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# Synthesis of the Nonatriacontapeptide corresponding to the Entire Amino Acid Sequence of Ostrich Adrenocorticotropic Hormone[1)]

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The nonatriacontapeptide corresponding to the entire amino acid sequence of ostrich adrenocorticotropic hormone (ACTH) was synthesized by successive condensations of four peptide fragments; Z(OMe)-(15—19)-OH, Z(OMe)-(11—14)-OH, Boc-(5—10)-OH and Z-(1—4)-$NHNH_2$, with H-(20—39)-OBzl, a synthetic intermediate of ostrich-type corticotropin-like intermediate lobe peptide, followed by deprotection with 1 M trifluoromethanesulfonic acid-thioanisole in TFA. The synthetic peptide exhibited an *in vivo* steroidogenic potency of 0.8 relative to synthetic human ACTH.

**Keywords**——ostrich ACTH; trifluoromethanesulfonic acid-thioanisole in TFA as a deprotecting system; *m*-cresol as a scavenger; pentachlorophenyl trichloroacetate condensation; mesitylene-2-sulfonylarginine; *in vivo* steroidogenic activity

In 1978, Li *et al.*[2)] determined the amino acid sequence of a new adrenocorticotropic hormone (ACTH) isolated from the pituitary gland of the ostrich, *Struthio camelus*. Thus, the primary structure of an ACTH from an avian species was disclosed for the first time. In comparison with the structure of human ACTH, among mammalian ACTHs so far known,[3)]

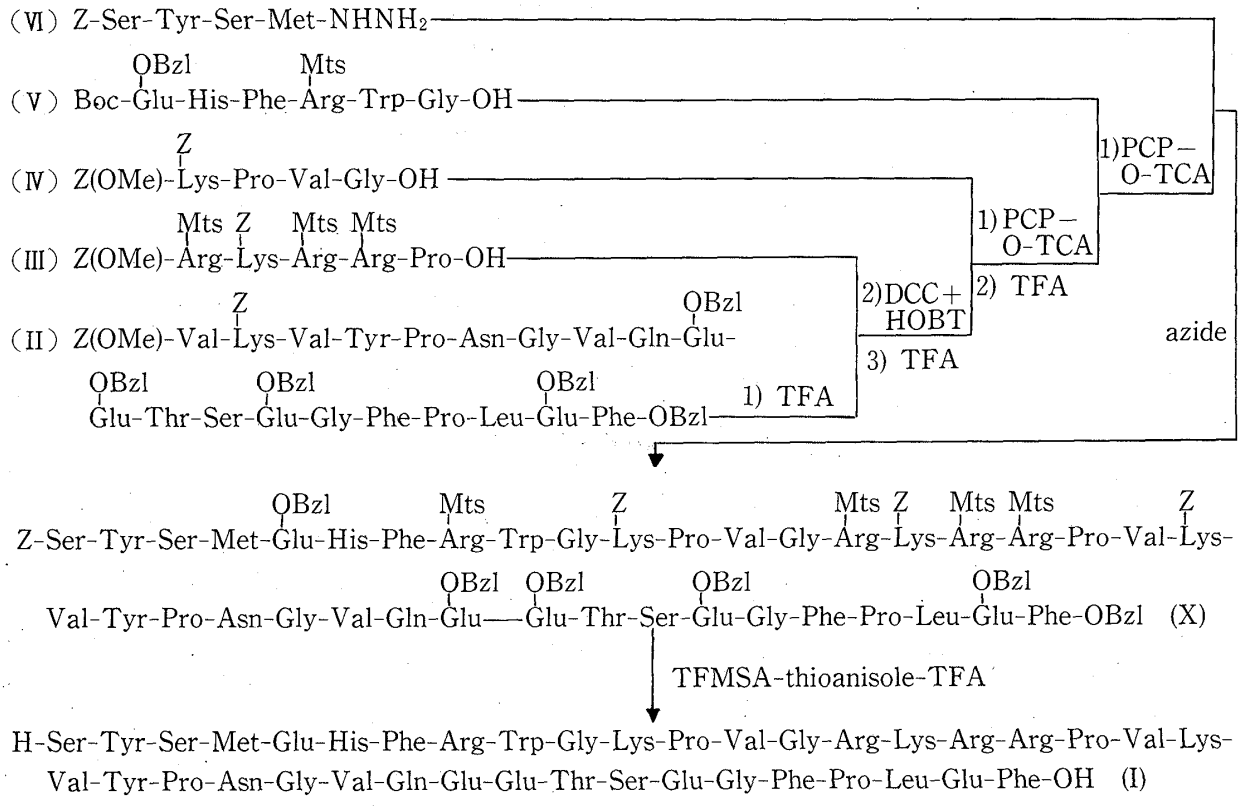


Fig. 1. Synthetic Route to Ostrich ACTH

6 amino acid differences are apparent in the 27—34 region (positions 27, 28, 29, 31, 32, 34) of the ostrich ACTH molecule. The amino acid sequence of the N-terminal portion (positions 1 to 26) of this hormone, however, is identical with that of mammalian ACTHs, except for a single amino acid substitution at position 15.

In this paper, we wish to report the synthesis of the nonatriacontapeptide corresponding to the entire amino acid sequence of ostrich ACTH (I). As shown in Fig. 1, the eicosapeptide ester[4] (II), a synthetic intermediate of ostrich-type corticotropin-like intermediate lobe peptide (CLIP), served as a starting amino component, and four peptide fragments, III (positions 15 to 19), IV (positions 11 to 14), V (positions 5 to 10) and VI (positions 1 to 4) were successively condensed with it.

In the present synthesis, trifluoromethanesulfonic acid (TFMSA)-thioanisole in TFA[5] was employed as a deprotecting reagent in the final step of the synthesis. Thus, amino acid derivatives bearing protecting groups removable by the combination of these two reagents were adopted; *i.e.*, Arg(Mts),[6] Lys(Z) and Glu(OBzl). The α-amino function of intermediates was protected by the TFA-labile Z(OMe) group.[7] For the $N^{\alpha}$-protection of Trp-containing peptides, however, the Boc group was selected, instead of the Z(OMe) group, since the extent of side reactions at the Trp residue was much less during the $N^{\alpha}$-deprotection with TFA,[8] compared with the TFA deprotection of the Z(OMe) group.[9] In addition, skatole and anisole containing ethanedithiol[10] were applied as cation scavengers to minimize side reactions at the Trp residue during the TFA treatment.

Of the four necessary fragments, the N-terminal tetrapeptide hydrazide, Z–Ser–Tyr–Ser–Met–$NHNH_2$[11] [VI, abbreviated as Z-(1—4)-$NHNH_2$] was used for our previous synthesis of dogfish ACTH.[12]

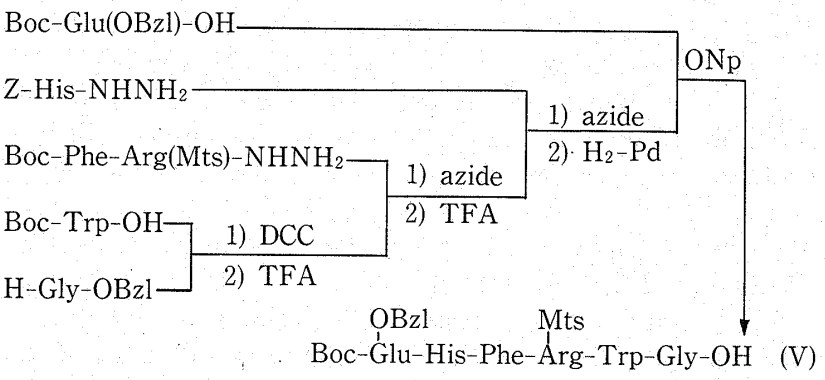


Fig. 2. Synthetic Route to the Protected Hexapeptide, Boc–(5—10)-OH (V)

The synthetic route to the protected hexapeptide, Boc–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–OH [V, abbreviated as Boc–(5—10)–OH] is illustrated in Fig. 2. In contrast to our previous synthesis of dogfish ACTH,[12] a new arginine derivative, Arg(Mts),[6] was employed for the preparation of this protected hexapeptide. Boc–Trp–Gly–OBzl was first prepared by DCC condensation,[13] and the Boc group was then removed by TFA with the precautions described above. Boc–Phe–Arg(Mts)–$NHNH_2$[14] and Z–His–$NHNH_2$ were introduced successively onto the resulting dipeptide ester by the modified azide procedure[15] to give the protected pentapeptide ester, Z–His–Phe–Arg(Mts)–Trp–Gly–OBzl. This, after hydrogenation, was coupled with Boc–Glu(OBzl)–OH by the Np method[16] to afford the fragment (V). The product was purified by column chromatography on silica and its homogeneity was assessed by thin-layer chromatography, elemental analysis and hydrolysis with 4 N methanesulfonic acid (MSA).[17]

The protected tetrapeptide, Z(OMe)–Lys(Z)–Pro–Val–Gly–OH [IV, abbreviated as Z(OMe)–(11—14)–OH] was prepared in the same manner as described for the synthesis of

porcine ACTH by Yajima *et al.*[18]

The protected pentapeptide, Z(OMe)–Arg(Mts)–Lys(Z)–Arg(Mts)–Arg(Mts)–Pro–OH [III, abbreviated as Z(OMe)–(15—19)–OH] was synthesized in essentially the same manner as described for our previous synthesis of dogfish ACTH,[12] except that Arg(Mts), instead of Arg(MBS),[19] was employed. After purification of III by column chromatography on silica, its homogeneity was confirmed by thin-layer chromatography, elemental analysis and 4 N MSA hydrolysis.

The five peptide subunits obtained as outlined above were then assembled according to the scheme illustrated in Fig. 1. 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 [II, abbreviated as Z(OMe)–(20—39)–OBzl],[4] was treated with TFA in the presence of anisole. The resulting trifluoroacetate was converted to the free base with $Et_3N$ and isolated by precipitation from DMF with ether. The resulting $N^{\alpha}$-deprotected eicosapeptide ester was condensed with Z(OMe)–Arg(Mts)–Lys(Z)–Arg(Mts)–Arg(Mts)–Pro–OH (III) by the DCC/HOBT procedure[20] to give the protected pentacosapeptide ester, Z(OMe)–Arg(Mts)–Lys(Z)–Arg(Mts)–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 [VII, abbreviated as Z(OMe)–(15—39)–OBzl]. The protected pentacosapeptide ester (VII), after TFA treatment followed by conversion to the free base as stated above, was condensed with Z(OMe)–Lys(Z)–Pro–Val–Gly–OH (IV) by the pentachlorophenyl trichloroacetate (PCP–O–TCA) procedure[21] to give the protected nonacosapeptide ester, Z(OMe)–Lys(Z)–Pro–Val–Gly–Arg(Mts)–Lys(Z)–Arg(Mts)–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 [VIII, abbreviated as Z(OMe)–(11—39)–OBzl]. This protected nonacosapeptide ester (VIII), after TFA treatment followed by conversion to the free base, was then coupled with Boc–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–OH (V). Rink and Riniker[22] pointed out that DCC has a tendency to mask the $N^{im}$-function of the His residue and this side reaction is accelerated in the presence of HOBT. Therefore, the PCP–O–TCA procedure was selected for this coupling reaction. The resulting protected pentatriacontapeptide ester, Boc–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–Lys(Z)–Pro–Val–Gly–Arg(Mts)–Lys(Z)–Arg(Mts)–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 [IX, abbreviated as Boc–(5—39)–OBzl] was treated with TFA in the presence of skatole and anisole containing 2% ethanedithiol as mentioned above, and then coupled with Z–Ser–Tyr–Ser–Met–$NHNH_2$ by the modified azide procedure to afford the fully protected nonatriacontapeptide ester, Z–Ser–Tyr–Ser–Met–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–Lys(Z)–Pro–Val–Gly–Arg(Mts)–Lys(Z)–Arg(Mts)–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 [X, abbreviated as Z–(1—39)–OBzl]. Purifications of the intermediates VII, VIII and IX, and the fully protected nonatriacontapeptide (X) were carried out by column chromatography on silica using the solvent system of $CHCl_3$–MeOH–$H_2O$ (120:10:1) or (100:10:1). The homogeneity of these protected peptides was confirmed by thin-layer chromatography, elemental analysis and 4 N MSA hydrolysis.

The protected nonatriacontapeptide ester (X) thus obtained was treated with 1 M TFMSA-thioanisole in TFA in an ice-bath for 60 min and at room temperature for 30 min to remove all the protecting groups. *m*-Cresol and skatole were added as scavengers. The former was employed to suppress a possible side reaction at the Tyr residue, *i.e.*, O-mesitylene-2-sulfonylation,[6] and the latter for further suppression of a side reaction at the Trp residue. The deprotected peptide, after conversion to the corresponding acetate with Amberlite IR-45 (acetate form), was subjected by gel-filtration on Sephadex G-25 to remove the scavengers and then purified by ion-exchange chromatography on CM-cellulose with gradient elution up to 0.2 M ammonium acetate buffer, pH 6.9. The absorbancy at 280 nm due to the Trp residue was

used to monitor the chromatographic purification. Ammonium acetate was mostly removed from the desired fractions by Sephadex G-25 chromatography and finally by repeated lyophilization. The fluffy powder thus obtained exhibited a single spot on thin-layer chromatography in two different solvent systems and behaved as a single component on polyacrylamide gel disc electrophoresis. The purity of the synthetic peptide was further checked by high-performance liquid chromatography (HPLC). Based on the calculation of the area under the major peak relative to the total integrated areas, the purity of this peptide was judged to be higher than 98%. The hydrolysate with 4 N MSA contained the constituent amino acids in the ratios predicted by theory. Despite the presence of the Pro residues,[23] complete digestion of this synthetic peptide with commercial aminopeptidase (AP-M)[24] was achieved and the presence of Asn and Gln residues in the product was thus confirmed.

The *in vivo* steroidogenic activity of our synthetic peptide was judged as 0.804, relative to synthetic human ACTH (100 IU/mg) as the standard (1.000). It has been reported by Naudé and Oelofsen[25] that natural ostrich ACTH has an activity of 73—77 IU/mg in an *in vitro* steroidogenic assay.

## Experimental

Melting points were determined using 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. Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 40 to 50°C. 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 (9: 1), $Rf_2$ $CHCl_3$–MeOH–$H_2O$ (18: 3: 1, lower phase), $Rf_3$ $CHCl_3$–MeOH–$H_2O$ (8: 3: 1, lower phase), $Rf_4$ *n*-butanol–AcOH–pyridine–$H_2O$ (4: 1: 1: 2) and $Rf_5$ *n*-butanol–AcOH–pyridine–$H_2O$ (5: 1: 5: 4). HPLC was conducted on a Waters Associates liquid chromatograph with a Waters Associates radial pack A (8 mm × 10 cm) column using 0.02 M, pH 3.0 triethylammonium phosphate–$CH_3CN$ (80: 20) as the eluent. (HPLC conditions: flow rate, 1.0 ml/min; detection, UV at 275 nm).

**Boc-Trp-Gly-OBzl**——Boc–Trp–OH (18.2 g) and DCC (12.4 g) were added to a solution of H–Gly–OBzl (prepared from 20.2 g of the tosylate and 8.3 ml of $Et_3N$) in THF (150 ml) under cooling with ice. The mixture was stirred at room temperature for 20 h, 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 treated with *n*-hexane to yield a solid, which was recrystallized from AcOEt and *n*-hexane; yield 22.8 g (84.1%), mp 115—116°C, $[\alpha]_D^{28}$ −7.62° ($c$=1.05, $CHCl_3$), $Rf_1$ 0.79. *Anal.* Calcd for $C_{25}H_{29}N_3O_5$: C, 66.50; H, 6.47; N, 9.31. Found: C, 66.68; H, 6.55; N, 9.21.

**Boc-Phe-Arg(Mts)-Trp-Gly-OBzl**——Boc–Trp–Gly–OBzl (8.05 g) was treated with TFA (20.0 ml) in the presence of skatole (0.05 g) and anisole (4.0 ml) containing 2% ethanedithiol at 0°C for 60 min, then dry ether–*n*-hexane (1: 1, v/v) was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (20 ml). The solution, after neutralization with $Et_3N$ (2.5 ml), was kept cool with ice, until the following azide was ready. Under cooling (−5°C), 3.43 N HCl/DMF (10.4 ml) and isoamyl nitrite (2.1 ml) were added successively to a solution of Boc–Phe–Arg(Mts)–$NHNH_2$[14] (11.0 g) in DMF (20 ml). The mixture was stirred for 10 min, until the hydrazine test of the solution became negative, then $Et_3N$ (4.6 ml) was added. This solution was then combined with the above solution containing the amino component. The mixture, after further addition of $Et_3N$ (2.5 ml), was stirred at 4°C for 40 h. 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 purified by column chromatography on silica (5.5 × 20.5 cm) using $CHCl_3$–MeOH (60: 1) as an eluent, and the product was recrystallized from MeOH and ether; yield 11.8 g (70.7%), mp 117—120°C, $[\alpha]_D^{28}$ −6.61° ($c$=0.61, DMF), $Rf_1$ 0.52. *Anal.* Calcd for $C_{49}H_{60}N_8O_9S \cdot 0.5H_2O$: C, 62.20; H, 6.50; N, 11.84. Found: C, 62.39; H, 6.38; N, 11.84.

**Z-His-Phe-Arg(Mts)-Trp-Gly-OBzl**——Boc–Phe–Arg(Mts)–Trp–Gly–OBzl (2.6 g) was treated with TFA (5.0 ml) in the presence of skatole (20 mg) and anisole (1.0 ml) containing 2% ethanedithiol at 0°C for 60 min, then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and dissolved in DMF (5.0 ml). To this ice-chilled solution, $Et_3N$ (0.89 ml) and the azide (prepared from 1.1 g of Z–His–$NHNH_2$ with 2.1 ml of 3.43 N HCl/DMF, 0.42 ml of isoamyl nitrite and 1.0 ml of $Et_3N$) in DMF (5.0 ml) were added, and the mixture was stirred at 4°C for 43 h. The solvent was evaporated off *in vacuo* and the residue was treated with $H_2O$ to yield a powder, which was purified by column chromato-

graphy on silica (2.8×18 cm) using $CHCl_3$–MeOH (30: 1) as an eluent, followed by precipitation from MeOH with ether; yield 1.95 g (62.9%), mp 146—149°C, $[\alpha]_D^{28}$ −24.6° ($c$=0.98, DMF), $Rf_1$ 0.33. *Anal.* Calcd for $C_{58}H_{65}N_{11}O_{10}S$: C, 62.86; H, 5.91; N, 13.90. Found: C, 62.68; H, 5.99; N, 13.82.

**Boc-Glu(OBzl)-His-Phe-Arg(Mts)-Trp-Gly-OH (V)**——In the usual manner, Z–His–Phe–Arg(Mts)–Trp–Gly–OBzl (0.65 g) in DMF (5.0 ml) containing AcOH (0.1 ml) was hydrogenated over a Pd catalyst for 5 h. The catalyst was removed by filtration and the filtrate was neutralized with $Et_3N$ (0.24 ml). To this solution, Boc–Glu(OBzl)–ONp (0.33 g) and $Et_3N$ (0.10 ml) were added and the mixture was stirred at room temperature for 50 h. The solvent was evaporated off *in vacuo* and ether was added to the residue to yield a powder, which was washed batchwise with 3% AcOH and $H_2O$. The crude product thus obtained was purified by column chromatography on silica (3.2×22 cm) using $CHCl_3$–MeOH–$H_2O$ (18: 3: 1, lower phase) as an eluent and further precipitated from THF with ether; yield 0.46 g (64.8%), mp 156—158°C, $[\alpha]_D^{18}$ −26.2° ($c$=1.03, DMF), $Rf_3$ 0.22. Amino acid ratios in 4 N MSA hydrolysate: Glu 1.07, His 1.04, Phe 1.04, Arg 1.11, Trp 1.09, Gly 1.00 (recovery of Gly, 90.5%). *Anal.* Calcd for $C_{60}H_{74}N_{12}O_{13}S$: C, 59.89; H, 6.20; N, 13.97. Found: C, 60.00; H, 6.38; N, 13.73.

**Z(OMe)-Lys(Z)-Pro-Val-Gly-OH (IV)**——The title compound was prepared according to the literature;[18] yield 86.9%, mp 145—148°C, $[\alpha]_D^{21}$ −58.8° ($c$=0.80, DMF). [lit.[18] mp 77—82°C, $[\alpha]_D^{25}$ −29.7° ($c$=0.8, DMF)]. $Rf_3$ 0.25. Amino acid ratios in 4 N MSA hydrolysate: Lys 1.04, Pro 0.96, Val 1.09, Gly 1.00 (recovery of Gly, 90.5%). *Anal.* Calcd for $C_{35}H_{47}N_5O_{10}$: C, 60.25; H, 6.79; N, 10.04. Found: C, 60.49; H, 6.98; N, 10.07.

**Z(OMe)-Arg(Mts)-Pro-OBzl**——Z(OMe)–Arg(Mts)–OH (13.0 g) and DCC (5.2 g) were added to a solution of H–Pro–OBzl (prepared from 6.1 g of the hydrochloride and 3.5 ml of $Et_3N$) in THF (30 ml) under cooling with ice. The mixture was stirred at room temperature for 18 h, 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 purified by column chromatography on silica (5.5×20 cm) with $CHCl_3$–MeOH (100: 1) as an eluent and precipitated from $CHCl_3$ with *n*-hexane; yield 15.1 g (85.3%), amorphous powder, $[\alpha]_D^{22}$ −48.5° ($c$=0.97, $CHCl_3$), $Rf_1$ 0.67. *Anal.* Calcd for $C_{36}H_{45}N_5O_8S$: C, 61.09; H, 6.41; N, 9.89. Found: C, 61.07; H, 6.46; N, 9.84.

**Z-Arg(Mts)-Arg(Mts)-Pro-OBzl**——Z(OMe)–Arg(Mts)–Pro–OBzl (13.5 g) was treated with TFA (30 ml) in the presence of anisole (7.0 ml) at 0°C for 60 min, then dry ether was added. The resulting oily product was washed with ether and dissolved in DMF (30 ml). The solution, after neutralization with $Et_3N$ (2.7 ml), was kept cool with ice until the following mixed anhydride was ready. Under cooling with ice-NaCl, $Et_3N$ (2.9 ml) and isobutyl chloroformate (2.8 ml) were added to a solution of Z–Arg(Mts)–OH (10.3 g) in DMF (20 ml) and the mixture was stirred for 10 min. This solution was combined with the above solution containing the amino component and the mixture was stirred at room temperature for 18 h. 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 purified by column chromatography on silica (5.5×22 cm) with $CHCl_3$–MeOH (80: 1) as an eluent and further precipitated from $CHCl_3$ with ether; yield 14.6 g (75.3%), amorphous powder, $[\alpha]_D^{22}$ −45.9° ($c$=0.98, $CHCl_3$), $Rf_1$ 0.62. *Anal.* Calcd for $C_{50}H_{65}N_9O_{10}S_2$: C, 59.09; H, 6.45; N, 12.40. Found: C, 58.84; H, 6.35; N, 12.21.

**Z(OMe)-Arg(Mts)-Lys(Z)-OMe**——A mixed anhydride [prepared from 12.6 g of Z(OMe)–Arg(Mts)–OH with 3.36 ml of $Et_3N$ and 3.19 ml of isobutyl chloroformate] in DMF (20 ml) was added to a solution of H–Lys(Z)–OMe (prepared from 8.01 g of the hydrochloride and 3.36 ml of $Et_3N$) in DMF (20 ml) and the mixture was stirred at room temperature for 24 h. 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 purified by column chromatography on silica (6.5×17.5 cm) with $CHCl_3$–MeOH (100: 1) as an eluent and further precipitated from AcOEt with *n*-hexane; yield 14.8 g (76.7%), amorphous powder, $[\alpha]_D^{20}$ −7.84° ($c$=1.02, $CHCl_3$), $Rf_1$ 0.61. *Anal.* Calcd for $C_{39}H_{52}N_6O_{10}S$: C, 58.78; H, 6.58; N, 10.55. Found: C, 58.89; H, 6.57; N, 10.27.

**Z(OMe)-Arg(Mts)-Lys(Z)-NHNH$_2$**——A solution of Z(OMe)–Arg(Mts)–Lys(Z)–OMe (4.6 g) in MeOH (20 ml) was treated with 80% hydrazine hydrate (1.4 ml). The mixture was stirred at room temperature for 40 h, then the solvent was evaporated off *in vacuo*. Addition of $H_2O$ to the residue afforded a gelatinous solid, which was recrystallized from EtOH and ether; yield 4.2 g (91.3%), mp 94—97°C, $[\alpha]_D^{22}$ −2.32° ($c$=1.12, DMF), $Rf_2$ 0.33. *Anal.* Calcd for $C_{38}H_{52}N_8O_9S$: C, 57.27; H, 6.58; N, 14.06. Found: C, 57.31; H, 6.78; N, 13.98.

**Z(OMe)-Arg(Mts)-Lys(Z)-Arg(Mts)-Arg(Mts)-Pro-OH (III)**——Z–Arg(Mts)–Arg(Mts)–Pro–OBzl (5.5 g) in MeOH (50 ml) containing AcOH (5.0 ml) was hydrogenated over a Pd catalyst in the usual manner. The catalyst was filtered off and the solvent was evaporated off *in vacuo*. The residue, after being dried over KOH pellets *in vacuo*, was dissolved in DMF (10 ml). To this ice-chilled solution, $Et_3N$ (1.5 ml) and the azide [prepared from 4.3 g of Z(OMe)–Arg(Mts)–Lys(Z)–$NHNH_2$ with 3.1 ml of 3.43 N HCl/DMF, 0.64 ml of isoamyl nitrite and 1.5 ml of $Et_3N$] in DMF (20 ml) were added, and the mixture was stirred at 4°C for 40 h. The solvent was evaporated off *in vacuo* and the residue was dissolved in $CHCl_3$. The solution was washed with 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 (5.5×22 cm) on silica using $CHCl_3$–MeOH (10: 1) as an eluent and further precipitated from $CHCl_3$ with ether; yield 5.5 g (65.5%), amorphous powder, $[\alpha]_D^{25}$ −2.04° ($c$=0.49, DMF), $Rf_3$

0.46. Amino acid ratios in 4 N MSA hydrolysate: Arg 2.87, Lys 0.86, Pro 1.00 (recovery of Pro, 89.1%). *Anal.* Calcd for $C_{73}H_{101}N_{15}O_{17}S_3 \cdot 2H_2O$: C, 55.04; H, 6.64; N, 13.19. Found: C, 55.07; H, 6.71; N, 13.03.

**Z(OMe)-Arg(Mts)-Lys(Z)-Arg(Mts)-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, Z(OMe)-(15—39)-OBzl (VII)**——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[4] (0.88 g) was treated with TFA (2.0 ml) in the presence of anisole (0.4 ml) at 0°C for 60 min, then dry ether was added. The resulting powder was collected by filtration and dissolved in DMF (5.0 ml) containing $Et_3N$ (0.04 ml). Addition of ether to this solution afforded the powder, which was collected by filtration and dried over KOH pellets *in vacuo*. Next, DCC (0.14 g) and HOBT (0.14 g) were added to an ice-chilled solution of Z(OMe)–Arg(Mts)–Lys(Z)–Arg(Mts)–Arg(Mts)–Pro–OH (0.55 g) in DMF (5.0 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 43 h. The solution, after filtration, was concentrated *in vacuo* and the residue was treated with ether. The resulting powder was recrystallized from DMF and EtOH and further purified by column chromatography on silica (3.2×25 cm) using $CHCl_3$–MeOH–$H_2O$ (120: 10: 1) as an eluent. The product was finally precipitated from DMF with EtOH; yield 0.82 g (64.6%), mp 165—168°C, $[\alpha]_D^{25}$ −26.3° ($c$=0.38, DMF), $Rf_2$ 0.19, $Rf_3$ 0.63. Amino acid ratios in 4 N MSA hydrolysate: Arg 3.34, Lys 1.97, Pro 3.36, Val 2.85, Tyr 0.87, Asp 1.11, Gly 2.03, Glu 5.56, Thr 1.08, Ser 1.01, Phe 2.14, Leu 1.00 (recovery of Leu, 83.0%). *Anal.* Calcd for $C_{220}H_{288}N_{38}O_{52}S_3$: C, 60.15; H, 6.61; N, 12.12. Found: C, 59.89; H, 6.85; N, 12.06.

**Z(OMe)-Lys(Z)-Pro-Val-Gly-Arg(Mts)-Lys(Z)-Arg(Mts)-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, Z(OMe)-(11—39)-OBzl (VIII)**——The above Z(OMe)-(15—39)-OBzl (1.55 g) was treated with TFA (3.5 ml) in the presence of anisole (0.8 ml) at 0°C for 60 min, then dry ether was added. The resulting TFA salt was collected by filtration, converted, as described above, to the free base by precipitation from DMF (5.0 ml) containing $Et_3N$ (0.05 ml) with ether, and dried over KOH pellets *in vacuo*. Next, $Et_3N$ (0.10 ml) and PCP–O–TCA (0.58 g) were added to an ice-chilled solution of Z(OMe)–Lys(Z)–Pro–Val–Gly–OH (0.49 g) in DMF (10 ml), and the mixture was stirred at room temperature for 4 h, until thin-layer chromatographic examination revealed the appearance of a new spot of $Rf_1$ 0.86 and the disappearance of the spot corresponding to the starting material. To this solution, the above pentacosapeptide ester was added, and the mixture was stirred at room temperature for 40 h. The solvent was evaporated off *in vacuo* and the residue was treated with ether. The resulting powder was recrystallized from DMF and EtOH and further purified by column chromatography on silica (3.2×18 cm) using $CHCl_3$–MeOH–$H_2O$ (100: 10: 1) as an eluent. The product was finally precipitated from DMF with EtOH; yield 1.01 g (58.4%), mp 161—163°C, $[\alpha]_D^{27}$ −27.8° ($c$=0.36, DMF), $Rf_2$ 0.15, $Rf_3$ 0.67. Amino acid ratios in 4 N MSA hydrolysate: Lys 3.30, Pro 4.50, Val 3.82, Gly 3.18, Arg 3.00, Tyr 0.81, Asp 1.05, Glu 5.47, Thr 0.99, Ser 0.93, Phe 2.13, Leu 1.00 (recovery of Leu, 88.2%). *Anal.* Calcd for $C_{246}H_{325}N_{43}O_{58}S_3$: C, 60.19; H, 6.67; N, 12.27. Found: C, 60.03; H, 6.95; N, 12.18.

**Boc-Glu(OBzl)-His-Phe-Arg(Mts)-Trp-Gly-Lys(Z)-Pro-Val-Gly-Arg(Mts)-Lys(Z)-Arg(Mts)-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, Boc-(5—39)-OBzl (IX)**——In the usual manner, the above Z(OMe)-(11—39)-OBzl (0.52 g) was treated with TFA (1.5 ml) in the presence of anisole (0.25 ml) at 0°C for 60 min, then dry ether was added. The resulting TFA salt was collected by filtration, converted to the free base, as stated above and dried over KOH pellets *in vacuo*. Next, $Et_3N$ (0.029 ml) and PCP–O–TCA (0.17 g) were added to an ice-chilled solution of Boc–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–OH (0.26 g) in DMF (5.0 ml), and the mixture was stirred at room temperature for 4 h. To this solution, the above nonacosapeptide ester was added, and the mixture was stirred at room temperature for 45 h. The solvent was evaporated off *in vacuo* and the residue was treated with ether. The resulting powder was recrystallized from DMF and EtOH and further purified by column chromatography on silica (2.2×20 cm) using $CHCl_3$–MeOH–$H_2O$ (120: 10: 1) as an eluent. The product was finally precipitated from DMF with EtOH; yield 0.49 g (77.8%), mp 159—161°C, $[\alpha]_D^{28}$ −21.6° ($c$=0.37, DMF), $Rf_2$ 0.15, $Rf_3$ 0.73. Amino acid ratios in 4 N MSA hydrolysate: Glu 6.50, His 0.88, Phe 3.09, Arg 4.28, Trp 0.86, Gly 4.09, Lys 3.00, Pro 4.35, Val 3.84, Tyr 0.85, Asp 1.00, Thr 0.95, Ser 0.90, Leu 1.00 (recovery of Leu, 81.4%). *Anal.* Calcd for $C_{297}H_{389}N_{55}O_{67}S_4 \cdot 3H_2O$: C, 59.61; H, 6.65; N, 12.87. Found: C, 59.55; H, 6.71; N, 12.59.

**Z-Ser-Tyr-Ser-Met-Glu(OBzl)-His-Phe-Arg(Mts)-Trp-Gly-Lys(Z)-Pro-Val-Gly-Arg(Mts)-Lys(Z)-Arg(Mts)-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, Z-(1—39)-OBzl (X)**——The above Boc-(5—39)-OBzl (IX) (0.23 g) was treated with TFA (0.50 ml) in the presence of skatole (10 mg) and anisole (0.15 ml) containing 2% ethanedithiol at 0°C 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 (0.50 ml) containing $Et_3N$ (0.022 ml). To this ice-cold solution, the azide (prepared from 0.075 g of Z–Ser–Tyr–Ser–Met–$NHNH_2$, 0.074 ml of 3.2 N HCl/DMF, 0.014 ml of isoamyl nitrite and 0.033 ml of $Et_3N$) in DMF (0.5 ml) was added, and the mixture was stirred at 4°C for 70 h. The solvent was evaporated off *in vacuo* and the residue was treated with $H_2O$. The resulting powder, after batchwise washing with $H_2O$ and EtOH, was precipitated from DMF with EtOH and further purified by column chromatography on silica (2.2×20 cm) using $CHCl_3$–MeOH–$H_2O$ (100: 10: 1) as an eluent.

The product was finally precipitated from DMF with EtOH; yield 0.19 g (77.4%), mp 198—201°C, $[\alpha]_D^{24}$ −20.7° ($c$=0.58, DMF), $Rf_2$ 0.17, $Rf_3$ 0.64. Amino acid ratios in 4 N MSA hydrolysate: Ser 2.99, Tyr 1.95, Met 0.90, Glu 6.49, His 0.83, Phe 3.13, Arg 3.68, Trp 0.64, Gly 4.22 Lys 3.34, Pro 4.29, Val 3.79, Asp 1.00, Thr 0.91, Leu 1.00 (recovery of Leu, 77.2%). *Anal.* Calcd for $C_{320}H_{415}N_{59}O_{74}S_5 \cdot 4H_2O$: C, 59.09; H, 6.56; N, 12.71. Found: C, 58.78; H, 6.29; N, 12.79.

**H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Arg-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Val-Gln-Glu-Glu-Thr-Ser-Glu-Gly-Phe-Pro-Leu-Glu-Phe-OH, Ostrich ACTH (I)**——The above Z-(1—39)-OBzl (X) (53.3 mg) was treated with 1 M TFMSA-thioanisole in TFA (1.7 ml) in the presence of *m*-cresol (0.12 ml) and skatole (22 mg) in an ice-bath for 60 min and at room temperature for 30 min. This reaction mixture was poured into dry ether–*n*-hexane (300 ml, 1: 1, v/v) and the resulting precipitate was separated by decantation. Addition of dry ether to the residual precipitate afforded a fine powder, which was collected by filtration, washed with dry ether and then dissolved in $H_2O$ (5 ml). The solution was treated with Amberlite IR-45 (acetate form, approximately 3 g) for 30 min with stirring and the resin was removed by filtration. The filtrate was lyophilized and the resulting powder was dissolved in 0.1 N AcOH (2 ml). The solution was applied to a column of Sephadex G-25 (3.5×64 cm), which was eluted with 0.1 N AcOH. Individual fractions (5 ml each) were collected and the absorbancy at 280 nm was determined. The fractions corresponding to the front peak (tube Nos. 43—61) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 33.2 mg (deblocking step 76.1%). This powder (32.7 mg) was dissolved in $H_2O$ (10 ml) and the solution was applied to a column of CM-cellulose (2.2×15 cm), which was eluted successively with $H_2O$ (100 ml), 0.05 M ammonium acetate buffer (pH 6.9, 100 ml) and then with a gradient formed from 0.2 M ammonium acetate buffer (pH 6.9) through a mixing flask containing 0.05 M ammonium acetate buffer (pH 6.9, 300 ml). Individual fractions (5 ml each) were collected and the absorbancy at 280 nm was determined. Fractions corresponding to the main peak (tube Nos. 71—82) were combined and the solution was concentrated to approximately 5 ml. This solution was then applied to a column of Sephadex G-25 (3.5×64 cm), which was eluted with 0.1 N AcOH. The desired fractions were collected as described above and the product was finally lyophilized to give a fluffy white powder; yield 8.0 mg (over-all yield 18.3%), $Rf_4$ 0.32, $Rf_5$ 0.58 positive to ninhydrin, methionine, Ehrlich and Sakaguchi tests. Amino acid ratios in 4 N MSA hydrolysate: Ser 2.81, Tyr 1.82, Met 0.72, Glu 5.95, His 0.93, Phe 2.91, Arg 3.83, Trp 0.66, Gly 4.18, Lys 2.65, Pro 3.87, Val 3.36, Asp 0.83, Thr 0.80, Leu 1.00 (recovery of Leu, 91.7%). Amino acid ratios in aminopeptidase (AP-M, Merck, lot No. 0040347) digest (numbers in parentheses are theoretical values): Ser+Asn 3.48 (3+1, calcd. as Ser), Tyr 1.89 (2), Met 0.77 (1), Glu 5.02 (5), His 0.91 (1), Phe 2.79 (3), Arg 4.29 (4), Trp 0.73 (1), Gly 3.94 (4), Lys 3.22 (3), Pro 4.06 (4), Val 3.86 (4), Gln+Thr 1.67 (1+1, calcd. as Thr), Leu 1.00 (1) (recovery of Leu, 84.4%). Disc electrophoretic mobility in 15% polyacrylamide gel (0.55×6.1 cm, 5 mA/tube) at pH 4.0 (0.3 M glycine–AcOH buffer) was 1.9 cm to the cathode after 100 min (the gel was stained with Amido Schwarz). $[\alpha]_D^{12}$ −81.1° ($c$=0.19, 1% AcOH). *Anal.* Calcd for $C_{210}H_{315}N_{59}O_{58}S \cdot 7CH_3COOH \cdot 12H_2O$: C, 51.12; H, 7.03; N, 15.70. Found: C, 50.98; H, 6.72; N, 15.46. HPLC: retention time, 4.55 min; peak area, 98.6%.

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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, Boc=*tert*-butyloxycarbonyl, OBzl=benzyl ester, ONp=*p*-nitrophenyl ester. Other abbreviations used are: Mts=mesitylene-2-sulfonyl, DCC=*N*,*N*′-dicyclohexylcarbodiimide, HOBT=*N*-hydroxybenzotriazole, TFA=trifluoroacetic acid, DMF=dimethylformamide, THF=tetrahydrofuran.
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