Simplified Pepstatins: Synthesis and Evaluation of N-Terminally Modified Analogues

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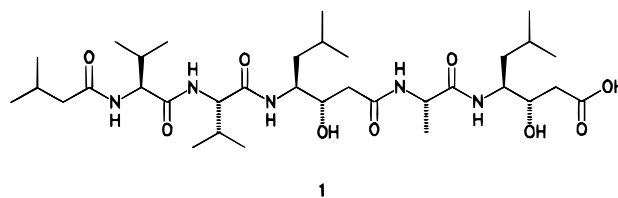
The promising strategy of gastric ulcer healing with perorally administered epidermal growth factor (EGF) is so far strongly limited by the pepsinic degradation of this therapeutic polypeptide in the stomach. The incorporation of EGF in a bioadhesive polymer−pepsin inhibitor conjugate used as drug carrier matrix, however, might provide sufficient protection toward pepsinic degradation. The synthesis of appropriate pepsin inhibitors represents a prerequisite for the development of such polymer−inhibitor conjugates. The presented study demonstrates that modifications at the N-terminus of simplified analogues of pepstatin which can be synthesized in a simple and straight way result only in slight variations of the inhibitory activity. These analogues display only 10-fold reduced inhibitory activity, compared to pepstatin A, when bearing a greater N-terminal group like isovaleryl, Boc, or Cbz. Compounds which are substituted at the N-terminus by a shorter *N*-acyl group like propionyl or cyclopropylcarbonyl show further reduced activity (0.01, compared to pepstatin A). The presence of an amide or a urethane moiety at the N-terminus has no considerable effect on enzyme inhibition. Therefore, the N-terminus of these analogues is able to be modified forming a covalent bond to various bioadhesive polymers via a suitable functionality.

Introduction

The peroral administration of therapeutic peptides and proteins is a great challenge to pharmaceutical sciences. So far, however, this route is of limited value because the gastrointestinal tract provides a hostile environment for (poly)peptides. In particular, luminally secreted and brush border membrane-bound enzymes lead to a rapid degradation of perorally administered peptide and protein drugs. A promising strategy to overcome this so-called 'enzymatic barrier' represents the use of auxiliary agents such as mucoadhesive polymers and enzyme inhibitors.[1] On the one hand, these polymers can provide intimate contact with the gastrointestinal mucosa, thereby excluding a presystemic metabolism of the therapeutic (poly)peptide on the way between the delivery system and the absorption membrane. Enzyme inhibitors, on the other hand, are able to inactivate proteases which penetrate into the polymeric carrier system. In addition, toxic side effects of enzyme inhibitors can be excluded by the covalent attachment of these auxiliary agents to the unabsorbable polymeric carrier matrix.

Among such polymer−inhibitor conjugates, the development of mucoadhesive polymer−pepsin inhibitor conjugates has gained considerable interest for the peroral administration of epidermal growth factor (EGF). Due to the oral administration of this polypeptide drug, an enhanced healing of gastric ulcers could already by verified in a double-blind controlled clinical study.[2] As EGF is rapidly degraded in the stomach by pepsin,[3] its ulcer-healing capability after oral dosing could be even drastically improved by an appropriate galenic. An initial step in this direction can be seen in the development of a bioadhesive drug delivery system containing a polymer−enzyme inhibitor conjugate, which provides a strong protective effect for the embedded therapeutic agent.[4] The efficient pepsin inhibitor pepstatin A (**1**) was selected. However, although this system turned out to be very successful in various studies,[4] its practical use is strongly limited by the extensive costs of pepstatin A. Following studies have been focused on the synthesis of simplified pepstatin A analogues which guarantee low production costs in large-scale preparation and form the basis for the development of practically usable polymer−pepsin inhibitor conjugates as vehicles for the peroral administration of EGF.[5]



1

Chemistry

McConnell et al. have described tripeptides (**2**) acting as pepsin inhibitors, which contain only the first three N-terminal amino residues of pepstatin A (**1**).[6] Recently, we have reported on comparably simplified analogues (**3**), which utilize a *vic*-amino alcohol in place of the C-terminal statine unit.[5] These compounds show comparable activities to pepstatin A itself. Due to synthetic considerations our compounds **3** have a Boc group at the N-terminus, while the analogues **2**, described by McConnell et al., are N-terminated by a Cbz unit.[6] It

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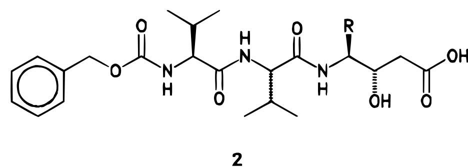

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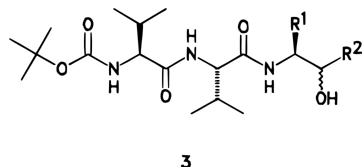
R = *iso*-butyl, benzyl, *p*-OH-benzyl, *n*-butyl

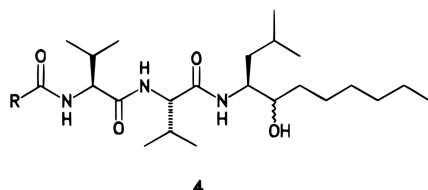


3

R^1 = *iso*-butyl, *sec*-butyl, *n*-propyl
R^2 = *n*-butyl, *n*-hexyl, *n*-octyl

seems to be that the protective group at the N-terminus has no considerable effect on enzyme inhibition. In the following, our aim was to produce analogues of our most active compounds with different groups at the N-terminus to elucidate structure–activity dependences.

Obviously, the shortest way to synthesize compounds of the general structure **4** is the synthesis of **3** (R^1 = isobutyl, R^2 = hexyl), cleavage of the Boc protecting



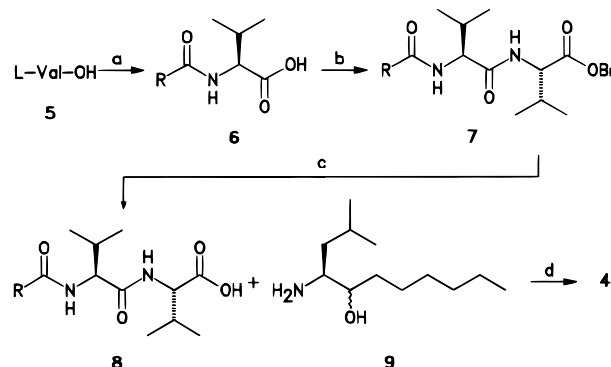
4

group, and finally N-acylation with various acid halides or chloroformates, respectively, though there is the risk of ester formation at the hydroxy group. To avoid additional steps caused by protection and deprotection of the hydroxy group, we decided to construct the target compounds starting from the N-terminus. The selected strategy is outlined in Scheme 1. L-Valine (**5**) was N-acylated with different acid chlorides (and chloroformates, respectively) (→**6**) and reacted with L-valine benzyl ester in the presence of *N,N'*-dicyclohexylcarbodiimide. Hydrogenolytic cleavage of the benzyl group (→**8**) and coupling with amino alcohol **9** afforded the target compounds **4**. The C-terminus of the described compounds, represented by **9**, has been derived from L-leucine, subsequent reaction (of the Weinreb amide) with hexylmagnesium bromide, and reduction with sodium borohydride.[5] For the synthesis of **4b** we directly used the commercially available dipeptidic acid Cbz-Val-Val-OH (**8b**).

Evaluation of the Inhibitory Activity

To evaluate the enzyme inhibitory activity, the IC_{50} of all compounds has been determined by an enzyme assay as previously described by our research group.[5] Horseradish peroxidase was thereby used as protein substrate for pepsin. The remaining activity of undegraded peroxidase was quantified by the capability of the enzyme to oxidize *o*-phenylenediamine leading to an orange-colored stain. The concentration of added inhibitors, at which peroxidase displayed half of its activity after incubation with pepsin, represented therefore the IC_{50}. Samples without pepstatin analogues and with neither pepstatin analogues nor pepsin served as references.

Scheme 1[a]



[a] (a) RCOCl, MgO, Et_2O/H_2O; (b) H_2N-Val-OBn, DCC, THF; (c) $Pd/C/H_2$, MeOH; (d) EEDQ, THF.

Table 1. Inhibitory Effect of Pepstatin A and Analogues toward the Pepsin Hydrolysis of Horseradish Peroxidase

compound	R	IC_{50} (M) ±SD (n=3)
pepstatin A		$3.1 \pm 1.3 \times 10^{-8}$
4a		$2.1 \pm 0.9 \times 10^{-7}$
4b		$6.9 \pm 1.7 \times 10^{-7}$
4c		$1.2 \pm 0.2 \times 10^{-6}$
4d		$1.1 \pm 0.2 \times 10^{-6}$
4e		$7.3 \pm 2.5 \times 10^{-7}$
4f		$9.2 \pm 2.4 \times 10^{-7}$
4g		$3.2 \pm 0.8 \times 10^{-6}$
4h		$3.6 \pm 1.5 \times 10^{-6}$
4i		$4.5 \pm 1.5 \times 10^{-6}$

Results and Discussion

The tested compounds possess a uniform backbone of two valine residues and a *vic*-aminol C-terminus; all analogues exhibit a strong inhibitory effect toward pepsin. Results of inhibition studies are listed in Table 1. Compared to the compound with the original *N*-isovaleryl moiety (of pepstatin A), derivatives with a shorter group at the nitrogen show a lower inhibiting activity. These smaller and similar groups comprised ethyl, isopropyl, and cyclopropyl. Elongation of the N-substituent does not have any adverse effect on the activity. Replacement of the N-terminating acyl group by a urethane moiety does not generally lead to more active compounds (see **4b** vs **4e** and **4c** vs **4f**) as might have been assumed by comparing the *N*-Boc derivative **4a** with the carba analogue **4d**. In the urethane series, the *N*-Cbz-terminated compound is surpassed by the *N*-Boc-protected analogue.

In summary, compared to the simplified pepstatin A analogues, recently described by Kratzel et al.,[5] derivatives with other groups at the N-terminus exhibit only slightly varying activities. Based on the results obtained within this study, the following structure−activity dependences could be concluded: (i) A substituent smaller than C_3 leads to a significant reduction of inhibitory activity. (ii) Compounds with N-substituents such as isovaleryl or longer carbon chains show similar activities. (iii) Amides and urethanes at the N-terminus display comparable activities.

Taking these results into consideration, a more generous variation of the N-substituent should be possible, e.g., incorporation of a terminating carboxy or hydroxy group. Hence, the prerequisites are given for the covalent attachment to a bioadhesive polymer also at the N-terminus.

Experimental Section

Melting points were determined on a Kofler hot plate apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer 1600 FTIR spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured on a Varian Unity Plus 300 spectrometer. ^{1}H NMR spectra were referenced to tetramethylsilane (δ 0.0); in ^{13}C NMR spectroscopy $CDCl_3$ (or MeOD-d_3, respectively) served as the internal standard [δ 77.0 (or δ 49.0, respectively)]. MS spectra were measured on a Shimadzu QP 5000 instrument. For inhibition studies a microtitration plate reader (Anthos Reader 2001) was used.

Flash chromatography was performed on Merck silica gel 60, TLC on plastic sheets (Merck silica gel 60 F_{254}). Tetrahydrofuran was dried over sodium/benzophenone, dichloromethane over phosphorus pentoxide.

***N*-Cyclopropylcarbonyl-L-valine (6i).** To a well-stirred mixture of L-valine (17 mmol, 2.0 g) and 1.8 g of magnesium oxide in a solvent mixture of diethyl ether (20 mL) and water (100 mL) was added cyclopropylcarbonyl chloride (18.7 mmol, 1.70 mL) at 0 °C. After stirring for 4 h at room temperature the mixture was acidified with HCl (6 N) to pH 2−3. The organic layer was separated, followed by extraction of the aqueous layer with ethyl acetate (2 × 20 mL). The combined organic extracts were washed with brine, dried (Na_2SO_4), and evaporated to afford 2.40 g (76%) of **6i** as a colorless oil; IR (KBr/liquid film, cm^{-1}) 3340 (OH), 1720, 1630 (C=O); ^{1}H NMR ($CDCl_3$) δ 0.65−0.74, 0.75−1.10 [each m, 2H, 8H, 2 × cyclopropyl-CH_2, CH(C*H*$_3$)$_2$], 1.35−1.62 (m, 1H, cyclopropyl-CH), 2.09 [m, 1H, C*H*(CH$_3$)$_2$], 4.51 (dd, 1H, *J* = 4.8 Hz, *J* = 8.6 Hz, α-H), 6.61 (br.d, 1H, NH), 9.70 (br, 1H, COOH); ^{13}C NMR ($CDCl_3$): δ 8.6 (2 × cyclopropyl-CH_2), 12.8 (cyclopropyl-CH), 17.6, 18.9 [CH(*C*H$_3$)$_2$], 31.0 [*C*H(CH$_3$)$_2$], 57.2 (α-C), 175.1, 176.8 (2 × C=O): MS *m*/*z* 185.1 (M$^+$).

General Procedure for the Preparation of Dipeptides 7. To a solution of *N*-acylated L-valine (5 mmol) and L-valine benzyl ester (5 mmol, 1.05 g) in 30 mL of tetrahydrofuran was added *N,N'*-dicyclohexylcarbodiimide (5.5 mmol, 1.14 g), dissolved in 10 mL of tetrahydrofuran, at 0 °C. The reaction mixture was stirred overnight, evaporated in vacuo, and redissolved in ethyl acetate. The solution was washed with citric acid (10%, 2 × 10 mL), saturated aqueous $NaHCO_3$ solution (2 × 10 mL), and brine (2 × 10 mL). The organic layer was dried (Na_2SO_4) and evaporated giving colorless solids.

***N*-Isopropoxycarbonyl-L-valyl-L-valine Benzyl Ester (7c).** Starting from 1.02 g (5 mmol) of *N*-isopropoxycarbonyl-L-valine (**6c**), 1.41 g (72%) of **7c** was obtained as colorless crystals: mp 107−110 °C; IR (KBr, cm^{-1}) 3285, 3260 (NH), 1735, 1690, 1655 (C=O); ^{1}H NMR ($CDCl_3$) δ 0.80−0.96, 1.14−1.22 [each m, 18H, 3 × CH(C*H*$_3$)$_2$], 1.70−2.05, 2.08−2.25 [each m, each 1H, 2 × C*H*(CH$_3$)$_2$], 4.08 (dd, 1H, *J* = 7.3 Hz, *J* = 8.8 Hz, α-H), 4.52 (dd, 1H, *J* = 5.0 Hz, *J* = 8.8 Hz, α-H), 4.70−4.92 [m, 1H, OC*H*(CH$_3$)$_2$], 5.07, 5.17 (AB-system, 2H, *J* = 12.2 Hz, benzyl-H), 5.38, 6.83 (each br.d, each 1H, 2 × NH), 7.31 (s, 5H, arom. H); ^{13}C NMR ($CDCl_3$) δ 17.6, 18.8, 19.1, 19.2, 21.9 [3 × CH(*C*H$_3$)$_2$], 30.9, 31.2 [2 × *C*H(CH$_3$)$_2$], 50.1, 57.0 (2 × α-C), 67.4 (benzyl-C), 68.3 [O*C*H(CH$_3$)$_2$], 128.2, 128.4 (arom. CH), 135.2 (arom. C), 156.6, 171.4, 171.6 (3 × C=O); MS *m*/*z* 392.3 (M$^+$).

***N*-(3,3-Dimethylbutyryl)-L-valyl-L-valine Benzyl Ester (7d).** Starting from 1.00 g (5 mmol) of *N*-(3,3-dimethylbutyryl)-L-valine (**6d**), 1.27 g (63%) of **7d** was obtained as colorless crystals: mp 160 °C; IR (KBr, cm^{-1}) 3290 (NH), 1735, 1690, 1655 (C=O); ^{1}H NMR ($CDCl_3$) δ 0.83−0.96 [m, 12H, 2 × CH(C*H*$_3$)$_2$], 1.05 [s, 9H, C(CH$_3$)$_3$], 1.85−2.10 [m, 2H, 2 × C*H*(CH$_3$)$_2$], 2.28 [s, 2H, COC*H*$_2$C(CH$_3$)$_3$], 4.55 (dd, 1H, *J* = 4.6 Hz, *J* = 8.6 Hz, α-H), 4.63 (dd, 1H, *J* = 5.0 Hz, *J* = 8.8 Hz, α-H), 5.11, 5.21 (AB-system, 2H, *J* = 12.4 Hz, benzyl-H), 5.90, 6.14 (each br.d, each 1H, 2 × NH), 7.35 (s, 5H, arom. H); ^{13}C NMR ($CDCl_3$) δ 17.5, 17.7, 18.9, 19.1 [2 × CH(*C*H$_3$)$_2$], 29.9 [C(*C*H$_3$)$_3$], 31.2 [2 × *C*H(CH$_3$)$_2$], 31.6 [*C*(CH$_3$)$_3$], 47.2 [CO*C*H$_2$C(CH$_3$)$_3$], 57.2, 58.3 (2 × α-C), 67.0 (benzyl-C), 128.3, 128.4, 128.5 (arom. CH), 135.2 (arom. C), 171.3, 171.4, 172.0 (3 × C=O); MS *m*/*z* 404.5 (M$^+$).

***N*-(3-Phenylpropionyl)-L-valyl-L-valine Benzyl Ester (7e).** Starting from 1.25 g (5 mmol) of *N*-(3-phenylpropionyl)-L-valine (**6e**), 1.88 g (86%) of **7e** was obtained as colorless crystals: mp 117−120 °C; IR (KBr, cm^{-1}) 3290 (NH), 1735, 1655, 1635 (C=O); ^{1}H NMR ($CDCl_3$) δ 0.76−0.96 [m, 12H, 2 × CH(C*H*$_3$)$_2$], 1.90−2.08, 2.10−2.25 [each m, each 1H, 2 × C*H*(CH$_3$)$_2$)], 2.48−2.63, 2.92−3.03 (each m, each 2H, 2 × CH_2), 4.40 (dd, 1H, *J* = 4.8 Hz, *J* = 8.6 Hz, α-H), 4.55 (dd, 1H, *J* = 7.2 Hz, *J* = 8.8 Hz, α-H), 5.11, 5.22 (AB-system, 2H, *J* = 12.0 Hz, benzyl-H), 6.46, 6.83 (each br.d, each 1H, 2 × NH), 7.14−7.28 (m, 5H, arom. H), 7.35 (s, 5H, arom. H); ^{13}C NMR ($CDCl_3$) δ 17.6, 18.1, 18.8, 18.9 [2 × CH(*C*H$_3$)$_2$], 30.8, 30.9 [2 × *C*H(CH$_3$)$_2$], 31.1, 38.1 (2 × CH_2), 57.2, 58.3 (2 × α-C), 67.0 (benzyl-C), 126.1, 126.3, 128.2, 128.3, 128.4, 128.5 (arom. CH), 135.2, 140.6 (arom. C), 171.4, 171.5, 172.1 (3 × C=O); MS *m*/*z* 438.4 (M$^+$).

***N*-Isovaleryl-L-valyl-L-valine Benzyl Ester (7f).** Starting from 1.00 g (5 mmol) of *N*-isovaleryl-L-valine (**6f**), 1.50 g (77%) of **7f** was obtained as colorless crystals: mp 130−132 °C; IR (KBr, cm^{-1}) 3285 (NH), 1735, 1655, 1635 (C=O); ^{1}H NMR ($CDCl_3$ + MeOD-d_3) δ 0.76−0.98 [m, 18H, 3 × CH(C*H*$_3$)$_2$], 1.80−2.24 [m, 4H, 2 × C*H*(CH$_3$)$_2$, COC*H*$_2$CH(CH$_3$)$_2$], 4.30, 4.51 (each m, each 1H, 2 × α-H), 5.03, 5.12 (AB-system, 2H, *J* = 12.2 Hz, benzyl-H), 6.28, 6.63 (each br.d, each 1H, 2 × NH), 7.29 (s, 5H, arom. H); ^{13}C NMR ($CDCl_3$ + MeOD-d_3) δ 17.6, 18.0, 18.3, 18.9, 22.3, 25.5 [3 × CH(*C*H$_3$)$_2$], 27.0 [COCH$_2$*C*H(CH$_3$)$_2$], 31.0, 31.2 [2 × *C*H(CH$_3$)$_2$], 57.2, 58.3 (2 × α-C), 66.9 (benzyl-C), 128.3, 128.5 (arom. CH), 135.4 (arom. C), 171.3, 171.6, 172.6 (3 × C=O); MS *m*/*z* 390.3 (M$^+$).

***N*-Propionyl-L-valyl-L-valine Benzyl Ester (7g).** Starting from 870 mg (5 mmol) of *N*-propionyl-L-valine (**6g**), 1.43 g (79%) of **7g** was obtained as colorless crystals: mp 180 °C; IR (KBr, cm^{-1}) 3290 (NH), 1730, 1640 (C=O); ^{1}H NMR ($CDCl_3$) δ 0.80−1.05 [m, 12H, 2 × CH(CH$_3$)$_2$], 1.12 (t, 3H, *J* = 7.2 Hz, CH$_2$C*H*$_3$), 1.15−1.45, 1.55−1.90 [each m, each 1H, 2 × C*H*(CH$_3$)$_2$)], 2.23 (q, 2H, *J* = 7.2 Hz, C*H*$_2$CH$_3$), 4.45, 4.55 (each m, each 1H, 2 × α-H), 5.13, 5.23 (AB-system, 2H, *J* = 12.0 Hz, benzyl-H), 6.57, 7.02 (each br.d, each 1H, 2 × NH), 7.34 (s, 5H, arom. H); ^{13}C NMR ($CDCl_3$) δ 9.9 (CH$_2$*C*H$_3$), 17.6, 18.0, 18.9, 19.3 [2 × CH(*C*H$_3$)$_2$], 29.0 (*C*H$_2$CH$_3$), 30.9, 31.2 [2 × *C*H(CH$_3$)$_2$], 55.6, 57.4 (2 × α-C), 66.9 (benzyl-C), 128.2, 128.3, 128.5 (arom. CH), 135.3 (arom. C), 171.3, 171.8, 173.9 (3 × C=O); MS *m*/*z* 362.2 (M$^+$).

***N*-Isobutyryl-L-valyl-L-valine Benzyl Ester (7h).** Starting from 935 mg (5 mmol) of *N*-isobutyryl-L-valine (**6h**), 1.33 g (71%) of **7h** was obtained as colorless crystals: mp 137 °C; IR (KBr, cm^{-1}) 3285 (NH), 1735, 1640 (C=O); ^{1}H NMR ($CDCl_3$) δ 0.82−0.98, 1.08−1.20 [each m, 18H, 3 × CH(C*H*$_3$)$_2$], 1.40−1.65, 1.70−1.95 [each m, each 1H, 2 × C*H*(CH$_3$)$_2$], 2.17 [hept, 1H, *J* = 6.8 Hz, COC*H*(CH$_3$)$_2$], 3.96 (dd, 1H, *J* = 7.0 Hz, *J* = 8.5 Hz, α-H), 4.21 (dd, 1H, *J* = 5.0 Hz, *J* = 8.5 Hz, α-H), 4.84, 4.93 (AB-system, 2H, *J* = 12.4 Hz, benzyl-H), 6.84, 7.55 (each br.d, each 1H, 2 × NH), 7.35 (s, 5H, arom. H); ^{13}C NMR ($CDCl_3$) δ 17.4, 17.8, 18.0, 18.7, 19.0, 19.6 [3 × CH(*C*H$_3$)$_2$], 30.9, 31.2, 35.8 [3 × *C*H(CH$_3$)$_2$], 57.3, 58.0 (2 × α-C), 66.8 (benzyl-C),

128.2, 128.3, 128.4 (arom. CH), 135.2 (arom. C), 171.4, 172.0, 177.8 (3 × C═O); MS *m*/*z* 376.2 (M^+).

***N*-Cyclopropylcarbonyl-L-valyl-L-valine Benzyl Ester (7i).** Starting from 925 mg (5 mmol) of *N*-cyclopropylcarbonyl-L-valine (**6h**), 1.25 g (66%) of **7h** was obtained as colorless crystals: mp 158−160 °C; IR (KBr, cm^{-1}) 3290 (NH), 1735, 1635 (C═O); ^1H NMR ($CDCl_3$) δ 0.70−0.82, 0.83−1.00 [each m, 2H, 10H, 2 × $CH(CH_3)_2$, 2 × cyclopropyl-CH_2], 1.50−1.65 (m, 1H, cyclopropyl-CH), 1.90−2.30 [m, 2H, 2 × $CH(CH_3)_2$], 4.33 (dd, 1H, *J* = 4.8 Hz, *J* = 8 4 Hz, α-H), 4.57 (t, 1H, *J* = 8.8 Hz, α-H), 5.13, 5.22 (AB-system, 2H, *J* = 12.2 Hz, benzyl-H), 6.45, 6.60 (each br.d, each 1H, 2 × NH), 7.36 (s, 5H, arom. H); ^{13}C NMR ($CDCl_3$) δ 7.3 (2 × cyclopropyl-CH_2), 14.2 (cyclopropyl-CH), 17.5, 18.2, 18.8, 19.1 [2 × $CH(CH_3)_2$], 30.9 [2 × $CH(CH_3)_2$], 56.1, 57.2 (2 × α-C), 67.0 (benzyl-C), 128.3, 128.4, 128.5 (arom. CH), 135.2 (arom. C), 171.4, 173.6 (3 × C═O); MS *m*/*z* 374.2 (M^+).

General Procedure for the Preparation of Dipeptidic Acids 8. Dipeptide **7** (3 mmol) was dissolved in 20 mL of methanol and hydrogenated with Pd/C (80 mg, 10%) as catalyst overnight. After filtration the solution was concentrated in vacuo, redissolved in ethyl acetate, and extracted with aqueous NaOH solution (0.5 N, 2 × 10 mL). The combined aqueous layers were washed with ethyl acetate (1 × 10 mL), acidified with HCl (2 N) to pH 2, and extracted with ethyl acetate (2 × 10 mL). The solution was dried (Na_2SO_4) and evaporated under reduced pressure yielding dipeptidic acids **8** as colorless oils or solids, respectively.

***N*-Isopropoxycarbonyl-L-valyl-L-valine (8c).** Starting from 1.18 g (3 mmol) of **7c**, 830 mg (92%) of **8c** was obtained as colorless crystals: mp 150 °C (methanol); IR (KBr, cm^{-1}): 3315 (NH, OH), 1720, 1690, 1640 (C═O); ^1H NMR ($CDCl_3$) δ 0.95−1.02, 1.23−1.28 [each m, 18H, 3 × $CH(CH_3)_2$], 2.05−2.20, 2.22−2.35 [each m, each 1H, 2 × $CH(CH_3)_2$], 4.06, 4.59 [each dd, each 1H, *J* = 4.6 Hz, *J* = 8.6 Hz, 2 × α-H], 4.82−5.00 [m, 1H, $OCH(CH_3)_2$], 5.45, 6.80 (each br.d, each 1H, 2 × NH), 10.80 (br, 1H, COOH); ^{13}C NMR ($CDCl_3$) δ 18.0, 18.4, 19.3, 19.6, 21.8, 22.4 [3 × $CH(CH_3)_2$], 31.1, 31.4 [2 × $CH(CH_3)_2$], 50.5, 57.8 (2 × α-C), 69.4 [$OCH(CH_3)_2$], 172.2, 172.4 (3 × C═O): MS *m*/*z* 302.2 (M^+).

***N*-(3,3-Dimethylbutyryl)-L-valyl-L-valine (8d).** Starting from 1.17 g (3 mmol) of **7d**, 580 mg (64%) of **8d** was obtained as colorless crystals: mp 157−160 °C (methanol); IR (KBr, cm^{-1}) 3360 (NH, OH), 1710, 1660, 1620 (C═O); ^1H NMR ($CDCl_3$) δ 0.92−1.08 (m, 21H, 7 × CH_3), 2.08−2.35 [m, 4H, $COCH_2C(CH_3)_3$, 2 × $CH(CH_3)_2$], 4.40, 4.66 (each m, each 1H, α-H), 6.19, 7.36 (each br.d, each 1H, 2 × NH), 10.87 (br, 1H, COOH); ^{13}C NMR ($CDCl_3$) δ 17.5, 17.7, 19.1, 19.3 [2 × $CH(CH_3)_2$], 29.8 [$C(CH_3)_3$], 30.9 [$C(CH_3)_3$], 31.0 [2 × $CH(CH_3)_2$], 50.5 [$COCH_2C(CH_3)_3$], 57.0, 59.0 (2 × α-C), 172.4, 174.1, 175.0 (3 × C═O); MS *m*/*z* 314.2 (M^+).

***N*-(3-Phenylpropionyl)-L-valyl-L-valine (8e).** Starting from 1.31 g (3 mmol) of **7e**, 950 mg (91%) of **8e** was obtained as colorless crystals: mp 149−152 °C (methanol); IR (KBr, cm^{-1}) 3295 (NH, OH), 1710, 1665, 1630 (C═O); ^1H NMR (MeOD-d_3): δ 0.72−0.90 [m, 12H, 2 × $CH(CH_3)_2$], 1.80−1.95, 1.97−2.15 [each m, each 1H, 2 × $CH(CH_3)_2$], 2.82 (t, 2H, *J* = 7.4 Hz, CH_2), 2.40−2.55 (m, 2H, CH_2), 4.09 (dd, 1H, *J* = 5.5 Hz, *J* = 8.6 Hz, α-H), 4.21 (dd, 1H, *J* = 4.6 Hz, *J* = 7.8 Hz, α-H), 7.06−7.14 (m, 5H, arom. H), 7.87, 7.94 (each br.d, each 1H, *J* = 8.4 Hz, 2 × NH); ^{13}C NMR (MeOD-d_3) δ 18.3, 18.8, 19.5, 19.6 [2 × $CH(CH_3)_2$], 31.5, 31.7 [2 × $CH(CH_3)_2$], 32.8, 38.5 (2 × CH_2), 58.8, 60.1 (2 × α-C), 66.8 (benzyl-C), 127.1, 129.3 (arom. CH), 141.9 (arom. C), 173.8, 174.5, 175.0 (3 × C═O); MS *m*/*z* 348.3 (M^+).

***N*-Isovaleryl-L-valyl-L-valine (8f).** Starting from 1.17 g (3 mmol) of **7f**, 810 mg (90%) of **8f** was obtained as colorless crystals. Physical and spectroscopic data are described in ref 7.

***N*-Propionyl-L-valyl-L-valine (8g).** Starting from 1.09 g (3 mmol) of **7g**, 750 mg (91%) of **8g** was obtained as a colorless oil: IR (KBr, cm^{-1}) 3295 (NH, OH), 1735, 1635 (C═O); ^1H NMR (MeOD-d_3) δ 0.80−0.95 [m, 12H, 2 × $CH(CH_3)_2$], 1.03 (t, 3H, *J* = 7.5 Hz, CH_2CH_3), 1.80−2.05 [m, 2H, 2 × $CH(CH_3)_2$], 2.18 (q, 2H, *J* = 7.5 Hz, CH_2CH_3), 4.12 (dd, 1H, *J* = 4.8 Hz, *J* = 7.8 Hz, α-H), 4.22 (m, 1H, α-H), 7.83, 8.00 (each br.d, each 1H, 2 × NH); ^{13}C NMR (MeOD-d_3) δ 10.5 (CH_2CH_3), 18.3, 18.8, 19.5, 19.8 [2 × $CH(CH_3)_2$], 29.9 (CH_2CH_3), 31.6, 31.8 [2 × $CH(CH_3)_2$], 58.9, 59.0 (2 × α-C), 174.0, 174.6, 177.1 (3 × C═O); MS *m*/*z* 272.2 (M^+).

***N*-Isobutyryl-L-valyl-L-valine (8h).** Starting from 1.13 g (3 mmol) of **7h**, 805 mg (94%) of **8h** was obtained as colorless crystals: mp 148−151 °C (methanol); IR (KBr, cm^{-1}) 3420 (NH, OH), 1715, 1650, 1635 (C═O); ^1H NMR (MeOD-d_3): δ 0.95−1.08 [m, 12H, 2 × $CH(CH_3)_2$], 1.08−1.23 [m, 6H, $COCH(CH_3)_2$], 2.05, 2.20 [each m, each 1H, 2 × $CH(CH_3)_2$], 2.61 [hept, 1H, *J* = 7.4 Hz, $COCH(CH_3)_2$], 4.26 (dd, 1H, *J* = 3.2 Hz, *J* = 8.0 Hz, α-H), 4.37 (m, 1H, α-H), 7.92, 8.14 (each br.d, each 1H, 2 × NH); ^{13}C NMR (MeOD-d_3) δ 18.3, 18.9, 19.5, 19.6, 19.7, 20.2 [3 × $CH(CH_3)_2$], 31.7, 31.8 [2 × $CH(CH_3)_2$], 36.0 [$COCH(CH_3)_2$], 58.8, 59.0 (2 × α-C), 174.0, 174.5, 180.1 (3 × C═O); MS *m*/*z* 286.2 (M^+).

***N*-Cyclopropylcarbonyl-L-valyl-L-valine (8i).** Starting from 1.12 g (3 mmol) of **7i**, 1.25 g (66%) of **8i** was obtained as colorless crystals: mp 200−203 °C; IR (KBr, cm^{-1}) 3490 (NH, OH), 1705, 1660, 1640 (C═O); ^1H NMR (MeOD-d_3) δ 0.76−0.85 (m, 2H, cyclopropyl-CH_2), 0.90−1.08 [m, 14H, 2 × $CH(CH_3)_2$, cyclopropyl-CH_2], 1.68−1.83 (m, 1H, cyclopropyl-CH), 2.02−2.30 [m, 2H, 2 × $CH(CH_3)_2$], 4.28 (dd, 1H, *J* = 5.1 Hz, *J* = 7.8 Hz, α-H), 4.36 (m, 1H, α-H), 8.13, 8.18 (each br.d, each 1H, 2 × NH); ^{13}C NMR (MeOD-d_3): δ 7.4, 7.6 (2 × cyclopropyl-CH_2), 14.5 (cyclopropyl-CH), 18.3, 18.8, 19.5, 19.7 [2 × $CH(CH_3)_2$], 31.7, 32.0 [2 × $CH(CH_3)_2$], 58.9, 60.2 (2 × α-C), 174.0, 174.6, 176.5 (3 × C═O); MS *m*/*z* 284.2 (M^+).

General Procedure for Amide Formation Yielding Compounds 4. A mixture of amino alcohol **9** (1 mmol, 200 mg) (diastereoisomeric ratio: 4*S*,5*S*:4*S*,5*R* = 2.5:1)[5] and the selected dipeptidic acid **8** (1 mmol) in a solvent mixture of tetrahydrofuran and dichloromethane was treated with EEDQ (2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline) (1.2 mmol, 300 mg) and stirred at room temperature for 24 h. Then the solution was evaporated, redissolved in ethyl acetate, and washed with HCl (1 N, 2 × 10 mL), saturated aqueous $NaHCO_3$ solution (2 × 20 mL), and brine (2 × 20 mL). The organic phase was dried (Na_2SO_4) and evaporated. Flash chromatography of the residue (ethyl acetate−hexane, 1:1) afforded the pure products (R_f: 0.45−0.76).

4-(*N*-Benzyloxycarbonylamino-L-valyl-L-valylamino)-2-methylundecan-5-ol (4b). Starting from 350 mg (1 mmol) of Cbz-L-Val-L-Val-OH (**8b**), 395 mg (74%) of **4b** was obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3310 (NH, OH), 1715, 1650 (C═O); ^1H NMR ($CDCl_3$) δ 0.70−0.95 (m, 21H, 7 × CH_3), 1.05−1.60 [m, 13H, chain-CH_2, $CH_2CH(CH_3)_2$], 1.98, 2.10 [each m, each 1H, 2 × $CH(CH_3)_2$], 3.60, 3.95, 4.08, 4.15 (each m, each 1H, 2 × α-H, 4-H, 5-H), 5.05 (s, 2H, benzyl-H), 5.80, 7.03 (each br.d, each 1H, 2 × NH), 7.13 (s, 5H, arom. H); MS *m*/*z* 533.4 (M^+).

4-(*N*-Isopropoxycarbonylamino-L-valyl-L-valylamino)-2-methylundecan-5-ol (4c). Starting from 300 mg (1 mmol) of **8c**, 315 mg (65%) of **4c** were obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3305 (NH, OH), 1700, 1650 (C═O); MS *m*/*z* 485.4 (M^+).

4-[*N*-(3,3-Dimethylbutyryl)-L-valyl-L-valylamino]-2-methylundecan-5-ol (4d). Starting from 315 mg (1 mmol) of **8d**, 310 mg (62%) of **4d** was obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3295 (NH, OH), 1725, 1645 (C═O); ^1H NMR ($CDCl_3$) δ 0.70−0.90 (m, 21H, 7 × CH_3), 1.05−1.70 [m, 15H, chain-CH_2, $CH_2CH(CH_3)_2$], 1.25 [s, 9H, $C(CH_3)_3$], 2.00−2.20 [m, 2H, 2 × $CH(CH_3)_2$], 2.80 (br, 1H, OH), 3.58, 3.80, 3.95, 4.08 (each m, each 1H, 2 × α-H, 4-H, 5-H), 5.06, 6.28 (each br.d, each 1H, 2 × NH); MS *m*/*z* 497.4 (M^+).

4-[*N*-(3-Phenylpropionyl)-L-valyl-L-valylamino]-2-methylundecan-5-ol (4e). Starting from 350 mg (1 mmol) of **8e**, 355 mg (67%) of **4e** was obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3290 (NH, OH), 1715, 1640 (C═O); ^1H NMR ($CDCl_3$) δ 0.70−1.00 (m, 21H, 7 × CH_3), 1.05−1.65 [m, 13H, chain-CH_2, $CH_2CH(CH_3)_2$], 2.05, 2.20 [each m, each 1H, 2 × $CH(CH_3)_2$], 2.45−2.58, 2.80−2.95 [each m, each 2H,

PhCH_2CH_2CO], 3.60, 3.90, 4.10, 4.28 (each m, each 1H, 2 × α-H, 4-H, 5-H), 6.25, 6.80 (each br.d, each 1H, 2 × NH), 7.05−7.12 (m, 5H, arom. H); MS *m*/*z* 531.4 (M^+).

4-(*N*-Isovaleryl-L-valyl-L-valylamino)-2-methylundecan-5-ol (4f). Starting from 300 mg (1 mmol) of **8f**, 305 mg (63%) of **4f** was obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3290 (NH, OH), 1720, 1640 (C=O); ^1H NMR ($CDCl_3$ + MeOD-*d*₃): δ 0.78−1.05 (m, 21H, 7 × CH_3), 1.10−1.75 [m, 13H, chain-CH_2, $CH_2CH(CH_3)_2$], 2.00, 2.15 [each m, each 1H, 2 × $CH(CH_3)_2$], 3.60, 3.85, 4.05, 4.40 (each m, each 1H, 2 × α-H, 4-H, 5-H), 6.80, 7.00 (each br.d, each 1H, 2 × NH); MS *m*/*z* 483.4 (M^+).

4-(*N*-Propionyl-L-valyl-L-valylamino)-2-methylundecan-5-ol (4g). Starting from 270 mg (1 mmol) of **8g**, 305 mg (61%) of **4g** was obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3290 (NH, OH), 1735, 1645 (C=O); MS *m*/*z* 455.4 (M^+).

4-(*N*-Isobutyryl-L-valyl-L-valylamino)-2-methylundecan-5-ol (4h). Starting from 300 mg (1 mmol) of **8h**, 305 mg (63%) of **4h** was obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3290 (NH, OH), 1735, 1640 (C=O); MS *m*/*z* 469.4 (M^+).

4-(*N*-Cyclopropylcarbonyl-L-valyl-L-valylamino)-2-methylundecan-5-ol (4i). Starting from 300 mg (1 mmol) of **8i**, 305 mg (63%) of **4i** was obtained as a colorless oil: IR (KBr/liquid film, cm^{-1}) 3290 (NH, OH), 1735, 1640 (C=O); ^1H NMR ($CDCl_3$) δ 0.66−0.80 (m, 2H, cyclopropyl-CH_2), 0.80−1.05 (m, 23H, 7 × CH_3, cyclopropyl-CH_2), 1.15−1.75 (m, 14H, $CH_2CH(CH_3)_2$, CH_2-chain, cyclopropyl-CH), 1.95−2.20 [m, 2H, 2 × $CH(CH_3)_2$], 3.60, 3.95, 4.20, 4.35 (each m, each 1H, 2 × α-H, 4-H, 5-H), 5.80, 6.25 (each br.d, each 1H, 2 × NH); MS *m*/*z* 467.4 (M^+).

Evaluation of the Inhibitory Activity. The enzyme inhibitory activity of all compounds was determined in a slightly modified way as described previously by our research group.[5] Each test compound was dissolved in dimethyl sulfoxide (DMSO) in a final concentration of 1.00 mg/mL and diluted in 1:10 (v:v) steps with the same solvent; 100 μL of an appropriate dilution was transferred to the first well of a microtitration plate (96-well, not binding) and diluted in 1:2 (v:v) steps with DMSO in the following five vertical wells. To each 50 μL of diluted pepstatin analogue, 75 μL of a fresh prepared pepsin solution [10 μg of pepsin equivalent to 35 units (Sigma, St. Louis, MO) per mL of 0.05 N HCl] was added; 60 μg of horseradish peroxidase (Sigma, St. Louis, MO) in 75 μL of 0.05 N HCl was transferred to each well, and the reaction mixtures were incubated for 2 h at 37 °C. Thereafter, samples were diluted in 1:2 (v:v) steps with 0.05 N HCl in the following 11 horizontal wells. To each 100 μL of diluted reaction mixture, 100 μL of the substrate medium containing 24 mg of *o*-phenylenediamine dihydrochloride, 12 mL of 0.2 M phosphate buffer (pH 6.5), and 24 μL of 30% H_2O_2 was added, and the enzymatic reaction was allowed to proceed at room temperature for 15 min. Optical density was read at 492 nm with a microtitration plate reader. Reaction mixtures containing increasing pepstatin A (Sigma, St. Louis, MO) concentrations instead of test compounds were used as references. For negative and positive controls, the enzyme assay was carried out as described above, but omitting test compounds or test compounds as well as pepsin, respectively.

Statistical Data Analysis. Statistical data analysis was performed using the Student *t*-test with $p < 0.05$ as the minimal level of significance.

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