Design and Synthesis of Hydrazinopeptides and Their Evaluation as Human Leukocyte Elastase Inhibitors

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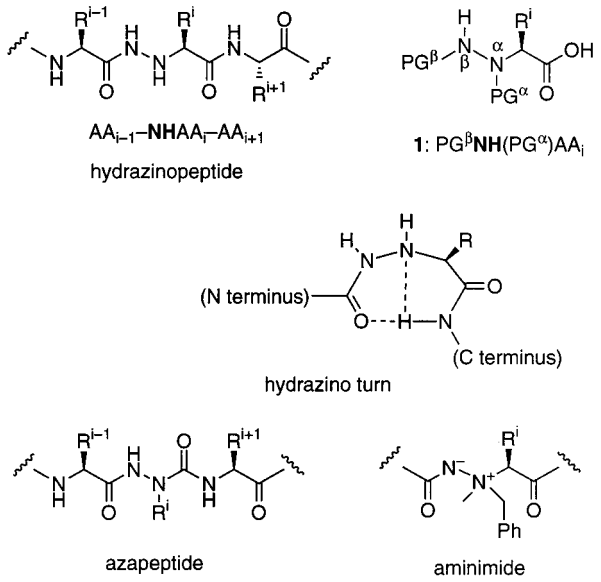
The name *hydrazinopeptide* designates peptidic structures in which one of the native CONH links is replaced by a CONHNH (hydrazido) fragment. In this paper, we report the synthesis of such hydrazinohexapeptides (**3**−**5**) analogous to Z-Ala-Ala-Pro-Val-Ala-Ala-NH^{i}Pr (**6**), a substrate of human leukocyte elastase (HLE; EC 3.4.21.37), cleaved by this serine protease between the Val4 and Ala5 residues. In hydrazinopeptides **3**−**5**, the Ala5, Val4, or Pro3 residue, respectively, of the model peptide, has been replaced by the corresponding α-L-hydrazino acid. In **3**, the bond likely to be cleaved by HLE is the one involving the CONHNH link, while in **4** and **5**, this link is normally shifted away by one or two amino acid units from the catalytic serine. On incubation with HLE, hydrazinopeptide **3** proved to be a substrate and was cleaved between Val4 and NHAla5, like peptide **6**. In contrast, **4** and **5** proved to bind to HLE without being cleaved, featuring properties consistent with reversible competitive inhibition. General guidelines for the synthesis of hydrazinopeptides are also reported in this paper. These guidelines take into account the chemical specificity of hydrazino acids, while being fully compatible with the conventional peptide coupling techniques. The utilization of orthogonally bisprotected hydrazino acids **1** where the N_β and N_α atoms bear a Boc and a Bzl group, respectively, is recommended for the easy construction of such hydrazinopeptides.

Introduction

Among the variety of pseudopeptide structures currently investigated in organic and medicinal chemistry,[1] little attention has been devoted to hydrazinopeptides, in which one of the native amino acids has been replaced by the corresponding α-hydrazino acid, to form a −CO−NH−NH−C* (hydrazide) link (Chart 1). This modification of the peptide bond was first introduced by Niedrich[2] in the mid-1960s for a series of eledoisin analogues, to increase the resistance toward proteolysis while keeping the physiological activity of the natural peptide unaffected. This goal has been met, at least partially, in these eledoisin mimics.[3] The subsequent lack of interest for this class of pseudopeptides is probably due to the difficulty of the synthesis of L- or D-α-hydrazino acids and to the absence of efficient methodologies for their insertion in peptide chains.

During the past decade, several methods allowing the preparation of L- or D-α-hydrazino acids **1** either free,[4] N_β-protected,[5,6] or N_β,N_α-orthogonally bisprotected[7] have been reported. This circumstance has renewed the interest for hydrazinopeptides in making these compounds more easily accessible. Short hydrazinopeptide models incorporating one hydrazino acid coupled with one or two amino acids have been synthesized. Structural studies (X-rays, NMR and IR spectroscopies, molecular modeling)[8−10] have revealed in these compounds a common conformational bias, called a hydrazino turn,[11] which folds the peptide backbone locally by way of a well-defined intramolecular bifurcated H-bonding pattern (see Chart 1).

Chart 1. Hydrazinopeptides and Related Pseudopeptides



We address here the problem of the development of hydrazinopeptidic inhibitors for serine proteases. Human leukocyte elastase (HLE; EC 3.4.21.37) which is involved in the pathogenesis of chronic inflammatory diseases such as pulmonary emphysema was chosen as

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Chart 2. Hydrazinopeptides **2**−**5** and the HLE Peptidic Substrate **6**[a]

2: Z-Ala–Ala–Pro–Val–//–**NH**Ile–Leu-OMe

3: Z-Ala–Ala–Pro–Val–//–**NH**Ala–Ala-NH^{i}Pr

4: Z-Ala–Ala–Pro–**NH**Val–Ala–Ala-NH^{i}Pr

5: Z-Ala–Ala–**NH**Pro–Val–Ala–Ala-NH^{i}Pr

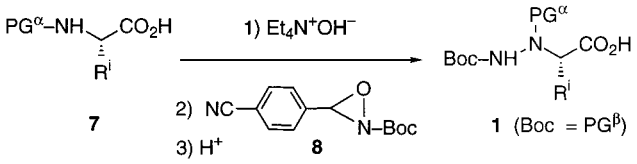
6: Z-Ala–Ala–Pro–Val–//–Ala–Ala-NH^{i}Pr

[a] The cleavage site of **2**, **3**, and **6** in the presence of HLE is indicated by -//-.

the target enzyme.[12−14] These diseases are associated with a functional or genetic deficiency of the major plasmatic inhibitor of HLE, α_1-PI, which normally regulates the activity of this enzyme. The replacement of the scissile bond of a peptidic substrate by a pseudopeptidic linkage that may resist proteolytic action has been a successful approach to the design of potent synthetic inhibitors for non-serine proteases such as HIV protease.[15] In contrast, little attention has been directed to the development of pseudopeptidic inhibitors for serine proteases such as HLE. The best-characterized pseudopeptidic inhibitors of HLE are the azapeptides[16] (Chart 1), in which a nitrogen atom replaces the α-carbon of an amino acid residue. Excellent titrants of the elastase active site have been obtained with azapeptides displaying a good leaving group.[17] Recently, a peptidomimetic aminimide (Chart 1) has been shown to inhibit porcine pancreatic elastase (PPE; EC 3.4.21.36), another serine protease closely related to HLE.[18] All these pseudopeptides contain a CO−N−N fragment like the hydrazinopeptides. In preliminary work we have shown that the hydrazinohexapeptide **2** (Chart 2) was a substrate of HLE and PPE and was cleaved by these enzymes in the same way as the corresponding hexapeptide.[19] This result prompted us to explore more systematically the interaction of HLE with tailored hydrazinopeptides. We report here the discovery of the first compounds of this class (**4** and **5**) which bind to HLE without being cleaved and feature properties consistent with reversible competitive inhibition.

Another general aspect of this work is the development of guidelines for the synthesis of hydrazinopeptides, which take into account the chemical specificity of hydrazino acids while being fully compatible with the conventional peptide coupling techniques. In particular, we wished to examine how the presence of a potentially nucleophilic N_α site may affect the C-coupling and N_β-coupling reactions of hydrazinoalanine (NHAla), hydrazinovaline (NHVal), hydrazinoisoleucine (NHIle), and hydrazinoproline (NHPro) with normal amino acids. Our conclusion is that the incorporation of α-hydrazino acids into pseudopeptidic chains is generally best accomplished by means of orthogonally bisprotected hydrazino acids **1**, where the N_β and N_α atoms bear a Boc and a Bzl protecting group, respectively. These hydrazino acid building blocks are themselves accessible in one step from the corresponding *N*-benzyl amino acids.[20] Thus further developments of this class of pseudopeptides, and more generally of bioactive structures containing hydrazino acids,[21] should no longer be restricted by synthetic issues.

Scheme 1. Preparation of N_β-Boc- and N_β-Boc,N_α-Bzl-L-α-hydrazino Acids



Product	R^i	PG$^\alpha$	yield
1a: Boc**NH**Ala	CH_3	H	50%
1b: Boc**NH**Val	$CH(CH_3)_2$	H	21%
1c: Boc**NH**Ile	$CH(CH_3)C_2H_5$	H	0%
1d: Boc**NH**Pro	$-CH_2CH_2CH_2-$		95%
1e: Boc**NH**(Bzl)Ala	CH_3	CH_2Ph	88%
1f: Boc**NH**(Bzl)Val	$CH(CH_3)_2$	CH_2Ph	69%
1g: Boc**NH**(Bzl)Ile	$CH(CH_3)C_2H_5$	CH_2Ph	75%

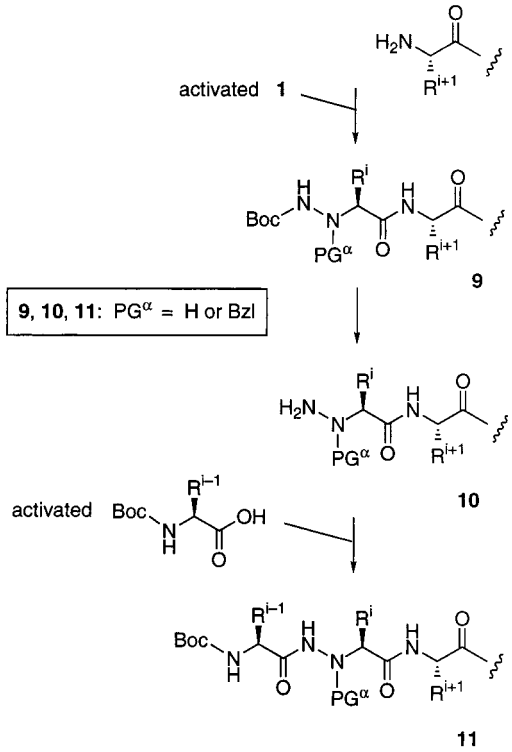
Results and Discussion

Design Principles. Peptide **6** was chosen as the model substrate because its Ala1-Ala2-Pro3-Val4 fragment appears to be one of the best known sequences for binding to the S_1-S_4 sites of HLE.[22] This sequence places the substrate in the active site in a way that promotes a selective cleavage of the peptide bond involving the Val4 carbonyl[23] (Chart 2). The Ala5-Ala6 fragment on the C-terminus side was preferred to the Ile5-Leu6 one used in our preliminary study (see **2**); the purpose of this change was to reduce the hydrophobicity, responsible for a poor solubility of the peptide in water, and to decrease the steric hindrance around the expected cleavage site. This last requirement is important if one wants to probe the sensitivity of the hydrazide link toward HLE hydrolysis. The choice of a CONH^{i}Pr end group instead of a CO_2Me group was made principally to avoid possible complications due to ester hydrolysis. Finally, a benzyloxycarbonyl group (Z) was fixed on the N-terminus side to serve as a chromophoric group for monitoring the HLE interaction experiments by HPLC. The nature of the N-terminus group seems to have little influence on the enzymatic hydrolysis parameters in the case of HLE and related elastases.[22] The model peptide **6** was prepared by conventional liquid-phase peptide coupling techniques.[24]

Hydrazinopeptides **3**−**5** were derived from structure **6** by replacing the Ala5, Val4, or Pro3 unit, respectively, by the corresponding hydrazino acid. In **3**, the bond likely to be cleaved by the protease is precisely the one involving the hydrazide link, while in **4** and **5** the hydrazide group is normally shifted away by one or two amino acid units from the catalytic serine, if the positioning of the three molecules in the enzyme is the same.

Hydrazino Acids. The L-hydrazino acid building blocks **1** required for the syntheses of **2**−**5** were prepared by reaction of the corresponding L-amino acids **7** (Et_4N^+ salts) with oxaziridine **8**, following our recently established methodology (Scheme 1).[7] The transfer of the N-Boc group from the oxaziridine to the nucleophilic amino acid nitrogen (Ala, Val, Ile, Pro) was generally

Scheme 2. Hydrazinopeptide Synthesis



fast (1 h at −15 °C in CH_2Cl_2). The reaction worked quite well with proline (**1d**: 95%) and with *N*-benzyl amino acids[25] (**7**: PG$^{\alpha}$ = Bzl) bearing a secondary amino group (**1e**,**f**,**g**: 88%, 69%, 75%). With unprotected amino acids (**7**: PG$^{\alpha}$ = H), the results were not as good. In fact, BocNHAla (**1a**) and BocNHVal (**1b**) could only be isolated in modest yield by this method, while no BocNHIle (**1c**) could be recovered. This is likely due to the fact that the 4-cyanobenzaldehyde released when oxaziridine **8** has transferred its N-Boc group can in turn react either with the starting material (**7**) to give the corresponding Schiff base or with the product (**1**) to give oxazolidinone heterocycles. These N$_{\beta}$-Boc-monoprotected derivatives **1a**−**c** could be easily obtained by catalytic hydrogenolysis of the *N*$_{\alpha}$-benzyl group of **1e**−**g** (H_2, 1 atm, EtOH, Pd/C). The reaction was quantitative, and no detectable cleavage of the hydrazide N−N bond occurred under these conditions.[26]

In the following sections we describe the construction of the hydrazinopeptidic chain of **2**−**5**. The strategy outlined in Scheme 2 is similar to that used in peptide synthesis (Boc methodology, liquid-phase synthesis), and we only focus here on the coupling steps involving the hydrazino acid units.

Coupling of N-Protected Hydrazino Acids to N-Free Peptide Derivatives. We have summarized in Table 1 some representative results on the activation of mono- or bis-N-protected hydrazino acids **1a**−**g** and on their coupling with appropriate precursors of **2**−**5** on the C-terminus side. The coupling of N$_{\alpha}$-unprotected derivatives **1a**,**c** to Ala-NH^{i}Pr and Leu-OMe was realized in modest yield in the presence of DCC and suitable additives (HOSu and HOBt) to give **9a**,**c** (entries 1 and 3). Despite its weak basicity (pK_b = 10.2 in H_2O for the related MocNHVal, Moc = methoxycarbonyl), the unprotected N$_{\alpha}$ is sufficiently nucleophilic to make the intermediate activated ester unstable (**12** in Scheme 3), leading to undesired products such as the diketopiperazine **13a** (FAB M + H = 373) and the mixture of related oligomers **13b** (n = 2−5, FAB M + Na = 599.2, 785.2, 971.4, 1157.9, respectively). This is why this coupling, in the presence of DCC, can only be successful when the activated ester is formed in situ; under these conditions, hindered hydrazino acids such as **1c** may give acceptable yields probably because the N$_{\alpha}$ is partially masked. The DCC activation worked reasonably well for the coupling of Boc-NHPro **1d**, where the N$_{\alpha}$ is tertiary. This is illustrated by the synthesis of **9d** (entry 4).

Mixed anhydrides prepared from ClCO$_2$^{i}Bu (^{i}BocCl) cannot be used for the activation of N$_{\alpha}$-unprotected hydrazino acids in hydrazinopeptide synthesis (e.g., see entry 2). Once the anhydride has been formed, its acyl group is immediately transferred to the N$_{\alpha}$ to give **14** (Scheme 3). The coupling can nevertheless be effected if 2 equiv of ^{i}BocCl is employed. This is of little practical interest, however, because the ^{i}Boc group fixed on the N$_{\alpha}$ is not as easy to remove as a conventional peptide protecting group.

Most of the above problems can be circumvented by using N$_{\beta}$-Boc,N$_{\alpha}$-Bzl-bisprotected hydrazino acids. Several activation methods were tested for the coupling of Boc-NH(Bzl)Ala (**1e**) to Ala-Ala-NH^{i}Pr to give **9e** (entries 5−8). The PyBop activation[27] was found to be the most efficient method here. The mixed anhydride activation also worked well, but a significant amount of unreacted starting material was always recovered. This is possibly due to a too short activation time (2 min at −15 °C); this question was not investigated further.

No detectable racemization of the N$_{\beta}$-Boc, N$_{\alpha}$-Bzl hydrazino acid units occurred under these coupling conditions. This was demonstrated by the fact that the diastereomer of **9e**, Boc-NH(Bzl)(D)Ala-(L)Ala-NH^{i}Pr, which was prepared separately to serve as a reference, was not detected by 500-MHz ^{1}H NMR in any of the coupling reactions listed in Table 1, leading to **9e**.

It was important to make sure that the N$_{\alpha}$-Bzl group in these hydrazinopeptide precursors could be removed by catalytic hydrogenolysis, without cleavage of the N−N bond. This was checked with **9f** which, on reaction with H_2 in EtOH (1 atm) in the presence of 10% Pd/C, at room temperature, was quantitatively converted to Boc-NHVal-Ala-Ala-NH^{i}Pr (**9b**).

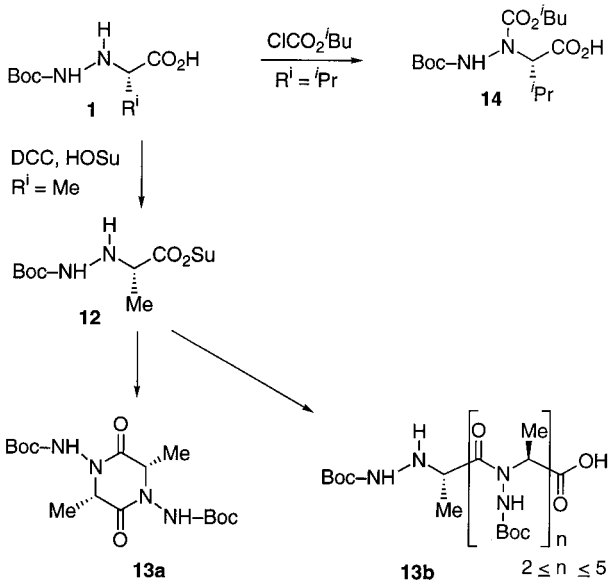
Formation of the Hydrazide Link. Experiments on the coupling of N-protected amino acids to the N$_{\beta}$-terminus of substrates **10** to give hydrazinopeptide precursors **11** are assembled in Table 2. Compounds **10a**−**e** were prepared from the corresponding **9a**−**e** by cleavage of the N$_{\beta}$-Boc under usual conditions (3 M HCl in ethyl acetate). It had been observed in previous works[2,3,8−11,19] that the coupling of completely deprotected hydrazines such as **10a**−**c** took place regioselectively on the N$_{\beta}$ when both the incoming amino acid and the hydrazino acid bear bulky side chains. The present results support this trend. In the cases we have investigated, fair coupling yields were obtained by using the mixed anhydride activation method (entries 3 and 4). This method allowed a short access to hydrazinopeptide **4**, which was obtained in 55% yield by coupling **10b** with the tripeptidic fragment Z-Ala-Ala-Pro. In contrast, the coupling of valine to **10a**, bearing

Table 1. Activation of the Carboxylic Group and Coupling of N-Protected Hydrazino Acids to Amino Derivatives

entry	hydrazino acid	**1**	activation	product	no.	yield (%)
1	Boc-**NH**Ala	**1a**	DCC/HOSu	Boc-**NH**Ala-Ala-NH^{i}Pr	**9a**	44
2	Boc-**NH**Val	**1b**	ClCO$_2{}^i$Bu	Boc-**NH**(^{t}Boc)Val	**14**	*a*
3	Boc-**NH**Ile	**1c**	DCC/HOBt	Boc-**NH**Ile-Leu-OMe	**9c**	67
4	Boc-**NH**Pro	**1d**	DCC/HOSu	Boc-**NH**Pro-Val-Ala-Ala-NH^{i}Pr	**9d**	75
5	Boc-**NH**(Bzl)Ala	**1e**	DCC/HOSu	Boc-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**9e**	46
6	Boc-**NH**(Bzl)Ala	**1e**	DCC/HOPfp	Boc-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**9e**	67
7	Boc-**NH**(Bzl)Ala	**1e**	ClCO$_2{}^i$Bu	Boc-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**9e**	64[b]
8	Boc-**NH**(Bzl)Ala	**1e**	PyBop	Boc-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**9e**	82
9	Boc-**NH**(Bzl)Val	**1f**	DCC/HOSu	Boc-**NH**(Bzl)Val-Ala-Ala-NH^{i}Pr	**9f**	60[c]
10	Boc-**NH**(Bzl)Ile	**1g**	ClCO$_2{}^i$Bu	Boc-**NH**(Bzl)Ile-Leu-OMe	**9g**	57[d]
11	Boc-**NH**(Bzl)Ile	**1g**	PyBop	Boc-**NH**(Bzl)Ile-Leu-OMe	**9g**	72

[a] ^{i}Boc, Isobutyloxycarbonyl; Viret, J. Thèse de Doctorat, Université Paris VI, 1988. [b] 26% of the starting material **1e** was recovered; the corrected yield is therefore 90%. [c] The diastereomeric Boc-**NH**(Bzl)(D)Ala-(L)AlaNH^{i}Pr was not detected by ^{1}H NMR (500 MHz). [d] 24% of the starting material **1g** was recovered; the corrected yield is 81%.

Scheme 3. Side Reactions in the Coupling of N_α-Unprotected Hydrazino Acids

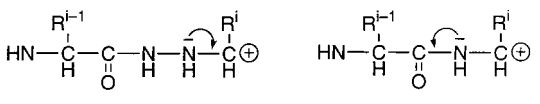


a NHAla terminus, proved to be less satisfactory (entry 1). To improve on this, we examined the utilization of the corresponding N_α-Bzl derivative **10e**, using Boc-Val as the acylating substrate (entries 6-9). Although the bulkiness of the benzyl group seems to make the N_β-acylation more difficult, the Boc-*N*-carboxyanhydride (UNCA) activation method[28] proved quite satisfactory (entry 9).

Hydrazinopeptides 2−5. The final coupling steps are summarized in Scheme 4. For **5**, a linear synthesis was followed throughout. At the last step, the coupling of Z-Ala-OSu with **15b** required the use of 2 equiv of Et_3N, because the acidolysis of the Boc group in **9b** left **15b** with its hydrazide N_α protonated. For **2**−**4**, as well as for the model hexapeptide **6**, a 3+3 segment synthesis was preferred. This choice, which significantly shortens the synthesis, was made because there is little racemization risk in a segment synthesis when the activated amino acid is a proline, such as in **16**.[29] Moreover, the bulkiness of the acylating segment **16** allowed the utilization of N_α-unprotected hydrazines (**10b**, **15a**,**c**) on the C-terminus side.[30] It is noteworthy that the hydrazinopeptides **2**−**4** were eventually isolated in better yields than the peptide **6**. This is not due to the chemistry but rather to the fact that **6** is difficult to purify because of its poor solubility and its tendency to form gels in organic solvents.

Characterization. Hydrazinopeptides **2**−**5** were purified by chromatography on silica gel and isolated as amorphous or microcrystalline solids. They were homogeneous by HPLC. The hydrazinopeptides proved to be much more soluble in water and organic solvents than the corresponding hexapeptides. They were fully characterized by high-field NMR (1D, COSY, and ROESY) and by mass spectroscopy (FAB and electrospray techniques). The ^{1}H NMR 500-MHz spectra of **2**−**5** all showed the presence of a minor species (<15%), in slow exchange with the major one. This was demonstrated by 2D experiments showing among other things the existence of correlations between the NH of the two forms. The most likely interpretation of this equilibrium is the presence of two conformational isomers associated with the Pro3 amide bond.[31] The ^{1}H NMR spectra of hydrazinopeptides **2**−**5** are in almost identical with those of the normal peptide, except for the hydrazino acid residues which exhibit the following specific features in DMSO-d_6 (Table 3): (i) the N_βH of the hydrazide link is shifted downfield (+1.1 to +1.5 ppm) with respect to the NH of a normal amide link; (ii) the N_αH is observed between 4 and 5.2 ppm; (iii) the C_αH is shifted upfield (−0.8 to −1.1 ppm) with respect to the corresponding amino acid. The FAB mass spectra of hydrazinopeptides **2**−**5** and **11a** showed prominent $(M + H)^+$ ions with little fragmentation. Among the weak fragment ions, the ion bearing the hydrazide link showed in general a remarkable intensity (10−20% of the molecular base peak in **2**, **3**, and **11a** to 100% in **5**), certainly due to its stabilization by the N_α nitrogen of the hydrazide link (Chart 3).

Chart 3. Possible Stabilization Mechanism of the Ion Bearing the Hydrazide Link (left) in Mass Spectroscopy



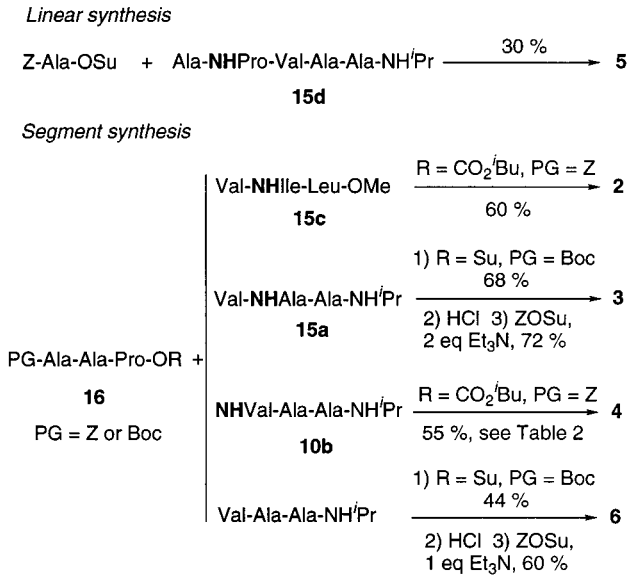
Interaction with HLE. To determine the potential of peptide **6** and hydrazinopeptides **3**−**5** to behave as substrates, compounds **3**−**6** were spectrophotometrically scanned between 220 and 400 nm in the presence of HLE or PPE at pH 8.0 and 25 °C. A significant time-dependent variation of the absorption spectrum was observed only in the case of hydrazinopeptide **3** and peptide **6**. This experiment suggested that **3** was enzymatically cleaved. The reaction mixtures were analyzed by HPLC with UV detection at 220 nm. The retention times (t_R) of **3**, **4**, **5**, and **6** were 14.6, 15.0,

Table 2. Formation of the Hydrazide Link

entry	activated substrate	N_β-free hydrazine (a)	**10**	hydrazinopeptide product	no.	yield (%)
1	Z-Val-OSu	**NH**Ala-Ala-NH^{i}Pr	**10a**	Z-Val-**NH**Ala-Ala-NH^{i}Pr	**11a**	40[b]
2	Boc-Ala-Ala-Pro-OSu	**NH**Val-Ala-Ala-NH^{i}Pr[c]	**10b**	Boc-Ala-Ala-Pro-**NH**Val-Ala-Ala-NH^{i}Pr	**11b**	25[d]
3	Z-Ala-Ala-Pro-OCO$_2{}^i$Bu	**NH**Val-Ala-Ala-NH^{i}Pr	**10b**	Z-Ala-Ala-Pro-**NH**Val-Ala-Ala-NH^{i}Pr	**4**	55
4	Z-Val-OCO$_2{}^i$Bu	**NH**Ile-Leu-OMe	**10c**	Z-Val-**NH**Ile-Leu-OMe	**11c**	60
5	Boc-Ala-OSu	**NH**Pro-Val-Ala-Ala-NH^{i}Pr	**10d**	Boc-Ala-**NH**Pro-Val-Ala-Ala-NH^{i}Pr	**11d**	42
6	Boc-Val-OSu	**NH**(Bzl)Ala-Ala-NH^{i}Pr	**10e**	Boc-Val-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**11e**	no reaction
7	Boc-Val-OCO$_2{}^i$Bu	**NH**(Bzl)Ala-Ala-NH^{i}Pr	**10e**	Boc-Val-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**11e**	22
8	Boc-Val/PyBop	**NH**(Bzl)Ala-Ala-NH^{i}Pr	**10e**	Boc-Val-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**11e**	28
9	Boc-Val-NCA	**NH**(Bzl)Ala-Ala-NH^{i}Pr	**10e**	Boc-Val-**NH**(Bzl)Ala-Ala-NH^{i}Pr	**11e**	60

[a] Obtained after cleavage of the Boc group in **9** (Table 1) by anhydrous HCl in AcOEt. [b] The coupling between Z-Val-OSu and Ala-Ala-NH^{i}Pr gave the corresponding tripeptide in 32% yield. [c] Hydrogenolysis of **9f** (H_2, EtOH, 1 atm, Pd/C) led to Boc-NHVal-Ala-Ala-NH^{i}Pr (**9b**) quantitatively. [d] The coupling between Boc-Ala-Ala-Pro-OSu and Val-Ala-Ala-NH^{i}Pr gave the corresponding hexapeptide in 44% yield.

Scheme 4. Final Steps for the Synthesis of Hydrazinopeptides **2**−**5** and Peptide **6**



14.5, and 15.4 min, respectively (20-min 0−100% acetonitrile gradient). In the presence of HLE or PPE, the peak areas corresponding to **3** and **6** decreased with the incubation time while a new peak (t_R = 13.7 min) appeared. The retention time of this peak was the same as that of peptide Z-Ala-Ala-Pro-Val. Amino acid analysis of the isolated fractions corresponding to the 13.7-min peak observed in the incubation of **3** and **6** yielded two alanyl, one prolyl, and one valyl residue. This means that hydrazinopeptide **3** and peptide **6** behave as substrates for both elastases and are cleaved by these enzymes after the valyl residue (i.e., between Val4 and Ala5, see Chart 2). In contrast, HPLC analyses did not reveal any significant change in the chromatograms of hydrazinopeptides **4** and **5** after a 4-h incubation with HLE or PPE, which indicated that neither **4** nor **5** was hydrolyzed by these enzymes under these conditions.

The kinetic parameters of the HLE-catalyzed hydrolysis of hydrazinopeptide **3** were derived from absorption data at 250 nm ($\Delta\epsilon$ = 629 M^{-1} cm^{-1} between **3** and the end products; 25 °C and pH 8.0). The kinetic parameters k_{cat} and K_m were calculated by iterative least-squares fits to the Michaelis equation (Table 4).[32] The catalytic efficiency (k_{cat}/K_m) of the HLE-catalyzed hydrolysis of peptide **6** was determined by HPLC at concentrations of **6** below K_m to allow linearization of the Michaelis equation: $v \approx k_{cat}/K_m \times ([HLE] \times [\mathbf{6}])$. The Michaelis constant K_m was then established by evaluating the competitive effect of **6** on the HLE amidolysis of the chromogenic substrate MeOSuc-Ala-Ala-Pro-Val-*p*NA;[33] k_{cat} was deduced from the values of k_{cat}/K_m and K_m (Table 4).

Although the replacement of the amide bond of **6** by a hydrazide bond in **3** does not prevent HLE hydrolysis, the catalytic efficiency is decreased by a factor of 2.2, as a result of a 5.4 times lower affinity of **3** compared to **6**. Both **6** and **3** behaved as normal substrates with no accumulation of acyl-enzyme, as confirmed experimentally, indicating that neither compound behaved as an acylating agent of HLE or PPE. This distinguishes hydrazinopeptides from azapeptides, which are efficient acylating agents of serine proteases.

Unlike **6** and **3**, hydrazinopeptides **4** and **5** did not behave as substrates of HLE and PPE. Their potential inhibitory properties toward HLE and PPE were studied using suitable chromogenic substrates (MeOSuc-Ala-Ala-Pro-Val-*p*NA and Suc-Ala-Ala-Ala-*p*NA, respectively). Both **4** and **5** acted as competitive inhibitors of HLE and PPE (Table 5). The total recovery of the enzyme activities after separation of the enzyme from the inhibitor by filtration indicated that the inhibitions were reversible. Hydrazinopeptide **5** was a better inhibitor than **4** toward both elastases and was more potent toward HLE.

It is noteworthy that the affinity of HLE for hydrazinopeptide **5** is 2-fold higher than its affinity for peptide **6** ($K_m(\mathbf{6})/K_i(\mathbf{5})$ = 2.0). The presence of the hydrazide bond in **5** therefore slightly enhances the binding to the enzyme active site with respect to the normal peptide (this is not true for **4**, however). The reason the Val4-Ala5 CONH bond is not cleaved in these compounds is not yet clear. Earlier X-ray, NMR, and IR studies on short models indicate that the hydrazinopeptide bond of **4** and **5** could form a hydrazino turn[8−11] (see Chart 1). This feature may modify the position of the Val4-Ala5 peptide bond within the enzyme active site in such a way as to make its hydrolysis more difficult. In short hydrazinopeptidic fragments, the existence of a hydrazino turn has been inferred from the observation that the ^{1}H NMR chemical shift of the CONH hydrogen of the i+1 amino acid linked to the (i)-hydrazino acid unit was almost insensitive to the solvent polarity (on going from $CDCl_3$ to DMSO-d_6).[11] This feature has been ascribed to the existence of the bifurcated H-bond sketched in Chart 1. Things are a little bit more complicated in the present hydrazinohexapeptides, which must be much more flexible than the previously investigated short models. Nevertheless, careful ^{1}H NMR

Table 3. Selected ^{1}H NMR Chemical Shifts for Hydrazinopeptides and Related Peptides (DMSO-d_6, 30 °C)[a]

			δ (ppm)		
entry	hydrazinopeptide or peptide	no.	$N_\alpha H$	$N_\beta H$ *or* NH	$C_\alpha H$
1	Boc-**NHAla-**Ala-NH^{i}Pr	**9a**	4.83	8.16	3.30
2	Boc-**NH(Bzl)Ala-**Ala-NH^{i}Pr	**9e**		7.96	3.46
3	Boc-**Ala-**Ala-NH^{i}Pr			7.00	3.91
4	Z-Val-**NHAla-**Ala-NH^{i}Pr	**11a**	5.21	9.36	3.13
5	Boc-Val-**NH(Bzl)Ala-**Ala-NH^{i}Pr	**11e**		8.98	3.50
6	Boc-Val-**Ala-**Ala-NH^{i}Pr			7.90	4.26
7	Z-Ala-Ala-Pro-Val-**NHAla-**Ala-NH^{i}Pr	**3**	5.17	9.30	3. 34
8	Z-Ala-Ala-Pro-Val-**Ala-**Ala-NH^{i}Pr	**6**		7.93	4.21
9	Boc-**NHVal-**Ala-Ala-NH^{i}Pr	**9b**	4.73	8.12	3.07
10	Boc-**NH(Bzl)Val-**Ala-Ala-NH^{i}Pr	**9f**		7.70	2.97
11	Boc-**Val-**Ala-Ala-NH^{i}Pr			6.71	3.77
12	Z-Ala-Ala-Pro-**NHVal-**Ala-Ala-NH^{i}Pr	**4**	4.03	9.16	2.99
13	Z-Ala-Ala-Pro-**Val-**Ala-Ala-NH^{i}Pr	**6**		7.70	4.07
14	Boc-**NHIle-**Leu-OMe	**9c**	4.70	8.01	3.18
15	Boc-**NH(Bzl)Ile-**Leu-OMe	**9g**		7.70	3.03
16	Boc-**Ile-**Leu-OMe			6.63	3.71
17	Z-Val-**NHIle-**Leu-OMe	**11c**	5.00	9.12	3.22
18	Boc-Val-**Ile-**Leu-OMe			8.30	4.24
19	Z-Ala-Ala-Pro-Val-**NHIle-**Leu-OMe	**2**	5.00	9.16	3.23
20	Z-Ala-Ala-Pro-Val-**Ile-**Leu-OMe			7.75	4.18
21	Boc-Ala-**NHPro-**Val-Ala-Ala-NH^{i}Pr	**11d**		9.33	3.44
22	Z-Ala-Ala-**NHPro-**Val-Ala-Ala-NH^{i}Pr	**5**		9.12	3.41
23	Z-Ala-Ala-**Pro-**Val-Ala-Ala-NH^{i}Pr	**6**			4.50

[a] The data correspond to the residues shown in bold.

Table 4. Kinetic Parameters for the Hydrolysis of Peptide **6** and Hydrazinopeptide **3** Catalyzed by HLE at 25 °C and pH 8.0[a]

compd	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
6	0.88 ± 0.14	131 ± 7	6776 ± 1016
3	2.24 ± 0.28	712 ± 171	3146 ± 865

[a] For chromatographic reasons, sodium chloride was omitted from the buffer used (0.05 M Hepes, 10% (v/v) DMSO).

Table 5. Inhibition Constants by Hydrazinopeptides **4** and **5** toward HLE and PPE at pH 8.0 and 25 °C (0.05 M Hepes, 10% (v/v) DMSO)

	K_i (μM)	
compd	HLE	PPE
4	595 ± 85	397 ± 44
5	65 ± 4	146 ± 5

studies on **3**−**6** as a function of the solvent polarity (Supporting Information) suggest that a hydrazino turn structuration may exist in **3** (cleaved by HLE) and **5** (not cleaved), but not in **4** (not cleaved). This suggests that the resistance of **4** and **5** toward enzymatic cleavage might have different origins.

Conclusion

In this study, we have described the synthesis of a series of hydrazinohexapeptides analogous to a HLE substrate. Two of these compounds behave as normal substrates, while the other two proved to be reversible inhibitors of this enzyme, at the micromolar level. Even though the mechanistic details of the enzyme−hydrazinopeptide interactions are still lacking, such a simple transformation of a HLE peptidic substrate into a protease-resisting inhibitor with increased affinity (at least for **5**) opens interesting prospects in the search for new classes of bioactive principles. Other families of proteases such as HIV proteases, for which potent azadipeptide inhibitors have recently been discovered,[34] are obvious targets for such studies. Moreover, as suggested by the pioneering works of Niedrich and Oehme, hydrazinopeptides may find applications in many other aspects of medicinal chemistry. Future works in this area will be facilitated by the availability of building units such as N_α-benzyl-N_β-Boc-protected L- and D-hydrazino acids and by the development of synthetic guidelines such as those described here, allowing their incorporation in peptide chains or in other potentially interesting structures.

Experimental Section

Chemistry. General Methods. Melting points were measured on a Perkin-Elmer DSC7 microcalorimeter, with simultaneous check of purity. Rotations were measured on a Perkin-Elmer 241 polarimeter at 25 °C using a 1-dm quartz cell. ^{1}H NMR spectra were recorded at 30 °C on a Bruker AC 200 (200 MHz) or a Varian Unity$^+$ (500 MHz) spectrometer using the solvent as internal standard (δ = 0 ppm for TMS); s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, dd = double doublet, br = broad peak. ^{1}H NMR peaks were assigned from 2D experiments at 500 MHz (COSY and ROESY sequences). FAB and electrospray mass spectra were obtained respectively on Fison ZAB VG and Hewlet Packard 5989A MS Engine mass spectrometers at the Service Central d'Analyses du CNRS (Vernaison, France), where the elemental analyses were also performed. Flash chromatography was performed on columns of silica gel 60 (Merck; 0.040−0.063 mm). Dimethoxyethane (DME) and *N*,*N*-dimethylformamide (DMF) were distilled over CaH_2 under atmospheric and reduced pressure, respectively. Tetrahydrofuran (THF) was dried and distilled over Na/benzophenone. Dry CH_2Cl_2 was obtained after filtration over basic alumina (activity I). Peptides (**6**, **16**, dipeptides or tripeptides described in Table 1) were prepared by conventional liquid-phase peptide coupling techniques (see details and characterization in Supporting Information) using *N*-Boc or *N*-Z-L-α-amino *N*′-hydroxysuccinimide esters (Boc-Ala-OSu, Z-Ala-OSu, Boc-Val-OSu, or Z-Val-OSu), purchased from Bachem (Switzerland). Z or Bzl protective groups were removed by atmospheric hydrogenolysis in methanol or 95% ethanol using 5% palladium on wet activated carbon (Degussa type E101). Boc protective group was removed by acidolysis with dry hydrogen chloride (3 M) in AcOEt (1 mL/mmol). N_β-Boc-N_α-Bzl-L-α-hydrazino acids **1a**−**f** were prepared according to ref 7.

L-*N*-Benzyl-*N*-(*tert*-butoxycarbonylamino)isoleucine (1g). The reaction[7] of *N*-benzyl-L-isoleucine (0.884 g, 4 mmol)

with oxaziridine **8** (1.03 g, 4.2 mmol) afforded 1.01 g (75%) of **1g** as a white solid: mp = 104 °C; $[\alpha]^{25}_D = +34.3$ (*c* 1, MeOH); ^{1}H NMR (200 MHz, $CDCl_3$) (two conformers in a 55:45 ratio) δ 0.83 (m, 3H, $CH_3\delta$), 0.92 (d, *J* = 7 Hz, 3H, $CH_3\gamma$), 1.25 (m, 1H, CHβ), 1.35 (s, 9H, Boc), 1.87 (m, 2H, $CH_2\gamma$), 3.21 (d, *J* = 6 Hz, 1H, CHα), 3.91 (m, 2H, CH_2N), 6.57 (0.55 H, NH), 7.04 (0.45 H, NH), 7.30 (m, 5H, Ph), 11.08 (br s, 1H, COOH). Anal. ($C_{14}H_{28}N_4O_4$) C, H, N.

Boc-NHAla-Ala-NH^{i}Pr (9a). General Procedure A, Using the DCC−HOSu Coupling Method. *N*-Hydroxysuccinimide (0.299 g, 2.6 mmol) and DCC (0.536 g, 2.6 mmol) were added to a cooled (5 °C) solution of BocNHAla (**1a**) (0.530 g, 2.6 mmol) in dry DME (5 mL). After stirring for 22 h at 5 °C and filtration, the filtrate was reacted with Ala-NH^{i}Pr (prepared by hydrogenolysis of 0.687 g of Z-Ala-NH^{i}Pr, 0.270 g, 2.6 mmol) for 18 h at room temperature. Filtration and washing of the solid by CH_2Cl_2 afforded 0.510 g of crude **9a**. A second crop of **9a** (0.749 g) was obtained after concentration of the filtrate under reduced pressure and washing of the solid by water. After dissolution in boiling AcOEt, filtration of insoluble impurities, and purification by flash chromatography [CH_2Cl_2/MeOH (98/2)], 0.365 g of pure **9a** was obtained (44%) as a white amorphous solid: $[\alpha]^{25}_D = -76.2$ (*c* 1.0, CH_2Cl_2); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.04 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.06 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.10 (d, *J* = 7.5 Hz, 3H, CH_3 NHAla), 1.19 (d, *J* = 5.5 Hz, 3H, CH_3 Ala), 3.35 (m, 1H, CHα NHAla), 3.81 (quint, *J* = 7.5 Hz, 1H, CH ^{i}Pr), 4.14 (m, 1H, CH Ala), 4.83 (br s, 1H, NHα NHAla), 7.54 (br s, 1H, NH ^{i}Pr), 7.85 (br s, 1H, NH Ala), 8.17 (br s, 1H, NHβ NHAla). Anal. ($C_{14}H_{28}N_4O_4\cdot 0.75H_2O$) C, H; N: calcd, 16.98; found, 16.40.

Boc-NHVal-Ala-Ala-NH^{i}Pr (9b). Compound **9b** was obtained by catalytic hydrogenation of **9f** (100%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.89 (d, *J* = 6.5 Hz, 3H, CH_3 NHVal), 0.91 (d, *J* = 6.5 Hz, 3H, CH_3 NHVal), 1.03 (d, *J* = 6.0 Hz, 3H, CH_3 ^{i}Pr), 1.04 (d, *J* = 6.0 Hz, 3H, CH_3 ^{i}Pr), 1.18 (d, *J* = 7.5 Hz, 3H, CH_3 Ala3), 1.23 (d, *J* = 7.5 Hz, 3H, CH_3 Ala1), 1.83 (m, 1H, CHβ NHVal), 3.08 (d, *J* = 5.5 Hz, 1H, CHα NHVal), 3.80 (o, *J* = 7.5 Hz, 1H, CH ^{i}Pr), 4.19 (m, 2H, CHα Ala2, CHα Ala3), 4.74 (br s, 1H, NHα NHVal), 7.58 (d, *J* = 7.5 Hz, 1H, NH ^{i}Pr), 7.71 (br s, 1H, NH Ala3), 7.94 (br s, 1H, NH Ala2), 8.12 (br s, 1H, NHβ NHVal).

Boc-NHPro-Val-Ala-Ala-NH^{i}Pr (9d). This compound was prepared by procedure A using HOSu (0.184 g, 1.8 mmol), DCC (0.371 g, 1.8 mmol), BocNHPro (0.414 g, 1.8 mmol) in DMF (1.8 mL) and then HCl Val-Ala-Ala-NH^{i}Pr (0.606 g, 1.8 mmol) and Et_3N (0.250 mL, 1.8 mmol). The mixture was stirred for 2 days at room temperature. The solvent was removed, and the crude material was precipitated in water and then filtered and recrystallized (DME) to afford 0.490 g of **9d**. Purification of the mother liquors by flash chromatography [CH_2Cl_2/MeOH (95/5)] afforded 0.210 g of **9d** as a white solid (75%): mp > 200 °C; $[\alpha]^{25}_D = -55.8$ (*c* 0.55, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.86 (d, *J* = 6.5 Hz, 6H, CH_3 Val), 1.01 (d, *J* = 6.0 Hz, 3H, CH_3 ^{i}Pr), 1.02 (d, *J* = 6.0 Hz, 6H, CH_3 ^{i}Pr), 1.14 (d, *J* = 7.0 Hz, H, CH_3 Ala3), 1.17 (d, *J* = 7.0 Hz, 3H, CH_3 Ala4), 1.37 (s, 9H, Boc), 1.58 (m, 1H, $CH_2\gamma$ NHPro), 1.73 (m, 1H, $CH_2\gamma$ NHPro), 2.09 (m, 2H, $CH_2\beta$ NHPro), 2.76 (m, 1H, $CH_2\delta$ NHPro), 3.14 (m, 1H, $CH_2\delta$ NHPro), 3.36 (dd, *J* = 10.0 Hz, *J* = 5.5 Hz, $CH_2\alpha$ NHPro), 3.77 (o, *J* = 6.5 Hz, 1H, CH ^{i}Pr), 4.03 (br s, 1H, CHα Val), 4.13 (quint, *J* = 7.0 Hz, 1H, CHα Ala3), 4.23 (quint, *J* = 7.0 Hz, 1H, CHα Ala4), 7.57 (d, *J* = 7.5 Hz, 1H, NH ^{i}Pr), 7.72 (d, *J* = 7.0 Hz, 1H, NH Ala3), 7.76 (br s, 1H, NH Ala4), 8.27 (br s, 1H, NH Val), 8.33 (br s, 1H, NHβ NHPro). Anal. ($C_{24}H_{44}N_6O_6$) C, H, N.

Boc-NH(Bzl)Ala-Ala-NH^{i}Pr (9e). This compound was prepared by procedure A using HOSu (0.391 g, 3.4 mmol), DCC (0.700 g, 3.4 mmol), Boc-NH(Bzl)Ala (**1e**) (0.999 g, 3.4 mmol), and then Ala-NH^{i}Pr (0.353 g, 3.4 mmol) in DME (2 mL). After stirring for 4 days at room temperature and removal of the solvent, the crude product was dissolved in CH_2Cl_2. The organic phase was washed three times with water, dried over Na_2SO_4, and concentrated in vacuo. Flash chromatography [CH_2Cl_2/Et_2O (8/2)] afforded 0.500 g (36%) of **9e** as a white amorphous solid: $[\alpha]^{25}_D = -21.1$ (*c* 1.0, CH_2Cl_2); ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.04 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.05 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.15 (d, *J* = 6.5 Hz, 3H, CH_3 Ala), 1.19 (d, *J* = 6.5 Hz, 3H, CH_3 NHAla), 1.24 (s, 9H, Boc), 3.46 (m, 1H, CHα NHAla), 3.80 (m, 3H, CH ^{i}Pr, CH_2Ph), 4.23 (m, 1H, CHα Ala), 7.24−7.43 (m, 5H, Ph), 7.64 (d, *J* = 6.5 Hz, 1H, NH ^{i}Pr), 7.96 (s, 1H, NHβ NHAla), 8.36 (br s, 1H, NH Ala). Anal. ($C_{21}H_{34}N_4O_4\cdot 0.75H_2O$) C, H, N.

Boc-NH(Bzl)Ala-Ala-NH^{i}Pr (9e). General Procedure B, Using the DCC−HOPfp Coupling Method. Compound **9e** was prepared by a procedure similar to A, using pentafluorophenol (HOPfp) (0.092 g, 0.5 mmol), DCC (0.103 g, 0.5 mmol), Boc-NH(Bzl)Ala (**1e**) (0.147 g, 0.5 mmol), and then Ala-NH^{i}Pr (0.052 g, 0.5 mmol) in DMF (0.6 mL). After removal of DMF and dissolution in CH_2Cl_2, the organic layer was washed by water, dried over Na_2SO_4, and concentrated in vacuo. Flash chromatography [CH_2Cl_2/Et_2O (1/1)] afforded 0.136 g (67%) of **9e**.

Boc-NH(Bzl)Ala-AlaNH^{i}Pr (9e). General Procedure C, Using the Mixed Anhydride Coupling Method. To Boc-NH(Bzl)Ala (**1e**) (0.151 g, 0.5 mmol) in dry THF (2.5 mL) under Ar at −20 °C were added Et_3N (0.069 mL, 0.5 mmol) and isobutyl chloroformate (0.065 mL, 0.5 mmol). After the mixture stirred for 5 min, a solution of Ala-NH^{i}Pr (0.052 g, 0.5 mmol) in dry THF (3 mL) was added. The mixture was allowed to warm and was stirred for 2 h at room temperature. After removal of THF and dissolution in CH_2Cl_2, the organic layer was washed twice with water, dried over Na_2SO_4, concentrated in vacuo, and then flash chromatographed [CH_2Cl_2/MeOH (95/5)], affording 0.133 g (64%) of pure **9e** and 0.039 g (26%) of starting material **1e** (corrected yield: 90%).

Boc-NH(Bzl)Ala-Ala-NH^{i}Pr (9e). General Procedure D, Using the PyBop Coupling Method. To a cooled (0 °C) solution of Ala-NH^{i}Pr (0.0965 g, 0.74 mmol) and Boc-NH(Bzl)-Ala (**1e**) (0.212 g, 0.72 mmol) in dry CH_2Cl_2 (1 mL) were added PyBop (0.388 g, 0.74 mmol) and Et_3N (0.205 mL, 1.48 mmol). The mixture was allowed to warm and was stirred for 18 h at room temperature. After evaporation of the solvent, the crude material was purified by flash chromatography [CH_2Cl_2/MeOH (97/3)] to yield 0.239 g (82%) of **9e**.

Boc-NH(Bzl)(D)Ala-(L)Ala-NH^{i}Pr. This compound was prepared by procedure D from Boc-NH(Bzl)(D)Ala (0.0227 g, 0.081 mmol) and Ala-NH^{i}Pr (0.0105 g) in 64% (0.020 g) yield: ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.04 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.05 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.18 (d, *J* = 6.5 Hz, 3H, CH_3 Ala), 1.20 (d, *J* = 6.5 Hz, 3H, CH_3 NHAla), 1.24 (s, 9H, Boc), 3.45 (m, 1H, CHα NHAla), 3.81 (m, 1H, CH ^{i}Pr), 3.74 and 3.88 (AB system, J_{AB} = 13 Hz, 2H, CH_2Ph), 4.18 (m, 1H, CHα Ala), 7.24−7.39 (m, 5H, Ph), 7.57 (d, *J* = 6.5 Hz, 1H, NH ^{i}Pr), 7.96 (s, 1H, NHβ NHAla), 8.20 (br s, 1H, NH Ala).

Boc-NH(Bzl)Val-Ala-Ala-NH^{i}Pr (9f). This compound was prepared by procedure A using HOSu (0.230 g, 2 mmol), DCC (0.412 g, 2 mmol), Boc-NH(Bzl)Ala (**1e**) (0.588 g, 2 mmol), and then HCl Ala-Ala-NH^{i}Pr (0.475 g, 2 mmol) and Et_3N (0.273 mL, 2 mmol). The mixture was stirred for 3 days at room temperature. The solvent was removed and the crude material was precipitated in water and then filtered and washed with Et_2O and pentane. Recrystallization (acetone) afforded 0.605 g of **9f** (60%) as a hygroscopic solid: $[\alpha]^{25}_D = -18.3$ (*c* 1.0, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.83 (d, *J* = 6.5 Hz, 3H, CH_3 NHVal), 1.04 (m, 3H, CH_3 Val, CH_3 ^{i}Pr), 1.18 (d, *J* = 10.0 Hz, 3H, CH_3 Ala3), 1.26 (m, 12H, CH_3 Ala2, Boc), 1.90 (m, 1H, CHβ NHVal), 2.97 (d, *J* = 9.5 Hz, 1H, CHα NHVal), 3.81 (m, 3H, CH ^{i}Pr, CH_2Ph), 4.18 (m, 1H, CHα Ala3), 4.36 (m, 1H, CHα Ala2), 7.20−7.42 (m, 5H, Ph), 7.65 (d, *J* = 6.5 Hz, 1H, CH ^{i}Pr), 7.70 (br s, 1H, NHβ NHVal), 7.81 (br s, 1H, NH Ala3), 8.29 (br s, 1H, NH Ala2). Anal. ($C_{26}H_{43}N_5O_5\cdot 0.5H_2O$) C, H, N.

Boc-NH(Bzl)Ile-Leu-OMe (9g). This compound was prepared by procedure C starting from Boc-NH(Bzl)Ile (**1g**) (0.291 g, 0.86 mmol), *N*-methylmorpholine instead of Et_3N (0.095 mL, 0.86 mmol), and HCl LeuOMe (0.157 mg, 0.86 mmol) dissolved in dry DMF (2 mL). After removal of THF and dissolution in CH_2Cl_2, the organic layer was washed with water, aqueous

5% $KHSO_4$, and aqueous 5% $KHCO_3$. Purification by flash chromatography [CH_2Cl_2/MeOH (98/2)] afforded 0.230 g of **9g** (57%) followed by 0.070 g (24%) of the starting material **1g** (corrected yield: 81%).

Compound **9g** was also prepared by procedure D using Boc-NH(Bzl)Ile (**1g**) (0.084 g, 0.25 mmol), HCl LeuOMe (0.545 g, 0.30 mmol), PyBop (0.130 g, 0.25 mmol), and Et_3N (0.105 mL, 0.75 mmol) in CH_2Cl_2 (0.5 mL). Flash chromatography [CH_2Cl_2/MeOH (98/2)] afforded 0.083 g of **9g** (72%): mp = 74 °C; $[\alpha]^{25}_D$ +13.9 (*c* 1.0, MeOH); ^{1}H NMR (200 MHz, DMSO-d_6) δ 0.76−0.93 (m, 12H), 1.19−1.28 (m, 10H), 1.45−1.91 (m, 5H), 3.03 (d, *J* = 8.7 Hz, 1H, CHα NHIle), 3.61 (s, 3H), 3.77 (br s, 2H), 4.35 (br s, 1H, CHα Leu), 7.06−7.32 (m, 5H, Ph), 7.70 (br s, 1H, NHBoc), 8.57 (d, *J* = 6.5 Hz, 1H, NH). Anal. ($C_{25}H_{41}N_3O_5$) C, H, N.

Z-Val-NHAla-Ala-NH^{i}Pr (11a). General Procedure E, for Acylation with Activated Esters. A mixture of Z-Val-OSu (0.392 g, 1.02 mmol), HCl NHAla-Ala-NH^{i}Pr (**10a**) (0.257 g, 1.02 mmol), and Et_3N (0.286 mL, 2.04 mmol) in dry DMF (2 mL) was stirred for 4 days at room temperature. After evaporation of the solvent, the crude product was precipitated by addition of water, filtered, and washed several times with pentane. Extraction of the aqueous phase with CH_2Cl_2 gave another crop of crude **11a**. Recrystallization (^{i}PrOH) yielded 0.183 g (40%) of **11a** as a white solid: dec at 190 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.81 (d, *J* = 12.5 Hz, 3H, CH_3 Val), 0.82 (d, *J* = 12.5 Hz, 3H, CH_3 Val), 1.03 (d, *J* = 11.0 Hz, 3H, CH_3 ^{i}Pr), 1.05 (d, *J* = 11.0 Hz, 3H, CH_3 ^{i}Pr), 1.12 (d, *J* = 7.0 Hz, 3H, CH_3 NHAla), 1.18 (d, *J* = 7.0 Hz, 3H, CH_3 Ala), 1.88 (m, 1H, CHβ Val), 3.37 (m, 1H, CHα NHAla), 3.75 (m, 1H, CHα Val), 3.81 (m, 1H, CH ^{i}Pr), 4.16 (m, 1H, CHα Ala), 5.01 (s, 2H, CH_2Ph), 5.21 (m, 1H, NHα NHAla), 7.29 (m, 1H, NH Val), 7.35 (br s, 5H, Ph), 7.65 (d, *J* = 7.5 Hz, 1H, NH ^{i}Pr), 7.92 (m, 1H, NH Ala), 9.37 (d, *J* = 5.0 Hz, 1H, NHβ NHAla). Anal. ($C_{22}H_{35}N_5O_5$) C, H, N.

Boc-Ala-Ala-Pro-NHVal-Ala-Ala-NH^{i}Pr (11b). This compound was prepared by procedure E using Boc-Ala-Ala-Pro-OSu (0.227 g, 0.5 mmol), HCl NHVal-Ala-Ala-NH^{i}Pr (**10b**) (1.756 g, 0.5 mmol), and Et_3N (0.069 mL, 0.5 mmol) in DMF (1.5 mL). The mixture was stirred for 6 days. Purification by flash chromatography [CH_2Cl_2/MeOH (95/5)] afforded 0.083 g of **11b** (25%): ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 86:14 ratio) δ 0.87 (d, *J* = 7.0 Hz, 3H, CH_3 Val), 0.90 (d, *J* = 7.0 Hz, 3H, CH_3 Val), 1.01 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.02 (d, *J* = 6.5 Hz, 3H, CH_3 ^{i}Pr), 1.15 (m, 9H, CH_3 Ala1, CH_3 Ala2, CH_3 Ala6), 1.22 (d, *J* = 7.0 Hz, 3H, CH_3 Ala5), 1.36 (m, 9H, CH_3 Boc), 1.70−2.00 (m, 5H, $CH_2\beta$ Pro, $CH_2\gamma$ Pro, CHβ NHVal), 2.91 (br s, 1H, CHα NHVal), 3.52 (m, 2H, $CH_2\delta$ Pro), 3.78 (m, 1H, CH ^{i}Pr), 3.94 (m, 1H, CHα Ala1), 4.27−4.34 (m, 3H, CHα Pro, CHα NHVal, CHα Ala6), 4.51 (m, 1H, CHα Ala2), 5.02 (m, 1H, NHα NHVal), 6.86 (d, *J* = 7.0 Hz, 1H, NH Ala1), 7.62 (d, *J* = 7.5 Hz, 1H, NH ^{i}Pr), 7.78 (d, *J* = 7.5 Hz, 1H, NH Ala6), 7.83 (d, *J* = 7.5 Hz, 1H, NH Ala2), 7.96 (d, *J* = 7.0 Hz, NH Ala5), 9.17 (d, *J* = 5.5 Hz, NHβ NHVal).

Z-Ala-Ala-Pro-NHVal-Ala-Ala-NH^{i}Pr (4). This compound was prepared by procedure C using Z-Ala-Ala-Pro-OH (0.128 g, 0.3 mmol, obtained after treatment of Z-Ala-Ala-Pro-O^{t}Bu by TFA in CH_2Cl_2) in THF (4.5 mL) and a solution of HCl NHVal-Ala-Ala-NH^{i}Pr (**10b**) (0.105 g, 0.3 mmol) and Et_3N (0.041 mL, 0.3 mmol) in DMF (1.3 mL). The mixture was stirred for 18 h at 5 °C. After concentration in vacuo and dissolution in CH_2Cl_2, the organic layer was washed twice by water, dried over Na_2SO_4, and concentrated in vacuo. Flash chromatography [CH_2Cl_2/MeOH (95/5)] afforded 0.115 g of **4** (55%): dec at 130 °C; $[\alpha]^{25}_D$ −124.9 (*c* 1.0, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 86:14 ratio) δ 0.86 (d, *J* = 6.5 Hz, 3H, CH_3 NHVal), 0.89 (d, *J* = 6.5 Hz, 3H, CH_3 NHVal), 1.00 (d, *J* = 7.0 Hz, 3H, CH_3 ^{i}Pr), 1.01 (d, *J* = 7.0 Hz, 3H, CH_3 ^{i}Pr), 1.15 (m, 9H, CH_3 Ala1, CH_3 Ala2, CH_3 Ala6), 1.20 (d, *J* = 7.0 Hz, 3H, CH_3 Ala5), 1.65−2.00 (m, 5H, $CH_2\beta$ Pro, $CH_2\gamma$ Pro, CHβ NHVal), 2.99 (m, 1H, CHα NHVal), 3.52 (m, 2H, CHδ Pro), 3.76 (m, 1H, CH ^{i}Pr), 4.03 (m, 1H, CHα Ala1), 4.20 (m, 3H, CHα Pro, CHα Ala5, CHα Ala6), 4.47 (m, 1H, CHα Ala2), 4.91 (m, 3H, CH_2Ph, NHα NHVal), 7.32 (m, 6H, Ph, NH Ala1), 7.61 (d, *J* = 7.5 Hz, 1H, NH ^{i}Pr), 7.77 (d, *J* = 7.5 Hz, 1H, NH Ala6), 7.94 (d, *J* = 7.5 Hz, 2H, NH Ala2, NH Ala5), 9.16 (d, *J* = 5.5 Hz, 1H, NHβ NHVal); MW $C_{33}H_{52}N_8O_8$ calcd 689.3986 (M + H), found 689.3998 (M + H, HRFABMS). Anal. ($C_{33}H_{52}N_8O_8\cdot H_2O$) C, H, N.

Boc-Ala-NHPro-Val-Ala-Ala-NH^{i}Pr (11d). This compound was prepared by procedure E using Boc-Ala-OSu (0.360 g, 1.6 mmol), HCl NHPro-Val-Ala-Ala-NH^{i}Pr (**10e**) (0.564 g, 1.26 mmol), and Et_3N (0.351 mL, 2.52 mmol) in DMF (2 mL). After the mixture stirred for 2 days, the solvent was concentrated in vacuo and **11d** was precipitated by addition of water. Recrystallization (^{i}PrOH) gave 0.313 g (42%) of **11d** as a white solid: dec > 240 °C; ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers) δ 0.84 (m, 6H, CH_3 Val), 1.01 (d, *J* = 5.5 Hz, 3H, CH_3 ^{i}Pr), 1.03 (d, *J* = 5.5 Hz, 3H, CH_3 ^{i}Pr), 1.14−1.21 (m, 9H, CH_3 Ala1, CH_3 Ala4, CH_3 Ala5), 1.61 (m, 1H, $CH_2\gamma$ Pro), 1.77 (m, 2H, $CH_2\gamma$ Pro, $CH_2\beta$ Pro), 2.14 (m, 2H, $CH_2\beta$ Pro, CHβ Val), 2.87 (m, 1H, $CH_2\delta$ Pro), 3.14 (m, 1H, $CH_2\delta$ Pro), 3.44 (m, 1H, CHα Pro), 3.78 (m, 1H, CH ^{i}Pr), 3.89 (m, 1H, CHα Ala1), 4.08 (m, 1H, CHα Val), 4.14 (m, 1H, CHα Ala5), 4.23 (m, 1H, CHα Ala4), 6.80 (br s, 1H, NH Ala1), 7.50 (d, *J* = 7.5 Hz, 1H, NH ^{i}Pr), 7.72 (m, 2H, NH Ala4, NH Ala5), 8.60 (d, *J* = 8.5 Hz, 1H, NH Val), 9.33 (s, NHβ Pro). Anal. ($C_{33}H_{52}N_8O_8\cdot H_2O$) C, H, N.

Boc-Val-NH(Bzl)Ala-Ala-NH^{i}Pr (11e). To a solution of Boc-Val-NCA (0.100 g, 0.46 mmol) in dry THF (1 mL) under Ar and at room temperature were added a solution of HCl NH-(Bzl)Ala-Ala-NH^{i}Pr (**10e**) (0.134 g, 0.46 mmol) in dry THF (5 mL) and then Et_3N (0.064 mL, 0.46 mmol). The mixture was stirred for 2 h at room temperature. After evaporation of the solvent and dilution with CH_2Cl_2, the organic layer was washed with aqueous 5% $KHCO_3$. Purification by flash chromatography [CH_2Cl_2/MeOH (95/5)] afforded 0.140 g (60%) of pure **11e**.

This compound was also prepared by procedure C using Boc-Val (0.115 g, 0.53 mmol) and HCl NH(Bzl)Ala-Ala-NH^{i}Pr (**10e**) (0.151 g, 0.44 mmol). After stirring for 19 h at 5 °C, concentration in vacuo, and dissolution in CH_2Cl_2, the organic layer was washed twice with water, dried over Na_2SO_4, and concentrated in vacuo. Flash chromatography [CH_2Cl_2/MeOH (95/5)] afforded 0.050 g (22%) of pure **11e**.

This compound was also prepared by procedure D using Boc-Val (0.0212 g, 0.097 mmol) and HCl NH(Bzl)Ala-Ala-NH^{i}Pr (**10e**) (from 0.0378 g **9e**, 0.093 mmol). After stirring for 36 h at room temperature and diluting with CH_2Cl_2, the organic layer was washed by aqueous 5% citric acid, aqueous 5% $KHCO_3$, and water. Purification by flash chromatography [CH_2Cl_2/MeOH (98/2)] afforded 0.013 g (28%) of pure **11e**: dec at 110 °C; $[\alpha]^{25}_D$ +1.3 (*c* 0.75, CH_2Cl_2); ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.61 (d, *J* = 7.0 Hz, 3H, CH_3 Val), 0.70 (d, *J* = 7.0 Hz, 3H, CH_3 Val), 1.05 (d, *J* = 7.5 Hz, 3H, CH_3 ^{i}Pr), 1.06 (d, *J* = 7.5 Hz, 3H, CH_3 ^{i}Pr), 1.18 (d, *J* = 6.0 Hz, 3H, CH_3 Ala), 1.21 (d, *J* = 5.5 Hz, 3H, CH_3 NHAla), 1.37 (s, 9H, Boc), 1.70 (o, *J* = 6.0 Hz, 1H, CHβ Val), 3.50 (q, *J* = 7.5 Hz, 1H, CHα NHAla), 3.59 (t, *J* = 9.0 Hz, 1H, CHα Val), 3.82 (o, *J* = 7.5 Hz, 1H, CH ^{i}Pr), 3.89 (s, 2H, CH_2Ph), 4.21 (quint, *J* = 7.0 Hz, 1H, CHα Ala), 6.57 (d, *J* = 9.0 Hz, 1H, NH Val), 7.21−7.32 (m, 4H, Ph), 7.42 (d, *J* = 9.0 Hz, 1H, Ph), 7.66 (d, *J* = 7.0 Hz, 1H, NH ^{i}Pr), 8.45 (br s, 1H, NH Ala), 8.98 (s, 1H, NHβ NHAla). Anal. ($C_{26}H_{43}N_5O_5\cdot H_2O$) C, H, N.

Z-Ala-Ala-NHPro-Val-Ala-Ala-NH^{i}Pr (5). This compound was prepared by procedure E using Z-Ala-OSu (0.170 g, 0.45 mmol) and HCl Ala-NHPro-Val-Ala-Ala-NH^{i}Pr (0.275 g, 0.45 mmol). After the mixture stirred for 60 h at room temperature, the solvent was removed and the precipitate washed with hot acetone. Flash chromatography [CH_2Cl_2/MeOH (95/5)] gave 0.120 g (30%) of **5**: ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 91:9 ratio) δ 0.83 (m, 6H, CH_3 Val), 0.99 (m, 6H, CH_3 ^{i}Pr), 1.20−1.08 (m, 12H, CH_3 Ala1, CH_3 Ala2, CH_3 Ala5, CH_3 Ala6), 1.60 (m, 1H, CHα NHPro), 1.74 (m, 1H, $CH_2\beta$ NHPro), 1.79 (m, 2H, $CH_2\gamma$ NHPro), 2.08 (m, 1H, CHβ Val), 2.15 (m, 1H, $CH_2\beta$ NHPro), 2.81 (d, *J* = 8.0 Hz, 1H, $CH_2\delta$ NHPro), 3.13 (m, 1H, $CH_2\delta$ NHPro), 3.41 (m, 1H, CHα NHPro), 3.75 (o, *J* = 6.5 Hz, 1H, CH ^{i}Pr), 4.03 (m, 2H, CHα Ala1, CHα

Val), 4.11 (quint, $J = 6.5$ Hz, 1H, CHα Ala6), 4.18 (quint, $J = 7.0$ Hz, 1H, CHα Ala2), 4.27 (m, 1H, CHα Ala5), 4.93 (d, $J_{AB} = 12.5$ Hz, 1H, NH Ala1), 5.03 (d, $J_{AB} = 12.5$ Hz, 2H, CH_2Ph), 7.32 (m, 5H, Ph), 7.45 (d, $J = 7.0$ Hz, 1H, NH Ala1), 7.51 (d, $J = 7.0$ Hz, 1H, NH ^{i}Pr), 7.72 (d, $J = 7.5$ Hz, 1H, NH Ala5), 7.84 (d, $J = 7.5$ Hz, 1H, NH Ala6), 7.98 (d, $J = 7.5$ Hz, 1H, NH Ala2), 8.49 (d, $J = 8.0$ Hz, 1H, NH Val), 9.12 (s, 1H, NHβ NHPro); MW $C_{33}H_{52}N_8O_8$ calcd 689.3986 (M + H), found 689.3985 (M + H, HRFABMS). Anal. ($C_{33}H_{52}N_8O_8 \cdot 0.5H_2O$) C, H, N.

Z-Ala-Ala-Pro-Val-NHIle-Leu-OMe (2). This compound was prepared by procedure C using Z-Ala-Ala-Pro-OH (0.074 g, 0.19 mmol, obtained after treatment of Z-Ala-Ala-Pro-O^{t}Bu by TFA in CH_2Cl_2), HCl Val-NHIle-Leu-OMe (0.074 g, 0.19 mmol), and *N*-methylmorpholine (0.022 mL, 0.2 mmol). After the mixture stirred for 18 h, the precipitated *N*-methylmorpholine hydrochloride was filtered off. THF was concentrated in vacuo, and the residue was dissolved in CH_2Cl_2. This organic layer was washed with aqueous 5% $KHSO_4$, aqueous 5% $KHCO_3$, and water, dried over Na_2SO_4, and concentrated. Flash chromatography [CH_2Cl_2/MeOH (98/2)] afforded 0.081 g (60%) of pure **2** as a white solid: $[\alpha]^{25}_D$ −136.3 (*c* 0.65, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 84:16 ratio) δ 0.79 (d, $J = 6.5$ Hz, 3H, CH_3 Val), 0.80 (d, $J = 6.5$ Hz, 3H, CH_3 Val), 0.81−0.90 (m, 12H, CH_3 NHIle, CH_3 Leu), 1.16 (m, 8H, CH_3 Ala1, CH_3 Ala2, $CH_2\gamma$ NHIle), 1.47 (m, 2H, CHγ Leu, $CH_2\beta$ Leu), 1.61 (m, 2H, $CH_2\beta$ Leu, CHβ NHIle), 1.89 (m, 5H, $CH_2\beta$ Val, $CH_2\beta$ Pro, $CH_2\gamma$ Pro), 3.21 (m, 1H, CHα NHIle), 3.48 (m, 2H, $CH_2\delta$ Pro), 3.57 (s, 3H, OMe), 4.04 (m, 2H, CHα Ala1, CHα Val), 4.23 (m, 1H, CHα Leu), 4.69 (m, 2H, CHα Ala2, CHα Pro), 4.98 (br s, 1H, NHα NHIle), 7.29−7.39 (m, 6H, NH Ala1, Ph), 7.69 (d, $J = 9.5$ Hz, 1H, NHVal), 7.97 (d, $J = 7.0$ Hz, 1H, NH Ala2), 8.12 (d, $J = 7.0$ Hz, 1H, NH Leu), 9.16 (d, $J = 4.5$ Hz, 1H, NHβ NHIle). MW $C_{37}H_{60}N_7O_9$ calcd 746.4452 (M + H), found 746.4453 (M + H, HRFABMS). Anal. ($C_{37}H_{60}N_7O_9 \cdot 0.5H_2O$) C, H, N.

Boc-Ala-Ala-Pro-Val-NHAla-Ala-NH^{i}Pr. This compound was prepared by procedure E using Boc-Ala-Ala-Pro-OSu (0.181 g, 0.4 mmol) and Val-NHAla-Ala-NH^{i}Pr (0.126 g, 0.4 mmol). After stirring for 5 days and concentration of the solvent in vacuo, the crude material was precipitated by addition of water and filtered. Extraction of the aqueous layer with CH_2Cl_2 gave a further crop. Flash chromatography [CH_2Cl_2/MeOH (80/20)] afforded 0.110 g of pure Boc-Ala-Ala-Pro-Val-NHAla-Ala-NH^{i}Pr (40%): $[\alpha]^{25}_D$ −56.4 (*c* 1.0, CH_2Cl_2); ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 88:12 ratio) δ 0.76 (d, $J = 7.0$ Hz, 3H, CH_3 Val), 0.79 (d, $J = 7.0$ Hz, 3H, CH_3 Val), 1.01 (d, $J = 6.0$ Hz, 6H, CH_3 ^{i}Pr), 1.08 (d, $J = 7.0$ Hz, 3H, CH_3 NHAla), 1.11 (d, $J = 7.0$ Hz, 3H, CH_3 Ala1), 1.15 (d, $J = 7.0$ Hz, 6H, CH_3 Ala2, CH_3 Ala6), 1.34 (s, 9H, Boc), 1.70−2.00 (m, 5H, $CH_2\beta$ Pro, $CH_2\gamma$ Pro, CHβ Val), 3.34 (m, 1H, CHα Val), 3.48 (m, 1H, $CH_2\delta$ Pro), 3.56 (m, 1H, $CH_2\delta$ Pro), 3.76 (m, 1H, CH ^{i}Pr), 3.93 (m, 1H, CHα Ala1), 3.99 (m, 1H, CHα NHAla), 4.13 (m, 1H, CHα Ala6), 4.40 (m, 1H, $CH_2\alpha$ Pro), 4.47 (m, 1H, $CH_2\alpha$ Ala2), 5.17 (br s, 1H, NHα NHAla), 6.86 (d, $J = 7.0$ Hz, 1H, NH Ala1), 7.64 (d, $J = 7.5$ Hz, 1H, NH^{i}Pr), 7.71 (d, $J = 8.5$ Hz, 1H, NH Val), 7.84 (d, $J = 7.0$ Hz, 1H, NH Ala2), 7.88 (d, $J = 7.5$ Hz, 1H, NH Ala6), 9.29 (d, $J = 5.0$ Hz, 1H, NHβ NHAla); MW $C_{30}H_{54}N_8O_8$ calcd 655.4143 (M + H), found 655.4161 (M + H, HRFABMS). Anal. ($C_{30}H_{54}N_8O_8 \cdot H_2O$) C, H, N.

Z-Ala-Ala-Pro-Val-NHAla-Ala-NH^{i}Pr (3). The replacement of the protective group was done following procedure E starting from Z-OSu (0.021 g, 0.08 mmol) and 2HCl Ala-Ala-Pro-Val-NHAla-Ala-NH^{i}Pr (0.053 g, 0.08 mmol) in the presence of Et_3N (0.022 mL, 0.16 mmol). After stirring for 72 h and solvent concentration in vacuo, the product was precipitated by addition of water. The solid was washed with Et_2O to give 0.038 g of **3** (72%): ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 87:13 ratio) δ 0.77 (d, $J = 7.0$ Hz, 3H, CH_3 Val) 0.79 (d, $J = 7.0$ Hz, 3H, CH_3 Val), 1.01 (d, $J = 6.5$ Hz, 6H, CH_3 ^{i}Pr), 1.09 (d, $J = 7.0$ Hz, 3H, CH_3 NHAla), 1.15 (d, $J = 7.0$ Hz, 9H, CH_3 Ala1, CH_3 Ala2, CH_3, Ala6), 1.70−2.02 (m, 5H, $CH_2\beta$ Pro, $CH_2\gamma$ Pro, CHβ Val), 3.34 (m, 1H, CHα NHAla), 3.48 (m, 1H, $CH_2\delta$ Pro), 3.57 (m, 1H, $CH_2\delta$ Pro), 3.79 (m, 1H, CH ^{i}Pr), 4.02 (m, 2H, CHα Ala1, CHα Val), 4.13 (m, 1H, CHα Ala6), 4.13 (m, 1H, CHα Pro), 4.50 (m, 1H, $CH_2\alpha$ Ala2), 4.98 (s, 1H, CH_2Ph), 5.17 (br s, 1H, NHα NHAla), 7.32 (m, 6H, NH Ala1, Ph), 7.65 (d, $J = 7.5$ Hz, 1H, NH^{i}Pr), 7.73 (d, $J = 8.5$ Hz, 1H, NH Val), 7.88 (d, $J = 7.5$ Hz, 1H, NH Ala6), 7.95 (d, $J = 7.5$ Hz, 1H, NH Ala2), 9.30 (d, $J = 5.0$ Hz, 1H, NHβ NHAla); MW $C_{33}H_{52}N_8O_8$ calcd 689.3986 (M + H), found 689.3994 (M + H, HRFABMS). Anal. ($C_{33}H_{52}N_8O_8 \cdot H_2O$) C, H.

Boc-Ala-Ala-Pro-Val-Ala-Ala-NH^{i}Pr. This compound was prepared by procedure E using Boc-Ala-Ala-Pro-Osu (0.122 g, 0.27 mmol) and HCl Val-Ala-Ala-NH^{i}Pr (0.090 g, 0.27 mmol). After stirring for 3 days and solvent concentration in vacuo, the product was precipitated by addition of water. Extraction of the aqueous phase with CH_2Cl_2 gave a further crop of Boc-Ala-Ala-Pro-Val-Ala-Ala-NH^{i}Pr. A solution of the crude product in CH_2Cl_2/MeOH was decolorized over activated charcoal. Chromatography on a column of silica gel [CH_2Cl_2/MeOH (1/1)] afforded 0.055 g of pure Boc-Ala-Ala-Pro-Val-Ala-Ala-NH^{i}Pr as a white solid which was found to gelify MeOH and ^{i}PrOH (45%): ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 90:10 ratio) δ 081 (d, $J = 7.0$ Hz, 3H, CH_3 Val), 0.84 (d, $J = 7.0$ Hz, 3H, CH_3 Val), 1.01 (d, $J = 6.5$ Hz, 6H, CH_3 ^{i}Pr), 1.03 (d, $J = 6.5$ Hz, 6H, CH_3 ^{i}Pr), 1.10−1.20 (m, 12H, CH_3 Ala1, CH_3 Ala2, CH_3 Ala5, CH_3 Ala6), 1.86 (m, 4H, $CH_2\beta$ Pro, $CH_2\gamma$ Pro), 1.96 (m, 1H, CHβ Val), 3.52 (m, 1H, $CH_2\delta$ Pro), 3.57 (m, 1H, $CH_2\delta$ Pro), 3.77 (m, 1H, CH ^{i}Pr), 3.95 (m, 1H, CHα Ala1), 4.08 (m, 1H, CHα Val), 4.13 (m, 1H, CHα Ala6), 4.22 (m, 1H, CHα Ala5), 4.41 (m, 1H, CHα Pro), 4.49 (m, 1H, CHα Ala2), 6.69 (d, $J = 7.5$ Hz, 1H, NH Ala1), 7.58 (d, $J = 7.5$ Hz, 1H, NH ^{i}Pr), 7.73 (m, 2H, NH Val, NH Ala6), 7.90 (d, $J = 7.0$ Hz, 1H, NH Ala2), 7.96 (d, $J = 7.0$ Hz, 1H, NH Ala5); MW $C_{30}H_{53}N_7O_8$ calcd 640.4034 (M + H), found 640.4028 (M + H, HRFABMS). Anal. ($C_{30}H_{53}N_7O_8 \cdot H_2O$) C, H.

Z-Ala-Ala-Pro-Val-Ala-Ala-NH^{i}Pr (6). The replacement of the protective group was done following general procedure E starting from Z-OSu (0.016 g, 0.06 mmol) and HCl Ala-Ala-Pro-Val-Ala-Ala-NH^{i}Pr (0.053 g, 0.05 mmol) in the presence of Et_3N (0.007 mL, 0.05 mmol). After 72 h of stirring and solvent concentration in vacuo, the product was precipitated by addition of water. The solid was washed with Et_2O to give 0.021 g of **6** (58%): ^{1}H NMR (500 MHz, DMSO-d_6) (two conformers in a 88:12 ratio) δ 0.81 (d, $J = 7.0$ Hz, 3H, CH_3 Val), 0.83 (d, $J = 7.0$ Hz, 3H, CH_3 Val), 1.00 (d, $J = 6.0$ Hz, 6H, CH_3 ^{i}Pr), 1.02 (d, $J = 6.0$ Hz, 6H, CH_3 ^{i}Pr), 1.18 (m, 12H, CH_3 Ala1, CH_3 Ala2, CH_3 Ala5, CH_3 Ala6), 1.85 (m, 4H, $CH_2\beta$ Pro, $CH_2\gamma$ Pro), 1.96 (m, 1H, CHβ Val), 3.52 (m, 1H, $CH_2\delta$ Pro), 3.57 (m, 1H, $CH_2\delta$ Pro), 3.77 (m, 1H, CH ^{i}Pr), 4.04 (m, 1H, CHα Ala1), 4.07 (m, 1H, CHα Val), 4.13 (m, 1H, CHα Ala6), 4.21 (m, 1H, CHα Ala5), 4.50 (m, 2H, CHα Pro, CHα Ala2), 4.99 (s, 2H, CH_2Ph), 7.37 (m, 6H, NH Ala1, Ph), 7.55 (d, $J = 7.5$ Hz, 1H, NH ^{i}Pr), 7.70 (m, 2H, NH Ala6, NH Val), 7.93 (d, $J = 7.0$ Hz, 1H, NH Ala5), 7.96 (d, $J = 7.0$ Hz, 1H, NH Ala2); MW $C_{33}H_{51}N_7O_8$ calcd 674.3877 (M + H), found 674.3885 (M + H, HRFABMS). Anal. ($C_{33}H_{51}N_7O_8 \cdot 1.5H_2O$) C, H, N.

Biochemical Procedures. General. HLE and PPE were purchased from Elastin Products Co. and Serva, respectively. The chromogenic substrates, methoxysuccinyl-alanyl-alanyl-prolyl-valyl-*p*-nitroanilide (MeO-Suc-Ala-Ala-Pro-Val-*p*NA) for HLE and *N*-succinyl-alanyl-alanyl-alanyl-*p*-nitroanilide (Suc-Ala_3-*p*NA) for PPE, were obtained from Sigma. HPLC quality acetonitrile was purchased from Touzart et Matignon (Vitry-sur-Seine, France). Active site titrations of both elastases[17,35] were carried out with *N*-benzyloxycarbonyl-alanyl-alanyl-prolyl-azaalanyl *p*-nitrophenyl ester. Spectrophotometric measurements were made with a lambda 5 Perkin-Elmer UV−vis spectrophotometer. Reverse-phase HPLC was performed using a Waters model 510 system on a Lichrosorb C_8, 5-μm column (Interchim; 250 × 4.6 mm). The enzymatic activities of HLE and PPE were measured at 25 °C in 0.05 M Hepes (pH 8.0, 10% v/v DMSO).

Enzymatic Hydrolysis. Reaction mixtures containing 100 μM of compounds **3**−**6** in the presence or absence of enzyme

(200 nM HLE) were initially scanned by spectrophotometry (from 230 to 400 nm) at different times of incubation to look for an evolution of absorption spectrum resulting from enzymatic activity. The kinetic parameters of the enzymatic hydrolyses of **3** (5−50 μM) were determined by spectrophotometry at 250 nm ($[E]_0 = 200$ nM, pH 8.0 and 25 °C).

Samples of the above reaction mixtures were taken at different incubation times ranging from 0 to 4 h. To quench enzyme activity, their pH was dropped to 3.5 by adding 10% (v/v) TFA. The reaction products were then separated from the enzyme by centrifugation/filtration on a Centricon 10 instrument (Amicon). The filtrates were concentrated by Speed-vac and fractionated by reverse-phase HPLC on a Lichrosorb C_8, 5-μm column in Milli-Q water (0.1% TFA; solvent A) using a 20-min (0−100%) linear gradient of acetonitrile (0.07% TFA; solvent B). The flow rate was 1 mL/min. The absorbance of the effluent was monitored at 220 nm. The absence of spontaneous hydrolysis was also checked by HPLC. This chromatographic technique was used to determine the kinetic parameters for the HLE-catalyzed hydrolysis of **6** (5−20 μM). The concentrations of the end products after 2−5 min of incubation were quantified from their peak areas precalibrated with peptide Z-Ala-Ala-Pro-Val.

Sites of Enzyme Cleavage. The end products obtained after elastase-catalyzed hydrolysis of **3** and **6** were isolated by HPLC as described above. Their amino acid composition was determined after hydrolysis in vacuo in 6 M HCl for 24 h at 110 °C and determination of phenylthiocarbamyl amino acids by HPLC techniques using a Waters model 510 system apparatus (Pico-Tag method).

Enzyme Inhibition Procedure. The inhibitory effects of **4** (50−250 μM), **5** (25−300 μM), and **6** (40−200 μM) toward HLE (20 nM) or PPE (22 nM) were determined spectrophotometrically at 405 nm by continuous monitoring of the release of *p*-nitroaniline from the respective chromogenic substrate.

The double-reciprocal plots of v vs substrate concentration gave straight lines with x intercepts of $-1/K_{m(app)}$. The apparent Michaelis constants $K_{m(app)}$ {equal to $K_m(1 + [I]_o/K_i)$} and the maximum velocities V_m were calculated with the software package Kaleidagraph 3.0 (Abelbeck, Reading, PA) by iterative least-squares fits to the equation for competitive inhibition: $v = V_m \cdot [S]_o/\{[S]_o + K_{m(app)}\}$, where v is the initial rate. The constant K_i was determined from the linear plot of $K_{m(app)}/V_m$ vs inhibitor concentration (least-squares analysis). Initial estimates of K_i were calculated from Dixon plots. The reversible character of the inhibitions was checked by removal of inhibitor after buffer exchange on a Centricon 10 instrument (Amicon) followed by determination of the remaining activity of the enzyme fraction.

Supporting Information Available: Preparation of peptides **6**, **16**, and dipeptides or tripeptides described in Table 1; Lineweaver−Burk representation of the HLE-catalyzed hydrolysis of hydrazinopeptide **3**; Dixon plot of the inhibition of HLE by **5** with the substrate MeO-Suc-Ala-Ala-Pro-Val-*p*NA; ^{1}H NMR spectra (500 MHz) of hydrazinohexapeptides **2**−**5** and hexapeptide **6**; ^{1}H NMR chemical shift of NH in hydrazinopeptides **3**−**5** and peptide **6** as a function of DMSO-d_6 content in the solvent (11 pages). Ordering information is given on any current masthead page.

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