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Studies on α_2-Plasmin Inhibitor Fragment T-11. III.[1a,b,2] Structure–Activity Relationships among the Fragments of T-11, the Plasminogen Binding Site(s) of Human α_2-Plasmin Inhibitor

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Twelve shortened fragments of peptide T-11, which is a part of human α_2-plasmin inhibitor and contains its plasmin(ogen)-binding site(s) were synthesized by a conventional solution method. The dissociation constants for the interaction between these synthetic fragments and plasmin were determined. Among these fragments, the octadecapeptide containing C-terminal lysine of T-11, which consists of 26 amino acids, was found to be the smallest active fragment of T-11. The N-terminal and the central portion fragments of T-11 possessed no binding activity. The heptadecapeptide which has the same sequence as the octadecapeptide but lacks the C-terminal lysine showed no binding activity. The hexadecapeptide containing the C-terminal lysine but lacking the 10th lysine in T-11 scarcely exhibited the activity. Thus, the lysine residues at positions 10 and 26 in T-11 must be important for the activity.

Keywords——α_2-plasmin inhibitor; α_2-plasmin inhibitor fragment; peptide synthesis; thioanisole–mediated TFMSA deprotection; dissociation constant; structure–activity relationship

α_2-Plasmin inhibitor is the most important inhibitor of plasmin in mammalian blood. Peptide T-11, which consists of 26 amino acid residues, was isolated from human α_2-plasmin inhibitor and shown to be the plasmin(ogen)-binding site(s) of the inhibitor.[3] A study on the structure activity relationship of T-11 has been carried out. This paper deals with the synthesis and activity of many shortened fragments of T-11.

Previously we demonstrated that peptide T-11 binds to the plasmin(ogen) lysine-binding site(s).[3] ω-Amino acids, for example, ε-aminocaproic acid[4] and *trans*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA),[5] also bind to plasmin(ogen). Amino and carboxyl groups of these ω-amino acids are reported to be essential for their binding activity to the lysine-binding site(s).[6] The fragment T-11 and *t*-AMCHA have been shown to be competitive inhibitors of binding to the lysine-binding site(s).[7] Thus, at least one pair of acidic and basic amino acids among three lysines and two glutamic acids or three aspartic acids in this peptide T-11 seems to be essential for binding to the same site(s) as that of ω-amino acids. To identify the pair of amino acids, twelve shortened fragments of T-11 lacking those basic and/or acidic amino acid residues were synthesized and their activities were determined.

In order to obtain fragments II, III, V and VI of T-11, deprotection of all protecting groups of their intermediates (I-15, I-14, I-10 and I-9), which were obtained in the course of the total synthesis of T-11,[1a,b] was carried out, and the deprotected products were purified. The protected N-terminal fragments (XII-4 and XIII-1), central fragments (X-7 and XI-2) and C-terminal fragments (IV-1 and VII-2) were newly synthesized and deprotected, follow-

ed by purification to give the peptides (XII, XIII, X, XI, IV and VII, respectively). Fragments VIII and IX, lacking C-terminal lysine were prepared from III and IV, respectively, by carboxypeptidase B[8] digestion.

1 5 10 15 20 25		K_d (μM)
G-D-K-L-F-G-P-D-L-K-L-V-P-P-M-E-E-D-Y-P-Q-F-G-S-P-K	(I) (T-11)	4—7
L·····K	(II)	18
D·····K	(III)	13
L·····K	(IV)	35
L·····K	(V)	250
M·····K	(VI)	250
F·····K	(VII)	700
D·····P	(VIII)	—
L·····P	(IX)	—
Ac-L·····P-NH_2	(X)	—
Ac-D·····P-NH_2	(XI)	—
L·····P-NH_2	(XII)	—
G·····P-NH_2	(XIII)	—
Ac-Lys-OH		800
ε-aminocaproic acid		100—200

Fig. 1. Amino Acid Sequences and Dissociation Constants (K_d) of the Synthesized Fragments of T-11

The standard IUPAC one-letter codes[9] for amino acid residues are used.

The strategy for the synthesis of the peptides was essentially the same as the method previously described.[1a,b] Asp(OBzl), Glu(OBzl) and Lys(Z) were employed as side chain-protected amino acid derivatives so that deprotection by thioanisole–mediated TFMSA treatment in TFA[10] could be performed at the final stage. The TFA–labile Boc-group[11] was employed as a temporary protector of α-amino groups.

The protected octadecapeptide ester (IV-1) was synthesized by coupling of Boc–Leu–Lys(Z)–OH[1b] with a TFA–treated sample of Boc–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (I-10)[1a] by the DCC–HOBT procedure[12] and purified by batchwise washing with dilute acid and base, followed by precipitation.

The protected hexapeptide ester (I-6)[1a] was treated with TFA and hydrogenated with Pd catalyst. After purification, three spots were detected on TLC. Therefore the shortened peptide fragment VII lacking the N-terminal Gln was synthesized. Fragment VII was prepared by condensation of a TFA–treated sample of Z(OMe)–Ser–Pro–Lys(Z)–OBzl[1a] and Boc–Phe–Gly–$NHNH_2$ obtained from Boc–Phe–Gly–OMe[1a] using the azide procedure,[13] followed by deprotection with TFA and hydrogenation with a Pd catalyst (Fig. 2).

As described before, fragments VIII and IX were prepared by the removal of the C-terminal lysine residue of III and IV, respectively, with carboxypeptidase B.[8] As the second amino acid from the C-terminal of III or IV was proline, the enzyme reaction stopped at this residue, releasing only lysine. Preparative HPLC was used for purification of VIII and IX.

Fragment X-7 was synthesized starting with Boc–Pro–NH_2 as shown in Fig. 3. The Tyr residue was introduced into a TFA–treated sample of the starting material by the azide procedure and the Asp(OBzl) and the Glu(OBzl) residues were introduced stepwise by the *p*-nitrophenyl ester procedure.[14] The resulting protected tetrapeptide amide, Boc–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-4), was exposed to TFA and condensed with Boc–Met(O)–Glu(OBzl)–OH (I-4)[1a] by the DCC–HOBT procedure. Then Boc–Leu–Val–Pro–Pro–$NHNH_2$ (I-5)[1a] was condensed with a TFA–deprotected sample of the above hexapeptide

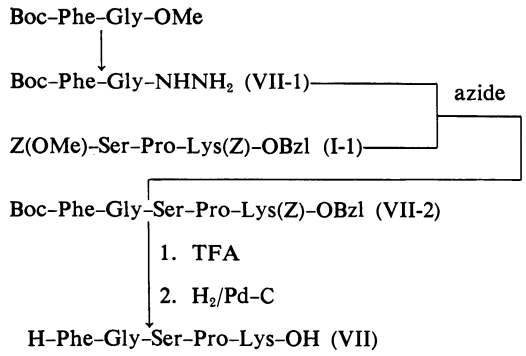

Fig. 2. Synthetic Scheme for the Pentapeptide (VII)

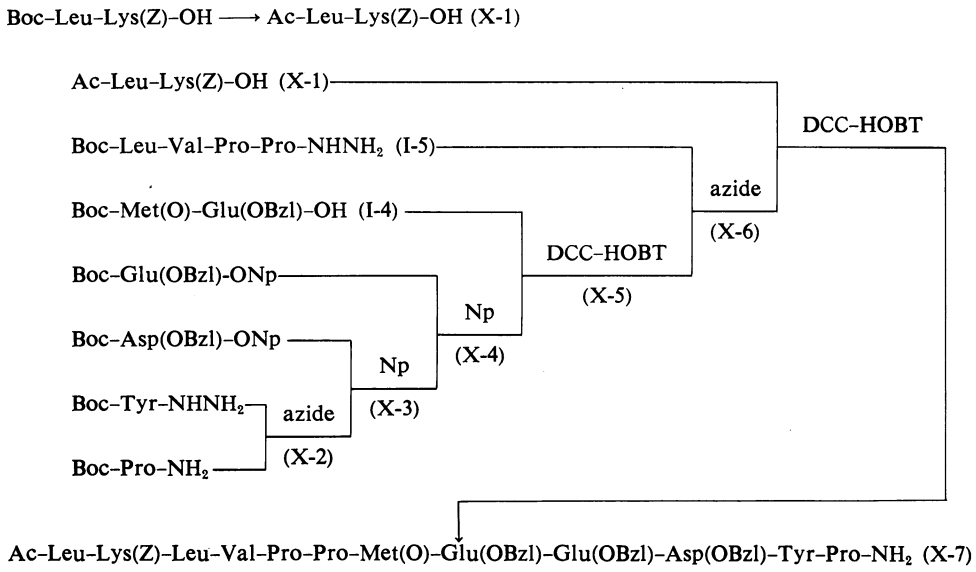

Fig. 3. Synthetic Scheme for the Protected Dodecapeptide Amide (X-7)

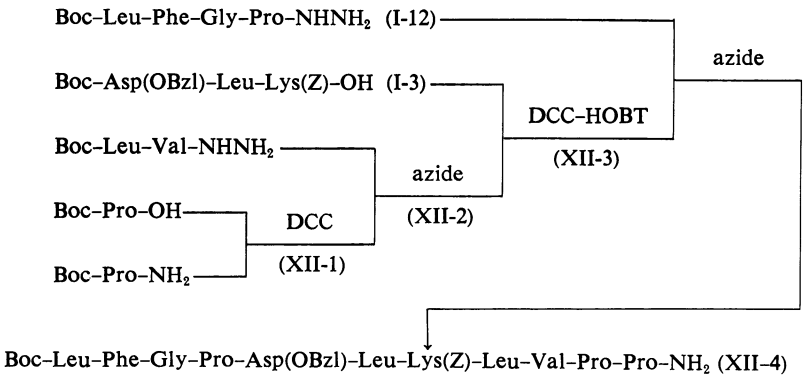

Fig. 4. Synthetic Scheme for the Protected Undecapeptide Amide (XII-4)

amide by the azide procedure. Finally, Ac–Leu–Lys(Z)–OH, which was prepared by the acetylation of a TFA–treated sample of Boc–Leu–Lys(Z)–OH[1b] with AcONp, was combined with the N^{α}-deprotected decapeptide amide.

Fragment XI-2 was prepared as follows. The above decapeptide amide (X-6) was treated with TFA and condensed by the DCC–HOBT method with Ac–Asp(OBzl)–Leu–Lys(Z)–OH, which was obtained by acetylation in the same manner as described for Ac–Leu–Lys(Z)–OH, after TFA treatment of Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11).[1b]

Fragment XII-4 was synthesized as shown in Fig. 4. Boc–Pro–Pro–NH_2 (XII-1), which was synthesized by the DCC condensation of Boc–Pro–OH with a TFA–treated sample of Boc–Pro–NH_2, was exposed to TFA and coupled with Boc–Leu–Val–$NHNH_2$[1b] by the azide procedure. After removal of the Boc group of the resulting tetrapeptide amide (XII-2), Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-3)[1b] was condensed by the DCC–HOBT procedure. The azide procedure was employed to prepare the protected fragment (XII-4) for the condensation of Boc–Leu–Phe–Gly–Pro–$NHNH_2$ (I-12)[1b] and a TFA–treated sample of the heptapeptide amide (XII-3) obtained above.

The condensation of a TFA–treated sample of fragment XII-4 and Boc–Gly–Asp(OBzl)–Lys(Z)–OH (I-13)[1b] by the DCC–HOBT technique afforded fragment XIII-1.

The deprotection and subsequent purification of these fragments were carried out by essentially the same procedures as described for the synthesis of T-11.[1b] The product was purified by ion-exchange chromatography on DEAE–cellulose or CM–cellulose. If necessary, to remove small amounts of impurities, preparative reversed-phase HPLC on a Senshupack $N5C_{18}$ column was carried out for further purification.

Each peptide thus obtained exhibited a single peak on HPLC and a sharp single spot on TLC. Its purity was further confirmed by amino acid analysis after acid hydrolysis and elemental analysis. The yield and the deprotection and purification procedures used for these peptides are shown in Table I, and the analytical data are given in Tables II and III.

The dissociation constants for the complexes between human plasmin and the T-11 fragments obtained were determined as the peptide concentrations that caused a 50% decrease of the apparent rate constant, according to the method of Wiman *et al.*[15] The values of the dissociation constants (K_d) determined for the peptides are summarized in Fig. 1. The K_d values for fragments II, III and IV were determined to be 18, 13 and 35 μM, respectively, and were of the same order as that of the fragment I (T-11).[1b,3] On the other hand, the values for fragments V, VI and VII were estimated to be 250, 250 and 700 μM, respectively, indicating that these fragments have weak binding activities similar to those of ε-aminocaproic acid and Ac–Lys–OH used as the controls. The N-terminal and central fragments, X to XIII, and fragments VIII and IX, lacking the C-terminal lysine, had no ability to bind to plasmin. Among these T-11 fragments prepared, fragment IV was the smallest one that exhibited binding activity to plasmin.

T-11 or its fragment III competed with *t*-AMCHA for binding to the lysine-binding site(s) of plasmin.[7] Therefore, some of the ε-amino and ω-carboxyl groups of T-11 might be involved in binding to the lysine-binding site(s) by the same mechanism as in the case of ω-amino acids, *e.g.*, ε-aminocaproic acid and *t*-AMCHA. Thus at least one pair of basic and acidic amino acid residues in T-11 seems to be essential for the binding.

Concerning basic amino acids, T-11 contains three lysines at positions 3, 10 and 26. The binding activity of fragment V, which contains only one lysine at position 26 in T-11, was greatly decreased, and its dissociation constant value was almost identical to that of the control as shown in Fig. 1. Fragments VIII and IX, lacking the C-terminal lysine at position 26, had no activity. However, fragment IV, which contains two lysines at positions 10 and 26 and consists of 18 amino acids, retained the binding activity. Thus, fragment IV might be the smallest active fragment of T-11, suggesting that the two lysines at positions 10 and 26 in T-11

are essential for the activity. Previously, we showed that the T-11 derivative amidinated at positions 3 and 10, but intact at position 26, lost its binding activity completely.[3] Therefore, the 10th lysine in T-11 might play an important role in the binding.

In relation to the acidic amino acids in T-11, fragment IV, which contains two glutamic acids at positions 16 and 17 and aspartic acid at position 18, retained the activity. However, fragments V and VI containing these acidic amino acids and fragment VII containing no acidic amino acid showed very small binding activity similar to that of the controls. Further study will be necessary to clarify which acidic amino acid residues and which pair of basic and acidic amino acid residues in T-11 are important for binding activity to plasmin.

Experimental

Melting points are uncorrected. Optical rotations were determined with a JASCO DIP 140 digital polarimeter. The amino acid compositions of 6 N HCl hydrolysates were determined with a Hitachi amino acid analyzer 835 and are not corrected for amino acid destruction. TLC was performed on silica gel plates (Kiesel gel 60 F_{254}, Merck) and *Rf* values refer to the following solvent systems: Rf^1 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf^2 $CHCl_3$–MeOH–acetic acid (85 : 10 : 5), Rf^3 *n*-butanol–acetic acid–pyridine–H_2O (4 : 1 : 1 : 2), Rf^4 *n*-butanol–pyridine–H_2O (1 : 1 : 1).

Analytical HPLC was conducted with a JASCO TWINCLE apparatus equipped with a Cosmosil $5C_{18}$-P (5 μ, Nakarai Chem. Co.) column (4.6 × 150 mm) by linear gradient elution with acetonitrile (10% to 50%; 15 min) in 0.05% TFA at a flow rate of 1.0 ml/min with monitoring at 230 nm. Preparative HPLC was conducted with the same apparatus but with a Senshupack $N5C_{18}$ column (8 × 250 mm), using the same linear gradient system as that used for analytical HPLC at a flow rate of 2.0 ml/min with monitoring at 275 nm.

Carboxypeptidase B (Lot J1K698) was obtained from Worthington Biochemical Corp.

The final deprotection and purification procedures that were used for the synthesis of fragment III are described in detail as a representative example.

Boc–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (IV-1)——The hexadecapeptide ester (I-10)[1a] (0.49 g) was treated with TFA (3 ml) at 0 °C for 1 h. The excess TFA was removed by evaporation *in vacuo*, and dry ether was added to the residue. The resulting powder was dried over KOH pellets *in vacuo* for 1 h and then dissolved in DMF (3 ml) and neutralized with Et_3N. DCC (62 mg) and HOBT·H_2O (34 mg) were added to a solution of Boc–Leu–Lys(Z)–OH[1b] (0.23 g) in DMF (2 ml), then the above N^{α}-deprotected peptide solution was added to this mixture. The whole was stirred for 18 h, then filtered. The filtrate was evaporated, and the residue was treated with ether. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and precipitated from MeOH with ether; yield 0.53 g (94%), mp 128—130 °C, $[\alpha]_D^{28}$ −72.4° (c = 1.0, MeOH), Rf^1 0.72. Amino acid ratios in 6 N HCl hydrolysate: Asp 0.95, Ser 0.83, Glu 2.76, Pro 4.15, Gly 0.98, Val 1.06, Met 0.63, Leu 2.23, Tyr 0.80, Phe 1.00, Lys 1.83 (recovery 91%). *Anal.* Calcd for $C_{145}H_{191}N_{21}O_{35}S \cdot 4H_2O$: C, 60.21; H, 6.94; N, 10.17. Found: C, 60.33; H, 6.77; N, 10.17.

Boc–Phe–Gly–$NHNH_2$ (VII-1)——Hydrazine hydrate (80%, 8.38 ml) was added to a solution of Boc–Phe–Gly–OMe[1a] (4.65 g) in MeOH (30 ml). After standing overnight at room temperature, the mixture was concentrated *in vacuo* at room temperature and the residue was extracted with AcOEt. The AcOEt layer was washed with NaCl-saturated H_2O, dried over Na_2SO_4 and evaporated. The resulting product was recrystallized from MeOH and ether; yield 3.70 g (80%), mp 131—132 °C, $[\alpha]_D^{28}$ −7.6° (c = 1.1, MeOH), Rf^1 0.67. *Anal.* Calcd for $C_{16}H_{24}N_4O_4$: C, 57.13; H, 7.19; N, 16.66. Found: C, 57.46; H, 7.17; N, 16.60.

Boc–Phe–Gly–Ser–Pro–Lys(Z)–OBzl (VII-2)——Z(OMe)–Ser–Pro–Lys(Z)–OBzl[1a] (1.08 g) was treated with TFA (6 ml) and anisole (0.81 ml) at 0 °C for 1 h. TFA was removed *in vacuo* and ether was added to the residue. The resulting powder was dried over KOH pellets, dissolved in DMF (5 ml) and neutralized with Et_3N. To this ice-chilled solution, the azide (prepared from 0.61 g of Boc–Phe–Gly–$NHNH_2$ with 0.61 ml of 7.62 N HCl–DMF, 0.31 ml of isoamyl nitrite and 0.86 ml of Et_3N) in DMF (1 ml) was added. The mixture was stirred at 4 °C for 48 h, then evaporated, and the residue was dissolved in AcOEt. The AcOEt phase was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and then dried over Na_2SO_4. Evaporation gave the desired product, which was crystallized with ether and recrystallized from MeOH and ether; yield 1.03 g (80%), mp 119—121 °C, $[\alpha]_D^{28}$ −53.8° (c = 1.0, MeOH), Rf^1 0.77. *Anal.* Calcd for $C_{45}H_{58}N_6O_{11} \cdot 0.5H_2O$: C, 62.27; H, 6.85; N, 9.68. Found: C, 62.26; H, 6.75; N, 9.89.

H–Phe–Gly–Ser–Pro–Lys–OH (VII)——Compound VII-2 (400 mg) was treated with TFA (2 ml) at 0 °C for 1 h, then evaporated and ether was added to the residue. The resulting powder was dissolved in MeOH (5 ml) and a few drops of acetic acid and 5% palladium carbon (200 mg) were added. After hydrogenation, the catalyst was removed by filtration, and the filtrate was evaporated *in vacuo*. Then 1 N acetic acid was added to the residue, and the solvent was removed by lyophilization. The lyophilized product was treated with ether to give a white powder; yield 240 mg (96%), $[\alpha]_D^{28}$ −47.1° (c = 0.62, 1 N AcOH), Rf^3 0.21, Rf^4 0.51. Amino acid ratios in a 6 N HCl hydrolysate: Ser 0.89, Pro 1.17, Gly 1.00, Phe 1.00, Lys 0.94 (average recovery 90%). *Anal.* Calcd for $C_{25}H_{38}N_6O_7 \cdot 2AcOH \cdot 3H_2O$: C, 49.14;

H, 7.40; N, 11.86. Found: C, 48.85; H, 7.18; N, 12.13.

H–Asp–Leu–Lys–Leu–Val–Pro–Pro–Met–Glu–Glu–Asp–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–OH (VIII)——Peptide III (40.15 mg) was dissolved in 0.2 M NH_4HCO_3 (4.0 ml; pH 7.9). Carboxypeptidase B (1.60 mg) was added to the peptide solution and incubation was carried out at 37 °C for 3 d. The reaction mixture was subjected directly to preparative HPLC on a Senshupack $N5C_{18}$ column (8 × 250 mm). The main peak portions were collected and lyophilized. The lyophilized product was dissolved in 1 N AcOH and the solvent was removed by lyophilization to give a white fluffy powder; yield 25.3 mg (67%), $[\alpha]_D^{27}$ −145.7° (c = 0.07, 1 N AcOH), Rf^4 0.77. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.95, Ser 0.83, Glu 2.76, Pro 4.15, Gly 0.98, Val 1.06, Met 0.63, Leu 2.23, Tyr 0.80, Phe 1.00, Lys 0.83 (average recovery 95%). *Anal.* Calcd for $C_{94}H_{140}N_{20}O_{30}S \cdot 2AcOH \cdot 18H_2O$: C, 46.95; H, 7.40; N, 11.18. Found: C, 47.32; H, 7.58; N, 11.03.

H–Leu–Lys–Leu–Val–Pro–Pro–Met–Glu–Glu–Asp–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–OH (IX)——This was obtained in the same manner as described for fragment VIII. Thus, fragment IV (16.0 mg) dissolved in 0.2 M NH_4HCO_3 (1.60 ml; pH 7.9) was treated with carboxypeptidase B (1.07 mg) and incubated at 37 °C for 2 d. Purification of the mixture by preparative HPLC as described above, followed by lyophilization, gave a white fluffy powder; yield 8.48 mg (57%), $[\alpha]_D^{27}$ −80.0° (c = 0.12, 1 N AcOH), Rf^4 0.77. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.06, Ser 0.90, Glu 2.96, Pro 3.95, Gly 1.01, Val 0.99, Met 0.97, Leu 1.91, Tyr 1.00, Phe 1.00, Lys 0.91 (average recovery 95%). *Anal.* Calcd for $C_{90}H_{135}N_{19}O_{27}S \cdot 2AcOH \cdot 16.5H_2O$: C, 47.74; H, 7.50; N, 11.26. Found: C, 47.42; H, 7.38; N, 11.22.

Ac–Leu–Lys(Z)–OH (X-1)——Boc–Leu–Lys(z)–OH[1b] (2.13 g) was treated with TFA (6 ml) in the usual manner. IPE was added to the residue. The resulting powder was collected by filtration, dissolved in DMF (10 ml) and neutralized with Et_3N. AcONp (0.97 g) and Et_3N (0.62 ml) were added to this solution, followed by overnight stirring at room temperature. The solvent was removed by evaporation, and the residue was dissolved in AcOEt. This solution was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and evaporated. Addition of IPE to the residue gave a powder, which was recrystallized from MeOH and IPE; yield 1.12 g (56%), mp 132—134 °C, $[\alpha]_D^{26}$ −15.3° (c = 1.1, MeOH), Rf^2 0.56. *Anal.* Calcd for $C_{22}H_{33}N_3O_6$: C, 60.67; H, 7.64; N, 9.65. Found: C, 60.50; H, 7.54; N, 9.67.

Boc–Tyr–Pro–NH_2 (X-2)——Boc–Pro–NH_2 (5.22 g) was treated with TFA (15 ml) in the usual manner. The resulting powder was dissolved in DMF (10 ml) and neutralized with Et_3N. To this solution, the azide (prepared from 6.58 g of Boc–Tyr–$NHNH_2$ with 7.61 ml of 7.62 N HCl–DMF, 4.20 ml of isoamyl nitrite and 11.13 ml of Et_3N) in DMF (10 ml) was added, and the mixture was stirred at 4 °C for 48 h. After evaporation of the solvent, the residue was dissolved in AcOEt. This solution was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O, dried over Na_2SO_4, and evaporated. Treatment of the residue with IPE gave white crystals, which were recrystallized from MeOH and ether; yield 5.25 g (62%), mp 105—108 °C, $[\alpha]_D^{28}$ −27.8° (c = 1.0, MeOH), Rf^1 0.57. *Anal.* Calcd for $C_{19}H_{27}N_3O_5$: C, 60.46; H, 7.21; N, 11.13. Found: C, 60.56; H, 7.35; N, 11.43.

Boc–Asp(OBzl)–Tyr–Pro–NH_2 (X-3)——Compound X-2 (5.14 g) was treated with TFA (20 ml) in an ice-bath for 1h. A white powder that formed on addition of ether was collected by filtration, dried over KOH pellets *in vacuo*, dissolved in DMF (20 ml), and neutralized with *N*-methylmorpholine. Boc–Asp(OBzl)–ONp (6.35 g) and *N*-methylmorpholine (1.20 ml) were added to the solution and the mixture was stirred at room temperature overnight. Removal of the solvent by evaporation gave a residue, which was dissolved in AcOEt. This solution was washed as usual with acid and base, dried over Na_2SO_4, and evaporated to give a residue, which was used for the next step without further purification; yield 5.23 g (66%), Rf^1 0.60.

Boc–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-4)——Compound X-3 (2.00 g) was treated with TFA (8 ml) in the usual manner, and coupled with Boc–Glu(OBzl)–ONp (1.73 g) as described above. The residue was further purified by silica-gel column chromatography (150 g, 4.5 × 23 cm) with $CHCl_3$–MeOH (20 : 1) as an eluent to afford the desired product after recrystallization from MeOH and ether; yield 1.33 g (48%), mp 88—90 °C, $[\alpha]_D^{26}$ −34.0° (c = 1.1, MeOH), Rf^1 0.60. *Anal.* Calcd for $C_{42}H_{51}N_5O_{11}$: C, 62.91; H, 6.41; N, 8.73. Found: C, 63.03; H, 6.65; N, 8.46.

Boc–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-5)——The above tetrapeptide amide (1.27 g) was treated with TFA (5 ml) as usual. The residue obtained by evaporation of the TFA was dissolved in DMF (6 ml) and neutralized with Et_3N. DCC (0.37 g) and HOBT·H_2O (0.27 g) were added to a solution of Boc–Met(O)–Glu(OBzl)–OH[1a] (0.81 g), and the N^{α}-deprotected peptide solution was added to this mixture. The reaction mixture was stirred at room temperature overnight. After filtration to remove DCurea, the filtrate was evaporated, and the residue was dissolved in *n*-butanol. This solution was washed with the usual acid and base, dried over $MgSO_4$ and evaporated. The residue was treated with ether and precipitated from MeOH with ether; yield 1.81 g (98%), mp 103—106 °C, $[\alpha]_D^{26}$ −30.5° (c = 1.1, MeOH), Rf^1 0.60. *Anal.* Calcd for $C_{59}H_{73}N_7O_{16}S \cdot 2H_2O$: C, 58.84; H, 6.44; N, 8.14. Found: C, 58.78; H, 6.28; N, 8.20.

Boc–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp–(OBzl)–Tyr–Pro–NH_2 (X-6)——The above hexapeptide amide (1.79 g) was treated with TFA (8 ml) as usual, and coupled with the azide (prepared from 0.94 g of Boc–Leu–Val–Pro–Pro–$NHNH_2$ with 0.55 ml of 7.62 N HCl–DMF, 0.28 ml of isoamyl nitrite and 0.79 ml of Et_3N) in DMF (3 ml). Work-up as described for X-2 gave a white powder, which was precipitated from MeOH–AcOEt with ether; yield 1.81 g (76%), mp 119—121 °C, $[\alpha]_D^{26}$ −77.6° (c = 1.1, MeOH), Rf^1 0.62. *Anal.* Calcd for $C_{80}H_{107}N_{11}O_{20}S \cdot 4H_2O$: C, 58.34; H, 7.04; N, 9.36. Found: C, 58.37; H, 6.84; N, 9.43.

Ac–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (X-7)——The above decapeptide amide (0.60 g) was treated with TFA (3 ml) in the usual manner. The TFA salt was dissolved in DMF (3 ml), neutralized with Et_3N and treated with a solution of DCC (0.15 g), HOBT·H_2O (0.10 g) and Ac–Leu–Lys(Z)–OH (X-1) (0.20 g) in DMF (2 ml). The mixture was stirred at room temperature overnight, then filtered. The filtrate was concentrated *in vacuo* and the residue was treated with H_2O and ether. The resulting powder was washed batchwise with acid and base as usual, followed by precipitation from MeOH with ether; yield 0.66 g (87%), mp 152—155 °C, $[\alpha]_D^{26}$ −70.4° (c=1.1, MeOH), Rf^1 0.67. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.93, Glu 1.86, Pro 2.94, Val 1.00, Met 0.55, Leu 2.09, Tyr 0.72, Lys 1.09 (average recovery 92%). *Anal.* Calcd for $C_{97}H_{130}N_{14}O_{23}S \cdot 2H_2O$: C, 60.42; H, 7.01; N, 10.17. Found: C, 60.45; H, 7.03; N, 9.78.

Ac–Asp(OBzl)–Leu–Lys(Z)–OH (XI-1)——This compound was prepared from Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11)[1b] (0.40 g) in the same manner as described for the synthesis of Ac–Leu–Lys(Z)–OH (X-1). TFA treatment of I-11 and coupling with AcONp (0.13 g) gave the product, which was recrystallized from MeOH and IPE; yield 0.34 g (89%), mp 123—127 °C, $[\alpha]_D^{26}$ −25.6° (c=1.1, MeOH), Rf^2 0.62. *Anal.* Calcd for $C_{33}H_{44}N_4O_9$: C, 61.86; H, 6.92; N, 8.75. Found: C, 61.89; H, 6.84; N, 8.86.

Ac–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–Met(O)–Glu(OBzl)–Glu(OBzl)–Asp(OBzl)–Tyr–Pro–NH_2 (XI-2)——This compound was prepared by the condensation of a TFA (3 ml)–treated sample of X-6 and XI-1 (0.29 g) with DCC (0.10 g) and HOBT·H_2O (0.07 g) in DMF (3 ml) as usual. After work-up as described for X-7, the resulting powder was precipitated from MeOH and ether; yield 0.79 g (98%), mp 166—169 °C, $[\alpha]_D^{26}$ −79.2° (c=0.5, MeOH), Rf^1 0.66. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.82, Glu 1.86, Pro 2.95, Val 1.00, Met 0.60, Leu 2.03, Tyr 0.76, Lys 1.11 (average recovery 91%). *Anal.* Calcd for $C_{108}H_{141}N_{15}O_{26}S \cdot 3H_2O$: C, 60.29; H, 6.89; N, 9.77. Found: C, 59.73; H, 6.41; N, 9.76.

Boc–Pro–Pro–NH_2 (XII-1)——In the usual manner, DCC (2.14 g) and HOBT·H_2O (1.0 g) were added to a solution of a TFA (8 ml)–treated sample of Boc–Pro–NH_2 (2.28 g) and Boc–Pro–OH (2.05 g) in THF (10 ml). After usual work-up, a low-melting mass was obtained; yield 1.88 g (57%), Rf^1 0.76.

Boc–Leu–Val–Pro–Pro–NH_2 (XII-2)——A TFA (8 ml) treated sample of XII-1 (1.88 g) was condensed with the azide (prepared from 2.25 g of Boc–Leu–Val–$NHNH_2$ with 4.45 ml of 3.87 N HCl–DMF, 1.16 ml of isoamyl nitrite and 3.23 ml of Et_3N) in DMF (10 ml). After usual work-up, the residue was treated with ether and IPE to afford a white powder, which was recrystallized from ether and IPE–*n*-hexane; yield 1.12 g (40%), mp 110—114 °C, $[\alpha]_D^{26}$ −104.3° (c=1.2, MeOH), Rf^1 0.71. *Anal.* Calcd for $C_{26}H_{45}N_5O_6$: C, 59.63; H, 8.66; N, 13.38. Found: C, 59.56; H, 8.45; N, 13.12.

Boc–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–NH_2 (XII-3)——The above protected tetrapeptide amide (XII-2) (1.13 g) was treated with TFA (5 ml). The resulting TFA salt was neutralized and condensed with Boc–Asp(OBzl)–Leu–Lys(Z)–OH (I-11)[1b] (1.64 g) with DCC (0.49 g) and HOBT·H_2O (0.36 g). After usual work-up, ether was added to the residue and the product was precipitated from MeOH–THF with ether; yield 1.72 g (72%), mp 140—142 °C, $[\alpha]_D^{26}$ −52.2° (c=1.1, DMF), Rf^1 0.63. *Anal.* Calcd for $C_{57}H_{85}N_9O_{13}$: C, 60.99; H, 7.81; N, 11.23. Found: C, 60.98; H, 7.72; N, 11.06.

Boc–Leu–Phe–Gly–Pro–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–NH_2 (XII-4)——A TFA (8 ml)–treated sample of XII-3 (1.61 g) was condensed with the azide (prepared from 0.96 g of Boc–Leu–Phe–Gly–Pro–$NHNH_2$ with 1.36 ml of 3.87 N HCl–DMF, 0.29 ml of isoamyl nitrite and 0.93 ml of Et_3N) in DMF (4 ml). After usual work-up, treatment of the residue with IPE gave a fine powder, which was precipitated from MeOH–THF with IPE; yield 2.00 g (90%), mp 146—148 °C, $[\alpha]_D^{26}$ −54.5° (c=1.2, DMF), Rf^1 0.58. Amino acid ratios in a 6 N HCl hydrolysate: Asp 0.98, Pro 2.59, Gly 0.99, Val 0.85, Leu 2.81, Phe 1.00, Lys 1.03 (average recovery 92%). *Anal.* Calcd for $C_{79}H_{115}N_{13}O_{17} \cdot 1.5H_2O$: C, 61.38; H, 7.69; N, 11.78. Found: C, 61.31; H, 7.61; N, 11.57.

Boc–Gly–Asp(OBzl)–Lys(Z)–Leu–Phe–Gly–Pro–Asp(OBzl)–Leu–Lys(Z)–Leu–Val–Pro–Pro–NH_2 (XIII-1)——This compound was prepared by the condensation of a TFA (5 ml)–treated sample of XII-4 (0.50 g) and Boc–Gly–Asp(OBzl)–Lys(Z)–OH (I-13)[1b] (0.32 g) with DCC (0.12 g) and HOBT·H_2O (0.08 g) in DMF (3 ml). After usual work-up, the resulting powder was washed batchwise with the usual acid and base and precipitated from MeOH–THF with ether; yield 0.62 g (92%), mp 201—205 °C, $[\alpha]_D^{26}$ −54.7° (c=0.6, DMF), Rf^1 0.69. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.99, Pro 2.50, Gly 2.03, Val 0.93, Leu 3.20, Phe 1.00, Lys 2.02 (average recovery 92%). *Anal.* Calcd for $C_{106}H_{147}N_{17}O_{24} \cdot 4H_2O$: C, 60.18; H, 7.39; N, 11.26. Found: C, 60.32; H, 7.09; N, 11.01.

H–Asp–Leu–Lys–Leu–Val–Pro–Pro–Met–Glu–Glu–Asp–Tyr–Pro–Gln–Phe–Gly–Ser–Pro–Lys–OH (III)——The protected nonadecapeptide ester (I-14)[1b] (817 mg) was treated with 1 M TFMSA–thioanisole in TFA (15.88 ml) in the presence of *m*-cresol (2.27 ml) in an ice-bath for 30 min, and then at room temperature for 1 5.h. The solvent was removed by evaporation *in vacuo* and then dry ether was added to the residue. The resulting powder was collected by filtration, washed with ether, and dried over KOH pellets *in vacuo* for 1 h. The deprotected crude peptide was dissolved in H_2O (8 ml) containing a few drops of acetic acid, and treated with Amberlite IRA-410 (acetate form) for 30 min in an ice-bath. After filtration, the filtrate was adjusted to pH 10.0 with 5% NH_4OH, stirred for 30 min in an ice-bath,[16] then readjusted to pH 4.0 with 1 N acetic acid and lyophilized to give a hygroscopic powder. This was dissolved in 1 N acetic acid (3 ml). The solution was applied to a Sephadex G-25 column (3.3 × 136 cm), which was eluted with 1 N acetic acid at a flow rate of 81.2 ml/h. The UV absorption at 275 nm was

TABLE I. Deprotection, Purification Methods and Physical Properties of the Peptides

	II	IV	V	VI	X	XI	XII	XIII
Protected peptide (mg)	120	280	200	200	200	200	200	200
Deprotection method[a]	A	A	A	A	A	A	C	C
Purification method[b]	B	B	A	A	C	C	D	D
Yield (mg)	16.8	31.2	28.8	30.0	38.0	23.0	40.0	25.0
(%)	(18%)	(15%)	(19%)	(21%)	(25%)	(14%)	(23%)	(16%)
Rf	Rf^4 0.74	Rf^4 0.70	Rf^3 0.33	Rf^3 0.32	Rf^3 0.31	Rf^3 0.21	Rf^3 0.42	Rf^3 0.11
$[\alpha]_D^{25}$ in 1 N AcOH	−91.4°	−122.0°	−107.2°	−78.8°	−137.9°	−148.0°	−141.4°	−136.9°
	$c=0.1$	$c=0.1$	$c=0.3$	$c=0.2$	$c=0.2$	$c=0.2$	$c=0.1$	$c=0.1$
Retention time on HPLC[c] (min)	18.5 (1)	13.0 (2)	16.0 (3)	19.5 (1)	23.0 (4)	24.0 (4)	15.7 (3)	16.0 (3)

a) Deprotection method A: 1 M TFMSA–thioanisole in TFA with *m*-cresol. Method B: 1) TFA, 2) H_2/Pd–C in MeOH. Method C: 1 M TFMSA–thioanisole in TFA. *b*) Purification method A: 1) Amberlite IRA 410 (acetate form), 2) 5% NH_4OH, 3) Sephadex G-25, 4) 2-mercaptoethanol, 5) DEAE–cellulose. Method B: 1—5) are the same procedures as described above in A, 6) reversed-phase HPLC. Method C: the same procedures as in B, but step 2) is omitted. Method D: 1) Amberlite IRA 410 (acetate form), 2) Sephadex G-25, 3) CM–cellulose, 4) reversed-phase HPLC. *c*) HPLC solvent system A: 0.05% TFA–H_2O. System B: 0.05% TFA–CH_3CN. (1) B: 10%—50%/30 min (1.0 ml/min). (2) B: 10%—50%/15 min (1.0 ml/min). (3) B: 10%—50%/20 min (1.0 ml/min). (4) B: 0%—50%/35 min (1.0 ml/min).

TABLE II. Elemental Analysis of the Peptides

	Formula	Analysis (%) Calcd (Found)		
		C	H	N
II	$C_{122}H_{182}N_{26}O_{35}S \cdot 3AcOH \cdot 24H_2O$	47.78 (47.96)	7.58 (7.83)	11.32 (11.03)
IV	$C_{96}H_{147}N_{21}O_{28}S \cdot 3AcOH \cdot 19H_2O$	47.16 (47.32)	7.64 (7.48)	11.32 (11.02)
V	$C_{84}H_{124}N_{18}O_{26}S \cdot 2AcOH \cdot 18H_2O$	46.39 (46.68)	7.43 (7.35)	11.07 (10.78)
VI	$C_{63}H_{90}N_{14}O_{22}S \cdot 2AcOH \cdot 15.5H_2O$	44.05 (43.94)	7.12 (7.21)	10.73 (10.58)
X	$C_{68}H_{106}N_{14}O_{20}S \cdot 2AcOH \cdot 20H_2O$	44.30 (43.66)	7.95 (8.01)	10.05 (10.23)
XI	$C_{72}H_{111}N_{15}O_{23}S \cdot 2AcOH \cdot 19H_2O$	44.54 (44.48)	7.72 (7.85)	10.25 (10.52)
XII	$C_{59}H_{95}N_{13}O_{13} \cdot 2AcOH \cdot 14.5H_2O$	48.02 (47.98)	8.44 (8.23)	11.56 (11.62)
XIII	$C_{71}H_{115}N_{17}O_{18} \cdot AcOH \cdot 17H_2O$	46.68 (46.81)	8.19 (8.25)	12.02 (11.82)

TABLE III. Amino Acid Analysis of the Peptides (6 N HCl Hydrolysis)

	II	IV	V	VI	X	XI	XII	XIII
Asp	2.07 (2)	1.01 (1)	0.95 (1)	1.06 (1)	1.02 (1)	2.14 (2)	1.03 (1)	1.87 (2)
Ser	0.84 (1)	0.91 (1)	0.63 (1)	0.78 (1)				
Glu	3.15 (3)	2.92 (3)	2.67 (3)	3.01 (3)	1.95 (2)	2.13 (2)		
Pro	5.30 (5)	4.01 (4)	3.78 (4)	2.00 (2)	3.24 (3)	3.15 (3)	2.79 (3)	2.80 (3)
Gly	1.95 (2)	0.96 (1)	1.00 (1)	1.03 (1)			1.06 (1)	1.92 (2)
Val	1.10 (1)	0.99 (1)	0.99 (1)		1.08 (1)	1.14 (1)	1.00 (1)	1.00 (1)
Met	1.08 (1)	0.96 (1)	0.85 (1)	0.98 (1)	1.00 (1)	1.02 (1)		
Leu	3.30 (3)	2.06 (2)	1.22 (1)		2.08 (2)	2.11 (2)	3.13 (3)	2.80 (3)
Tyr	0.88 (1)	0.98 (1)	0.76 (1)	0.96 (1)	1.04 (1)	1.07 (1)		
Phe	2.00 (2)	1.00 (1)	1.00 (1)	1.00 (1)			1.07 (1)	1.00 (1)
Lys	2.10 (2)	1.96 (2)	0.94 (1)	1.06 (1)	1.00 (1)	1.00 (1)	0.99 (1)	1.80 (2)
Rec. (%)	90	91	90	93	92	90	93	91

determined for each fraction (10 ml). The fractions corresponding to the front main peak (tube Nos. 66—84) were combined and the solvent was removed by lyophilization to give a white fluffy powder.

The above product was dissolved in H_2O (10 ml), then incubated with 2-mercaptoethanol (0.70 ml) under an N_2 atmosphere at 70 °C for 18 h.[17] H_2O was added to this mixture, and the solvent was lyophilized. The crude product was dissolved in H_2O (4 ml), and the solution was applied to a column of DEAE-cellulose (2.5 × 40 cm), which was eluted first with 0.02 M NH_4HCO_3, and then with a linear gradient formed from 0.02 M NH_4HCO_3 (800 ml) and 0.25 M NH_4HCO_3 (800 ml) at a flow rate of 79.2 ml/h. The UV absorption in each fraction (11.3 ml) was determined. The fractions corresponding to the main peak (tube Nos. 97—110) were combined and the solvent and ammonium salt were removed by repeated lyophilization. The powder obtained was dissolved in 1 N acetic acid and lyophilized to give the acetate as a white fluffy powder; yield 114 mg (19.3%), $[\alpha]_D^{25}$ −109.6° (c = 0.3, 1 N AcOH), Rf^4 0.77. The synthetic peptide (III) thus purified exhibited a single peak on HPLC at a retention time of 14.9 min on a Cosmosil $5C_{18}$-P column (4.6 × 150 mm). Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.04 (2), Ser 0.77 (1), Glu 2.88 (3), Pro 3.68 (4), Gly 1.02 (1), Val 1.05 (1), Met 0.78 (1), Leu 2.05 (2), Tyr 0.86 (1), Phe 1.00 (1), Lys 2.01 (2) (recovery 91%). *Anal.* Calcd for $C_{100}H_{152}N_{22}O_{31}S \cdot 3AcOH \cdot 19H_2O$: C, 46.93; H, 7.51; N, 11.36. Found: 47.01; H, 7.59; N, 11.13.

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References and Notes

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2) Amino acids and peptides and their derivatives mentioned in this paper are of the L-configuration. The following abbreviations were used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Boc = *tert*-butoxycarbonyl, Np = *p*-nitrophenyl, CM = carboxymethyl, DCC = dicyclohexylcarbodiimide, DCurea = dicyclohexylurea, DEAE = diethylaminoethyl, DMF = dimethylformamide, DMSO = dimethylsulfoxide, AcOEt = ethyl acetate, HPLC = high performance liquid chromatography, IPE = isopropyl ether, MeOH = methanol, THF = tetrahydrofuran, TLC = thin-layer chromatography, Et_3N = triethylamine, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, UV = ultraviolet.
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