Notes

NAcSDKP Analogues Resistant to Angiotensin-Converting Enzyme

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Two series of analogues of the tetrapeptide NAcSDKP, an inhibitor of hematopoietic stem cell proliferation, were prepared, and their enzymatic stability toward rabbit lung angiotensin-converting enzyme (ACE) was evaluated as well as their capacity to inhibit NAcSDKP hydrolysis by this enzyme. In the first series, each of the peptide bonds has been successively replaced by an aminomethylene bond. In the second one, the C-terminus of the peptide has been modified by decarboxylation or amidation. The results reported here indicate that all of these molecules but one have good stability toward the enzyme but none of the compounds is able to inhibit NAcSDKP hydrolysis by ACE.

The peptide NAcSDKP (**1**), first isolated from foetal calf bone marrow, has been demonstrated to be a negative regulator of stem cell proliferation.[1] Thus its use during chemotherapy has been proposed to prevent the hematopoietic stem cells from proliferating and being destroyed by the cytotoxic phase-dependent drugs. Clinical trials using cytosine arabinoside and cyclophosphamide as anti-cancer drugs and NAcSDKP as a protecting drug have been initiated.[2]

In vitro stability studies of NAcSDKP (**1**) in human plasma have demonstrated the role of angiotensin-converting enzyme (ACE) in the catabolism of the peptide[3] resulting in cleavage of the Asp−Lys bond. Further studies have shown **1** to be a natural and specific substrate for the N-terminal active site of human ACE.[4]

In vivo, peptides are subject to proteolysis and rapid elimination. Many strategies have been devised during the past decade to design biologically active peptides resistant to proteolysis.[6] Among these, cyclization or replacement of the scissile bond by diverse amide bond surrogates have been most often used.[5,7,8] In order to get more stable molecules for therapeutic use and to gain structural information about the peptide backbone, each of the amide bonds of the tetrapeptide **1** has been successively replaced by the so-called reduced bond giving the analogues **2**, **3**, and **4**.

The proposed mechanism for the hydrolysis of angiotensin I by ACE involves an ionic interaction between the C-terminal carboxylate group and a protonated arginine side chain in the active site of ACE. This model has been successfully exploited to design inhibitors of ACE.[9] We hypothesized that the suppression of this ionic bond would reduce the affinity of the substrate for the enzyme and therefore increase the half-life of the molecule in physiological medium. Thus, two analogues lacking the carboxylate moiety have been synthesized: one with a carboxamide C-terminus **5** and the other lacking the C-terminal carboxylate group **6**. The present paper deals with the synthesis and the stability studies toward ACE of the five following peptides and pseudopeptides analogues of **1** (Figure 1).

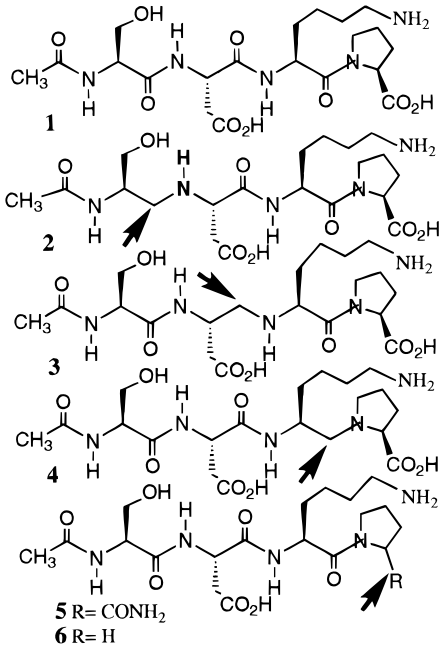

Figure 1. NAcSDKP analogues. The arrows indicate the site of modification.

As such manipulations of the peptide bond have sometimes yielded inhibitors, and also in view of the resemblance of NAcSDKP with lisinopril, a well-known inhibitor of ACE, the inhibitory potency of these molecules toward the hydrolysis of NAcSDKP by ACE has also been assessed.

Chemistry

The different syntheses were realized step by step using mainly the mixed anhydride methodology.[10] α-amino groups were protected with Boc, the side chain groups and the C-terminus with benzyl alcohol-derived protecting groups in the case of **2** and **4**; α-amino groups were protected with Z and the side chain groups and C-terminus with *tert*-butyl alcohol-derived protecting groups in the case of **3**.

Several methods are available for the replacement of amide bond with the aminomethylene bond $\Psi(CH_2NH)$.

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Table 1. Analytical Properties of the Analogues

	HPLC[a]		FAB-HRMS[b]		
compd	k'_1	k'_2	formula	calculated	found
2	9.28	8.3	$C_{20}H_{36}N_5O_8$	474.2564	474.2554
3	8.17	7.6	$C_{20}H_{36}N_5O_8$	474.2564	474.2562
4	4.11	3.92	$C_{20}H_{36}N_5O_8$	474.2564	474.2562
5	8.4	7.1	$C_{20}H_{35}N_6O_8$	487.2516	487.2518
6	10.1	8.9	$C_{19}H_{34}N_5O_7$	444.2458	444.2461

[a] k'_1 and k'_2 refer to the HPLC gradient described in the Experimental Section. [b] High-resolution fast atom bombardment mass spectroscopy.

However, the method in general use involves reductive alkylation of the α-amine of an amino acid (or peptide) ester with an aldehyde derived from a Weinreb amide.[12] The latter compound was obtained by coupling the acid group of suitably protected amino acids with *N*,*O*-dimethylhydroxylamine. The hydroxamate derivatives of Boc-Ser(Bzl)-OH, Z-Asp(O-Bu^t)-OH, and Boc-Lys(Z)-OH were prepared and converted into aldehydes as described in refs l3 and 14. Reduction conditions (temperature, amount of $LiAlH_4$) should be carefully controlled to prevent overreduction or epimerization of the aldehyde.[11]

The amide analogue **5** was prepared *via* amidation of the tripeptide Z-Asp(O-Bu^t)-Lys(Boc)-Pro-OH by reacting the tripeptide, activated through its mixed anhydride, with aqueous ammonia. Hydrogenolysis of Z-Ser-Asp(O-Bu^t)-Lys(Boc)-Pro-NH_2 and subsequent acetylation were performed very efficiently in a single step by adding acetylimidazole to the catalyst in ethyl acetate.

The decarboxylated analogue **6** was prepared from the pyrrolidide derivative of Boc-Lys(Z)-OH which was obtained through the mixed anhydride of Boc-Lys(Z)-OH treated with pyrrolidine. The following reactions consisted of a classical peptide synthesis sequence.

Acetylation of the N-terminus serine proceeded very smoothly with acetylimidazole in DMF, in presence of triethylamine in the case of trifluoroacetate salt. Final deprotection of benzyl alcohol-derived protecting groups was performed by hydrogenolysis with 10% Pd/C, and *tert*-butyl alcohol-derived protecting groups were cleaved off by acidolysis with trifluoroacetic acid. Crude peptides were purified by preparative reverse phase HPLC using mixtures of acetonitrile and water containing 0.1% trifluoroacetic acid as an eluent. The purified peptides were characterized by their FAB or LSI mass spectrum, and their purity was checked by HPLC analysis in two systems of solvents (Table 1).

ACE Degradation Study

The stability of each of the analogues was checked by incubating them with rabbit lung ACE for 30 min or 24 h in comparison to NAcSDKP (**1**). The conditions used for the hydrolysis are those described by Rousseau and al.[4] HPLC analysis of the reaction mixture was performed. The amount of residual peptide was assessed by measuring the area of the peak corresponding to the residual tetrapeptide **1** or analogues recorded at 215 nm.

ACE Inhibition in Vitro Assays

The ability of ACE resistant analogues to inhibit the hydrolysis of NAcSDKP (**1**) was studied by preincubating each one of them at different concentrations (0.5, 5, or 50 μM) with rabbit lung angiotensine-converting enzyme before adding the radiolabeled NAcSD[^{3}H]KP diluted with some cold peptide (5 μM). The tritium label was located in the lysine side chain.[15] The conditions used for incubation are those used in the stability study. Two control experiments were carried out: the first one was a simple hydrolysis of NAcSD[^{3}H]KP and the second one was an hydrolysis of NAcSD[^{3}H]KP in the presence of captopril (1 μM). After 30 min at 37 °C, the incubation medium was analyzed by high-voltage electrophoresis.[4] Comparison of the radioactivity associated with residual NAcSD[^{3}H]KP in the different sets of experiments was used to quantify the effect of the analogue on the hydrolysis of NAcSDKP.

Table 2. Stability toward Enzymatic Degradation by Rabbit Lung ACE of **1** and Analogues

	% residual peptide[a]	
peptide	$t = 30$ min	$t = 24$ h
AcSDKP	43.6 ± 1.6	0
2	99.7 ± 0.3	97.6 ± 2.4
3	100 ± 0	100 ± 0
4	99.7 ± 0.3	99.4 ± 0.6
5	99.9 ± 0.1	96.7 ± 3.3
6	36.2 ± 0.5	0

[a] Amount of intact peptide after 30 min or 24 h incubation time was estimated from the area of the corresponding peak observed at 215 nm in HPLC and compared to the value given at $t = 0$.

Results and Discussion

HPLC analysis of the incubation media of **1** (or each one of the pseudopeptides) with ACE showed, after 30 min, 44% of residual peptide **1** and 100% of residual pseudopeptides (Table 2). After a 24 h incubation, there was still 98% of residual **2** whereas 100% and 99% of pseudopeptides **3** and **4** were remaining (Table 2). These results illustrate the fact that when a peptide bond is modified even at a distance from the hydrolyzed bond, improved stability of the resulting analogue toward proteolysis can be observed.

The same stability can be seen for the amido analogue (100% after 30 min 98% residual analogue after 24 h) (Table 2). Conversely, the decarboxylated peptide is hydrolyzed with a time course similar to that observed for the parent peptide **1** (36% after 30 min, 0% of residual **6** after 24 h) (Table 2). This result led us to conclude that our hypothesis relying upon an ionic interaction between the C-terminal carboxylate group of angiotensin I and a protonated arginine side chain in the active site of ACE was not valid in the case of our compounds and that the interaction of **1** or the analogues with the enzyme is probably different from the one proposed for angiotensin I.

As shown in Table 3, the addition of the analogues **2**, **3**, **4**, or **5** did not change the extent of hydrolysis of NAcSDKP, suggesting that these molecules have weaker affinities than the parent peptide **1** for the active site of ACE or are not recognized by the enzyme. The peculiar properties of pseudopeptides, especially the ability of the peptide amide bond surrogate to be protonated at physiological pH, might explain the weak binding to the active site of the enzyme.

Nonetheless, all of the analogues described here retain full biological activity as inhibitors of the entry into cycle of two different primitive hematopoietic cells: HPP-CFC and CFU-GM.[16]

Table 3. Inhibitory Potency of Captopril and Analogues in NAcSDKP Hydrolysis by Rabbit Lung ACE

compound	% residual peptide **1**[a]
1	54.4 ± 2.3
captopril	99.2 ± 0.4
2, 50 μM	46.7 ± 6.2
2, 51 μM	54.0 ± 0.7
2, 0.5 μM	46.9 ± 3.8
3, 50 μM	41.7 ± 3.7
3, 5 μM	49.3 ± 9.4
3, 0.5 μM	46.0 ± 1.8
4, 50 μM	50.3 ± 6.5
4, 5 μM	48.8 ± 2.0
4, 0.5 μM	47.3 ± 7.5
5, 50 μM	49.7 ± 5.1
5, 5 μM	50.1 ± 8.4
5, 0.5 μM	46.0 ± 7.5

[a] Amount of remaining NAcSDKP after a 30 min incubation time following a 30 min preincubation with analogues or captopril at 37 °C.

The good stability of the three pseudopeptides and of the analogue **5** along with their full biological activity turns these compounds into attractive candidates for clinical trials and useful tools for further studies aimed at elucidating the still elusive mechanism of action of NAcSDKP.

Experimental Section

General Methods. All protected amino acids were purchased from Bachem AG or Novabiochem and are of L-configuration. Thin-layer chromatography (TLC) were run on silica gel precoated plates (60 F-254, Merck). Solvent systems were (A) dichloromethane/methanol, 98/2; (B) dichloromethane/methanol, 95/5; (C) dichloromethane/methanol, 9/1; (D) ethyl acetate/heptane, 1/1; (E) 1-butanol/acetic acid/eau, 4/1/1. UV light, ninhydrin, and/or Pataki reagent were used for detection. Protected peptides were purified by column chromatography on Merck silica gel 60 (40−63 μm).

All reagents and solvents were of analytical grade and used as supplied except for THF, which was either distilled from sodium/benzophenone or filtered through a column of basic alumina immediately prior to use, and DMF, which was distilled from ninhydrin under reduced pressure and stored over 4 Å molecular sieves. Protected peptides were characterized by their FAB or LSI mass spectrum. Mass spectra were obtained using a Kratos MS 80 mass spectrometer using a xenon FAB gun with glycerol, thioglycerol, or nitrobenzyl alcohol as a matrix. HPLC purifications were performed on a C-18 Beckmann Ultrasphere column (5 μm, 10 × 250 mm) using either a gradient or an isocratic elution with a mixture of acetonitrile and water containing 0.1% trifluoracetic acid at a flow rate of 3 mL min^{-1}. Elution was monitored by recording absorbance at 215 nm. The fractions were pooled, concentrated, and lyophilized. Pure peptides were characterized by their high-resolution LSI mass spectrum recorded on a Fisons (VG ZabSpec-T) mass spectrometer. HPLC analysis for purity control was performed on a Waters Nova-Pak column C-18 (4 μm particle size, 3.9 × 150 mm) with a solvent system consisting of a binary system of water and acetonitrile containing 0.1% TFA at a 1 mL min^{-1} flow rate with monitoring at 215 nm. The solvent programs involved the following linear gradients: (1) 0−50% acetonitrile over 50 min, (2) 0−80% acetonitrile over 40 min. k' values are reported in these two solvent systems.

Chemistry: Typical Procedures. Boc Acidolysis. The protected peptide was dissolved in a 1/1 mixture of dichloromethane and trifluoroacetic acid (10−30 equiv). After the mixture was stirred for 1−2 h at room temperature, the solvents were removed in vacuum after dilution with dichloromethane. The trifluoracetate salt was generally triturated with a mixture of dry ether and petroleum ether (1/3, v/v) and dried under vacuum for several hours.

Hydrogenolysis. The protected peptide was dissolved in either ethanol or methanol/water (9/1) for final deprotection. To this solution was added 10% Pd/C (about 20% by weight). The reaction was stirred under an atmosphere of hydrogen at atmospheric pressure and room temperature. The catalyst was removed by filtration through a filter paper and the filtrate concentrated under reduced pressure.

Coupling Reactions. Mixed Anhydride Method. The N-protected amino acid was dissolved in tetrahydrofuran (5 mL/mmol). To this solution cooled to −15 °C was added *N*-methylmorpholine (1 equiv) and isobutyl chloroformate (1 equiv). After 5 min, the temperature was lowered to −20 °C and the cooled solution of C-protected amino acid or peptide (1.1 equiv) dissolved in a minimum amount of dimethylformamide or dichloromethane was added along with *N*-methylmorpholine (1.1 equiv) in the case of the trifluoracetate salt. After 1 h at −10 °C, the temperature was allowed to warm to room temperature. Completion of the reaction was monitored by TLC. The reaction mixture was then concentrated under reduced pressure, and the residue was taken up with ethyl acetate and 5% citric acid. The organic layer was washed with water, 5% sodium bicarbonate, water, and brine and dried over sodium sulfate. Evaporation of the solvent yielded the crude product which was either purified through column chromatography or used in the next step when homogeneous by TLC.

Acetylation Reaction. The N-deprotected peptide or its trifluoroacetate salt was dissolved in DMF (2 mL/mmol). Acetylimidazole (1.1 equiv) was added to the solution, cooled on a ice bath. Triethylamine (1.1 equiv) was added in the case of the salt. The reaction mixture was stirred at room temperature. Completion of the reaction was monitored by TLC. The reaction mixture was evaporated down and the residue was taken up with 0.5 N HCl and ethyl acetate. The organic layer was washed with water until the pH was neutral and then with brine and then was dried over sodium sulfate. Evaporation of the solvent yielded the crude product which was purified through column chromatography before the last deprotection step.

N^{α}-Acetyl-L-seryl-Ψ(CH_2NH)-L-aspartyl-L-lysyl-L-proline (2). The dipeptide Boc-Lys(Z)-Pro-Bzl was obtained by coupling of Boc-Lys(Z)-OH with the HCl,Pro-OBzl via the mixed anhydride method. The crude product obtained as a syrup was purified on silica gel using CH_2Cl_2/MeOH (99/1) as an eluent. Yield: 68%. R_f(B) = 0.65, R_f(D) = 0.23. FAB-MS: *m/z* 590 $(MNa)^+$, 568 $(MH)^+$, 468 $(MH)^+$ − Boc, 434 $(MH)^+$ − Z.

Boc-Lys(Z)-Pro-Bzl was deprotected by treatment with TFA for 2 h and 30 min at room temperature. Boc-Asp(OBzl)-OH was coupled with the trifluoracetate salt for 5 h via the mixed anhydride method and purified on column (AcOEt/heptane, 1/1). Yield: 81%. R_f(B) = 0.4, R_f(D) = 0.10. FAB-MS: *m/z* 795 $(M + Na)^+$, 773 $(M + H)^+$, 673 $(M + H)^+$ − Boc, 639 $(M + H)^+$ − Z.

Boc-Ser(OBzl)-$N(CH_3)OCH_3$ was prepared as described by Martinez[5] and purified on column (CH_2Cl_2/MeOH, 99/1). Yield: 47%. R_f(D) = 0.43, R_f (A) = 0.4. CI-MS: *m/z* 338 $(MH)^+$, 282 $(MH)^+$ − Bu^t, 238 $(MH)^+$ − Boc.

The tripeptide Boc-Asp(OBzl)-Lys(Z)-Pro-Bzl (0.372 g, 0.5 mmol) was deprotected by treatment with TFA for 2 h at room temperature. The salt was dried under reduced pressure. The aldehyde prepared by the reduction of Boc-Ser(Bzl)-$N(CH_3)$-OCH_3 (0.75 mmol) according to the method of Martinez[5] was dissolved in a mixture of MeOH/AcOH (99/1) containing the trifluoroacetate salt obtained from the deprotection of Boc-Asp-(OBzl)-Lys(Z)-Pro-Bzl (0.50 mmol). Sodium cyanoborohydride (0.036 g) was added portionwise. After 1 h, the reaction mixture was worked up as described in ref 5. The crude product (0.490 g) was purified on column (CH_2Cl_2/MeOH, 98.5/1.5). Yield: 70%. R_f(B) = 0.20, R_f(C) = 0.50. FAB-MS: *m/z* 936 $(MH)^+$, 836 $(MH)^+$ − Boc, 882 $(MH)^+$ − Z.

Boc-Ser(Bzl)-Asp(OBzl)-Lys(Z)-Pro-Bzl (0.290 g, 0.31 mmol) was deprotected by treatment with TFA for 1 h and 30 min at room temperature. The trifluoroacetate salt (0.110 g, 0.31 mmol) dissolved in DMF (0.6 mL) containing Et_3N (0.047 mL, 0.031 mmol) was reacted with acetylimidazole (0.034 g, 0.031 mmol). After 6 h and 30 min of stirring, the reaction mixture was concentrated under reduced pressure. Workup afforded

the crude product (0.235 g) purified on column (CH_2Cl_2/MeOH (98/2)). Yield: 62%. R_f(B) = 0.36, R_f(C) = 0.68. FAB-MS: *m/z* 878 $(MH)^+$, 778 $(MH)^+ - Boc$, 744 $(MH)^+ - Z$.

The peptide Ac-Ser(Bzl)-Ψ(CH_2NH)-Asp(OBzl)-Lys(Z)-Pro-Bzl was dissolved in 10% aqueous EtOH with 10% Pd/C catalyst (0.030 g). The suspension was stirred for 20 h under an atmosphere of hydrogen and purified by RP-HPLC; isocratic elution with CH_3CN/H_2O/TFA (95/5/0.1), flow rate 3 mL min^{-1}.

***N*$^{\alpha}$-Acetyl-L-seryl-L-aspartyl-Ψ(CH_2N)-L-lysyl-L-proline (3).** Z-Lys(Boc)-Pro-OBu^t was obtained by coupling Z-Lys(Boc)-OH (2.66 g, 7 mmol) with H-Pro-OBu^t (1.32 g, 7.7 mmol) via the mixed anhydride method. After 6 h of stirring, the reaction mixture was worked up as usual to afford an oil (3.6 g). Yield: 96%. R_f(B) = 0.26, R_f(D) = 0.21. The crude product (1.08 g, 2 mmol) was dissolved in EtOH (40 mL). Then 10% Pd/C (0.120 g) was added, and the suspension was stirred for 4 h and 30 min under an atmosphere of hydrogen. Yield: 0.672 g (84%). R_f(C) = 0.34. FAB-MS: *m/z* 422 $(MNa)^+$, 400 $(MH)^+$, 344 $(MH)^+ - Bu^t$, 300 $(MH)^+ - Boc$, 288 $(MH)^+ - 2Bu^t$, 244 $(MH)^+ - Bu^t - Boc$.

N$^{\alpha}$-(Benzyloxycarbonyl)-*O*-*tert*-butyl-L-aspartyl *N*,*O*-dimethylhydroxamate was prepared and reduced into aldehyde as described by Martinez.[5] The aldehyde (2 mmol) was dissolved in MeOH/AcOH (99/1) (7 mL) containing H-Lys(Boc)-Pro-OBu^t (1 mmol). Sodium cyanoborohydride (0.094 g) was added portionwise. After 2 h and 30 min, the reaction mixture was worked up. Chromatography on column (CH_2Cl_2/MeOH, 99/1, then CH_2Cl_2/MeOH, 98/2) gave Z-Asp(OBu^t)-Ψ(CH_2NH)-Lys(Boc)-Pro-OBu^t. Yield: 56%; R_f(B) = 0.28, R_f(C) = 0.61, R_f(AcOEt/heptane, 2/1) = 0.11. FAB-MS: *m/z* 691 $(MH)^+$, 635 $(MH)^+ - Bu^t$, 557 $(MH)^+ - Z$.

Z-Asp(OBu^t)-Ψ(CH_2NH)-Lys(Boc)-Pro-OBu^t (0.34 mmol) was dissolved in 10% aqueous EtOH (9.9 mL) with 10% Pd/C (0.044 g). The suspension was stirred under an atmosphere of hydrogen for 24 h. Yield: 0.277 g (100%). R_f(C) = 0.42. FAB-MS: *m/z* 557 $(MH)^+$, 501 $(MH)^+ - Bu^t$.

Z-Ser(Bu^t)-OH (0.132 g, 0.45 mmol) was coupled with the tripeptide so obtained via the mixed anhydride method. After 6 h, the usual workup gave Z-Ser(Bu^t)-Asp(OBu^t)-Ψ(CH_2NH)-Lys(Boc)-Pro-OBu^t as a white foam (0.351 g). Purification on column (AcOEt/hexane, 1/1). Yield: 0.233 g (70%). R_f(B) = 0.37, R_f(C) = 0.63. FAB-MS: *m/z* 834 $(M + H)^+$, 778 $(M + H)^+ - Bu^t$.

Z-Ser(Bu^t)-Asp(OBu^t)-Ψ(CH_2NH)-Lys(Boc)-Pro-OBu^t (0.2 mmol) was dissolved in 10% aqueous EtOH (4.4 mL) with 10% Pd/C (0.035 g). The suspension was stirred under an atmosphere of hydrogen overnight. Yield: 0.110 g (66%). R_f(B) = 0.32, R_f(C) = 0.48. The resulting peptide (0.140 g, 0.2 mmol) was dissolved in DMF (0.5 mL) and reacted with acetylimidazole (0.033 g, 0.3 mmol). After 4 h of stirring, the usual workup gave Ac-Ser(Bu^t)-Asp(OBu^t)-Ψ(CH_2NH)-Lys(Boc)-Pro-OBu^t. Purification was on column (AcOEt/MeOH, 99/1). Yield: 0.100 g (74%). R_f(B) = 0.37, R_f(C) = 0.55. FAB-MS: *m/z* 742 $(MH)^+$, 686 $(MH)^+ - Bu^t$.

Ac-Ser(Bu^t)-Asp(OBu^t)-Ψ(CH_2NH)-Lys(Boc)-Pro-OBu^t (0.056 g, 0.067 mmol) was dissolved in TFA/H_2O (300 μL/30 μL). The solution was stirred for 5 h at room temperature. The reaction mixture was concentrated under reduced pressure after addition of H_2O (1 mL) and then lyophylized. Purification by RP-HPLC, gradient solvent system: 0% to 3% CH_3CN over 10 min, 3% CH_3CN over l5 min, flow rate 3 mL min^{-1}.

***N*$^{\alpha}$-Acetyl-L-seryl-L-aspartyl-L-lysyl-Ψ(CH_2-N)-L-proline (4).** *N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*$^{\epsilon}$-(benzyloxycarbonyl)-L-lysyl *N*,*O*-dimethylhydroxamate: Boc-Lys(Z)-OH (2.28 g, 6 mmol) was dissolved in CH_2Cl_2 (30 mL) containing *N*,*O*-dimethylhydroxylamine hydrochloride (0.702 g, 7.2 mmol). DCC (1.238 g, 6 mmol) and DMAP (0.366 g, 3 mmol) were added at 0 °C followed by DIPEA (1.24 mL, 7.2 mmol). The reaction mixture was stirred overnight, and then the solvent was evaporated under reduced pressure. The same workup as for the coupling reaction yielded Boc-Lys(Z)-N(CH_3)OCH_3. Purification was on column (solvent A). Yield: 1.7 g (66%). R_f(CH_2Cl_2/MeOH, 97/3) = 0.25, R_f(D) = 0.2. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.4 (s, 5H), 5.25 (broad s, 1H), 5.1 (s, 2H), 4.9 (broad s, 1H), 4.65 (broad s, 1H), 3.75 (s, 3H), 3.2 (m, 5H), 1.8−1.4 (m, 6H), 1.4 (s, 9H). FAB-MS: *m/z* 446 $(MNa)^+$, 424 $(MH)^+$, 324 $(MH)^+ - Boc$, 290 $(MH)^+ - Z$. IR (film): 3332, 2975, 2937, 2869, 1708−1702, 1655−1650, 1523−1519, 1500, 1456, 1391, 1367, 1250, 1170 cm^{-1}. Anal. ($C_{21}H_{33}O_6N_3$) C, H, N, O. $[\alpha]_D = -13.1$ (*c* 1.0, MeOH).

The hydroxamate derivative (l.44 g, 3.96 mmol) dissolved in dry THF (7 mL) was reduced with $AlLiH_4$ (0.325 g, 8.8 mmol) at 0 °C under stirring as described in ref 5. Boc-Lys(Z)-H was obtained as an oil (1.224 g, 85%), which was immediately used without purification. R_f(A) = 0.18, R_f(D) = 0.42. ^{1}H-NMR (200 MHz, $CDCl_3$): δ 9.55 (s, 1H), 7.35 (s, 5H), 5.35 (s, 1H), 5.1 (s, 2H), 4.9 (broad s, 1H), 3.2 (m, 2H), 2−1.4 (m, 6H), 1.4 (s, 9H). MS (CI): *m/z* 365 $(MH)^+$, 309 $(MH)^+ - Bu^t$, 265 $(MH)^+ - Boc$. IR (film): 3342, 3006, 2976, 2935, 2867, 1718−1686, 1533−1509, 1456, 1392, 1367, 1251, 1166 cm^{-1}.

HCl,H-Pro-OBzl (1.136 g, 4.7 mmol) and the aldehyde Boc-Lys(Z)-H (1.2 g, 3.36 mmol) were dissolved in a solution of MeOH-AcOH (99:1). $NaBH_3CN$ (0.252 g, 4 mmol) was added portionwise over 45 min. The reaction was monitored by TLC. After 1 h, the reaction mixture was worked up. Chromatography on column (solvent A) gave Boc-Lys(Z)-Ψ(CH_2N)-Pro-OBzl. Yield: 0.985 g (53%). R_f(A) = 0.3, R_f(D) = 0.29. FAB-MS: *m/z* 576 $(MNa)^+$, 554 $(MH)^+$, 454 $(M+H)^+ - Boc$, 418 $(MH)^+ - Z$.

Boc-Lys(Z)-Ψ(CH_2N)-Pro-OBzl (0.744 g, 1.3 mmol) was deprotected by treatment by TFA (20 equiv). The trifluoroacetate salt was triturated with petroleum ether and dried under vacuum. Boc-Asp(OBzl)-OH (0.394 g, 1.2 mmol) was coupled with the dipeptide salt via the mixed anhydride method. After 6 h, the usual workup gave Boc-Asp(OBzl)-Lys(Z)-Ψ(CH_2N)-Pro-OBzl as an oil homogeneous in TLC. Yield: 0.577 g (57%). R_f (CH_2Cl_2/MeOH, 97/3) = 0.23, R_f(D) = 0.22. FAB-MS: *m/z* 781 $(MNa)^+$, 759 $(MH)^+$, 623 $(MH)^+ - Z$.

Boc-Asp(OBzl)-Lys(Z)-Ψ(CH_2N)-Pro-OBzl (0.577 g, 0.76 mmol) was treated by a 1/1 mixture of CH_2Cl_2/TFA. Boc-Ser(Bzl)-OH (0.204 g, 0.69 mmol) was coupled with the tripeptide salt via the mixed anhydride method. After 5 h of stirring, the usual workup gave Boc-Ser(Bzl)-Asp(OBzl)-Lys(z)-Ψ(CH_2N)-Pro-OBzl as an oil. Yield: 0.495 g (70%). R_f(CH_2Cl_2/MeOH, 97/3) = 0.23, R_f(D) = 0.1. FAB-MS: *m/z* 958 $(MNa)^+$, 936 $(MH)^+$, 800 $(MH)^+ - Z$.

Boc-Ser(Bzl)-Asp(OBzl)-Lys(Z)-Ψ(CH_2N)-Pro-OBzl (0.480 g, 0.5 mmol) was treated by a 1/1 mixture of TFA/CH_2Cl_2. The trifluoracetate salt was dissolved in DMF (3 mL). Acetylimidazole (0.062 g, 0.56 mmol) followed by triethylamine (0.078 mL, 0.56 mmol) was added at room temperature. After 4 h and 30 min of stirring, the reaction mixture was worked up. Purification on column (AcOEt/MeOH, 99/1) gave Ac-Ser(Bzl)-Asp(OBzl)-Lys(Z)-Ψ(CH_2N)-Pro-OBzl. Yield: 0.187 g (42%). R_f(CH_2Cl_2/MeOH, 97/3) = 0.2, R_f(AcOEt) = 0.25. FAB-MS: *m/z* 900 $(MNa)^+$, 878 $(M + H)^+$, 788 $(MH)^+ - Bzl$, 742 $(MH)^+ - Z$.

Ac-Ser(Bzl)-Asp(OBzl)-Lys(Z)-Ψ(CH_2N)-Pro-OBzl (0.180 g, 0.2 mmol) was dissolved in 10% aqueous MeOH (10 mL). 10% Pd/C (0.036 g) was added, and the suspension was stirred overnight under an atmosphere of hydrogen. The crude peptide was purified by RP-HPLC using the following gradient solvent system: 0% to 5% CH_3CN over 20 min, flow rate 3 mL min^{-1}.

***N*$^{\alpha}$-Acetyl-L-seryl-L-aspartyl-L-lysyl-L-prolinamide (5).** Z-Lys(Boc)-Pro-OBzl was obtained from Z-Lys(Boc)-OH (1.54 g, 4 mmol), and HCl,Pro-OBzl (1.06 g, 4.4 mmol) was coupled via the mixed anhydride method. The crude product was purified on silica gel using CH_2Cl_2/MeOH (99/1) as an eluent to give Z-Lys(Boc)-Pro-OBzl as a white foam. Yield: 1.76 g (77%). R_f(A) = 0.22; R_f(D) = 0.24. FAB-MS: *m/z* 590 $(MNa)^+$, 568 $(MH)^+$, 512 $(MH)^+ - Bu^t$, 468 $(MH)^+ - Boc$, 434 $(MH)^+ - Z$, 378 $(MH)^+ - Boc - Bzl$, 334 $(MH)^+ - Boc - Z$.

Z-Lys(Boc)-Pro-OBzl (1 g, 1.75 mmol) was dissolved in 10% aqueous MeOH (33 mL); 10% Pd/C (0.2 g) was added, and the suspension was stirred under an atmosphere of hydrogen overnight. Yield: 0.565 g (94%). R_f(E) = 0.55. FAB-MS: *m/z* 366 $(MNa)^+$, 344 $(MH)^+$, 288 $(MH)^+ - Bu^t$, 244 $(MH)^+ - Boc$. Z-Asp(OBu^t)-OH (0.323 g, 1 mmol) was coupled with the dipeptide H-Lys(Boc)-Pro-OH via the mixed anhydride method. After 5 h of stirring, the reaction mixture was worked up as usual. The crude product was purified on silica gel using (CH_2-

Cl_2/MeOH/AcOH, 97/3/0.5) as an eluent. Yield: 0.450 g (60%). R_f(CH_2Cl_2/MeOH/AcOH, 97/3/0.5) = 0.11. FAB-MS: *m/z* 693 $(M2Na)^+ - H$, 671 $(MNa)^+$, 615 $(MNa)^+ - Bu^t$, 549 $(MH)^+ -$ Boc, 537 $(MNa)^+ - Z$, 515 $(MNa)^+ - Bu^t -$ Boc, 493 $(MH)^+ - Bu^t -$ Boc, 437 $(MNa)^+ -$ Boc $- Z$.

To a stirred solution of the tripeptide Z-Asp(OBu^t)-Lys(Boc)-Pro-OH (0.129 g, 0.2 mmol) in THF (5 mL) cooled to −15 °C was added *N*-methylmorpholine (0.022 mL, 0.2 mmol) followed by isobutyl chloroformate (0.028 mL, 0.2 mmol). After 5 min of stirring at −15 °C, a cold 34% ammonia solution (0.2 mL) was added at −20 °C. After 1 h of stirring at a temperature below −10 °C and an additional hour at a temperature below 0 °C, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in ethyl acetate and 5% citric acid. The aqueous phase was extracted with ethyl acetate. The combined organic layers were washed with H_2O and brine, then dried over Na_2SO_4, and concentrated under reduced pressure to afford Z-Asp(OBu^t)-Lys(Boc)-Pro-NH_2 as a white foam. Purification was on column with CH_2Cl_2/MeOH (95/5) as an eluent. Yield: 0.105 g (81%). R_f(B) = 0.24, R_f(AcOEt/MeOH, 99/1) = 0.45. FAB-MS: *m/z* 670 $(MNa)^+$, 648 $(MH)^+$, 614 $(MNa)^+ - Bu^t$, 548 $(MH)^+ -$ Boc, 534 $(MH)^+ -$ $ProNH_2$, 514 $(MH)^+ - Z$, 492 $(MH)^+ - Bu^t -$ Boc.

Z-Asp(OBut)-Lys(Boc)-Pro-NH_2 (0.152 g, 0.23 mmol) was dissolved in MeOH (6 mL); 10% Pd/C(0.030 g) was added, and the suspension was stirred under an atmosphere of hydrogen for 2 h. Yield: 0.111 g (94%). R_f(B) = 0.08, R_f(C) = 0.35. FAB-MS: *m/z* 536 $(MNa)^+$, 514 $(MH)^+$, 480 $(MNa)^+ - Bu^t$, 458 $(MH)^+ - Bu^t$, 414 $(MH^+) -$ Boc, 400 $(MH)^+ -$ $ProNH_2$.

Z-Ser-OH (0.045 g, 0.19 mmol) was coupled with the tripeptide H-Asp(OBut)-Lys(Boc)-Pro-NH_2 (0.105 g, 0.2 mmol) via the mixed anhydride method. After 5 h of stirring, the reaction mixture was concentrated under reduced pressure. The usual workup gave Z-Ser-Asp(OBut)-Lys(Boc)-Pro-NH_2. Purification was on column (CH_2Cl_2/MeOH, 94/6). Yield: 0.140 g (80%). R_f(B) = 0.18, R_f(C) = 0.43. FAB-MS: *m/z* 757 $(MNa)^+$, 735 $(MH)^+$, 701 $(MNa)^+ - Bu^t$, 635 $(MH)^+ -$ Boc, 601 $(MH)^+ - Z$, 579 $(MH)^+ - Bu^t -$ Boc, 501 $(MH)^+ -$ Boc $- Z$, 465 $(MH)^+ -$ Boc $- Bu^t -$ $ProNH_2$.

Z-Ser-Asp(OBu^t)-Lys(Boc)-Pro-NH_2 (0.080 g, 0.011 mmol) was dissolved in AcOEt (2 mL); 10% Pd/C (0.016 g) and acetylimidazole (0.014 g, 0.013 mmol) was added and the suspension was stirred under an atmosphere of hydrogen overnight. The catalyst was removed by filtration on a Celite pad, and the filtrate was concentrated under reduced pressure. Purification on column (CH_2Cl_2/MeOH, 9/1). Yield: 0.050 g (71%); R_f(C) = 0.29. FAB-MS: *m/z* 665 $(MNa)^+$, 643 $(MH)^+$, 609 $(MNa)^+ - Bu^t$, 543 $(MH)^+ -$ Boc, 487 $(MH)^+ - Bu^t -$ Boc, 373 $(MH)^+ -$ Boc $- Bu^t -$ $ProNH_2$.

Ac-Ser-Asp(OBu^t)-Lys(Boc)-Pro-NH_2 (0.039 g, 0.06 mmol) in solution in 200 μL of TFA containing 20 μL of H_2O was stirred at room temperature for 90 min. The reaction mixture was concentrated under reduced pressure and the residue was triturated twice with dry ether. After removal of ether, the solid white residue was taken up in 1.5 mL of H_2O and lyophilized. Purification by RP-HPLC, elution gradient consisting of two solvents (A, H_2O/0.1% TFA; B, CH_3CN/0.1% TFA; 100% to 80% A over 20 min; t_R = 13 min), flow rate 3 mL min^{-1}.

***N*$^{\alpha}$-Acetyl-L-seryl-L-aspartyl-L-lysylpyrrolidide (6).** Boc-Lys(Z)-OH (0.76 g, 2 mmol) in THF (10 mL) was coupled with pyrrolidine (0.18 mL, 2.2 mmol) via the mixed anhydride method. The usual workup gave Boc-Lys(Z)-pyrrolidide as a white foam. Yield: 0.786 g (91%). R_f(B) = 0.23. IC-MS: *m/z* 434 $(MH)^+$, 343 $(MH)^+ -$ Boc, 300 $(MH)^+ - Z$.

Boc-Lys(Z)-pyrrolidide (0.775 g, 1.34 mmol) was dissolved in CH_2Cl_2/TFA (20 equiv) for deprotection. Boc-Asp(OBzl)-OH (0.499 g, l.55 mmol) was coupled with the dipeptide salt (1.7 mmol) via the mixed anhydride method. The usual workup provided Boc-Asp(OBzl)-Lys(Z)-pyrrolidide as an oil. Yield: 0.771 g (78%). R_f(A) = 0.22.

The dipeptide (0.303 g, 0.47 mmol) was deprotected with trifluoroacetic acid. The trifluoracetate salt was coupled with Boc-Ser(Bzl)-OH via the mixed anhydride method. Purification of the crude product on silica gel with solvent A as an eluent yielded Boc-Ser(Bzl)-Asp(OBzl)-Lys(Z)-pyrrolidide as a white foam. Yield: 0.181 g (71%). R_f(A) = 0.19, R_f(AcOEt) = 0.39. FAB-MS: *m/z* 838 $(MNa)^+$, 816 $(MH)^+$, 716 $(MH)^+ -$ Boc, 682 $(MH)^+ - Z$, 582 $(MH)^+ -$ Boc $- Z$.

The tripeptide (0.181 g, 0.22 mmol) was deprotected with TFA. The trifluoracetate salt dissolved in DMF (0.5 mL) was treated with acetylimidazole (0.026 g, 0.24 mmol) and triethylamine (0.034 mL, 0.23 mmol). After 4 h at room temperature, the usual workup and purification of the crude product on column (CH_2Cl_2/MeOH, 96/4) gave Ac-Ser(Bzl)-Asp(OBzl)-Lys(Z)-pyrrolidide as an oil. Yield: 0.121 g (74%). R_f(B) = 0.31, R_f(AcOEt/MeOH, 95/5) = 0.16. FAB-MS: *m/z* 780 $(MNa)^+$, 758 $(MH)^+$.

Ac-Ser(Bzl)-Asp(OBzl)-Lys(Z)-pyrrolidide (0.100 g, 0.13 mmol) was dissolved in 10% aqueous MeOH (7.7 mL). Pd/C (10%, 0.020 g) was added, and the suspension was stirred overnight under an atmosphere of hydrogen. Purification by RP-HPLC gradient elution: 0−10% CH_3CN over 35 min. t_R = 25 min. FAB-MS: *m/z* 466 $(MNa)^+$, 444 $(MH)^+$.

ACE Degradation Study. NAcSDKP was a gift from IPSEN-BIOTECH (Paris, France). ACE from lung rabbit was a commercial enzyme purchased from Sigma. A 150 μL of a solution of ACE (0.12 unit/mL) in Tris-maleate buffer (100 mM Tris, 50 mM NaCl, 10^{-5} M $ZnSO_4$) at pH 7 was preincubated at 37 °C for 30 min, and 30 μL of a solution of the tetrapeptide NAcSDKP (**1**) or the analogues (300 μM) in buffer were added. Incubation of the medium containing the enzyme (0.1 unit/mL) and **1** or one of the analogues (50 μM) was carried out at 37 °C for either 30 min or 24 h. At the end of incubation time, aliquots (50 μL) were withdrawn and enzymatic hydrolysis was stopped by addition of 50 μL of a 1% TFA solution. RP-HPLC analysis of these aliquots was performed twice using a Nova-Pak C-18 column (Waters) (3.9 × 150 mm, 4 μM, 80 Å). Elution was monitored by recording UV absorbance at 215 nm. The solvents system used for elution consisted of H_2O/0.1% TFA and CH_3CN/0.1% TFA. Different conditions of elution were used: for **2**, **3**, and **6**, a linear gradient of 0−5% CH_3CN over 25 min, for **5** a linear gradient of 0−5% CH_3CN over 15 min, and for **4** which is the most polar, an isocratic elution with water/CH_3CN/TFA (90/10/0.1, v/v/v) containing octane sulfonic acid (5 mM). A calibration curve has been set up for the six compounds in the 5−50 μm range to check the linearity of the response at 215 nm and deduce the concentration of the residual compound from the area of the corresponding peak. The results shown in Table 2 are the mean values of two experiments run in triplicate.

ACE Inhibition Assay. A 30 μL portion of the solutions of **2**, **3**, **4**, or **5** (at the following concentrations: 1.5, 15, or 150 μM) or 30 μL of a 3 μM solution of captopril was preincubated with ACE (45 μL, 0.12 unit/mL) in Tris-maleate buffer (100 mM Tris, 50 mM NaCl, 10^{-5} M $ZnSO_4$) at pH 7 at 37 °C for 30 min. At this point, the hydrolysis was initiated by adding NAcSD[^{3}H]KP (3.6 μCi) and 15 μL of a 30 μM solution of **1**. Incubation of the medium containing the enzyme (0.1 unit/mL) with **1** (5 μM) and one of the analogue (0.5, 5, or 50 μM) or captopril (1 μM) as a control, was carried out for 30 min at 37 °C. Enzymatic hydrolysis was stopped by freezing the incubation medium at −80 °C. It was analyzed by high-voltage paper electrophoresis as described by Rousseau et al.[4] The residual radioactivity associated with NAcSD[^{3}H]KP at t = 30 min was compared with the one at t = 0. Each experiment was run in duplicate. The results shown in Table 3 are the mean values of two experiments.

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(16) Biological activities will be reported in detail elsewhere.

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