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Synthesis of the Docosapeptide corresponding to the Amino Acid Sequence of Ostrich-type Corticotropin-like Intermediate Lobe Peptide (CLIP)[1)]

Koichi Yasumura,*,[a] Kenji Okamoto,[a] Shinichi Shimamura[a] and Haruaki Yajima[b]

Kyoto College of Pharmacy,[a] Yamashina-ku, Kyoto, 607, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,[b] Sakyo-ku, Kyoto, 606, Japan

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The docosapeptide, H–Arg–Pro–Val–Lys–Val–Tyr–Pro–Asn–Gly–Val–Gln–Glu–Glu–Thr–Ser–Glu–Gly–Phe–Pro–Leu–Glu–Phe–OH, corresponding to positions 18 to 39 of ostrich ACTH (ostrich-type corticotropin-like intermediate lobe peptide, ost-CLIP), was synthesized, using 1 M trifluoromethanesulfonic acid–thioanisole in TFA as a deprotecting reagent.

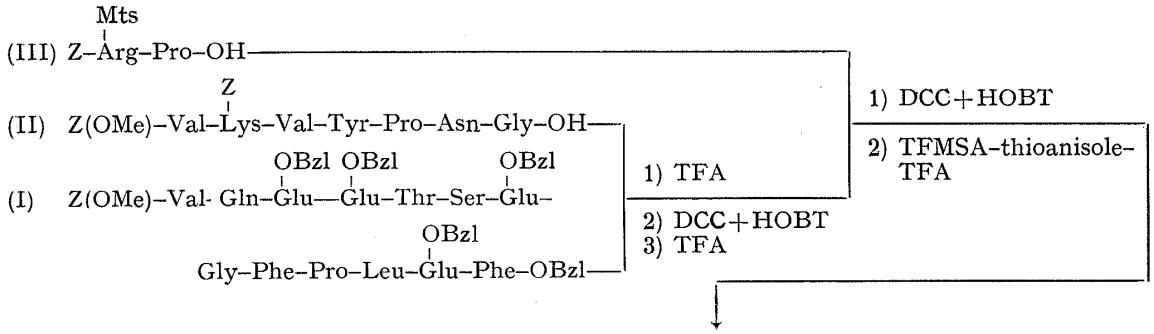
Keywords——ostrich ACTH; ostrich-type CLIP; trifluoromethanesulfonic acid–thioanisole in TFA as a deprotecting system; *m*-cresol as a scavenger; triphenylphosphite–imidazole condensation; mesitylene-2-sulfonylarginine

In 1973, Scott *et al.*[2)] reported the isolation of the docosapeptide corresponding to positions 18 to 39 of adrenocorticotropin (ACTH) from the pars intermedia of pig pituitary, and suggested that this immunoreactive peptide, termed corticotropin-like intermediate lobe peptide (CLIP), is formed by the intracellular cleavage of ACTH as a precursor. Subsequently, Lowry, Scott *et al.*[3)] isolated CLIP from dogfish pituitary, and recently Kawauchi *et al.*[4)] isolated CLIP from salmon pituitary. So far, in addition to porcine CLIP[5)] and dogfish CLIP,[6)] bovine-type[7)] and human-type[8)] CLIPs have also been synthesized.

In 1978, Li *et al.*[9)] determined the amino acid sequence of ostrich (*Struthio camelus*) ACTH. The C-terminal portion of this hormone is somewhat different from that of ACTHs[3,10)] so far known. Most of the structural difference between ostrich ACTH and mammalian ACTHs resides within this segment. Amino acid replacement from mammalian species can be seen at positions, 15, 27, 28, 29, 31, 32 and 34 in ostrich ACTH.

As a step towards its total synthesis, we wish to report the synthesis of the docosapeptide corresponding to positions 18 to 39 of ostrich ACTH, which we designate as ostrich-type CLIP.

In the present synthesis, trifluoromethanesulfonic acid (TFMSA)–thioanisole in TFA[11)] was employed as a deprotecting reagent in the final step of the synthesis. Thus, amino acid derivatives bearing protecting groups removable by a combination of these two reagents were adopted; *i.e.*, Arg(Mts),[12)] Lys(Z) and Glu(OBzl). These side-chain protecting groups survive mostly intact under careful TFA treatment for the removal of the Z(OMe) group,[13)]



H–Arg–Pro–Val–Lys–Val–Tyr–Pro–Asn–Gly–Val–Gln–Glu–Glu–Thr–Ser–Glu–Gly–Phe–Pro–Leu–Glu–Phe–OH (ostrich-type CLIP)

Fig. 1. Synthetic Route to Ostrich-type CLIP

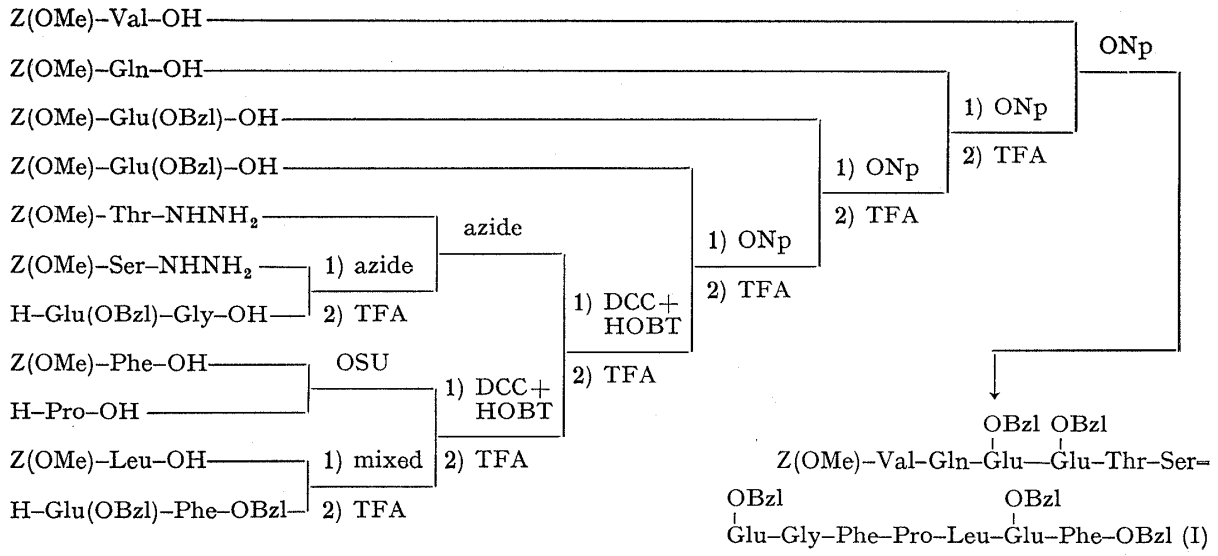


Fig. 2. Synthetic Route to the C-Terminal Tridecapeptide Ester, Z(OMe)-(ostrich ACTH 27—39)-OBzl (I)

employed as a temporary α-amino protecting group. As shown in Fig. 1, three fragments served to construct the entire sequence of the ostrich-type CLIP, *i.e.*, Z-Arg(Mts)-Pro-OH (III), Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-OH (II) and Z(OMe)-Val-Gln-Glu-(OBzl)-Glu(OBzl)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl (I).

The synthetic route to the C-terminal tridecapeptide ester (I), abbreviated as Z(OMe)-(ost-ACTH 27—39)-OBzl, is illustrated in Fig. 2. In order to synthesize this fragment (I), the C-terminal protected pentapeptide ester, Z(OMe)-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, was first prepared by the DCC/HOBT condensation[14] of two peptide subunits, Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl[7] and Z(OMe)-Phe-Pro-OH. The former was alternatively prepared by condensation of Z(OMe)-Leu-OH and H-Glu(OBzl)-Phe-OBzl[7] by the mixed anhydride procedure[15] and the latter was prepared by the N-hydroxysuccinimide ester procedure.[16] The protected pentapeptide ester was obtained in analytically pure form after purification by column chromatography on silica. Z(OMe)-Thr-Ser-Glu(OBzl)-Gly-OH (positions 31 to 34) was next prepared. Starting with the known dipeptide, Z(OMe)-Glu(OBzl)-Gly-OH,[17] Z(OMe)-Ser-$NHNH_2$ and Z(OMe)-Thr-$NHNH_2$ were introduced stepwisely by the modified azide procedure[18] without particular difficulty. This tetrapeptide was then condensed with a TFA-treated sample of the protected pentapeptide ester obtained above by DCC in the presence of HOBT to give the protected nonapeptide ester, Z(OMe)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, in nearly quantitative yield. Next, the Np method[19] was employed to introduce stepwisely 2 residues of Z(OMe)-Glu(OBzl)-OH, and one residue each of Z(OMe)-Gln-OH and Z(OMe)-Val-OH to afford the desired fragment (I). All intermediates were purified simply by precipitation from AcOEt or THF with ether.

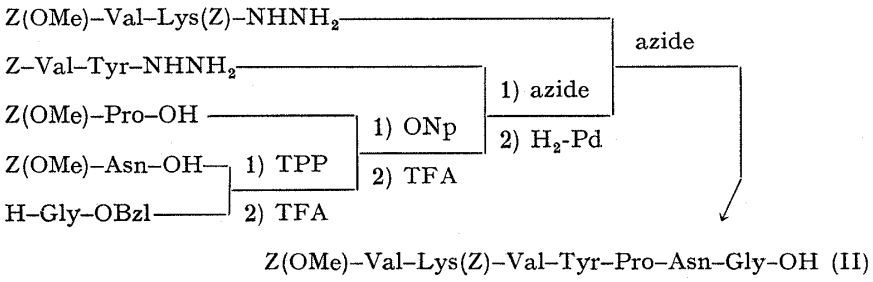


Fig. 3. Synthetic Route to the Protected Heptapeptide, Z(OMe)-(ostrich ACTH 20—26)-OH (II)

The next fragment (II),[8] abbreviated as Z(OMe)–(ost–ACTH 20—26)–OH, is identical with the one employed for the synthesis of mammalian CLIPs. The previous route was slightly modified as shown in Fig. 3. Z(OMe)–Asn–Gly–OBzl was prepared by the direct condensation of Z(OMe)–Asn–OH with H–Gly–OBzl by the triphenylphosphite (TPP)-imidazole procedure,[20] without accompanying formation of the nitrile derivative, which is a known by-product of the Asn residue.[19,21] Z(OMe)–Pro–OH and Z–Val–Tyr–$NHNH_2$[22] were then introduced stepwisely into this dipeptide ester by the Np method and the modified azide method, respectively, to give the protected pentapeptide ester, Z–Val–Tyr–Pro–Asn–Gly–OBzl. At this stage, two protecting groups of the pentapeptide ester, Z and Bzl, were removed by catalytic hydrogenation to give the known free pentapeptide, H–Val–Tyr–Pro–Asn–Gly–OH.[8] This peptide was then elaborated into the fragment (II) by condensation with Z(OMe)–Val–Lys(Z)–$NHNH_2$[23] as reported previously.

Next, for the synthesis of the N-terminal dipeptide unit, a new arginine derivative, Arg(Mts), was used. Z–Arg(Mts)–OH was smoothly condensed with H–Pro–OMe by the mixed anhydride procedure and the resulting dipeptide ester was subsequently saponified to afford Z–Arg(Mts)–Pro–OH (III).

The three fragments thus obtained were then assembled according to the route illustrated in Fig. 1. For the condensation of II and I, the Z(OMe) group of the latter was cleaved by TFA treatment in the presence of anisole as usual and the resulting TFA salt was converted to the free base by precipitation from DMF with H_2O containing Et_3N. The resulting N^{α}-deprotected tridecapeptide ester was coupled with the fragment II by the DCC/HOBT procedure to give the protected eicosapeptide ester, Z(OMe)–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–Val–Gln–Glu(OBzl)–Glu(OBzl)–Thr–Ser–Glu(OBzl)–Gly–Phe–Pro–Leu–Glu(OBzl)–Phe–OBzl, abbreviated as Z(OMe)–(ost–ACTH 20—39)–OBzl, which was purified by recrystallization from DMF and ethanol. Its purity was assessed by thin-layer chromatography, 4 N MSA hydrolysis[24] and elemental analysis.

Condensation of the protected eicosapeptide ester, Z(OMe)–(ost–ACTH 20—39)–OBzl, and the fragment III was then carried out essentially in the same manner. The resulting protected docosapeptide ester, Z–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–Val–Gln–Glu(OBzl)–Glu(OBzl)–Thr–Ser–Glu(OBzl)–Gly–Phe–Pro–Leu–Glu(OBzl)–Phe–OBzl, was purified by column chromatography on silica and its homogeneity was again confirmed by three criteria; *i.e.*, thin-layer chromatography, elemental analysis and acid hydrolysis with 4 N MSA.

The protected docosapeptide ester thus obtained was treated with 1 M TFMSA–thioanisole (1 : 1) in TFA in an ice-bath for 60 min and at room temperature for 30 min to remove all the protecting groups. As a cation scavenger, *m*-cresol was employed to suppress a possible side reaction at the Tyr residue, *i.e.*, O-mesitylene-2-sulfonylation.[12] The deprotected peptide was converted to the corresponding acetate by treatment with Amberlite IR-45 (acetate form) and purified by ion-exchange chromatography on CM-cellulose with gradient elution up to 0.05 M ammonium acetate buffer, pH 6.9, followed by gel-filtration on Sephadex G-25. The absorbancy at 275 nm due to the Tyr residue was used as a marker during this purification. The homogeneity of the synthetic ostrich-type CLIP thus obtained was assessed by thin-layer chromatography, elemental analysis and 4 N MSA hydrolysis. Despite the presence of the Pro residues,[25] complete digestion of this synthetic peptide with commercial aminopeptidase (AP–M)[26] was achieved and the presence of Asn and Gln residues in the desired product was thus confirmed.

The physiological roles of CLIP remain to be clarified. Our synthetic peptide may thus aid in the clarification of complex pituitary functions in future studies.

Experimental

Melting points were determined on a Yamato melting point apparatus, model MP-21, and are uncorrected. Rotations were measured with a Union automatic polarimeter, model P-101 (cell length: 1 cm). The amino

acid compositions of acid hydrolysates and aminopeptidase digests were determined with a Hitachi liquid chromatograph, model 034. Concentration of solutions was carried out in a rotary evaporator under reduced pressure, unless otherwise mentioned, at a temperature of 40 to 50°. Thin-layer chromatography was performed on silica gel (Kieselgel 60 F 254, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (29: 1), Rf_2 $CHCl_3$–MeOH (9: 1), Rf_3 $CHCl_3$–MeOH–H_2O (18: 3: 1, lower phase), Rf_4 $CHCl_3$–MeOH–H_2O (8: 3: 1, lower phase), Rf_5 *n*-butanol–AcOH–pyridine–H_2O (4: 1: 1: 2) and Rf_6 *n*-butanol–AcOH–pyridine–H_2O (15: 3: 10: 12).

Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl——Z(OMe)–Glu(OBzl)–Phe–OBzl[7] (23.0 g) was treated with TFA (25 ml) in the presence of anisole (12 ml) at 0° for 60 min, then dry ether–petroleum ether (1: 1, v/v) was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (70 ml). The solution was neutralized with Et_3N (4.9 ml). Next, Et_3N (5.0 ml) and isobutyl chloroformate (4.8 ml) were added to a solution of Z(OMe)–Leu–OH (10.6 g) in DMF (50 ml) at −10°. After being stirred for 10 min, this solution was added to the above solution containing the amino component and the mixture was stirred at room temperature for 40 hr. The solvent was evaporated off *in vacuo* and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was recrystallized from AcOEt and petroleum ether; yield 23.1 g (86.5%), mp 106—109°, $[\alpha]_D^{20}$ −21.6° (c=1.02, MeOH), (lit.[7] prepared by the DCC procedure, mp 87—88°, $[\alpha]_D^{25}$ −10.0° (c=1.0, MeOH)). Rf_1 0.82. *Anal.* Calcd for $C_{43}H_{49}N_3O_9$: C, 68.69; H, 6.57; N, 5.59. Found: C, 68.67; H, 6.55; N, 5.45.

Z(OMe)-Phe-Pro-OH——A solution of Z(OMe)–Phe–OSU (17.0 g) in THF (40 ml) was added to a solution of H–Pro–OH (5.52 g) and Et_3N (12.2 ml) in H_2O (40 ml), and the mixture was stirred at room temperature for 44 hr. The solvent was evaporated off *in vacuo* and the residue was dissolved in H_2O. This solution was washed with AcOEt, then acidified with citric acid and the resulting precipitate was extracted with AcOEt. The organic phase was washed with 0.5 M citric acid, H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. The solid formed by addition of petroleum ether was recrystallized from AcOEt and petroleum ether; yield 15.6 g (91.8%), mp 125—127°, $[\alpha]_D^{25}$ −35.8° (c=1.20, DMF). Rf_4 0.50. *Anal.* Calcd for $C_{23}H_{26}N_2O_6$: C, 64.78; H, 6.15; N, 6.57. Found: C, 64.59; H, 6.14; N, 6.28.

Z(OMe)-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——Z(OMe)–Leu–Glu(OBzl)–Phe–OBzl (22.0 g) was treated with TFA (30 ml) in the presence of anisole (8.5 ml) at 0° for 60 min, then the excess TFA was evaporated off *in vacuo* at room temperature. Next, 5% $NaHCO_3$ was added to the residue under cooling with ice–NaCl. The resulting precipitate was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo* (bath temperature 30°). DCC (6.63 g) and HOBT (4.67 g) were added to an ice-chilled solution of Z(OMe)–Phe–Pro–OH (12.5 g) in DMF (40 ml), and the whole was stirred in an ice-bath for 30 min. Next, a solution of the above tripeptide ester in DMF (20 ml) was added and the mixture was stirred at room temperature for 24 hr. After filtration, the solvent was evaporated off *in vacuo* and the residue was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was purified by column chromatography on silica (6.5 × 20 cm) using the solvent system of $CHCl_3$–MeOH (70: 1); yield 21.3 g (72.9%), oily product, $[\alpha]_D^{18}$ −35.0° (c=1.2, DMF), Rf_1 0.71. Amino acid ratios in 4 N MSA hydrolysate: Phe 1.89, Pro 0.85, Leu 1.00, Glu 1.04 (recovery of Leu, 98.0%). *Anal.* Calcd for $C_{57}H_{65}N_5O_{11}$: C, 68.73; H, 6.58; N, 7.03. Found: C, 68.98; H, 6.53; N, 6.76.

Z(OMe)-Ser-Glu(OBzl)-Gly-OH——Z(OMe)–Glu(OBzl)–Gly–OH[17] (45.8 g) was treated with TFA (80 ml) in the presence of anisole (25 ml) at 0° for 60 min and then dry ether–petroleum ether (1: 1, v/v) was added. The resulting oily precipitate was washed with ether, dried over KOH pellets *in vacuo* and then dissolved in DMF (50 ml). The solution, after neutralization with Et_3N (13.9 ml), was kept under cooling with ice, until the following azide was ready. Under cooling with ice–NaCl, Z(OMe)–Ser–$NHNH_2$ (34.0 g) was dissolved in DMF (30 ml) with the aid of 3.43 N HCl/DMF (70.0 ml). To this chilled solution (−5°), isoamyl nitrite (14.2 ml) was added. The mixture was stirred for 10 min, when the hydrazine test of the solution became negative, then Et_3N (33.4 ml) was added. This solution was then combined with the above solution containing the amino component and the mixture, after further addition of Et_3N (16.7 ml), was stirred at 4° for 42 hr then filtered. The filtrate was concentrated *in vacuo* and the residue was treated with 0.5 M citric acid. The resulting solid was washed batchwisely with 0.5 M citric acid and H_2O and then recrystallized from AcOEt and ether; yield 39.6 g (72.5%), mp 113—117°, $[\alpha]_D^{28}$ +9.00° (c=1.00, DMF), Rf_4 0.16. *Anal.* Calcd for $C_{26}H_{31}N_3O_{10}\cdot H_2O$: C, 55.41; H, 5.90; N, 7.46. Found: C, 55.00; H, 5.49; N, 7.40.

Z(OMe)-Thr-Ser-Glu(OBzl)-Gly-OH——Z(OMe)–Ser–Glu(OBzl)–Gly–OH (10.0 g) was treated with TFA (20 ml) in the presence of anisole (5.0 ml) at 0° for 60 min, then dry ether–petroleum ether (1: 1, v/v) was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (10 ml). To this ice-chilled solution, Et_3N (5.6 ml) and the azide (prepared from 6.5 g of Z(OMe)–Thr–$NHNH_2$ with 12.8 ml of 3.43 N HCl/DMF, 2.6 ml of isoamyl nitrite and 6.1 ml of Et_3N) in DMF (10 ml) were added and the mixture was stirred at 4° for 20 hr. The solution, after filtration, was concentrated *in vacuo*. The residue was dissolved in 3% NH_4OH. This solution was washed with AcOEt, then acidified with citric acid and the resulting precipitate was extracted with AcOEt. The organic phase was washed with H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was recrystallized from AcOEt

and ether; yield 9.0 g (76.3%), mp 133—136°, $[\alpha]_D^{28}$ +2.78° (c=0.72, DMF), Rf_4 0.12. Amino acid ratios in 4 N MSA hydrolysate: Thr 1.02, Ser 0.96, Glu 1.00, Gly 0.93 (recovery of Glu, 93.6%). *Anal.* Calcd for $C_{30}H_{38}N_4O_{12}\cdot 1.5\,H_2O$: C, 53.49; H, 6.13; N, 8.32. Found: C, 53.77; H, 5.80; N, 8.15.

Z(OMe)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——Z(OMe)–Phe–Pro–Leu–Glu(OBzl)-Phe–OBzl (13.5 g) was treated with TFA (30 ml) in the presence of anisole (7.0 ml) at 0° for 60 min. The excess TFA was evaporated off *in vacuo* (bath temperature 20°) and 5% $NaHCO_3$ was added to the residue under cooling with ice–NaCl. The resulting precipitate was extracted with AcOEt. The extract was washed with 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo* (bath temperature 30°). The residue was converted to a powder with ether. Next, DCC (3.34 g) and HOBT (2.93 g) were added to an ice-chilled solution of Z(OMe)–Thr–Ser–Glu(OBzl)–Gly–OH (9.65 g) in DMF (20 ml), and the mixture was stirred in an ice-bath for 30 min. To this solution, the above pentapeptide ester was added and the mixture was stirred at room temperature for 40 hr, then filtered. The solvent was evaporated off *in vacuo* and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was precipitated from $CHCl_3$ with ether; yield 18.6 g (94.4%), mp 89—94°, $[\alpha]_D^{29}$ −27.3° (c=1.03, DMF), Rf_2 0.54. Amino acid ratios in 4 N MSA hydrolysate: Thr 0.99, Ser 0.95, Glu 2.11, Gly 1.00, Phe 2.11, Pro 1.06, Leu 1.00 (recovery of Leu, 93.0%). *Anal.* Calcd for $C_{78}H_{93}N_9O_{19}$: C, 64.14; H, 6.42; N, 8.63. Found: C, 64.11; H, 6.29; N, 8.56.

Z(OMe)-Glu(OBzl)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——The above protected nonapeptide ester (18.5 g) was treated with TFA (40 ml) in the presence of anisole (10 ml) at 0° for 60 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (30 ml). To this solution, Et_3N (3.9 ml), Z(OMe)–Glu(OBzl)–ONp (7.9 g) and HOBT (60 mg) were added and the mixture was stirred at room temperature for 40 hr. The solvent was evaporated off *in vacuo* and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was precipitated from AcOEt with ether; yield 20.7 g (97.2%), mp 96—99°, $[\alpha]_D^{29}$ −19.8° (c=1.06, DMF), Rf_2 0.58. Amino acid ratios in 4 N MSA hydrolysate: Glu 3.35, Thr 1.07, Ser 0.99, Gly 1.04, Phe 2.08, Pro 1.01, Leu 1.00 (recovery of Leu, 89.9%). *Anal.* Calcd for $C_{90}H_{106}N_{10}O_{22}$: C, 64.35; H, 6.36; N, 8.34. Found: C, 64.18; H, 6.30; N, 8.17.

Z(OMe)-Glu(OBzl)-Glu(OBzl)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——In the usual manner, the above protected decapeptide ester (20.5 g) was treated with TFA (40 ml) in the presence of anisole (10 ml) at 0° for 60 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (30 ml). Et_3N (3.7 ml), Z(OMe)–Glu(OBzl)–ONp (7.6 g) and HOBT (50 mg) were added to the resulting solution. The mixture was stirred at room temperature for 40 hr and then the solvent was evaporated off *in vacuo*. The residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$, 0.5 M citric acid and H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The product was precipitated from THF with ether; yield 21.1 g (90.9%), mp 154—155°, $[\alpha]_D^{28}$ −31.1° (c=0.52, DMF), Rf_2 0.53, Rf_3 0.64. Amino acid ratios in 4 N MSA hydrolysate: Glu 4.37, Thr 0.99, Ser 0.93, Gly 1.06, Phe 2.11, Pro 1.08, Leu 1.00 (recovery of Leu, 86.9%). *Anal.* Calcd for $C_{102}H_{119}N_{11}O_{25}$: C, 64.51; H, 6.32; N, 8.11. Found: C, 64.71; H, 6.41; N, 8.00.

Z(OMe)-Gln-Glu(OBzl)-Glu(OBzl)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——As usual, the above protected undecapeptide ester (20.8 g) was treated with TFA (40 ml) in the presence of anisole (10 ml) at 0° for 60 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (30 ml). Et_3N (3.2 ml), Z(OMe)–Gln–ONp (5.2 g) and HOBT (50 mg) were added to the resulting solution. After the solution had been stirred at room temperature for 24 hr, the solvent was evaporated off *in vacuo* and the residue was treated with ether. The resulting powder was washed with H_2O and then precipitated from THF with ether; yield 20.4 g (91.5%), mp 180—181°, $[\alpha]_D^{28}$ −21.7° (c=0.83, DMF), Rf_3 0.47. Amino acid ratios in 4 N MSA hydrolysate: Glu 5.38, Thr 0.97, Ser 0.92, Gly 1.04, Phe 2.09, Pro 1.09, Leu 1.00 (recovery of Leu, 81.5%). *Anal.* Calcd for $C_{107}H_{127}N_{13}O_{27}$: C, 63.39; H, 6.31; N, 8.98. Found: C, 63.19; H, 6.21; N, 8.81.

Z(OMe)-Val-Gln-Glu(OBzl)-Glu(OBzl)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl (I)——The above protected dodecapeptide ester (4.0 g) was treated with TFA (8.0 ml) in the presence of anisole (2.0 ml) as described above. The powder formed by addition of dry ether was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (10 ml). To this solution, Et_3N (0.57 ml), Z(OMe)–Val–ONp (0.87 g) and HOBT (10 mg) were added and the mixture was stirred at room temperature for 20 hr. After the solvent had been evaporated off *in vacuo*, addition of ether to the residue afforded a powder, which was precipitated from DMF with ether. For further purification, the product was applied to a column of silica (5.5×15 cm), which was eluted with the solvent system of $CHCl_3$–MeOH–H_2O (120: 10: 1). The desired compound, obtained from the column eluate, was precipitated from DMF with ether; yield 2.45 g (58.3%), mp 208—209°, $[\alpha]_D^{18}$ −18.0° (c=1.00, DMF), Rf_3 0.38. Amino acid ratios in 4 N MSA hydrolysate: Val 0.96, Glu 5.11, Thr 0.99, Ser 0.89, Gly 0.91, Phe 1.95, Pro 0.70, Leu 1.00 (recovery of Leu, 90.5%). *Anal.* Calcd for $C_{112}H_{136}N_{14}O_{28}$: C, 63.26; H, 6.45; N, 9.22. Found: C, 63.13; H, 6.48; N, 9.18.

Z(OMe)-Asn-Gly-OBzl——Imidazole (5.44 g) and triphenylphosphite (24.8 g) were added to a solution of Z(OMe)–Asn–OH (14.8 g), H–Gly–OBzl tosylate (16.9 g) and Et_3N (7.0 ml) in DMF (80 ml). After being

stirred at 40° for 48 hr, the solution was concentrated *in vacuo*. Ether was added to the residue to form a solid, which was collected by filtration, washed batchwisely with 0.5 M citric acid and H_2O and then recrystallized from MeOH and ether; yield 13.4 g (60.4%), mp 167—168°, $[\alpha]_D^{22}$ −3.00° (c=1.17, DMF). No CN absorption band was seen in infrared spectrum. (lit.[8] prepared by the Np method, mp 164—167°, $[\alpha]_D^{26}$ −3.1° (c=0.8, DMF)). Rf_3 0.43. *Anal.* Calcd for $C_{22}H_{25}N_3O_7$: C, 59.59; H, 5.68; N, 9.48. Found: C, 59.33; H, 5.78; N, 9.50.

Z(OMe)-Pro-Asn-Gly-OBzl——Z(OMe)–Asn–Gly–OBzl (23.9 g) was treated with TFA (50.0 ml) in the presence of anisole (12.0 ml) at 0° for 60 min. The gummy product precipitated by addition of dry ether–petroleum ether (1: 1, v/v) was treated with dry ether to give a fine powder, which was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (60 ml). To this solution, Et_3N (16.5 ml) and Z(OMe)–Pro–ONp (25.9 g) were added. The mixture was stirred at room temperature for 20 hr, the solvent was evaporated off *in vacuo*, and the residue was treated with H_2O. The resulting powder was washed batchwisely with 5% $NaHCO_3$, 0.5 M citric acid and H_2O and then recrystallized from DMF, MeOH and ether; yield 19.9 g (68.2%), mp 160—161°, $[\alpha]_D^{18}$ −43.1° (c=1.02, DMF). Rf_3 0.46. *Anal.* Calcd for $C_{27}H_{32}N_4O_8$: C, 59.99; H, 5.97; N, 10.36. Found: C, 60.21; H, 5.95; N, 10.34.

Z-Val-Tyr-Pro-Asn-Gly-OBzl——Z(OMe)–Pro–Asn–Gly–OBzl (17.2 g) was treated with TFA (35 ml) in the presence of anisole (8.0 ml) at 0° for 60 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (20 ml). To this ice-cold solution, Et_3N (4.42 ml) and the azide (prepared from 15.0 g of Z–Val–Tyr–$NHNH_2$[22] with 20.4 ml of 3.43 N HCl/DMF, 4.13 ml of isoamyl nitrite and 9.73 ml of Et_3N) in DMF (20 ml) were added and the mixture, after further addition of Et_3N (4.87 ml), was stirred at 4° for 75 hr. The solution was filtered, the filtrate was concentrated *in vacuo*, and the residue was treated with H_2O. The resulting powder was washed batchwisely with ether, 5% $NaHCO_3$, 0.5 M citric acid and H_2O and then recrystallized from DMF, MeOH and ether; yield 17.3 g (70.3%), mp 212—214°, $[\alpha]_D^{18}$ −32.7° (c=1.13, DMF), Rf_3 0.36. *Anal.* Calcd for $C_{40}H_{48}N_6O_{10}$: C, 62.16; H, 6.26; N, 10.87. Found: C, 62.38; H, 6.20; N, 10.70.

H-Val-Tyr-Pro-Asn-Gly-OH——In the usual manner, Z–Val–Tyr–Pro–Asn–Gly–OBzl (16.0 g) in a mixture of DMF (100 ml) and AcOH (20 ml) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. Addition of EtOH to the residue afforded a hygroscopic powder, which was precipitated from MeOH with ether; yield 11.0 g (96.5%), mp 195—198°, $[\alpha]_D^{25}$ −82.6° (c=0.46, 10% AcOH), (lit.[8] mp 194—197°, $[\alpha]_D^{27}$ −50.9° (c=0.8, 10% AcOH)). Rf_5 0.34. *Anal.* Calcd for $C_{25}H_{36}N_6O_8 \cdot 1.5\,H_2O$: C, 52.17; H, 6.83; N, 14.60. Found: C, 52.45; H, 7.13; N, 14.49.

Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-OH (II)——The title compound was synthesized as described in the literature[8] by the azide condensation of Z(OMe)–Val–Lys(Z)–$NHNH_2$[23] with H–Val–Tyr–Pro–Asn–Gly–OH. Yield 72.1%, mp 158—160°, $[\alpha]_D^{25}$ −32.3° (c=0.96, DMF). (lit.[8] mp 158—162°, $[\alpha]_D^{26}$ −32.6° (c=1.1, DMF)). Rf_4 0.09, Rf_5 0.67. Amino acid ratios in 4 N MSA hydrolysate: Val 2.03, Lys 0.99, Tyr 0.83, Pro 0.94, Asp 1.14, Gly 1.00 (recovery of Gly, 79.7%). *Anal.* Calcd for $C_{53}H_{71}N_9O_{15} \cdot 0.5H_2O$: C, 58.77; H, 6.70; N, 11.64. Found: C, 58.73; H, 6.75; N, 11.77.

Z-Arg(Mts)-Pro-OMe——Et_3N (8.26 ml) and isobutyl chloroformate (7.84 ml) were added to a solution of Z–Arg(Mts)–OH (29.1 g) in DMF (50 ml) at −10°. After being stirred for 10 min, the solution was added to a solution of H–Pro–OMe (prepared from 9.86 g of the hydrochloride and 8.26 ml of Et_3N) in DMF (30 ml) and the mixture was stirred at room temperature for 20 hr. The solvent was evaporated off *in vacuo* and the residue was dissolved in AcOEt. The solution was washed with 5% $NaHCO_3$, 1 N HCl and H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The product was purified by column chromatography on silica (4×24 cm) using $CHCl_3$–MeOH (100: 1) and then precipitated from $CHCl_3$ with petroleum ether; yield 28.3 g (79.1%), amorphous powder, $[\alpha]_D^{18}$ −31.4° (c=1.05, MeOH), Rf_2 0.52. *Anal.* Calcd for $C_{29}H_{39}N_5O_7S$: C, 57.89; H, 6.53; N, 11.64. Found: C, 57.76; H, 6.52; N, 11.56.

Z-Arg(Mts)-Pro-OH (III)——A solution of Z–Arg(Mts)–Pro–OMe (0.8 g) in MeOH (10 ml) was treated with 1 N NaOH (1.46 ml) at room temperature for 3 hr. The mixture was neutralized with 0.5 M citric acid, and the solvent was evaporated off *in vacuo*. The residue was extracted with 5% $NaHCO_3$. The extract was washed with AcOEt then acidified with citric acid, and the resulting precipitate was extracted with AcOEt. The organic phase was washed with H_2O, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was precipitated from AcOEt with petroleum ether; yield 0.67 g (85.9%), amorphous powder, $[\alpha]_D^{18}$ −30.4° (c=1.15, MeOH), Rf_4 0.22. *Anal.* Calcd for $C_{28}H_{37}N_5O_7S$: C, 57.22; H, 6.35; N, 11.92. Found: C, 57.21; H, 6.40; N, 11.72.

Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Val-Gln-Glu(OBzl)-Glu(OBzl)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——The above protected tridecapeptide ester (I) (5.10 g) was treated with TFA (10 ml) in the presence of anisole (2.5 ml) at 0° for 60 min. The excess TFA was evaporated off *in vacuo* at room temperature and the residue was dissolved in DMF (5.0 ml). To this solution, H_2O (200 ml) containing Et_3N (0.33 ml) was added and the resulting powder was collected by filtration, precipitated from THF with ether and then dried over KOH pellets *in vacuo*. Next, DCC (1.19 g) and HOBT (1.15 g) were added to an ice-chilled solution of Z(OMe)–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–OH (II) (3.09 g) in DMF (10 ml), and the solution was stirred in an ice-bath for 60 min. The above tridecapeptide ester was added to this solution and the mixture was stirred at room temperature for 40 hr. The solution, after filtration, was

concentrated *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwisely with 0.5 M citric acid and H_2O and then recrystallized twice from DMF and EtOH; yield 5.38 g (74.3%), mp 195—199°, $[\alpha]_D^{28}$ −15.7° (c=0.51, DMF), Rf_3 0.25, Rf_4 0.68. Amino acid ratios in 4 N MSA hydrolysate: Val 2.62, Lys 0.77, Tyr 0.85, Pro 2.18, Asp 1.04, Gly 2.14, Glu 5.38, Thr 0.97, Ser 0.91, Phe 2.11, Leu 1.00 (recovery of Leu, 81.4%). *Anal.* Calcd for $C_{156}H_{197}N_{23}O_{39}$: C, 62.08; H, 6.58; N, 10.67. Found: C, 61.83; H, 6.68; N, 10.64.

Z-Arg(Mts)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Val-Gln-Glu(OBzl)-Glu(OBzl)-Thr-Ser-Glu(OBzl)-Gly-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl——The above protected eicosapeptide ester (0.45 g) was treated with TFA (1.0 ml) in the presence of anisole (0.20 ml) at 0° for 60 min, then dry ether was added. The resulting powder was collected by filtration and dissolved in DMF (2.0 ml) containing Et_3N (0.02 ml). Ether was added to this solution and the resulting powder was collected by filtration and dried over KOH pellets *in vacuo*. Next, DCC (0.046 g) and HOBT (0.039 g) were added to an ice-chilled solution of Z–Arg(Mts)–Pro–OH (III) (0.105 g) in DMF (2.0 ml), and the solution was stirred in an ice-bath for 60 min. The above eicosapeptide ester was added to this solution, and the mixture was stirred at room temperature for 42 hr. The mixture, after filtration, was concentrated *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwisely with 5% $NaHCO_3$, 0.5 M citric acid and H_2O and then recrystallized from DMF and EtOH. The product was further purified by column chromatography on silica (2.2×20 cm) using the solvent system of $CHCl_3$–MeOH–H_2O (120: 10: 1). The product was finally precipitated from DMF with EtOH; yield 0.37 g (72.5%), mp 176—179°, $[\alpha]_D^{28}$ −10.2° (c=0.30, DMF), Rf_3 0.16, Rf_4 0.73. Amino acid ratios in 4 N MSA hydrolysate: Arg 1.05, Pro 3.23, Val 2.88, Lys 0.92, Tyr 0.80, Asp 1.01, Gly 2.04, Glu 5.39, Thr 1.07, Ser 1.02, Phe 2.05, Leu 1.00 (recovery of Leu, 86.7%). *Anal.* Calcd for $C_{175}H_{224}N_{28}O_{42}S \cdot 2H_2O$: C, 60.75; H, 6.64; N, 11.34. Found: C, 60.79; H, 6.61; N, 11.10.

H-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Val-Gln-Glu-Glu-Thr-Ser-Glu-Gly-Phe-Pro-Leu-Glu-Phe-OH, Ostrich-type CLIP——The above protected docosapeptide ester (61 mg) was treated with 1 M TFMSA–thioanisole (1: 1) in TFA (1.8 ml) in the presence of *m*-cresol (0.15 ml) in an ice-bath for 60 min and at room temperature for 30 min, and then dry ether was added. The resulting powder was collected by filtration, washed with ether and dissolved in H_2O (10 ml). The solution was treated with Amberlite IR-45 (acetate form, approximately 5 g) for 30 min with stirring, then the resin was removed by filtration. The filtrate was applied to a column of CM-cellulose (2.2×15 cm), which was eluted with H_2O (100 ml) and then with a gradient to 0.05 M ammonium acetate buffer (pH 6.9) through a mixing flask containing H_2O (100 ml). Individual fractions (10 ml each) were collected and the absorbancy at 275 nm was determined. The fractions corresponding to the main peak (tube Nos. 26—27) were combined and the solvent was removed by evaporation. The residue was dissolved in 0.1 N AcOH (2 ml) and the solution was applied to a column of Sephadex G-25 (3.5×64 cm), which was eluted with the same solvent. Ultraviolet (UV) absorption at 275 nm was measured in each fraction (5 ml). The fractions corresponding to the main peak (tube Nos. 51—57) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 18.4 mg (36.7%), $[\alpha]_D^{15}$ −100° (c=0.12, H_2O), Rf_5 0.30, Rf_6 0.35. Amino acid ratios in 4 N MSA hydrolysate: Arg 0.83, Pro 3.53, Val 2.79, Lys 0.93, Tyr 0.71, Asp 1.04, Gly 2.12, Glu 5.55, Thr 1.00, Ser 0.94, Phe 2.03, Leu 1.00 (recovery of Leu, 85.7%). Amino acid ratios in aminopeptidase (AP-M, Merck, Lot No. 0040347) digest (numbers in parentheses are theoretical values): Arg 1.02 (1), Pro 3.02 (3), Val 2.94 (3), Lys 1.21 (1), Tyr 0.80 (1), Asn+Ser 1.75 (1+1 calcd. as Ser), Gly 2.03 (2), Gln+Thr 1.76 (1+1 calcd. as Thr), Glu 4.45 (4), Phe 1.97 (2), Leu 1.00 (1) (recovery of Leu, 74.0%). *Anal.* Calcd for $C_{115}H_{172}N_{28}O_{36} \cdot 2CH_3COOH \cdot 10H_2O$: C, 50.63; H, 7.14; N, 13.89. Found: C, 50.31; H, 6.69; N, 14.22.

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References and Notes

1) Amino acids, peptides and their derivatives (except glycine) mentioned in this paper are of the L-configuration. Abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, OBzl=benzyl ester, ONp=*p*-nitrophenyl ester. Other abbreviations used are: Mts=mesitylene-2-sulfonyl, DCC=N,N′-dicyclohexylcarbodiimide, OSU=N-hydroxysuccinimide ester, HOBT=N-hydroxybenzotriazole, TFA=trifluoroacetic acid, MSA=methanesulfonic acid, DMF=dimethylformamide, THF=tetrahydrofuran.
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