-Q triple–quadruple electrospray mass spectrometer (Fisons Instruments, England). Elemental analyses were performed at the Department of Chemistry, Martin-Luther-University Halle-Wittenberg, Germany. ^{1}H and ^{13}C NMR spectra were recorded on Bruker ARX500 and Varian UNITY 500 spectrometers, respectively. Standard methods were used to perform one- and two-dimensional experiments, pulse programs having been taken from the Bruker and Varian software libraries. Resonance assignments were carried out by a combined analysis of H,H-COSY, APT, and HETCOR spectra.

3-Benzyloxycarbonylamino-2-hydroxy acids were prepared according to the procedure described by Fehrentz and Castro[14] and by Herranz et al.[15] starting from benzyloxycarbonyl-(*R*)-phenylalanine *N*-methyl-*O*-methylcarboxamide, benzyloxycarbonyl-(*S*)-phenylalanine *N*-methyl-*O*-methylcarboxamide, or benzyloxycarbonyl-(*R*)-leucine *N*-methyl-*O*-methylcarboxamide.

4.2. Synthesis of 3-amino-2-hydroxy acid pyrrolidide, 3-amino-2-hydroxy acid thiazolidides, 3-amino-2-hydroxyacyl-(*S*)-proline methyl ester, 3-amino-2-hydroxyacyl-(*S*)-proline, or 3-amino-2-hydroxyacyl-(*S*)-prolyl-(*S*)-phenylalanine methyl ester

4.2.1. General procedure. The corresponding *Z*-protected 3-amino-2-hydroxy acid (1 mmol) and an amino component [pyrrolidine, thiazolidine, (*S*)-proline methyl ester hydrochloride, or (*S*)-prolyl-(S)-phenylalanine methyl ester hydrochloride (1.1 mmol)] were dissolved in dichloromethane (10 ml). In the case of the amino components (*S*)-proline methyl ester hydrochloride or (*S*)-prolyl-(*S*)-phenylalanine methyl ester hydrochloride, triethylamine (154 μl, 1.1 mmol) was added. The solution, cooled to 4 °C, was treated with hydroxybenzotriazole (170 mg, 1.1 mmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (210 mg, 1.1 mmol). The mixture was allowed to stir for 1 h at 4 °C and overnight at room temperature. The organic layer was subsequently washed with water, 0.2 M HCl, brine, saturated $NaHCO_3$, and brine. The reaction mixture was dried over $MgSO_4$. The solvent was removed under reduced pressure and the amide was obtained as an oil or was crystallized from EtOAc/*n*-hexane.

4.2.2. (2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline methyl ester (1). Compound **1** was synthesized by coupling of (2*S*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (300 mg, 0.91 mmol) and (*S*)-proline methyl ester hydrochloride (165 mg, 1 mmol). Yield: 290 mg (72%); decomposition >136 °C; TLC: $R_f = 0.61$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −12.3 (*c* 1, $CHCl_3$); elemental analysis Calcd (%) for $C_{24}H_{28}N_2O_6 \cdot \frac{1}{2}H_2O$ (449.50): C, 64.13; H, 6.50; N 6.23. Found: C, 64.66; H, 6.48; N, 5.57; MS ESI: *m*/*z* = 441.45 [M+H$^+$]; ^{13}C NMR (500 MHz, $CDCl_3$, 30 °C, TMS) δ = 25.3 (C^{γ}H-Pro), 28.6 (C^{β}H-Pro), 38.5 (C^{δ}H-AHPB), 46.5 (C^{δ}H-Pro), 52.2 (OCH_3), 54.0 (C^{β}H-AHPB), 59.6 (C^{α}H-Pro), 66.8 (CH_2-Z), 68.5 (C^{α}H-AHPB), 126.7 (CH-Ph), 128.1 (CH-Ph), 128.5 (2 CH-Ph),128.7 (2 CH-Ph), 129.5 (2 CH-Ph), 129.6 (2 CH-Ph), 136.8 (CH-Ph), 138.0 (CH-Ph), 155.8 (CO-Z), 171.0 (CO-AHPB), 171.6 (CO-Pro).

4.2.3. (2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoyl-(*S*)-prolyl-(*S*)-phenylalanine methyl ester (2). Compound **2** was synthesized by coupling of (2*S*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (290 mg, 0.88 mmol) and (*S*)-prolyl-(*S*)-phenylalanine methyl ester hydrochloride (303 mg, 0.97 mmol). Yield: 336 mg (65%); mp 57–62 °C; TLC: $R_f = 0.59$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −28.8 (*c* 1, $CHCl_3$); elemental analysis Calcd (%) for $C_{33}H_{37}N_3O_7 \cdot \frac{1}{2}H_2O$ (596.68): C, 66.43; H 6.42; N 7.04. Found: C, 66.47; H, 6.65; N, 6.75; MS ESI: *m*/*z* = 588.17 [M+H$^+$].

4.2.4. (2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid pyrrolidide (3). Compound **3** was synthesized by coupling of (2*S*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (290 mg, 0.88 mmol) and pyrrolidine (80 μl, 0.97 mmol). Yield: 270 mg (80%); mp 137–143 °C; TLC: $R_f = 0.60$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ +1.6 (*c* 1, $CHCl_3$); elemental analysis Calcd (%) for $C_{22}H_{26}N_2O_4$ (382.46): C, 69.09; H, 6.85; N, 7.32; Found: C, 68.86; H, 6.99; N, 6.86; MS ESI: *m*/*z* = 383.30 [M+H]$^+$; ^{1}H NMR (500 MHz, $CDCl_3$, 30 °C, TMS) δ = 1.76 (A, 1H, C^3H_2-Pyrr; m, 2H, C^4H_2-Pyrr), 1.91 (B, 1H, C^3H_2-Pyrr), 2.96 (A, 1H, C^2H_2-Pyrr; m, 2H, $C^{\gamma}H_2$-AHPB), 3.22 (B, 1H, C^2H_2-Pyrr), 3.31 (A, 1H, C^5H_2-Pyrr), 3.44 (B, 1H, C^5H_2-Pyrr), 4.07 (br s, 1H, C^{α}H-AHPB), 4.13 (br s, 1H, OH), 4.20 (m, 1H, C^{β}H-AHPB), 5.01 (A, 1H, ^2J(H,H) = 12.4 Hz, CH_2-Z), 5.06 (B, 1H, ^2J(H,H) = 12.4 Hz, CH_2-Z), 5.14 (d, 1H, ^3J(H,H) = 9.8 Hz, NH), 7.22–7.36 (m, 10H, phenyl); ^{13}C NMR (500 MHz, $CDCl_3$, 30 °C, TMS) δ = 23.6 (C^4H_2-Pyrr), 26.0 (C^3H_2-Pyrr), 38.9 ($C^{\gamma}H_2$-AHPB), 45.5 (C^2H_2-Pyrr), 46.5 (C^5H_2-Pyrr), 53.2 (C^{β}H-AHPB), 66.7 (CH_2-Z), 68.4 (C^{α}H-AHPB), 126.7 (CH-Ph), 127.8 (CH-Ph), 128.0 (2 CH-Ph), 128.5 (2 CH-Ph), 128.7 (2 CH-Ph), 129.5 (2 CH-Ph), 136.6 (CH-Ph), 137.8 (CH-Ph), 156.0 (CO-Z), 170.0 (CO-AHPB).

4.2.5. (2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid thiazolidide (4). Compound **4** was synthesized by coupling of (2*S*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (290 mg, 0.88 mmol) and thiazolidine (91 mg, 0.97 mmol). Yield: 360 mg (90%, E/Z-isomers); decomposition >126 °C; TLC: $R_f = 0.66$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −5.7 (*c* 1, $CHCl_3$); elemental analysis Calcd (%) for $C_{21}H_{24}N_2O_4S$ (400.50): C, 62.98; H, 6.04; N, 6.99; S, 8.01. Found: C, 63.04; H, 6.28; N, 6.57; S, 8.12; MS ESI: *m*/*z* = 401.25 [M+H$^+$]; ^{1}H NMR (500 MHz, $CDCl_3$, 27 °C, TMS) major isomer: δ = 2.95 (m, 4H, C^4H_2-Thia, $C^{\gamma}H_2$-AHPB), 3.25 (A, 1H, C^5H_2-AHPB), 3.48 (B, 1H, C^5H_2-Thia), 4.00 (br s, 1H, C^{α}H-AHPB), 4.09 (br s, 1H, OH), 4.17 (m, 1H, C^{β}H-AHPB), 4.24 (A, 1H, C^2H_2-Thia), 4.67 (B, 1H, C^2H_2-Thia), 5.04 (m, 2H, CH_2-Z), 5.14 (br s, 1H, NH), 7.23–7.35 (m, 10H, phenyl); minor isomer: δ = 2.95 (m, 4H, C^4H_2-Thia, $C^{\gamma}H_2$-AHPB), 3.48 (A, 1H, C^5H_2-Thia), 3.90 (A, 1H, C^2H_2-Thia, B, 1H, C^5H_2-Thia), 4.00 (br s, 1H, C^{α}H-AHPB), 4.09 (br s, 1H, OH), 4.17 (m, 1H, C^{β}H-AHPB), 4.24 (B, 1H, C^2H_2-Thia), 5.04 (m, 2H, CH_2-Z), 5.14 (br s, 1H, NH), 7.23–7.35 (m, 10H, phenyl); ^{13}C NMR (500 MHz, $CDCl_3$, 30 °C, TMS) major isomer: δ = 30.9 (C^5H_2-Thia), 38.7 ($C^{\gamma}H_2$-AHPB), 47.7 (C^4H_2-Thia), 48.6 (C^2H_2-Thia), 53.6 (C^{β}H-AHPB), 66.8 (CH_2-Z), 68.7 (C^{α}H-AHPB), 126.9 (CH-Ph), 127.9 (CH-Ph), 128.1 (CH-Ph), 128.5 (CH-Ph), 128.8 (CH-Ph), 129.4 (CH-Ph), 136.5 (CH-Ph), 137.5 (CH-Ph), 137.6 (CH-Ph), 156.0 (CO-Z), 170.1 (CO-AHPB); minor isomer: δ = 29.0 (C^5H_2-Thia), 38.7 ($C^{\gamma}H_2$-AHPB), 47.2 (C^2H_2-Thia), 49.1 (C^4H_2-Thia), 53.6 (C^{β}H-AHPB), 66.8 (CH_2-Z), 68.4 (C^{α}H-AHPB), 126.9 (CH-Ph),

127.9 (CH-Ph), 128.1 (CH-Ph), 128.5 (CH-Ph), 128.8 (CH-Ph), 129.4 (CH-Ph), 136.5 (CH-Ph), 137.5 (CH-Ph), 137.6 (CH-Ph), 156.0 (CO-Z), 170.1 (CO-AHPB).

4.2.6. (2*R*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline methyl ester (5). Compound **5** was synthesized by coupling of (2*R*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (100 mg, 0.3 mmol) and (*S*)-proline methyl ester hydrochloride (58 mg, 0.35 mmol). Yield: 100 mg (76 %); oil; TLC: $R_f = 0.61$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −22.0 (*c* 2.9, $CHCl_3$); elemental analysis Calcd (%) for $C_{24}H_{28}N_2O_6 \cdot \frac{1}{2}H_2O$ (449.50): C, 64.13; H, 6.50; N, 6.23. Found: C, 64.37; H, 6.45; N, 5.89; MS ESI: $m/z = 441.45$ $[M+H^+]$; ^{13}C NMR (500 MHz, $CDCl_3$, 27 °C, $CHCl_3$) $\delta = 24.5$ ($C^{\gamma}H_2$-Pro), 28.6 ($C^{\beta}H_2$-Pro), 34.9 ($C^{\gamma}H_2$-AHPB), 46.3 ($C^{\delta}H_2$-Pro), 52.3 (OCH_3), 53.6 (C^{β}H-AHPB), 59.3 (C^{α}H-Pro), 66.8 (CH_2-Z), 71.0 (C^{α}H-AHPB), 126.6 (2 CH-Ph), 127.9 (4 CH-Ph), 129.3 (2 CH-Ph), 129.4 (2 CH-Ph), 136.4 (CH-Ph), 137.7 (CH-Ph), 156.1 (CO-Z), 169.9 (CO-AHPB), 172.2 (CO-Pro).

4.2.7. (2*R*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid thiazolidide (6). Compound **6** was synthesized by coupling of (2*R*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (220 mg, 0.67 mmol) and thiazolidine (70 mg, 0.74 mmol) Yield: 230 mg (86%, E/Z-isomers); mp 116–122 °C; TLC: $R_f = 0.65$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ +6.5 (*c* 1, $CHCl_3$); elemental analysis Calcd (%) for $C_{21}H_{24}N_2O_4S$ (400.50): C, 62.98; H, 6.04; N, 6.99; S, 8.01. Found: C, 62.60; H, 6.21; N, 6.64; S, 8.03; MS ESI: $m/z = 401.16$ $[M+H]^+$; ^1H NMR (500 MHz, $CDCl_3$, 30 °C, TMS) isomer 1: $\delta = 2.76$ (A, 1H, C^4H_2-Thia), 2.86 (m, 2H, $C^{\gamma}H_2$-AHPB), 3.02 (B, 1H, C^4H_2-Thia), 3.78 (A, 1H, C^5H_2-Thia), 3.90 (B, 1H, C^5H_2-Thia), 3.97 (br s, 1H, OH), 4.17 (m, 1H, C^{β}H-AHPB), 4.20 (A, 1H, $^2J(H,H) = 10.2$ Hz, C^2H_2-Thia), 4.50 (m, 1H, C^{α}H-AHPB), 4.65 (B, 1H, $^2J(H,H) = 10.0$ Hz, C^2H_2-Thia), 5.06 (s, 2H, CH_2-Z), 5.33 (d, 1H, $^3J(H,H) = 3.9$ Hz, NH), 7.15–7.36 (m, 10H, phenyl); isomer 2: $\delta = 2.86$ (A, 1H, C^4H_2-Thia, m, 2H, $C^{\gamma}H_2$-AHPB), 2.95 (B, 1H, C^4H_2-Thia), 3.46 (A, 1H, C^5H_2-Thia), 3.90 (B, 1H, C^5H_2-Thia), 3.97 (br s, 1H, OH), 4.17 (m, 1H, C^{β}H-AHPB), 4.52 (A, 1H, $^2J(H,H) = 9.4$ Hz, C^2H_2-Thia), 4.57 (m, 1H, C^{α}H-AHPB), 4.61 (B, 1H, $^2J(H,H) = 9.4$ Hz, C^2H_2-Thia), 5.06 (s, 2H, CH_2-Z), 5.33 (d, 1H, $^3J(H,H) = 3.9$ Hz, NH), 7.15–7.36 (m, 10H, phenyl); ^{13}C NMR (500 MHz, $CDCl_3$, 27 °C, TMS); isomer 1: $\delta = 31.1$ (C^5H_2-Thia), 34.3 ($C^{\gamma}H_2$-AHPB), 48.0 (C^4H_2-Thia), 48.9 (C^2H_2-Thia), 54.0 (C^{β}H-AHPB), 66.9 (CH_2-Z), 71.4 (C^{α}H-AHPB), 126.7 (CH-Ph), 127.9 (CH-Ph), 128.2 (CH-Ph), 128.5 (CH-Ph), 129.1 (CH-Ph), (CH-Ph), 136.1 (CH-Ph), 137.1 (CH-Ph), 137.2 (CH-Ph), 156.1 (CO-Z), 169.5 (CO-AHPB); isomer 2: $\delta = 29.0$ (C^5H_2-Thia), 34.3 ($C^{\gamma}H_2$-AHPB), 48.0 (C^4H_2-Thia), 48.5 (C^2H_2-Thia), 54.0 (C^{β}H-AHPB), 66.9 (CH_2-Z), 71.0 (C^{α}H-AHPB), 126.7 (CH-Ph), 127.9 (CH-Ph), 128.2 (CH-Ph), 128.5 (CH-Ph), 129.1 (CH-Ph), 136.1 (CH-Ph), 137.1 (CH-Ph), 137.2 (CH-Ph), 156.1 (CO-Z), 169.2 (CO-AHPB).

4.2.8. (2*R*,3*S*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline methyl ester (7). Compound **7** was synthesized by coupling of (2*R*,3*S*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (330 mg, 1 mmol) and (*S*)-proline methyl ester hydrochloride (182 mg, 1.1 mmol). Yield: 330 mg (75%); mp 158–164 °C; TLC: $R_f = 0.65$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −56.0 (*c* 1, $CHCl_3$); elemental analysis Calcd (%) for $C_{24}H_{28}N_2O_6 \cdot \frac{1}{2}H_2O$ (449.50): C, 64.13; H, 6.50; N, 6.23. Found: C, 64.76; H. 6.53; N, 5.82; ESI MS: $m/z = 441.31$ $[M+H]^+$; ^{13}C NMR (500 MHz, $CDCl_3$, 27 °C, TMS) $\delta = 24.6$ ($C^{\gamma}H_2$-Pro), 28.6 ($C^{\beta}H_2$-Pro), 38.6 ($C^{\gamma}H_2$-AHPB), 46.0 ($C^{\delta}H_2$-Pro), 52.3 (OCH_3), 52.9 (C^{β}HNH), 59.5 (C^{α}H-Pro), 66.7 (CH_2-Z), 68.4 (C^{α}HOH), 126.8 (CH-Ph), 127.8 (2 CH-Ph), 128.0 (CH-Ph), 128.5 (2 CH-Ph), 128.7 (2 CH-Ph), 129.4 (2 CH-Ph), 136.5 (CH-Ph), 137.5 (CH-Ph), 156.0 (CO-Z), 170.7 (CO-AHPB), 172.3 (CO-Pro).

4.2.9. (2*R*,3*S*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid thiazolidide (8). Compound **8** was synthesized by coupling of (2*R*,3*S*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (330 mg, 1 mmol) and thiazolidine (103 mg, 1.1 mmol). Yield: 330 mg (71%, E/Z-isomers); mp 133–137 °C; TLC: $R_f = 0.64$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ +4.5 (*c* 0.7, $CHCl_3$); elemental analysis Calcd (%) for $C_{21}H_{24}N_2O_4S \cdot 1\frac{1}{2}H_2O$ (427.52): C, 59.00; H, 6.36; N, 6.55; S, 7.50. Found: C, 58.53; H, 5.76; N, 6.05; S, 7.59; MS ESI: $m/z = 401.16$ $[M+H]^+$; ^1H NMR (500 MHz, $CDCl_3$, 30 °C, TMS) isomer 1: $\delta = 2.97$ (m, 4H, C^4H_2-Thia, $C^{\gamma}H_2$-AHPB), 3.26 (A, 1H, C^5H_2-Thia), 3.48 (B, 1H, C^5H_2-Thia), 3.97 (br s, 1H, OH), 4.10 (m, 1H, C^{α}H-AHPB), 4.16 (m, 1H, C^{β}H-AHPB), 4.24 (A, 1H, C^2H_2-Thia), 4.67 (B, 1H, C^2H_2-Thia), 5.03 (A, 1H, $^2J(H,H) = 16.2$ Hz, CH_2-Z), 5.06 (B, 1H, $^2J(H,H) = 15.9$ Hz, CH_2-Z), 5.10 (d, 1H, $^3J(H,H) = 8.7$ Hz, NH), 7.23–7.36 (m, 10H, phenyl); isomer 2: $\delta = 2.97$ (m, 4H, C^4H_2-Thia, $C^{\gamma}H_2$-AHPB), 3.48 (A, 1H, C^5H_2-Thia), 3.91 (A, 1H, C^2H_2-Thia; B, 1H, C^5H_2-Thia), 3.97 (br s, 1H, OH), 4.16 (m, 2H, C^{α}H-AHPB, C^{β}H-AHPB), 4.24 (B, 1H, C^2H_2-Thia), 5.03 (A, 1H, $^2J(H,H) = 16.2$ Hz, CH_2-Z), 5.06 (B, 1H, $^2J(H,H) = 15.9$ Hz, CH_2-Z), 5.10 (d, 1H, $^3J(H,H) = 8.7$ Hz, NH), 7.23–7.36 (m, 10H, phenyl); ^{13}C NMR (500 MHz, $CDCl_3$, 30 °C, TMS) isomer 1: $\delta = 30.9$ (C^5H_2-Thia), 38.7 ($C^{\gamma}H_2$-AHPB), 47.4 (C^4H_2-Thia), 48.7 (C^2H_2-Thia), 53.6 (C^{β}H-AHPB), 66.8 (CH_2-Z), 68.7 (C^{α}H-AHPB), 126.9 (CH-Ph), 127.9 (CH-Ph), 128.1 (2 CH-Ph), 128.5 (2 CH-Ph), 128.8 (2 CH-Ph), 129.4 (2 CH-Ph), 136.5 (CH-Ph), 137.5 (CH-Ph), 156.0 (CO-Z), 170.1 (CO-AHPB); isomer 2: $\delta = 29.0$ (C^5H_2-Thia), 38.7 ($C^{\gamma}H_2$-AHPB), 47.2 (C^2H_2-Thia), 49.1 (C^5H_2-Thia), 53.6 (C^{β}H-AHPB), 66.8 (CH_2-Z), 68.4 (C^{α}H-AHPB), 126.9 (CH-Ph), 127.9 (CH-Ph), 128.1 (2 CH-Ph), 128.5 (2 CH-Ph), 128.8 (2 CH-Ph), 129.4 (2 CH-Ph), 136.5 (CH-Ph), 137.5 (CH-Ph), 156.0 (CO-Z), 170.1 (CO-AHPB).

4.2.10. (2*S*,3*S*)-3-Benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline methyl ester (9). Compound **9** was synthesized by coupling of (2*S*,3*S*)-3-benzyloxycarbonylamino-2-hydroxy-4-phenylbutanoic acid (180 mg, 0.54 mmol) and (*S*)-proline methyl ester hydrochloride

(92 mg, 0.56 mmol). Yield: 150 mg (63%); mp 60–62 °C; TLC: $R_f = 0.60$ ($CHCl_3$/MeOH 9:1); elemental analysis Calcd (%) for $C_{24}H_{28}N_2O_6 \cdot \frac{1}{4}H_2O$ (444.78): C, 64.78; H, 6.45; N, 6.29. Found: C, 64.78; H, 6.14; N, 6.07; MS ESI: $m/z = 441.31$ $[M+H]^+$.

4.2.11. (2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-5-methylhexanoyl-(*S*)-proline methyl ester (10). Compound **10** was synthesized by coupling of (2*S*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-5-methylhexanoic acid (220 mg, 0.74 mmol) and (*S*)-proline methyl ester hydrochloride (135 mg, 0.82 mmol). Yield: 240 mg (79%, cis/trans-isomers); mp 112–117 °C; TLC: $R_f = 0.60$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −39.4 (*c* 1.1, $CHCl_3$); elemental analysis Calcd (%) for $C_{21}H_{30}N_2O_6$ (406.48): C, 62.05; H, 7.44; N, 6.52. Found: C, 61.88; H, 7.48; N, 6.52; MS ESI: $m/z = 407.24$ $[M+H]^+$; ^{13}C NMR (500 MHz, $CDCl_3$, 27 °C, TMS) major isomer: $\delta = 22.3$ ($C^{\varepsilon}H_3$-AHMH), 23.2 ($C^{\varepsilon}H'_3$-AHMH), 24.7 ($C^{\delta}H$-AHMH), 25.4 ($C^{\gamma}H_2$-Pro), 28.6 ($C^{\beta}H_2$-Pro), 40.9 ($C^{\gamma}H_2$-AHMH), 46.9 ($C^{\delta}H$-Pro), 50.2 ($C^{\beta}H$-AHMH), 52.2 (OCH_3), 59.5 ($C^{\alpha}H$-Pro), 66.9 (CH_2-Z), 70.8 ($C^{\alpha}H$-AHMH), 128.0 (CH-Ph), 128.1 (2 CH-Ph), 128.4 (2 CH-Ph), 136.7 (CH-Ph), 156.0 (CO-Z), 170.8 (CO-AHMH), 171.7 (CO-Pro); minor isomer: $\delta = 22.0$ ($C^{\varepsilon}H_3$-AHMH), 22.9 ($C^{\varepsilon}H_3'$-AHMH), 24.7 ($C^{\delta}H$-AHMH), 25.4 ($C^{\gamma}H_2$-Pro), 31.4 ($C^{\beta}H_2$-Pro), 41.3 ($C^{\gamma}H_2$-AHMH), 47.4 ($C^{\delta}H$-Pro), 50.1 ($C^{\beta}H$-AHMH), 52.8 (OCH_3), 58.4 ($C^{\alpha}H$-Pro), 66.9 (CH_2-Z), 71.1 ($C^{\alpha}H$-AHMH), 127.8 (CH-Ph), 128.1 (2 CH-Ph), 128.4 (2 CH-Ph), 136.6 (CH-Ph), 156.1 (CO-Z), 170.7 (CO-AHMH), 172.1 (CO-Pro).

4.2.12. (2*S*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-5-methylhexanoic acid thiazolidide (11). Compound **11** was synthesized by coupling of (2*S*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-5-methylhexanoic acid (380 mg, 1.3 mmol) and thiazolidine (130 mg, 1.4 mmol). Yield: 280 mg (60%, E/Z-isomers); mp 141–144 °C; TLC: $R_f = 0.65$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −42.0 (*c* 1.1, CH_3OH); elemental analysis Calcd (%) for $C_{18}H_{26}N_2O_4S$ (366.48): C, 58.99; H, 7.15; N, 7.64; S, 8.75. Found: C, 58.85; H, 7.21; N, 7.10; S, 8.63; MS ESI: $m/z = 367.17$ $[M+H]^+$; ^{13}C NMR (500 MHz, $CDCl_3$, 27 °C, TMS) major isomer: $\delta = 22.3$ ($C^{\varepsilon}H_3$-AHMH), 23.1 ($C^{\varepsilon}H'_3$-AHMH), 24.8 ($C^{\delta}H$-AHMH), 31.0 (C^5H_2-Thia), 41.5 ($C^{\gamma}H_2$-AHMH), 48.0 (C^2H_2-Thia), 48.7 (C^4H_2-Thia), 50.1 ($C^{\beta}H$-AHMH), 66.7 (CH_2-Z), 71.7 ($C^{\alpha}H$-AHMH), 127.8 (CH-Ph), 128.0 (2 CH-Ph), 128.5 (2 CH-Ph), 136.6 (CH-Ph), 156.1 (CO-Z), 170.0 (CO-AHMH); minor isomer: $\delta = 22.3$ ($C^{\varepsilon}H_3$-AHMH), 23.1 ($C^{\varepsilon}H'_3$-AHMH), 24.8 ($C^{\delta}H$-AHMH), 29.0 (C^5H_2-Thia), 41.5 ($C^{\gamma}H_2$-AHMH), 47.6 (C^4H_2-Thia), 49.2 (C^2H_2-Thia), 50.1 ($C^{\beta}H$-AHMH), 66.7 (CH_2-Z), 71.5 ($C^{\alpha}H$-AHMH), 127.8 (CH-Ph), 128.0 (2 CH-Ph), 128.5 (2 CH-Ph), 136.6 (CH-Ph), 156.1 (CO-Z), 169.7 (CO-AHMH).

4.2.13. (2*R*,3*R*)-3-Benzyloxycarbonylamino-2-hydroxy-5-methylhexanoyl-(*S*)-proline methyl ester (12). Compound **12** was synthesized by coupling of (2*R*,3*R*)-3-benzyloxycarbonylamino-2-hydroxy-5-methylhexanoic acid (140 mg, 0.47 mmol) and (*S*)-proline methyl ester hydrochloride (93 mg, 0.56 mmol). Yield: 110 mg (58%); oil; TLC: $R_f = 0.60$ ($CHCl_3$/MeOH 9:1); $[\alpha]_D^{20}$ −46 (*c* 0.6, $CHCl_3$); elemental analysis Calcd (%) for $C_{21}H_{30}N_2O_6 \cdot \frac{1}{2}H_2O$ (415.48): C, 60.71; H, 7.52; N, 6.74. Found: C, 60.82; H, 7.48; N, 6.53; MS ESI: $m/z = 407.24$ $[M+H]^+$.

4.2.14. (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline methyl ester hydrobromide (13). Compound **1** (100 mg, 0.23 mmol) was suspended in HBr/AcOH (33%, 0.5 ml) at room temperature. After completion of deprotection, controlled by TLC, the product was precipitated by addition of dry diethyl ether, filtered off, washed several times with diethyl ether, and recrystallized from MeOH/diethyl ether. Yield: 63 mg (52%, cis/trans-isomers); decomposition >104 °C; elemental analysis Calcd (%) for $C_{16}H_{22}N_2O_4 \cdot HBr \cdot \frac{1}{2}H_2O$ (396.28): C, 48.49; H, 6.10; N, 7.07. Found: C, 48.35; H, 6.00; N, 6.83; MS ESI: $m/z = 307.21$ $[M+H]^+$; ^{13}C NMR (500 MHz, $[D_6]$DMSO, 27 °C, DMSO) major isomer: $\delta = 24.5$ ($C^{\gamma}H_2$-Pro), 28.5 ($C^{\beta}H_2$-Pro), 34.6 ($C^{\gamma}H_2$-AHPB), 46.6 ($C^{\delta}H_2$-Pro), 52.0 (OCH_3), 54.2 ($C^{\beta}H$-AHPB), 58.8 ($C^{\alpha}H$-Pro), 66.8 ($C^{\alpha}H$-AHPB), 127.1 (CH-Ph), 128.6 (2 CH-Ph), 129.4 (2 CH-Ph), 136.0 (CH-Ph), 169.1 (CO-AHPB), 172.0 (CO-Pro); minor isomer: $\delta = 24.4$ ($C^{\gamma}H_2$-Pro), 28.4 ($C^{\beta}H_2$-Pro), 35.0 ($C^{\gamma}H_2$-AHPB), 46.3 ($C^{\delta}H_2$-Pro), 51.0 (OCH_3), 53.6 ($C^{\beta}H$-AHPB), 58.6 ($C^{\alpha}H$-Pro), 66.1 ($C^{\alpha}H$-AHPB), 127.0 (CH-Ph), 128.7 (2 CH-Ph), 129.5 (2 CH-Ph), 135.9 (CH-Ph), 169.3 (CO-AHPB), 172.6 (CO-Pro).

4.2.15. (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline (14) and (3*S*,4*R*,7*S*)-4-benzyl-3-hydroxy-1,5-diazabicyclo-[5.3.0]-decan-2,6-dion (15). Compound **1** (374 mg, 0.85 mmol) was dissolved in MeOH. The catalyst Pd/C was added. Subsequently, the mixture was hydrogenated at room temperature. After completion of the reaction controlled by TLC, the catalyst was deactivated and filtered off. The solvent was evaporated, and compounds **14** and **15** were isolated by fractionated crystallization from methanol/diethyl ether and dried over P_4O_{10}.

4.2.15.1. (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline (14). Yield: 87 mg (35%); mp 126–137 °C; elemental analysis Calcd (%) for $C_{15}H_{20}N_2O_4 \cdot 1\frac{1}{2}H_2O$ (319.355): C, 56.42; H, 7.26; N, 8.77. Found: C, 56.68; H, 7.16; N, 8.53; MS ESI: $m/z = 293.15$ $[M+H]^+$; ^{13}C NMR (500 MHz, $[D_6]$DMSO, 27 °C; DMSO) $\delta = 24.5$ ($C^{\gamma}H_2$-Pro), 28.6 ($C^{\beta}H_2$-Pro), 34.4 ($C^{\delta}H_2$-AHPB), 46.6 ($C^{\delta}H_2$-Pro), 54.4 ($C^{\beta}H$-AHPB), 58.9 ($C^{\alpha}H$-Pro), 66.7 ($C^{\alpha}H$-AHPB), 127.0 (CH-Ph), 128.7 (2 CH-Ph), 129.5 (2 CH-Ph), 136.2 (CH-Ph), 169.0 (CO-AHPB), 173.0 (COOH).

4.2.15.2. (3*S*,4*R*,7*S*)-4-Benzyl-3-hydroxy-1,5-diazabicyclo-[5.3.0]-decan-2,6-dion (15). Yield: 75 mg (27%); mp 135–143 °C; elemental analysis Calcd (%) for $C_{15}H_{18}N_2O_3 \cdot \frac{1}{2}H_2O$ (283.32): C, 63.59; H, 6.76; N, 9.89. Found: C, 63.42; H, 6.74; N, 9.58; MS ESI: $m/z = 275.13$ $[M+H]^+$; ^1H NMR (500 MHz, $[D_6]$DMSO, 30 °C, TMS) $\delta = 1.64$ (m, 2H, C^9H_2), 2.08 (A, 1H, C^8H_2), 2.33 (B, 1H, C^8H_2), 2.85 (A, 1H, CH_2-benzyl),

2.93 (B, 1H, CH_2-benzyl), 3.55 (m, 2H, $C^{10}H_2$), 3.58 (m, 1H, C^4H), 3.81 (d, 1H, $^3J(H,H) = 4.3$, C^3H), 4.99 (m, 1H, C^7H), 6.33 (d, 1H, $^3J(H,H) = 4.3$ Hz, OH), 7.23–7.33 (m, 6H, phenyl, NH); ^{13}C NMR (500 MHz, [D$_6$]DMSO, 30 °C, TMS) $\delta = 21.8$ (C^9H_2), 29.2 (C^8H_2), 38.0 (CH_2-benzyl), 46.8 ($C^{10}H_2$), 56.9 (C^4H), 57.3 (C^7H), 70.8 (C^3H), 126.1 (CH-Ph), 128.5 (2 CH-Ph), 129.2 (2 CH-Ph), 137.2 (C-Ph), 169.3 (C^2), 169.8 (C^6).

4.2.16. (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-prolyl-(*S*)-phenylalanine methyl ester hydrochloride (16). Compound **2** (200 mg, 0.34 mmol) was dissolved in MeOH. The catalyst Pd/C was added. Subsequently, the mixture was hydrogenated at room temperature. After completion of the reaction controlled by TLC, the catalyst was deactivated and filtered off. The crude product, obtained by evaporation, was treated with HCl/MeOH (4.6 m). Compound **16** was precipitated by dry diethyl ether, filtered off, and dried in vacuo over P_4O_{10}. Yield: 95 mg (57%, cis/trans-isomers); mp 98–106 °C; $[\alpha]_D^{20}$ −30.8 (*c* 1, CH_3OH); elemental analysis Calcd (%) for $C_{25}H_{31}N_3O_5 \cdot HCl \cdot \frac{1}{4}H_2O$ (494.50): C, 60.72; H, 6.62; N, 8.50. Found: C, 60.67; H, 6.59; N, 8.17; MS ESI: $m/z = 454.48$ $[M+H]^+$; ^{13}C NMR (500 MHz, [D$_6$]DMSO, 27 °C, DMSO) major isomer: $\delta = 24.2$ ($C^{\gamma}H_2$-Pro), 29.0 ($C^{\beta}H_2$-Pro), 34.5 ($C^{\gamma}H_2$-AHPB), 36.5 ($C^{\beta}H_2$-Phe), 46.8 ($C^{\delta}H_2$-Pro), 51.8 (OCH_3), 53.8 ($C^{\beta}H$-AHPB), 54.4 ($C^{\alpha}H$-Phe), 59.5 ($C^{\alpha}H$-Pro), 66.4 ($C^{\alpha}H$-AHPB), 126.6 (CH-Ph), 127.0 (CH-Ph), 128.2 (2 CH-Ph), 128.6 (2 CH-Ph), 129.3 (2 CH-Ph), 129.5 (2 CH-Ph), 136.2 (CH-Ph), 136.3 (CH-Ph), 169.1 (CO-AHPB), 171.5 (CO-Pro), 171.8 (CO-Phe); minor isomer: $\delta = 23.9$ ($C^{\gamma}H_2$-Pro), 28.3 ($C^{\beta}H_2$-Pro), 34.5 ($C^{\gamma}H_2$-AHPB), 36.5 ($C^{\beta}H_2$-Phe), 46.5 ($C^{\delta}H_2$-Pro), 51.9 (OCH_3), 53.8 ($C^{\beta}H$-AHPB), 54.4 ($C^{\alpha}H$-Phe), 59.4 ($C^{\alpha}H$-Pro), 66.2 ($C^{\alpha}H$-AHPB), 126.6 (CH-Ph), 127.0 (CH-Ph), 128.2 (2 CH-Ph), 128.6 (2 CH-Ph), 129.3 (2 CH-Ph), 129.5 (2 CH-Ph), 136.2 (CH-Ph), 136.3 (CH-Ph), 169.2 (CO-AHPB), 171.5 (CO-Pro), 171.9 (CO-Phe).

4.2.17. (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoic acid pyrrolidide hydrochloride (17). Compound **17** was obtained from compound **3** (170 mg, 0.44 mmol) by the same procedure described for **16**. Yield: 78 mg (62%); mp 167–172 °C; $[\alpha]_D^{20}$ −11.7 (*c* 1, CH_3OH); elemental analysis Calcd (%) for $C_{14}H_{20}N_2O_2 \cdot HCl \cdot \frac{1}{4}H_2O$ (289.29): C, 58.13; H, 7.49; N, 9.68. Found: C, 58.20; H, 7.33; N, 9.41; MS ESI: $m/z = 249.23$ $[M+H]^+$; ^1H NMR (500 MHz, [D$_6$]DMSO, 30 °C, DMSO) $\delta = 1.73$ (m, 2H, C^3H_2-Pyrr), 1.77 (m, 2H, C^4H_2-Pyrr), 2.89 (A, 1H, $C^{\gamma}H_2$-AHPB), 2.97 (B, 1H, $C^{\gamma}H_2$-AHPB), 3.24 (A, 1H, C^5H_2-Pyrr; m, 2H, C^2H_2-Pyrr), 3.49 (B, 1H, C^5H_2-Pyrr), 3.56 (m, 1H, $C^{\beta}H$-AHPB), 4.16 (m, 1H, $C^{\alpha}H$-AHPB), 6.47 (d, 1H, $^3J(H,H) = 6.0$ Hz, OH), 7.25–7.35 (m, 5H, phenyl), 8.01 (br s, 2H, NH_2); ^{13}C NMR (500 MHz, [D$_6$]DMSO, 27 °C, DMSO) $\delta = 23.5$ (C^4H_2-Pyrr), 25.5 (C^3H_2-Pyrr), 35.0 ($C^{\gamma}H_2$-Pyrr), 45.7 (C^2H_2-Pyrr), 45.8 (C^5H_2-Pyrr), 53.9 ($C^{\beta}H$-AHPB), 66.1 ($C^{\alpha}H$-AHPB), 126.9 (CH-Ph), 128.5 (2 CH-Ph), 129.4 (2 CH-Ph), 136.3 (CH-Ph), 168.8 (CO).

4.2.18. (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoic acid thiazolidide hydrobromide (18). Compound **18** was obtained by acidolytic deprotection of compound **4** (140 mg, 0.35 mmol), according to compound **13**. Yield: 100 mg (82%, E/Z-isomers); mp 98–106 °C; $[\alpha]_D^{20}$ +7.9 (*c* 1, CH_3OH); elemental analysis Calcd (%) for $C_{13}H_{18}N_2O_2S \cdot HBr \cdot 1\frac{1}{4}AcOH$ (422.34): C, 44.08; H, 5.73; N, 6.63; S, 7.59. Found: C, 43.91; H, 5.53; N, 7.12; S, 7.27; MS ESI: $m/z = 266.97$ $[M+H]^+$; ^{13}C NMR (500 MHz, [D$_6$]DMSO, 27 °C, TMS) major isomer: $\delta = 30.6$ (C^5H_2-Thia), 35.0 ($C^{\gamma}H_2$-AHPB), 48.1 (C^4H_2-Thia), 48.3 (C^2H_2-Thia), 53.7 ($C^{\beta}H$-AHPB), 67.0 ($C^{\alpha}H$-AHPB), 126.9 (CH-Ph), 128.5 (2 CH-Ph), 129.3 (2 CH-Ph), 136.0 (CH-Ph), 168.6 (CO); minor isomer: $\delta = 28.4$ (C^5H_2-Thia), 35.0 ($C^{\gamma}H_2$-AHPB), 47.5 (C^2H_2-Thia), 48.7 (C^4H_2-Thia), 53.7 ($C^{\beta}H$-AHPB), 64.8 ($C^{\alpha}H$-AHPB), 126.9 (CH-Ph), 128.5 (2 CH-Ph), 29.3 (2 CH-Ph), 136.0 (CH-Ph), 168.3 (CO).

4.2.19. (2*R*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline trifluoroacetate (19). Compound **5** (205 mg, 0.46 mmol) was dissolved in MeOH. The catalyst Pd/C was added. Subsequently, the mixture was hydrogenated at room temperature. After completion of the reaction controlled by TLC, the catalyst was deactivated and filtered off. After hydrogenolytic deprotection, the crude product, obtained by evaporation, was dissolved in dioxane (8 ml) and aqueous NaOH (0.1 m, 8 ml). The reaction mixture was stirred at room temperature. After completion of saponification, the reaction mixture was neutralized with 1 M HCl and evaporated to dryness. Purification was succeeded by RP-HPLC (column RP-8, HiBar® 25 μm, 250 mm Merck) with acetonitrile–water (45/55, v/v) containing trifluoroacetic acid (0.04%, v/v) as eluent. Compound **19** became amorphous. Yield: 147 mg (76%); decomposition >139 °C; elemental analysis Calcd (%) for $C_{15}H_{20}N_2O_4 \cdot 1\frac{1}{2}TFA \cdot \frac{1}{2}H_2O$ (472.38): C, 45.77; H, 4.80; N, 5.93. Found: C, 45.79; H, 5.06; N, 5.72; MS ESI: $m/z = 293.16$ $[M+H]^+$; ^{13}C NMR (500 MHz, [D$_6$]DMSO, 27 °C, DMSO) $\delta = 24.3$ ($C^{\gamma}H_2$-Pro), 28.3 ($C^{\beta}H_2$-Pro), 33.5 ($C^{\gamma}H_2$-AHPB), 46.3 ($C^{\delta}H_2$-Pro), 54.0 ($C^{\beta}H$-AHPB), 58.8 ($C^{\alpha}H$-Pro), 67.6 ($C^{\alpha}H$-AHPB), 126.8 (CH-Ph), 128.5 (2 CH-Ph), 129.3 (2 CH-Ph), 136.1 (CH-Ph), 168.3 (CO-AHPB), 172.9 (COOH).

4.2.20. (2*R*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoic acid thiazolidide hydrobromide (20). Compound **20** was obtained by acidolytic deprotection of **6** (130 mg, 0.32 mmol) by the same procedure as described for **13**. Yield: 82 mg (75%, E/Z-isomers); mp 106–114 °C; $[\alpha]_D^{20}$ −2.0 (*c* 1, CH_3OH); elemental analysis Calcd (%) for $C_{13}H_{19}N_2O_2S \cdot HBr\frac{1}{2}AcOH$ (378.31): C, 44.45; H, 5.86; N, 7.40; S, 8.48. Found: C, 44.81; H, 5.62; N, 7.35; S, 8.23; MS ESI: 267.15 $[M+H]^+$; ^{13}C NMR (500 MHz, CD_3OD, 27 °C, CH_3OH) major isomer: $\delta = 31.8$ (C^5H_2-Thia), 35.6 ($C^{\gamma}H_2$-AHPB), 50.2 (C^2H_2-Thia), 56.7 ($C^{\beta}H$-AHPB), 68.6 ($C^{\alpha}H$-AHPB), 128.6 (CH-Ph), 128.9 (CH-Ph), 130.3 (CH-Ph), (CH-Ph), 135.8 (CH-Ph), 136.6 (CH-Ph), 169.9 (CO); minor isomer: $\delta = 29.8$ (C^5H_2-Thia), 35.5 ($C^{\gamma}H_2$-AHPB), 49.9 (C^2H_2-Thia), 56.7 ($C^{\beta}H$-AHPB), 68.5 ($C^{\alpha}H$-AHPB), 128.6 (CH-Ph), 128.9 (CH-Ph), 130.3 (CH-Ph), 135.8

(CH-Ph), 136.6 (CH-Ph), 169.7 (CO). For both isomers the signal of C^4H_2 is buried under the CH_3OH signal at 49.0 ppm.

4.2.21. (2*R*,3*S*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline trifluoroacetate (21) and (3*R*,4S,7*S*)-4-benzyl-3-hydroxy-1,5-diazabicyclo-[5.3.0]-decan-2,6-dion (22). From compound **7** (154 mg, 0.35 mmol), the N-terminal protecting group was removed by catalytic hydrogenolysis, following the same procedure as described for **14** and **15**. Compound **22** was isolated by crystallization from MeOH/diethyl ether. Compound **22** was filtered off and the solvent was evaporated. Dioxane (6 ml) and 0.1 M aqueous NaOH (6 ml) were added to the remaining residue. After completion of saponification, compound **21** was isolated and purified by RP-HPLC as described for **19**.

4.2.21.1. (2*R*,3*S*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline trifluoroacetate (21). Yield: 72 mg (49%, cis/trans-isomers); mp 91–99 °C; $[\alpha]_D^{20}$ −27.8 (*c* 0.8, CH_3OH); elemental analysis Calcd (%) for $C_{15}H_{20}N_2O_4 \cdot 1\frac{1}{2}TFA \cdot \frac{1}{2}H_2O$ (472.38): C, 45.77; H, 4.80; N, 5.93. Found: C, 45.44; H, 4.97; N, 5.92; MS ESI: *m*/*z* = 293.16 $[M+H]^+$; ^{13}C NMR (500 MHz, $[D_6]$DMSO, 27 °C, TMS) major isomer: δ = 28.4 ($C^{\beta}H_2$-Pro), 34.9 ($C^{\gamma}H_2$-AHPB, $C^{\gamma}H_2$-Pro), 46.2 ($C^{\delta}H_2$-Pro), 53.7 ($C^{\beta}H$-AHPB), 58.7 ($C^{\alpha}H$-Pro), 66.1 ($C^{\alpha}H$-AHPB), 126.9/127.0 (CH-Ph), 128.6/128.7 (2 CH-Ph), 129.4/129.5 (2 CH-Ph), 136.0/136.1 (CH-Ph), 169.1 (CO-AHPB), 173.0 (COOH); minor isomer: δ = 28.5 ($C^{\beta}H_2$-Pro), 34.9 ($C^{\gamma}H_2$-AHPB, $C^{\gamma}H_2$-Pro), 46.4 ($C^{\delta}H_2$-Pro), 53.6 ($C^{\beta}H$-AHPB), 58.9 ($C^{\alpha}H$-Pro), 66.8 ($C^{\alpha}H$-AHPB), 126.9/127.0 (CH-Ph), 128.6/128.7 (2 CH-Ph), 129.4/129.5 (2 CH-Ph), 136.0/136.1 (CH-Ph), 169.0 (CO-AHPB), 173.3 (COOH).

4.2.21.2. (3*R*,4*S*,7*S*)-4-Benzyl-3-hydroxy-1,5-diazabicyclo-[5.3.0]-decan-2,6-dion (22). Yield: 15 mg (16%); decomposition >234 °C; elemental analysis Calcd (%) for $C_{15}H_{18}N_2O_3 \cdot \frac{1}{2}H_2O$ (283.32): C, 63.59; H, 6.76; N, 9.89. Found: C, 63.65; H, 6.72; N, 9.65; MS ESI: *m*/*z* = 275.10 $[M+H]^+$; ^1H NMR (500 MHz, $[D_6]$DMSO, 30 °C, TMS) δ = 1.69 (m, 2H, C^9H_2), 1.95 (A, 1H, C^8H_2), 2.30 (B, 1H, C^8H_2), 2.66 (A, 1H, ^2J(H,H) = 13.7 Hz, CH_2-benzyl), 2.88 (B, 1H, ^2J(H,H) = 13.6, CH_2-benzyl), 3.48 (B, 1H, $C^{10}H_2$), 3.91 (m, 1H, C^4H), 4.16 (m, 1H, C^3H), 4.57 (t, 1H, ^3J(H,H) = 7.3 Hz, C^7H), 5.44 (d, 1H, ^3J(H,H) = 5.1 Hz, OH), 7.18–7.30 (m, 5H, phenyl), 7.74 (d, 1H, ^3J(H,H) = 6.3 Hz, NH). The signal of the second proton of $C^{10}H_2$ is buried under the water signal from solvent at 3.29 ppm. ^{13}C NMR (500 MHz, $[D_6]$DMSO, 30 °C, TMS) δ = 21.9 (C^9H_2), 28.3 (C^8H_2), 36.3 (CH_2-benzyl), 47.6 ($C^{10}H_2$), 54.1 (C^4H), 57.5 (C^7H), 70.5 (C^3H), 126.2 (CH-Ph), 128.1 (2 CH-Ph), 129.5 (2 CH-Ph), 138.3 (C-Ph), 169.5 (C^2), 169.8 (C^6).

4.2.22. (2*R*,3*S*)-3-Amino-2-hydroxy-4-phenylbutanoic acid thiazolidide hydrobromide (23). Compound **23** was obtained by deprotection of compound **8** (59 mg, 0.15 mmol) according to **13**. Yield: 40 mg (77%, E/Z-isomers); mp 108–116 °C; $[\alpha]_D^{20}$ +4.6 (*c* 1.1, CH_3OH); elemental analysis Calcd (%) for $C_{13}H_{18}N_2O_2S \cdot 1\frac{1}{4}HBr \cdot AcOH$ (427.55): C, 42.14; H, 5.48; N, 6.55; S, 7.52. Found: C, 42.42; H, 5.30; N, 6.99; S, 7.49; MS ESI: *m*/*z* = 266.97 $[M+H]^+$; ^{13}C NMR (500 MHz, $[D_6]$DMSO, 27 °C, DMSO) major isomer: δ = 30.7 (C^5H_2-Thia), 35.1 ($C^{\gamma}H_2$-AHPB), 48.3 (C^4H_2-Thia), 48.4 (C^2H_2-Thia), 53.7 ($C^{\beta}H$-AHPB), 67.0 ($C^{\alpha}H$-AHPB), 127.0 (CH-Ph), 128.6 (2 CH-Ph), 129.3 (2 CH-Ph), 136.0 (CH-Ph), 168.7 (CO); minor isomer: δ = 28.5 (C^5H_2-Thia), 35.1 ($C^{\gamma}H_2$-AHPB), 47.6 (C^2H_2-Thia), 48.9 (C^4H_2-Thia), 53.7 ($C^{\beta}H$-AHPB), 67.0 ($C^{\alpha}H$-AHPB), 127.0 (CH-Ph), 128.6 (2 CH-Ph), 129.3 (2 CH-Ph), 136.0 (CH-Ph), 168.3 (CO).

4.2.23. (2*S*,3*S*)-3-Amino-2-hydroxy-4-phenylbutanoyl-(*S*)-proline methyl ester (24). Dioxane (3 ml) and aqueous NaOH (0.1 M, 3 ml) were added to **9** (140 mg, 0.32 mmol). The reaction mixture was stirred at room temperature. After completion of saponification, the dioxane was removed under reduced pressure and the residue was diluted with water (10 ml). The aqueous solution was extracted with ethyl acetate to remove remaining **9** and, subsequently, acidified to pH 2–3 with 1 M HCl. The C-terminal free dipeptide was extracted with ethyl acetate. The combined extracts were washed with brine and dried over $MgSO_4$. The solvent was removed under reduced pressure. The N-terminal deprotection was carried out by Pd-catalyzed hydrogenolysis as described for **14** and **15**. (2*S*,3*S*)-AHPB-(*S*)-Pro-OMe was obtained, instead of the expected acid. Esterification by the solvent methanol took place. Yield: 60 mg (61%, cis/trans-isomers); decomposition >121 °C; elemental analysis Calcd (%) for $C_{16}H_{22}N_2O_4 \cdot H_2O$ (324.37): C, 59.24; H, 7.46; N, 8.64. Found: C, 59.28; H, 7.49; N, 8.43; MS ESI: *m*/*z* = 307.16 $[M+H]^+$; ^1H NMR (500 MHz, $[D_6]$DMSO, 30 °C, TMS) major isomer: δ = 1.90 (m, 4H, $C^{\beta}H_2$-Pro, $C^{\gamma}H_2$-Pro), 2.91 (m, 2H, $C^{\gamma}H_2$-AHPB), 3.51 (m, 1H, $C^{\beta}H$-AHPB), 3.64 (B, $C^{\delta}H_2$-Pro), 3.67 (s, 3H, OCH_3), 4.33 (m, 2H, $C^{\alpha}H$-AHPB, $C^{\alpha}H$-Pro), 6.17 (br s, 1H, OH), 7.22–7.37 (m, 5H, phenyl), 7.95 (br s, 2H, NH_2); minor isomer: δ = 1.90 (m, 2H, $C^{\gamma}H_2$-Pro), 2.23 (m, 2H, $C^{\beta}H_2$-Pro), 2.79 (m, 2H, $C^{\gamma}H_2$-AHPB), 3.51 (m, 1H, $C^{\beta}H$-AHPB), 3.64 (B, 1H, $C^{\delta}H_2$-Pro), 3.67 (s, 3H, OCH_3), 4.33 (m, 2H, $C^{\alpha}H$-AHPB, $C^{\alpha}H$-Pro), 6.17 (br s, 1H, OH), 7.22–7.37 (m, 5H, phenyl), 7.95 (br s, 2H, NH_2). For both isomers, the signal of A (1H) of $C^{\delta}H_2$-proline is buried under the water signal at 3.29 ppm.

4.2.24. (2*S*,3*R*)-3-Amino-2-hydroxy-5-methylhexanoyl-(*S*)-proline trifluoroacetate (25). The deprotection of **10** (100 mg, 0.25 mmol) to yield compound **25** succeeded by the procedure as described for **19**. Yield: 57 mg (48%, cis/trans-isomers); mp 117–122 °C; elemental analysis Calcd (%) for $C_{12}H_{22}N_2O_4 \cdot \frac{1}{4}TFA \cdot 1\frac{1}{2}H_2O$ (427.87): C, 40.70; H, 6.18; N, 6.55. Found: C, 40.63; H, 6.25; N, 6.39; MS ESI: *m*/*z* = 259.20 $[M+H]^+$; ^{13}C NMR (500 MHz, $[D_6]$DMSO, 27 °C, DMSO) major isomer: δ = 22.0 ($C^{\varepsilon}H_3$-AHMH), 22.7 ($C^{\varepsilon}H_3'$-AHMH), 23.3 ($C^{\delta}H$-AHMH), 24.7 ($C^{\gamma}H_2$-Pro), 28.6 ($C^{\beta}H_2$-Pro), 37.6 ($C^{\gamma}H_2$-AHMH), 46.5 ($C^{\delta}H_2$-Pro), 51.0 ($C^{\beta}H$-AHMH), 59.6 ($C^{\alpha}H$-Pro), 67.9 ($C^{\alpha}H$-AHMH), 169.1

(CO-AHMH), 173.2 (COOH); minor isomer: δ = 22.0 ($C^{\varepsilon}H_3$-AHMH), 22.8 ($C^{\varepsilon}H'_3$-AHMH), 23.3 ($C^{\delta}H$-AHMH), 24.7 ($C^{\gamma}H_2$-Pro), 30.6 ($C^{\beta}H_2$-Pro), 38.6 ($C^{\gamma}H_2$-AHMH), 46.2 ($C^{\delta}H$-Pro), 50.7 ($C^{\beta}H$-AHMH), 57.4 ($C^{\alpha}H$-Pro), 68.1 ($C^{\alpha}H$-AHMH), 170.0 (CO-AHMH), 173.5 (COOH).

4.2.25. (2*S*,3*R*)-3-Amino-2-hydroxy-5-methylhexanoic acid thiazolidide hydrobromide (26). Compound **26** was obtained by deprotection of **11** (40 mg, 0.11 mmol) according to compound **13**. Yield: 33 mg (96%, E/Z-isomers); mp 161–165 °C; $[\alpha]_D^{20}$ −6.1 (*c* 0.8, CH_3OH); elemental analysis Calcd (%) for $C_{10}H_{20}N_2O_2S{\cdot}HBr{\cdot}AcOH$ (373.31): C, 38.61; H, 6.75; N, 7.50; S, 8.59. Found: C, 38.67; H, 6.83; N, 7.23; S, 8.53; ESI MS: *m*/*z* = 233.04 $[M+H]^+$; ^1H NMR (500 MHz, [D$_6$]DMSO, 27 °C, TMS) major isomer: δ = 0.88 (d, 6H, ^3J(H,H) = 6.49 Hz, $C^{\varepsilon}H_3$, $C^{\varepsilon}H'_3$-AHMH), 1.41 (m, 2H, $C^{\gamma}H_2$-AHMH), 1.73 (m, 1H, $C^{\delta}H$-AHMH), 3.11 (t, 2H, ^3J(H,H) = 6.24 Hz, C^4H_2-Thia), 3.82 (A, 1H, C^5H_2-Thia), 3.91 (B, 1H, C^5H_2-Thia), 4.31 (br s, 1H, $C^{\alpha}H$-AHMH), 4.48 (A, 1H, ^2J(H,H) = 10.22 Hz, C^2H_2-Thia), 4.51 (B, 1H, ^2J(H,H) = 10.24 Hz, C^2H_2-Thia), 6.38 (br s, 1H OH) 7.69 (br s, 2H, NH_2); minor isomer: δ = 0.88 (d, 6H, ^3J(H,H) = 6.49 Hz, $C^{\varepsilon}H_3$, $C^{\varepsilon}H'_3$-AHMH), 1.41 (m, 2H, $C^{\gamma}H_2$-AHMH), 1.73 (m, 1H, $C^{\delta}H$-AHMH), 3.02 (t, 2H, ^3J(H,H) = 6.42 Hz, C^4H_2-Thia), 3.69 (dm, 2H, C^5H_2-Thia), 4.35 (br s, 1H, $C^{\alpha}H$-AHMH), 4.66 (A, 1H, ^2J(H,H) = 9.14 Hz, C^2H_2-AHMH), 4.72 (B, 1H, ^2J(H,H) = 9.12 Hz, C^2H_2-Thia), 6.38 (br s, 1H OH) 7.69 (br s, 2H, NH_2). For both isomers, the signal of $C^{\beta}H$-AHMH is buried under the water signal at 3.22 ppm.

4.3. Kinetic studies

4.3.1. Enzymes. Recombinant APP from *E. coli* was a gift from T. Yoshimoto, Nagasaki University, Japan. Membrane-bound rat APP was isolated from intestine, as described previously.[28] Lys(Abz)-Pro-Pro-4NA (Abz, 2-aminobenzoyl; 4NA, 4-nitroanilide) was synthesized, according to standard procedures.[29]

4.3.2. Enzyme assays. The activity of APP was determined by use of the substrate Lys(Abz)-Pro-Pro-4NA ($APP_{E.\ coli}$ K_m = 40.7 μM, APP_{rat} K_m = 3.6 μM) monitoring the fluorescence of released Lys(Abz) at an excitation wavelength of 310 nm and an emission wavelength of 410 nm at 30 °C on a Perkin-Elmer LS-50B luminescence spectrometer.[29]

4.3.2.1. Bacterial APP. At first, lyophilized *E. coli* APP (0.1 mg) was dissolved in Tricine–citrate buffer (3 ml, 40 mM Tricine, 0.6 mM citrate, pH 7.4) containing $MnCl_2$ (3 mM). APP was preincubated for 1 h at 30 °C. A typical reaction mixture (1 ml) consisted of Tris buffer (40 mM, pH 7.4), $MnCl_2$ (0.75 mM), and Lys(Abz)-Pro-Pro-4NA in various concentrations (4–15 μM) and different concentrations of inhibitor. The reaction was initiated by adding activated APP (10 μl). The final enzyme concentration was 24 nM in the case of mixed-type inhibition and 5 nM for slow-binding inhibition (MW: 49,650 Da).[30]

4.3.2.2. Rat APP. Rat intestine APP (25 μl) was incubated with Tricine–citrate buffer (275 μl, 40 mM Tricine, 0.6 mM citrate, pH 7.4) containing $MnCl_2$ (1.25 mM) for 1 h at 30 °C.[28] The activated enzyme was diluted with buffer (1.2 ml). The reaction mixture consisted of Tricine buffer (40 mM, pH 7.4), $MnCl_2$ (2.4 mM), various concentrations of Lys(Abz)-Pro-Pro-4NA (4–10 μM), and different concentrations of inhibitor around the expected K_i value. The reaction was initiated by adding activated enzyme (10 μl). The final concentration of APP was 0.24 and 0.12 nM for mixed-type inhibition and slow-binding inhibition, respectively (MW: 96 kDa).[31]

4.3.3. Determination of inhibition constants. All investigations were started by adding the enzyme and were run in duplicates. In general, the reaction velocities for both inhibition mechanisms were calculated over a time interval in which less than 10% cleavage of substrate took place.

4.3.3.1. Mixed-type inhibition. In the case of mixed-type inhibition, steady-state kinetics were analyzed using the equation

$$v = \frac{V_{max}[S]}{K_m(1+\frac{[I]}{K_i}) + [S](1+\frac{[I]}{\alpha K_i})}, \qquad (1)$$

where K_i is the competitive inhibition constant and K_i multiplied with factor α represents the uncompetitive inhibition constant.[18] Thus, the equation is applicable to the three classical reversible inhibition mechanisms, namely the competitive, uncompetitive, and noncompetitive inhibition. However, the Dixon plot (Eq. 2) of the velocity equation (Eq. 1) does not allow to distinguish between pure competitive and linear mixed-type inhibition, a special case of noncompetitive inhibition. In both cases, the straight lines have a common point of intersection within the second quadrant. For this reason, slopes and intercepts from Dixon plot (Eq. 2) were replotted versus 1/[*S*] to determine the inhibition mechanism and the constants K_i, as well as, α (Eqs. 3 and 4).

$$\frac{1}{v} = \frac{(1+\frac{\alpha K_m}{[S]})}{\alpha K_i V_{max}}[I] + \frac{1}{V_{max}}\left(1+\frac{K_m}{[S]}\right), \qquad (2)$$

$$\text{slope} = \frac{K_m}{K_i V_{max}}\frac{1}{[S]} + \frac{1}{\alpha K_i V_{max}}, \qquad (3)$$

$$\text{intercept} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}. \qquad (4)$$

The kinetic data were calculated using the FLWinLab software 1.10 (Perkin-Elmer, Germany) and GraFit 3.0 (Erithacus Software Ltd, England).

4.3.3.2. Slow-binding inhibition. The single progress curves of a slow-binding inhibition are described by the following Eq. 5.[20,32]

$$P = v_s \cdot t + (v_i - v_s)\cdot(1 - e^{-k_{obs}\cdot t})/k_{obs} + d. \qquad (5)$$

Eq. 5 allows the determination of the initial velocity v_i, the steady-state velocity v_s, and the apparent first-order rate constant k_{obs} for the approach of the steady state. The constant d is the displacement from zero at $t = 0$. P is the concentration of product at time t.

Plots of v_i versus $[I]$ and v_s versus $[I]$ according to Eq. 6 allowed the determination of the dissociation constant K_i and the overall inhibition constant K_i^*, respectively.

$$v = \frac{V_{max} \cdot [S]}{[S] + K_m(1 + [I]/K_i)}. \quad (6)$$

Eqs. 7 and 8 were used for determination of the detailed type of slow-binding inhibition, as well as the characteristic constants k_{off} and k_{on}, respectively.[20,32] The constants k_{on} and k_{off} represent the forward and reverse rate constants, respectively, for a slow conversion of the initial EI-complex into the tight-binding complex EI*.

$$k_{obs} = k_{off} + k_{on}\frac{[I]K_i}{1 + [S]/K_m + [I]/K_i^*}, \quad (7)$$

$$\frac{k_{on}}{k_{off}} = \left(\frac{K_i}{K_i^*}\right) - 1. \quad (8)$$

Moreover, the Dixon plots ($1/v_s$ versus $[I]$) and $1/v_i$ versus $[I]$, (Eq. 2) and the replots of the slopes from Dixon plots versus $1/[S]$ (Eq. 3) were used to prove the competitive character of the slow-binding inhibitors.

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