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Solution Synthesis of a Heptacosapeptide known as Bovine γ_3-Melanotropin (γ_3-MSH)[1]

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A heptacosapeptide (known as γ_3-MSH) which is located within the amino acid sequence of bovine corticotropin-β-lipotropin precursor protein was synthesized by fragment condensation of Z-(1—12)-OSu and H-(13—27)-OBzl followed by deprotection with trifluoromethanesulfonic acid-thioanisole in TFA. The synthetic peptide exhibited weak melanocyte-stimulating activity (1.5×10^6 U/g, *in vitro*).

Keywords——bovine corticotropin-β-lipotropin precursor protein; MSH core peptide, His–Phe–Arg–Trp; gel-filtration on Sephacryl S-200; deprotection by TFMSA-thioanisole in TFA; reduction of Met(O) by thioanisole; melanocyte-stimulating activity; digestion by carboxypeptidase P

In 1979, Nakanishi *et al.*[2] elucidated the primary structure of bovine corticotropin-β-lipotropin precursor protein (pro-opiomelanocortin) on the basis of the complete complementary deoxyribo-nucleic acid (cDNA) nucleotide sequence of precursor protein messenger-ribonucleic acid (m-RNA) isolated from the intermediate lobe of bovine pituitary gland. They pointed out the presence of a third melanotropin fragment (H–Tyr–Val–Met–Gly–His–Phe–Arg–Trp–Asp–Arg–Phe–Gly–OH termed as γ-MSH) near the *N*-terminal portion of pro-opiomelanocortin. This γ-MSH was synthesized by the authors[3] and by Guillemin *et al.*[4] Subsequently, Guillemin *et al.*[4] synthesized the heptacosapeptide [A], a longer peptide than the γ-MSH sequence, by the solid phase method. The amino acid sequence of this peptide, termed γ_3-MSH, corresponds to positions −55 to −29 of the precursor protein.

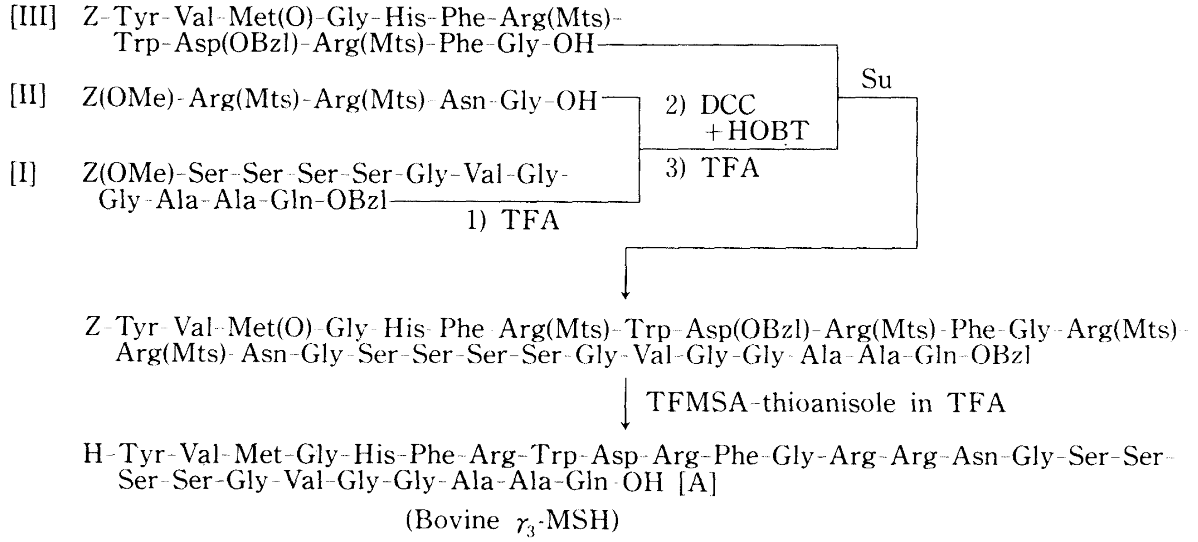

Fig. 1. Synthetic Route to Bovine γ_3-MSH

We have synthesized the heptacosapeptide [A] in a conventional manner in order to examine its physiological functions, if any.

For this synthesis, three peptide fragments, [I] (positions 17—27), [II] (positions 13—16) and [III] (positions 1—12), were selected as building blocks for construction of the entire amino acid sequence of γ_3-MSH as shown in Fig. 1. Of these, fragment [III] is one used for our previous synthesis of γ-MSH.3) Two other fragments, [I] and [II], were newly synthesized. Amino acid derivatives bearing protecting groups removable by TFMSA–thioanisole–TFA5) were employed; *i.e.*, Asp(OBzl) and Arg(Mts).6) In addition, Met(O)7) was employed to prevent partial oxidation during the synthesis.

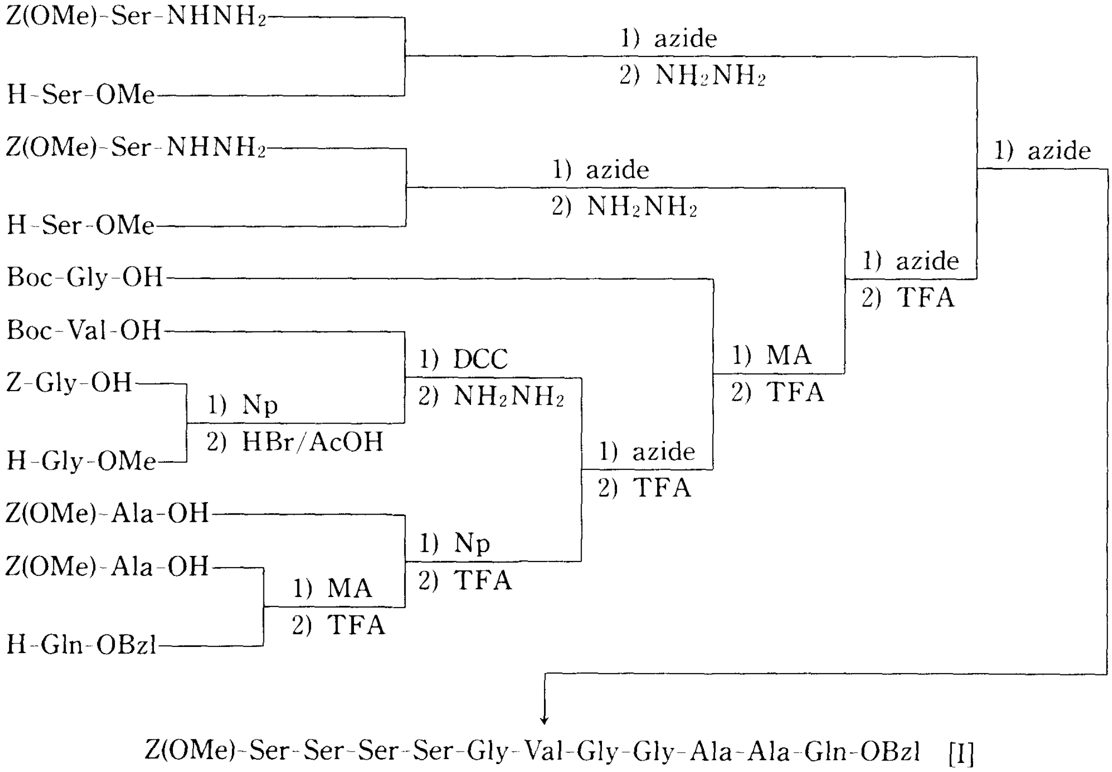

Fig. 2. Synthetic Route to Fragment [I]

First, fragment [I] was synthesized as shown in Fig. 2. Starting from H–Gln–OBzl,8) the C-terminal protected tripeptide, Z(OMe)–Ala–Ala–Gln–OBzl, was synthesized in a stepwise manner by the combination of N^{α}-deprotection with TFA and coupling reaction *via* the mixed anhydride9) or the *p*-nitrophenyl ester.10) The protected tripeptide, after treatment with TFA, was subsequently condensed with Boc–Val–Gly–Gly–$NHNH_2$ by the azide procedure11) to give the protected hexapeptide, Boc–Val–Gly–Gly–Ala–Ala–Gln–OBzl. The above tripeptide hydrazide was derived by hydrazinolysis from Boc–Val–Gly–Gly–OMe, which was synthesized by the DCC condensation12) of Boc–Val–OH and H–Gly–Gly–OMe. Next, in order to elongate the hexapeptide chain obtained above to the undecapeptide [I], the mixed anhydride method was employed for condensation of Boc–Gly–OH and the azide method for two successive units of Z(OMe)–Ser–Ser–$NHNH_2$. The product [I] was purified by the usual extraction procedure and its purity was ascertained by thin-layer chromatography (TLC), acid hydrolysis and elemental analysis.

Fragment [II] was synthesized as shown in Fig. 3. Z(OMe)–Arg(Mts)–OH was condensed with H–Arg(Mts)–OMe by the mixed anhydride method to give Z(OMe)–Arg(Mts)–Arg(Mts)–OMe. This dipeptide, after conversion to the corresponding hydrazide, was condensed *via* the azide with H–Asn–Gly–OH,13) prepared by condensation of Z–Asn–OH and H–Gly–OBzl with

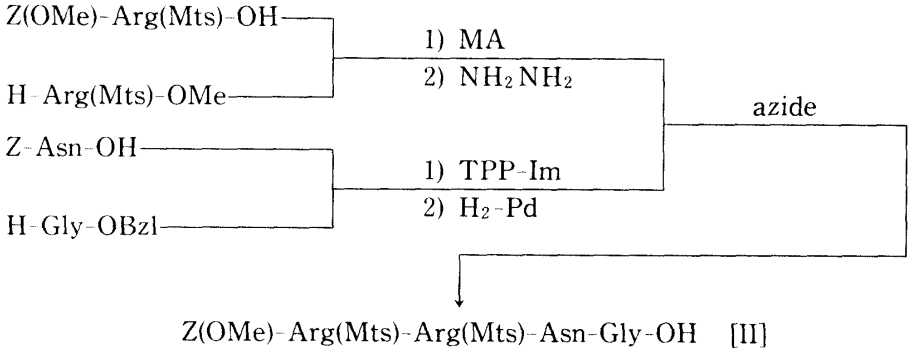

Fig. 3. Synthetic Route to Fragment [II]

triphenylphosphite–imidazole[14)] followed by catalytic hydrogenation. The product was purified by column chromatography on silica.

The three fragments thus obtained were then assembled according to the route illustrated in Fig. 1. The Z(OMe) group of fragment [I] was removed by TFA in the presence of anisole as usual and the resulting TFA salt was converted to the free base through the corresponding hydrochloride, followed by neutralization with Et_3N. The N^α-deprotected [I] thus obtained was then condensed with fragment [II] by DCC in the presence of HOBT[15)] to give the protected pentadecapeptide, Z(OMe)–Arg(Mts)–Arg(Mts)–Asn–Gly–Ser–Ser–Ser–Ser–Gly–Val–Gly–Gly–Ala–Ala–Gln–OBzl, which was purified by repeated precipitation from DMSO–MeOH with ether. Its purity was assessed by TLC, acid hydrolysis and elemental analysis. Subsequently, this protected pentadecapeptide, after TFA treatment, was condensed with fragment [III] by the *N*-hydroxysuccinimide ester method[16)] to give the protected heptacosapeptide, Z–Tyr–Val–Met (O)–Gly–His–Phe–Arg (Mts)–Trp–Asp (OBzl)–Arg (Mts)–Phe–Gly–Arg(Mts)–Arg(Mts)–Asn–Gly–Ser–Ser–Ser–Ser–Gly–Val–Gly–Gly–Ala–Ala–Gln–OBzl. The heptacosapeptide thus obtained was purified by gel-filtration on Sephacryl S-200 using 95% aqueous DMSO as the eluent and its purity was again assessed by three criteria; TLC, acid hydrolysis and elemental analysis.

Finally, the protected heptacosapeptide (protected γ_3-MSH) was treated with 1 M TFMSA-thioanisole in TFA to remove all the protecting groups. In a model experiment, most Met(O) was shown to be reduced back to Met residue under this treatment.[3)] The deprotected peptide was converted to the acetate form by treatment with Amberlite IRA-400 (acetate form) and subjected to gel-filtration on Sephadex G-25. In order to ensure the complete reduction of the Met(O) residue, the product was incubated with 2-mercaptoethanol at 50°C for 5 h under N_2 gas and the reduced product was purified by ion-exchange chromatography on carboxymethyl (CM)-cellulose with ammonium acetate buffer gradient elution. The heptacosapeptide thus purified exhibited a single spot on TLC in two different solvent systems and behaved as a single component on disc electrophoresis at pH 4.0 (0.37 M glycine acetate buffer). Amino acid ratios in a 4 N MSA hydrolysate were in good agreement with those predicted by theory. This peptide was digested completely by aminopeptidase M[17)] (AP–M, Lot. No. 0040347, Merck), but the recovery of C-terminal Gln was low. However, the presence of one mol of Gln in this peptide could be confirmed by digestion with carboxypeptidase P[18)] (CP–P, Lot. No. 241001, Peptide Institute, Inc.). The low recovery of Gln in the former case was presumably due to pyrrolidone formation. From the results cited above, we concluded that our synthetic γ_3-MSH has a high degree of homogeneity.

The *in vitro* melanocyte-stimulating activity[19,20)] of our synthetic γ_3-MSH was judged to be 1.5×10^6 U/g, when synthetic α-MSH (2.0×10^{10} U/g) was taken as a standard. This value is approximately $1/1.4 \times 10^{-4}$ of that of synthetic α-MSH on a weight basis. Guillemin *et al.*[4)] reported that the *in vitro* melanocyte-stimulating potency of solid-phase-synthetic γ_3-MSH was approximately $1/2.2 \times 10^{-6}$ of that of α-MSH on a molar basis. The steroidogenic activity of our synthetic γ_3-MSH was negligible. Physiological activities of this segment of pro-opiomelanocortin remain to be examined.

Experimental

General experimental methods employed were essentially the same as those described in the previous paper.[21] Unless otherwise mentioning, products were purified by either one of the following procedures. A: the residue was washed batchwisely with 10% citric acid, 5% $NaHCO_3$, and H_2O, then dried over P_2O_5 *in vacuo*. B: the residue was dissolved in AcOEt. The solution was washed with 10% citric acid, 5% $NaHCO_3$, and NaCl–H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. Thin-layer chromatography was performed on silica gel (DC-aluroll Kieselgel 60F 254, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (9:1), Rf_2 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_3 $CHCl_3$–MeOH–H_2O–pyridine (8:3:1:2), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2), Rf_5 AcOEt–*n*-BuOH–AcOH–H_2O (1: 1: 1: 1), Rf_6 *n*-BuOH–AcOH–pyridine–H_2O (6: 6: 1.2: 4.8), Rf_7 *n*-BuOH–AcOH–pyridine–H_2O (15: 3: 10: 12).

Z(OMe)-Ala-Gln-OBzl——Boc–Gln–OBzl[10] (20.2 g) was treated with TFA–anisole (36 ml–10 ml) at 0°C for 60 min and then ether was added. The oily product was washed with ether and dried over KOH pellets *in vacuo* for 3 h. H–Gln–OBzl·TFA thus obtained was dissolved in DMF (50 ml) containing Et_3N (8.34 ml). Separately, Et_3N (8.34 ml) and isobutyl chloroformate (7.92 ml) were added to a solution of Z(OMe)–Ala–OH (15.2 g) in DMF (50 ml) at −10°C. After being stirred for 10 min, this solution was added to the amine component in DMF. The mixture was stirred at room temperature for 13 h. The solvent was evaporated off *in vacuo* and the residue was purified by procedure A followed by recrystallization from EtOH and ether; yield 23.5 g (83.0%), mp 160—161°C, $[\alpha]_D^{30}$ −19.7° (c=0.61, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{24}H_{29}N_3O_7$: C, 61.14; H, 6.20; N, 8.91. Found: C, 61.11; H, 6.12; N, 8.80.

Z(OMe)-Ala-Ala-Gln-OBzl——Z(OMe)–Ala–Gln–OBzl (10.3 g) was treated with TFA–anisole (15 ml–5 ml) as described above. The resulting oily precipitate was dissolved in DMF (35 ml). To this solution, Et_3N (6.37 ml) and Z(OMe)–Ala–ONp (8.98 g) were added and the mixture was stirred at room temperature for 44 h. The solvent was evaporated off *in vacuo*. The residue was purified by procedure A followed by recrystallization from DMF–MeOH and ether; yield 10.3 g (87.3%), mp 204—205°C, $[\alpha]_D^{30}$ −13.5° (c=1.04, DMF), Rf_1 0.52. *Anal.* Calcd for $C_{27}H_{34}N_4O_8$: C, 59.77; H, 6.32; N, 10.33. Found: C, 59.53; H, 6.31; N, 10.07.

Boc-Val-Gly-Gly-OMe——Z–Gly–Gly–OMe[22] (23.8 g) was treated with 25% HBr in AcOH (220 ml) in an ice-bath for 60 min and then ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and dissolved in DMF (60 ml), then the solution was neutralized with Et_3N (11.0 ml). To this ice-chilled solution, Boc–Val–OH (16.0 g) and DCC (16.7 g) were added and the reaction mixture was stirred at room temperature for 15 h. After filtration, the filtrate was concentrated *in vacuo*. The residue was purified by procedure B followed by recrystallization from AcOEt and petroleum ether; yield 19.0 g (74.8%), $[\alpha]_D^{19}$ +3.1° (c=4.20, DMF), Rf_1 0.70. *Anal.* Calcd for $C_{15}H_{27}N_3O_6$: C, 52.16; H, 7.88; N, 12.17. Found: C, 52.26; H, 7.90; N, 12.40.

Boc-Val-Gly-Gly-NHNH$_2$——In the usual manner, Boc–Val–Gly–Gly–OMe (5.5 g) in MeOH (20 ml) was treated with 80% hydrazine hydrate (3.88 ml) overnight. The product was recrystallized from EtOH–H_2O and ether; yield 4.9 g (89.1%), mp 136—137°C, $[\alpha]_D^{19}$ −1.14° (c=3.50, DMF), Rf_1 0.17. *Anal.* Calcd for $C_{14}H_{27}N_5O_5$: C, 48.68; H, 7.88; N, 20.28. Found: C, 48.57; H, 7.85; N, 20.11.

Boc-Val-Gly-Gly-Ala-Ala-Gln-OBzl——Z(OMe)–Ala–Ala–Gln–OBzl (2.72 g) was treated with TFA–anisole (8 ml–1.5 ml) as described above. The resulting powder was dissolved in DMF (5 ml) containing Et_3N (0.7 ml). To this ice-chilled solution, Et_3N (0.7 ml) and the azide (prepared from Boc–Val–Gly–Gly–$NHNH_2$ (1.73 g) with 3.57 N HCl/DMF (2.81 ml), isoamyl nitrite (0.59 ml) and Et_3N (1.39 ml)) in DMF (5 ml) were added, and the mixture was stirred at 5°C for 40 h. The solvent was evaporated off *in vacuo*. The residue was purified by procedure A followed by recrystallization from DMF and ether; yield 2.5 g (72.0%), mp 205—207°C, $[\alpha]_D^{33}$ −16.9° (c=0.71, DMF), Rf_2 0.61. *Anal.* Calcd for $C_{32}H_{49}N_7O_{10}$: C, 55.56; H, 7.14; N, 14.17. Found: C, 55.27; H, 7.10; N, 14.04.

Boc-Gly-Val-Gly-Gly-Ala-Ala-Gln-OBzl——Boc–Val–Gly–Gly–Ala–Ala–Gln–OBzl (0.71 g) was treated with TFA-anisole (2.0 ml–0.5 ml) as described above. The resulting powder was dissolved in DMF–DMSO (5 ml–2 ml) containing Et_3N (0.14 ml). To this ice-chilled solution, the mixed anhydride (prepared from Boc–Gly–OH (0.22 g) with Et_3N (0.17 ml) and isobutyl chloroformate (0.16 ml)) in DMF (5 ml) was added and the mixture was stirred at room temperature for 23 h. The solvent was evaporated off *in vacuo*. The residue was purified by procedure A followed by recrystallization from DMF and AcOEt; yield 0.62 g (80.5%), mp 205—206°C, $[\alpha]_D^{33}$ −15.5° (c=0.71, DMF), Rf_2 0.50. *Anal.* Calcd for $C_{34}H_{52}N_8O_{11}$: C, 54.53; H, 7.00; N, 14.96. Found: C, 54.40; H, 7.09; N, 14.67.

Z(OMe)-Ser-Ser-OMe——The azide (prepared from Z(OMe)–Ser–$NHNH_2$ (28.3 g) with 3.1 N HCl/DMF (64.5 ml), isoamyl nitrite (13.0 ml) and Et_3N (27.8 ml)) in DMF (50 ml) and Et_3N (13.9 ml) were added to a solution of H–Ser–OMe (prepared from the hydrochloride (15.6 g) and Et_3N (13.9 ml)) in DMF (50 ml). The reaction mixture was stirred at 5°C for 41 h, then filtered. The filtrate was concentrated *in vacuo*.

The residue was purified by procedure B followed by recrystallization from AcOEt and petroleum ether; yield 23.8 g (64.3%), mp 129—130°C, $[\alpha]_D^{33}$ +11.9° (c=1.01, DMF), Rf_1 0.30. *Anal.* Calcd for $C_{16}H_{22}N_2O_8$: C, 51.89; H, 5.99; N, 7.56. Found: C, 52.01; H, 5.97; N, 7.30.

Z(OMe)-Ser-Ser-NHNH$_2$——Z(OMe)–Ser–Ser–OMe (22.0 g) in MeOH (100 ml) was treated with 80% hydrazine hydrate (14.4 ml) for 40 h in the usual manner. The product was recrystallized from DMF and ether; yield 21.0 g (95.5%), mp 200—201°C (dec.), $[\alpha]_D^{20}$ +6.7° (c=1.2, 95% aqueous DMSO), Rf_2 0.37. *Anal.* Calcd for $C_{15}H_{22}N_4O_7$: C, 48.65; H, 5.99; N, 15.13. Found: C, 48.35; H, 5.91; N, 15.04.

Z(OMe)-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OBzl——Boc–Gly–Val–Gly–Gly–Ala–Ala–Gln–OBzl (3.2 g) was treated with TFA–anisole (8 ml–2 ml) as described above. The N^{α}-deprotected peptide ester, obtained as a powder, was dissolved in DMF–DMSO (15 ml–15 ml). To this ice-chilled solution, Et_3N (1.14 ml) and the azide (prepared from Z(OMe)–Ser–Ser–$NHNH_2$ (1.6 g) with 3.5 N HCl/DMF (2.4 ml), isoamyl nitrite (0.51 ml) and Et_3N (1.2 ml)) in DMF (15 ml) were added and the reaction mixture was stirred at 5°C for 38 h. After filtration, the filtrate was poured into ether–petroleum ether (1: 2, v/v; 500 ml) and the resulting oily product was separated by decantation and then triturated with H_2O. The powder was purified by procedure A followed by recrystallization from DMF and MeOH; yield 2.8 g (72.7%), mp 215—216°C (dec.), $[\alpha]_D^{33}$ −19.7° (c=0.61, DMSO), Rf_2 0.39. *Anal.* Calcd for $C_{44}H_{62}N_{10}O_{16}$: C, 53.54; H, 6.33; N, 14.19. Found: C, 53.29; H, 6.39; N, 13.99.

Z(OMe)-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OBzl [I]——The above protected nonapeptide (3.45 g) was treated with TFA–anisole (10 ml–2 ml) as described above. The N^{α}-deprotected peptide ester was dissolved in DMF–DMSO (10 ml–10 ml) and allowed to react with the azide (prepared from Z(OMe)–Ser–Ser–$NHNH_2$ (1.42 g) as mentioned above) in DMF (10 ml) at 5°C for 38 h. To this reaction mixture, ether (150 ml) was added and the resulting powder was purified by procedure A followed by recrystallization from DMSO–MeOH and ether; yield 2.75 g (73.9%), mp 230—232°C (dec.), $[\alpha]_D^{16}$ −11.0° (c=0.73, 95% aqueous DMSO), Rf_3 0.26. Amino acid ratios in 4 N MSA hydolysate: $Ser_{3.84}Gly_{3.16}Val_{1.00}Ala_{2.09}Glu_{1.02}$ (recovery of Val, 87.7%). *Anal.* Calcd for $C_{50}H_{72}N_{12}O_{20}\cdot 2.5H_2O$: C, 49.79; H, 6.43; N, 13.93. Found: C, 49.97; H, 6.37; N, 13.50.

Z(OMe)-Arg(Mts)-Arg(Mts)-OMe——The mixed anhydride (prepared from Z(OMe)–Arg(Mts)–OH (5.0 g) with Et_3N (1.33 ml) and isobutyl chloroformate (1.27 ml) as mentioned above) in DMF (20 ml) was added to a solution of H–Arg(Mts)–OMe (prepared from the hydrochloride (3.9 g) and Et_3N (1.33 ml)) in DMF (20 ml). The reaction mixture was stirred at room temperature for 18 h. The solvent was evaporated off *in vacuo*. The residue was purified by procedure B followed by column chromatography on silica using a solvent system of $CHCl_3$–MeOH (40: 1) and further precipitated from $CHCl_3$ with petroleum ether; yield 7.0 g (83.3%), amorphous powder, $[\alpha]_D^{16}$ +4.0° (c=0.38, $CHCl_3$), Rf_1 0.43. *Anal.* Calcd for $C_{40}H_{56}N_8O_{10}S_2$: C, 55.03; H, 6.47; N, 12.83. Found: C, 54.80; H, 6.55; N, 12.61.

Z(OMe)-Arg(Mts)-Arg(Mts)-NHNH$_2$——In the usual manner, Z(OMe)–Arg(Mts)–Arg(Mts)–OMe (6.0 g) in MeOH (15 ml) was treated with 80% hydrazine hydrate (1.7 ml) for 48 h; yield 5.35 g (89.2%), amorphous powder, $[\alpha]_D^{18}$ +1.4° (c=0.70, DMF), Rf_1 0.15. *Anal.* Calcd for $C_{39}H_{56}N_{10}O_9S_2$: C, 53.65; H, 6.47; N, 16.04. Found: C, 53.41; H, 6.71; N, 15.98.

Z(OMe)-Arg(Mts)-Arg(Mts)-Asn-Gly-OH [II]——The azide (prepared from Z(OMe)–Arg(Mts)–Arg(Mts)–$NHNH_2$ (4.16 g) with 3.1 N HCl/DMF (3.07 ml), isoamyl nitrite (0.56 ml) and Et_3N (1.32 ml)) in DMF (20 ml) was added to an ice-chilled solution of H–Asn–Gly–OH[13] (0.9 g) in H_2O (10 ml) containing Et_3N (1.32 ml). The reaction mixture was stirred at 5°C for 48 h and the solvent was evaporated off *in vacuo*. The residue was treated with 10% citric acid and the resulting gummy product was purified by column chromatography on silica using a solvent system of $CHCl_3$–MeOH–H_2O (8: 3: 1); yield 2.67 g (54.5%), amorphous powder, $[\alpha]_D^{31}$ −2.9° (c=1.03, DMF), Rf_2 0.12. *Anal.* Calcd for $C_{45}H_{63}N_{11}O_{13}S_2\cdot 2H_2O$: C, 50.69; H, 6.33; N, 14.45. Found: C, 50.96; H, 6.54; N, 14.39.

Z(OMe)-Arg(Mts)-Arg(Mts)-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OBzl——The above protected undecapeptide (I, 1.74 g) was treated with TFA–anisole (5 ml–0.8 ml) as described above and then the excess TFA was removed by evaporation *in vacuo* at 0°C. The residue was treated with 3.6 N HCl/AcOEt (5 ml) and the AcOEt was evaporated off *in vacuo* below 30°C. The residue was triturated with ether. The $N\alpha$-deprotected undecapeptide ester thus obtained was dissolved in DMSO (7 ml) containing Et_3N (0.3 ml). To this solution, a mixture of Z(OMe)–Arg(Mts)–Arg(Mts)–Asn–Gly–OH (II, 1.85 g), DCC (0.46 g) and HOBT (0.33 g) in DMF (4 ml) was added and the reaction mixture was stirred at room temperature for 63 h. After filtration, ether was added to the filtrate and the resulting powder was purified by repeated precipitation from DMSO–MeOH and ether; yield 2.45 g (81.4%), mp 232—233°C (dec.), $[\alpha]_D^{18}$ −9.8° (c=0.72, 95% aqueous DMSO), Rf_2 0.12. Amino acid ratios in 4 N MSA hydrolysate: $Arg_{2.05}Asp_{1.03}Gly_{3.97}Ser_{3.52}Val_{1.00}Ala_{1.98}Glu_{1.08}$ (recovery of Val, 85.8%). *Anal.* Calcd for $C_{86}H_{125}N_{23}O_{29}S_2\cdot 3H_2O$: C, 50.06; H, 6.40; N, 15.61. Found: C, 50.15; H, 6.49; N, 15.43.

Z-Tyr-Val-Met(O)-Gly-His-Phe-Arg(Mts)-Trp-Asp(OBzl)-Arg(Mts)-Phe-Gly-OSu——Under cooling with ice, N-hydroxysuccinimide (54 mg) and DCC (89.4 mg) were added to a solution of Z–Tyr–Val–Met(O)–Gly–His–Phe–Arg(Mts)–Trp–Asp(OBzl)–Arg(Mts)–Phe–Gly–OH[3] (0.79 g) in DMF (10 ml). The reaction mixture was stirred at room temperature for 72 h. After filtration, the filtrate was concentrated *in vacuo*. The residue was purified by reprecipitation from DMF with MeOH; yield 550 mg (66.9%), mp 211—215°C,

$[\alpha]_D^{24}$ −18.0° (c=0.5, DMF). *Anal.* Calcd for $C_{111}H_{134}N_{22}O_{25}S_3 \cdot H_2O$: C, 58.20; H, 5.98; N, 13.45. Found: C, 57.90; H, 5.96; N, 13.24.

Z-Tyr-Val-Met(O)-Gly-His-Phe-Arg(Mts)-Trp-Asp(OBzl)-Arg(Mts)-Phe-Gly-Arg(Mts)-Arg(Mts)-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OBzl——The above protected pentadecapeptide (0.3 g) was treated with TFA–anisole (3 ml–0.6 ml) as described above. The N^{α}-deprotected pentadecapeptide ester, obtained as a powder, was dissolved in DMF–DMSO (4: 1, v/v; 12 ml) containing Et_3N (0.02 ml). To this solution, the above *N*-hydroxysuccinimide ester (318 mg) and Et_3N (0.02 ml) were added and the reaction mixture was stirred at room temperature for 72 h. The solvent was evaporated off *in vacuo* and ether was added. The resulting powder was reprecipitated from DMSO–DMF (1: 1) with ether and further purified by gel-filtration on Sephacryl S-200 (2.5 × 100 cm) using 95% aqueous DMSO as the eluent; yield 300 mg (51.5%), mp 234—238°C (dec.), $[\alpha]_D^{25}$ −17.6° (c=0.85, DMSO), Rf_5 0.64. Amino acid ratios in 4 N MSA hydrolysate: $Asp_{2.09}Ser_{4.11}Glu_{0.89}Gly_{6.61}Ala_{1.74}Val_{2.11}Met+Met(O)_{0.99}Tyr_{0.90}Phe_{2.00}Trp_{0.92}His_{1.06}Arg_{4.00}$ (recovery of Phe, 84.6%). *Anal.* Calcd for $C_{184}H_{246}N_{44}S_5 \cdot 5H_2O$: C, 54.00; H, 6.31; N, 15.06. Found: C, 53.80; H, 6.29; N, 14.73.

H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-Arg-Arg-Asn-Gly-Ser-Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OH——The above protected heptacosapeptide (130 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of skatole (20 ml) in an ice-bath for 2 h. Dry ether was then added and the resulting oily product was dissolved in H_2O (10 ml). The solution was treated with Amberlite IRA-400 (acetate form) for 30 min. After filtration, the filtrate was concentrated to approximately 3 ml. The solution was applied to a column of Sephadex G-25 (3.3 × 65 cm) and eluted with 0.1 N AcOH. Individual fractions (10 ml each) were collected and the absorbancy at 275 nm was determined. The fractions corresponding to the main peak (tube Nos. 33—37) were collected and the solvent was removed by lyophilization. The residue was dissolved in H_2O (10 ml) and the solution, after incubation with 2-mercaptoethanol (0.2 ml) at 50°C for 5 h under N_2 gas, was applied to a column of CM-cellulose (2.0 × 10 cm), which was eluted with H_2O (100 ml), 0.05 M ammonium acetate buffer (pH 6.9, 100 ml) and then with a gradient formed from 0.1 M ammonium acetate buffer (pH 6.9, 400 ml) through a mixing flask containing 0.05 M ammonium acetate buffer (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. 67—74) were collected and concentrated to approximately 3 ml. The solution was applied to a column of Sephadex G-10 (3.3 × 40 cm) and eluted with 0.1 N AcOH. The desired fractions were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 15 mg (13.7%), $[\alpha]_D^{18}$ −37.5° (c=0.24, 10% AcOH). [lit.[4] $[\alpha]_D^{23}$ −44.4° (c=1, 1% AcOH)]. Rf_4 0.08, Rf_6 0.38, Rf_7 (cellulose) 0.42. Single band in 15% polyacrylamide gel electrophoresis at pH 4.0 (mobility, 1.8 cm from the origin toward the cathode, after running at 5 mA per tube for 90 min. Amino acid ratios in 4 N MSA hydrolysate: $Tyr_{0.80}Val_{1.87}Met_{0.80}Gly_{6.39}His_{0.88}Phe_{2.00}Arg_{3.96}Trp_{0.80}Asp_{1.94}Ser_{3.64}Glu_{1.02}Ala_{2\ 00}$ (recovery of Ala, 85.5%). Amino acid ratios in AP–M digest: $Tyr_{0.83}Val_{1.88}Met_{0.93}Gly_{6.40}His_{1.07}Phe_{1.89}Arg_{4.15}Trp_{1.11}Asp_{1.25}Ser+Asn_{4.81}$ (4+1, Calcd as Ser) $Gln_{0.10}Glu_{0.39}Ala_{2.00}$ (recovery of Ala, 82.1%). Amino acid ratios in CP–P digest: $Ala_{2.00}Gln_{0.90}$ (recovery of Ala, 86.5%). *Anal.* Calcd for $C_{126}H_{188}N_{44}O_{37}S \cdot 4CH_3COOH \cdot 13H_2O$: C, 47.09; H, 6.78; N, 18.03. Found: C, 46.76; H, 6.14; N, 17.83.

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References and Notes

1) Amino acids, peptides and their derivatives mentioned in this report are of L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Mts = mesitylene-2-sulfonyl, Np = *p*-nitrophenyl ester, Su = *N*-hydroxysuccinimide ester, Im = imidazole DCC = dicyclohexylcarbodiimide, TPP = triphenylphosphite, TFA = trifluoroacetic acid, DMF = dimethylformamide, DMSO = dimethylsulfoxide, TFMSA = trifluoromethanesulfonic acid, MSA = methanesulfonic acid.
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