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Synthesis of the Docosapeptide Corresponding to the Entire Amino Acid Sequence of Dogfish Corticotropin-like Intermediate Lobe Peptide[1]

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The docosapeptide, H–Arg–Pro–Ile–Lys–Val–Tyr–Pro–Asn–Ser–Phe–Glu–Asp–Glu–Ser–Val–Glu–Asn–Met–Gly–Pro–Glu–Leu–OH, corresponding to dogfish corticotropin-like intermediate lobe peptide (CLIP), was synthesized using protecting groups removable by hydrogen fluoride.

Keywords——dogfish ACTH; dogfish CLIP; docosapeptide; hydrogen fluoride procedure; DCC plus HOBT procedure; modified azide procedure; *p*-nitrophenyl ester procedure; partition column chromatography

In 1973, Scott *et al.*[3] reported the isolation of a new adrenocorticotropin-like peptide, termed as corticotropin-like intermediate lobe peptide (CLIP) from the pars intermedia of pig pituitary, and clarified that this peptide is a fragment (position 18 to 39) formed by tryptic-like cleavage of the precursor, adrenocorticotropin (ACTH). Succeeding this finding, Lowry *et al.*[4] found that the identical pathway exists also in the pituitary of non-mammalian source, dogfish *squalus acanthias*.

They determined the amino acid sequences of ACTH and CLIP isolated from the pituitary of the dogfish. The structure of dogfish ACTH thus clarified is similar to that of mammalian

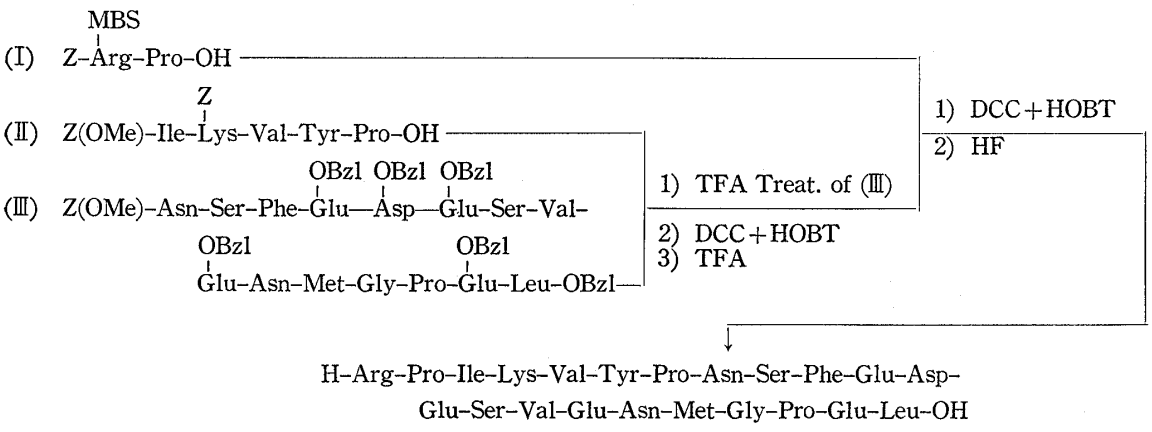

Fig. 1. Synthetic Route to the Dogfish CLIP

1) The amino acids (except glycine) employed in this paper are of the L-configuration. Abbreviation used are those recommended by IUPAC-IUB Commission of Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, MBS=*p*-methoxybenzenesulfonyl, OBzl=benzyl ester, ONP=*p*-nitrophenyl ester, DMF=dimethylformamide, THF=tetrahydrofuran.

2) Location: *a*) *Misasagi, Yamashina-ku, Kyoto*; *b*) *Sakyo-ku, Kyoto*.

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4) P.J. Lowry, H.P.J. Bennett, C. McMartin, and A.P. Scott, *Biochem. J.*, **141**, 427 (1974).

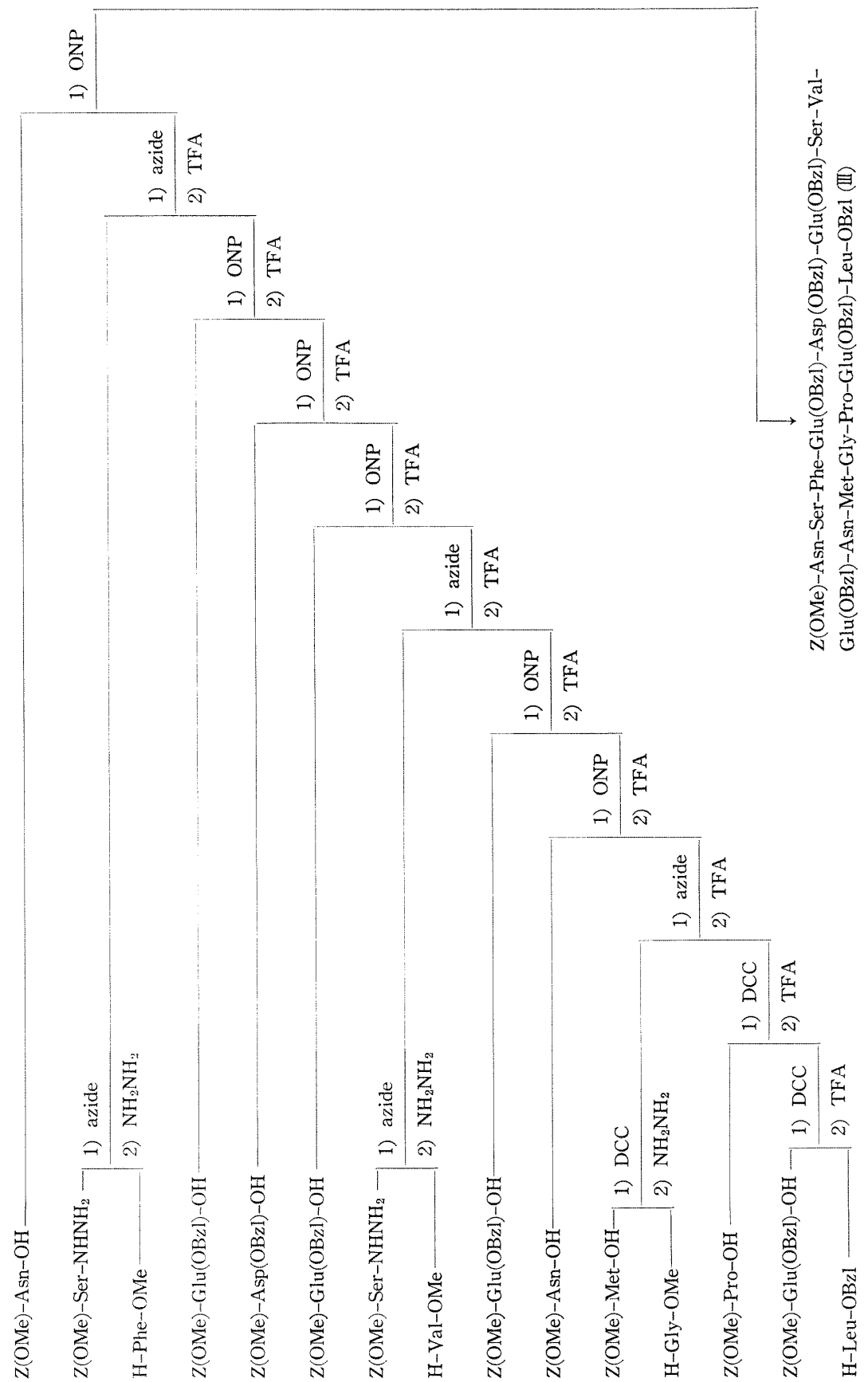

Fig. 2. Synthetic Route to the C-terminal Pentadecapeptide Ester, Z(OMe)-(d-ACTH 25—39)-OBzl

ACTHs[5] in respect of the chain length, but largely differs in amino acid compositions. Thus the structure of dogfish CLIP is different from that of the mammalian CLIPs so far known.[5]

In this paper, we wish to describe the synthesis of the docosapeptide corresponding to the entire amino acid sequence of dogfish CLIP, which covers the C-terminal portion of dogfish ACTH (position 18 to 39).

In the present synthesis, amino acid derivatives bearing protecting groups removable by hydrogen fluoride[6] were employed; *i.e.*, Arg(MBS),[7] Lys(Z), Glu(OBzl) and Asp(OBzl). These side chain protecting groups survive mostly intact under the careful trifluoroacetic acid(TFA) treatment for the removal of the Z(OMe) group,[8] employed as a temporary α-amino protecting group.

As shown in Fig. 1, three fragments served to construct the entire sequence of dogfish CLIP: Z-Arg(MBS)-Pro-OH (I), Z(OMe)-Ile-Lys(Z)-Val-Tyr-Pro-OH (II) and Z(OMe)-Asn-Ser-Phe-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl (III).

Synthetic route to the C-terminal pentadecapeptide ester (III), abbreviated as Z(OMe)-(d-ACTH 25—39)-OBzl, is illustrated in Fig. 2. The protected tripeptide ester, Z(OMe)-Pro-Glu(OBzl)-Leu-OBzl (IV) was synthesized in a stepwise manner by the dicyclohexylcarbodiimide (DCC) procedure[9] starting from H-Leu-OBzl. After the TFA treatment[8] of IV, the resulting tripeptide ester was condensed with Z(OMe)-Met-Gly-$NHNH_2$ by modified azide procedure[10] to give the protected pentapeptide, Z(OMe)-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl (V). Combination of the TFA treatment for deprotection of the Z(OMe) group and the *p*-nitrophenyl ester method[11] or the azide procedure was applied to elongate this pentapeptide chain (V) to the pentadecapeptide stage. The former condensation tool was employed for introduction of Asn, Asp(OBzl) and Glu(OBzl) residues and the latter for dipeptide units, Ser-Val and Ser-Phe. The purity of the protected pentadecapeptide ester, Z(OMe)-(d-ACTH 25—39)-OBzl, was confirmed by three criteria; *i.e.*, thin-layer chromatography (TLC), elemental and amino acid analyses.

Next, synthetic route to the protected pentapeptide (II), abbreviated as Z(OMe)-(d-ACTH 20—24)-OH, is illustrated in Fig. 3. Z-Val-Tyr-$NHNH_2$[12] was condensed with the triethyl-

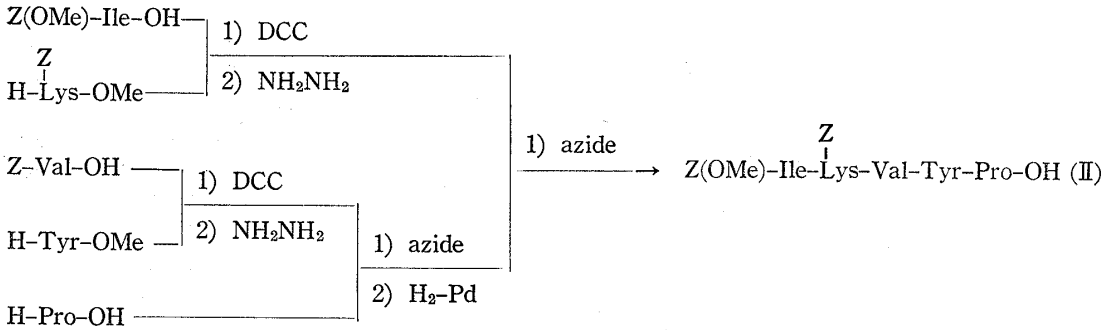

Fig. 3. Synthetic Route to the Protected Pentapeptide (II), Z(OMe)-(d-ACTH 20—24)-OH

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ammonium salt of H-Pro-OH by the modified azide method to give the protected tripeptide, Z-Val-Tyr-Pro-OH (VI), which after hydrogenation, was condensed with Z(OMe)-Ile-Lys(Z)-$NHNH_2$ again by the modified azide method. The purity of the product (II) thus obtained was confirmed by TLC, elemental and amino acid analyses.

The fragment (I), Z-Arg(MBS)-Pro-OH, was prepared by condensation of Z-Arg(MBS)-OH[7] and H-Pro-OBzl with DCC method followed by saponification.

Three fragments thus obtained were then assembled according to the scheme illustrated in Fig. 1. For condensation of Z(OMe)-(d-ACTH 20—24)-OH and Z(OMe)-(d-ACTH 25—39)-OBzl, the Z(OMe) group of the latter was cleaved by TFA in the presence of anisole, and the resulting TFA salt was neutralized with 5% $NaHCO_3$. The deblocked pentadecapeptide ester isolated as a powder was coupled with Z(OMe)-(d-ACTH 20—24)-OH by DCC in the presence of N-hydroxybenzotriazole (HOBT).[13] The resulting protected eicosapeptide ester, Z(OMe)-Ile-Lys(Z)-Val-Tyr-Pro-Asn-Ser-Phe-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl, abbreviated as Z(OMe)-(d-ACTH 20—39)-OBzl, was purified by batchwise washing with acid, base and methanol followed by reprecipitation from DMF with methanol.

The protected eicosapeptide ester, Z(OMe)-(d-ACTH 20—39)-OBzl, was treated with TFA in the presence of anisole as stated above and the resulting TFA salt was converted to the corresponding hydrochloride and subsequently neutralized with triethylamine. The deblocked eicosapeptide ester was condensed with Z-Arg(MBS)-Pro-OH (I) by DCC in the presence of HOBT. The docosapeptide ester thus obtained, Z-Arg(MBS)-Pro-Ile-Lys(Z)-Val-Tyr-Pro-Asn-Ser-Phe-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl (protected CLIP), was purified by batchwise washing followed by reprecipitation in essentially the same manner as mentioned above.

Finally, the protected docosapeptide ester thus obtained was treated with HF in the presence of anisole to remove all protecting groups. The deblocked product, after treatment with Amberlite IR-45 (acetate form), was purified by partition column chromatography on Sephadex G-25 according to Yamashiro.[14] The solvent system consisting of *n*-butanol, acetic acid and water (4: 1: 5) was applied to elute the desired compound. The absorbancy (275 nm) due to the Tyr residue was the guide for this chromatographic purification. Homogeneity of the synthetic dogfish CLIP thus obtained was confirmed by TLC, paper electrophoresis and elemental analysis. The hydrolysate by 6 N hydrochloric acid contained the constituent amino acids in ratios predicted by theory. In addition, complete digestion of the synthetic docosapeptide was achieved by aminopeptidase M (AP-M).[15] In amino acid analysis of the latter hydrolysate, Asn peak was overlapped with that of Ser. However, from the difference of Asp recoveries between acid and enzymatic hydrolysate, nearly quantitative amount of Asn could be estimated.

Our synthetic peptide may serve, together with synthetic mammalian CLIPs,[16] to clarify intrinsic physiological roles of this intracellular fragment derived from ACTH as a part of pituitary functions.

Experimental

The melting points are uncorrected. Rotations were determined in a Rex Photoelectric Polarimeter, Model NEP-2 (cell length: 10 cm). The amino acid compositions of the acid and enzymatic hydrolysates

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were determined with a Hitachi Amino Acid Analyzer, Model KLA-5. The solvents were evaporated *in vacuo* at a bath temperature of 40 to 50° in a rotary evaporator. High voltage paper electrophoresis, 33.3 volts per cm at 5° for 60 min, was performed with Toyo High Voltage Paper Electrophoresis Apparatus, Model HPE-V. TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (9: 1), Rf_2 $CHCl_3$–MeOH (29: 1), Rf_3 $CHCl_3$–MeOH–H_2O (90: 15: 5), Rf_4 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf_5 $CHCl_3$–MeOH–AcOH (9: 1: 0.5), Rf_6 $CHCl_3$–MeOH–AcOH (9: 1: 1), Rf_7 *n*-butanol–AcOH–pyridine–H_2O (15: 3: 10: 12), Rf_8 *n*-butanol–AcOH–pyridine–H_2O (4: 1: 1: 2) and Rf_9 *n*-butanol–AcOH–H_2O (2: 1: 1).

Z(OMe)-Glu(OBzl)-Leu-OBzl——Z(OMe)–Glu(OBzl)–OH (20.1 g) and DCC (10.3 g) were added to a solution of H–Leu–OBzl (prepared from 19.7 g of the tosylate and 7.0 ml of Et_3N) in THF (100 ml) under cooling with ice and the mixture was stirred at room temperature for 10 hr. After filtration, the filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 10% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was recrystallized from AcOEt and pet. ether; yield 23.0 g (75.9%), mp 87—89°, $[\alpha]_D^{31}$ −14.6° (c=1.78, $CHCl_3$), Rf_2 0.61. *Anal.* Calcd. for $C_{34}H_{40}N_2O_8$: C, 67.53; H, 6.67; N, 4.63. Found: C, 67.53; H, 6.65; N, 4.54.

Z(OMe)-Pro-Glu(OBzl)-Leu-OBzl——Z(OMe)–Glu(OBzl)–Leu–OBzl (20.0 g) was treated with TFA (40 ml) in the presence of anisole (7 ml) at 0° for 60 min. The excess TFA was evaporated *in vacuo*. The oily product was washed with pet. ether and then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and dissolved in THF (80 ml). To this solution, Et_3N (2.9 ml), Z(OMe)–Pro–OH (6.4 g) in THF (20 ml) and DCC (4.7 g) were added under cooling with ice and the mixture was stirred at room temperature for 20 hr. After filtration, the filtrate was evaporated *in vacuo* and the residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 10% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was recrystallized from AcOEt and pet. ether; yield 10.8 g (73.5%), mp 147—148.5°, $[\alpha]_D^{30}$ −57.4° (c=2.16, MeOH), Rf_3 0.84. *Anal.* Calcd. for $C_{39}H_{47}N_3O_9$: C, 66.75; H, 6.75; N, 5.99. Found: C, 66.97; H, 6.86; N, 6.12.

Z(OMe)-Met-Gly-OMe——Z(OMe)–Met–OH (20.0 g) and DCC (13.2 g) were added to a solution of H–Gly–OMe (prepared from 8.8 g of the hydrochloride and 9.8 ml of Et_3N) in THF (120 ml)–DMF (20 ml) under cooling with ice and the mixture was stirred at room temperature for 18 hr. After filtration, the filtrate was evaporated *in vacuo* and the residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 10% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated *in vacuo*. The residue was recrystallized from AcOEt and pet. ether; yield 16.5 g (64.2%), mp 96.5—97°, $[\alpha]_D^{30}$ −5.9° (c=1.05, $CHCl_3$), Rf_2 0.39. *Anal.* Calcd. for $C_{17}H_{24}N_2O_6S$: C, 53.11; H, 6.29; N, 7.29. Found: C, 53.38; H, 6.32; N, 7.35.

Z(OMe)-Met-Gly-NHNH$_2$——To a solution of Z(OMe)–Met–Gly–OMe (16.5 g) in MeOH (100 ml), 80% hydrazine hydrate (9.7 ml) was added and the mixture was kept on standing for 48 hr. The resulting solid was recrystallized from MeOH and H_2O; yield 15.0 g (90.9%), mp 142.5—145°, $[\alpha]_D^{17}$ −7.6° (c=1.95, DMF), Rf_3 0.58. *Anal.* Calcd. for $C_{16}H_{24}N_4O_5$: C, 49.99; H, 6.29; N, 14.57. Found: C, 50.08; H, 6.00; N, 14.45.

Z(OMe)-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——Z(OMe)–Pro–Glu(OBzl)–Leu–OBzl (20.0 g) was treated with TFA (40 ml) in the presence of anisole (10 ml) at 0° for 30 min and the excess TFA was evaporated *in vacuo*. The oily residue, after washing with *n*-hexane, was dried over KOH pellets *in vacuo* and then dissolved in THF (100 ml)–DMF (10 ml). To this ice-chilled solution, Et_3N (4.0 ml) and a solution of azide (prepared from 11.5 g of Z(OMe)–Met–Gly–$NHNH_2$/DMF (20 ml) with 28.5 ml of 2 N HCl/DMF, 4.0 ml of isoamyl nitrite and 4.0 ml of Et_3N) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and the residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 10% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated *in vacuo*. The residue was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH (100: 1)); yield 15.2 g (60.0%), mp 130—132°, $[\alpha]_D^{23}$ −45.8° (c=1.43, DMF), Rf_1 0.23. *Anal.* Calcd. for $C_{46}H_{59}N_5O_{11}S$: C, 62.07; H, 6.68; N, 7.87. Found: C, 62.29; H, 6.79; N, 7.81.

Z(OMe)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——Z(OMe)–Met–Gly–Pro–Glu(OBzl)–Leu–OBzl (15.0 g) was treated with TFA (35 ml) in the presence of anisole (7.5 ml) at 0° for 60 min and the TFA salt precipitated by addition of dry ether–pet. ether was collected by filtration and dried over KOH pellets *in vacuo*. This powder was dissolved in DMF (90 ml). To this solution, Z(OMe)–Asn–ONP (10.3 g), Et_3N (2.3 ml) and HOBT (20 mg) were added. The mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo* and the residue was washed batchwisely with 10% citric acid, 10% Na_2CO_3 and H_2O–NaCl. The product was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH (40: 1)); yield 10.2 g (67.0%), mp 200—203°, $[\alpha]_D^{22}$ −42.5° (c=1.04, DMF), Rf_4 0.66. *Anal.* Calcd. for $C_{50}H_{65}N_7O_{13}S$: C, 59.81; H, 6.52; N, 9.76. Found: C, 59.51; H, 6.63; N, 9.73.

Z(OMe)-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——Z(OMe)–Asn–Met–Gly–Pro–Glu(OBzl)–Leu–OBzl (15.0 g) was treated with TFA (30 ml) in the presence of anisole (7.5 ml) at 0° for 60 min. The TFA salt precipitated by addition of dry ether was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (60 ml). To this solution, Z(OMe)–Glu(OBzl)–ONP (8.2 g), Et_3N (1.82 ml) and HOBT (30 mg) were added and the mixture was stirred at room temperature for 72 hr. The solvent was evaporated *in vacuo* and the residue was washed batchwisely with 10% citric acid, 10% Na_2CO_3 and H_2O–

NaCl. The product was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH (30: 1)); yield 8.8 g (55.0%), mp 204.5—206.5°, $[\alpha]_D^{21}$ −36.4° (c=0.93, DMF), Rf_4 0.64. *Anal.* Calcd. for $C_{62}H_{78}N_8O_{16}S$: C, 60.87; H, 6.43; N, 9.16. Found: C, 61.17; H, 6.34; N, 9.07.

Z(OMe)-Ser-Val-OMe——Under cooling with ice-NaCl, Et_3N (5.6 ml) and a solution of azide (prepared from 9.0 g of Z(OMe)–Ser–$NHNH_2$/DMF (20 ml) with 40 ml of 2 N HCl/DMF, 5.9 ml of isoamyl nitrite and 11.2 ml of Et_3N) were added to a solution of H–Val–OMe (prepared from 6.8 g of the hydrochloride and 5.6 ml of Et_3N) in DMF (40 ml). The mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and then the residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 10% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated *in vacuo*. The residue was recrystallized from AcOEt and pet. ether; yield 12.2 g (79.7%), mp 98.5—99.5°, $[\alpha]_D^{28}$ −15.9° (c=1.17, $CHCl_3$), Rf_1 0.63. *Anal.* Calcd. for $C_{18}H_{26}N_2O_7$: C, 56.54; H, 6.85; N, 7.33. Found: C, 56.81; H, 6.88; N, 7.14.

Z(OMe)-Ser-Val-$NHNH_2$——To a solution of Z(OMe)–Ser–Val–OMe (11.5 g) in MeOH (100 ml), 80% hydrazine hydrate (7.3 ml) was added and the mixture was kept on standing for 72 hr. The resulting solid was recrystallized from DMF and ether; yield 11.0 g (95.7%), mp 228.5—229°, $[\alpha]_D^{29}$ +11.8° (c=0.47, DMF), Rf_4 0.66. *Anal.* Calcd. for $C_{17}H_{26}N_4O_6$: C, 53.39; H, 6.85; N, 14.65. Found: C, 53.65; H, 7.03; N, 14.57.

Z(OMe)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——Z(OMe)–Glu(OBzl)–Asn–Met–Gly–Pro–Glu(OBzl)–Leu–OBzl (8.0 g) was treated with TFA (16 ml) in the presence of anisole (4 ml) at 0° for 60 min and then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and then dissolved in DMF (25 ml). To this ice-chilled solution, Et_3N (1.8 ml) and a solution of azide (prepared from 3 g of Z(OMe)–Ser–Val–$NHNH_2$/DMF (13 ml) with 17.3 ml of 0.7 N HCl/DMF, 1.14 ml of isoamyl nitrite and 2.2 ml of Et_3N) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and the residue was washed batchwisely with 10% citric acid and H_2O–NaCl. The product was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH–H_2O (100: 10: 1)); yield 7.0 g (76.0%), mp 225—230°, $[\alpha]_D^{30}$ −31.0° (c=0.86, DMF), Rf_4 0.56. *Anal.* Calcd. for $C_{70}H_{92}N_{10}O_{19}S \cdot 0.5H_2O$: C, 59.27; H, 6.61; N, 9.87. Found: C, 59.17; H, 6.73; N, 9.85.

Z(OMe)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——The above protected nonapeptide ester (5.0 g) was treated with TFA (13 ml) in the presence of anisole (2.5 ml) at 0° for 60 min and then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and then dissolved in DMF (30 ml). To this solution, Et_3N (0.98 ml), Z(OMe)–Glu(OBzl)–ONP (2.0 g) and HOBT (30 mg) were added. The solvent, after stirring at room temperature for 72 hr, was evaporated *in vacuo* and the residue was washed batchwisely with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl. The product was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH–H_2O (90: 15: 5)); yield 4.2 g (73.0%), mp 234—239°, $[\alpha]_D^{19}$ −29.8° (c=0.61, DMF), Rf_3 0.47. Amino acid ratios in acid hydrolysate: $Glu_{3.16}Asp_{1.19}Ser_{0.96}Pro_{0.70}Gly_{1.00}Val_{1.04}Met_{0.50}Leu_{0.96}$ (average recovery 78.1%). *Anal.* Calcd. for $C_{82}H_{105}N_{11}O_{22}S \cdot 0.5H_2O$: C, 60.13; H, 6.52; N, 9.41. Found: C, 59.96; H, 6.55; N, 9.17.

Z(OMe)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——The above protected decapeptide ester (3.9 g) was treated with TFA (10 ml) in the presence of anisole (2 ml) at 0° for 60 min and then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and then dissolved in DMF (50 ml). To this solution, Et_3N (0.68 ml), Z(OMe)–Asp(OBzl)–ONP (1.5 g) and HOBT (40 mg) were added. The solution, after stirring at room temperature for 72 hr, was condensed *in vacuo* and the residue was washed batchwisely with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl. The product was reprecipitated from DMF with MeOH; yield 3.83 g (87.0%), mp 230—235°, $[\alpha]_D^9$ −28.2° (c=0.77, DMF), Rf_3 0.44. Amino acid ratios in acid hydrolysate: $Asp_{2.09}Glu_{3.50}Ser_{0.93}Val_{1.00}Met_{0.70}Gly_{1.00}Pro_{0.70}Leu_{1.00}$ (average recovery 90.4%). *Anal.* Calcd. for $C_{90}H_{116}N_{12}O_{25}S$: C, 60.90; H, 6.38; N, 9.16. Found: C, 60.68; H, 6.32; N, 9.16.

Z(OMe)-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——The above protected undecapeptide ester (4.1 g) was treated with TFA (10 ml) in the presence of anisole (2.0 ml) at 0° for 60 min and then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and dissolved in DMF (40 ml). To this solution, Et_3N (0.6 ml), Z(OMe)–Glu(OBzl)–ONP (1.36 g) and HOBT (30 mg) were added and the mixture was stirred at room temperature for 72 hr. The solvent was evaporated *in vacuo* and the residue was washed batchwisely with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl. The product was reprecipitated from DMF with MeOH; yield 3.7 g (82.0%), mp 232—237°, $[\alpha]_D^{10}$ −30.3° (c=0.66, DMF), Rf_3 0.53. Amino acid ratios in acid hydrolysate: $Glu_{4.69}Asp_{2.07}Ser_{0.90}Val_{1.00}Met_{0.62}Gly_{1.00}Pro_{0.66}Leu_{1.00}$ (average recovery 97.0%). *Anal.* Calcd. for $C_{105}H_{129}N_{13}O_{28}S$: C, 61.42; H, 6.33; N, 8.87. Found: C, 61.18; H, 6.29; N, 8.88.

Z(OMe)-Ser-Phe-OMe——To an ice-chilled solution of H–Phe–OMe (prepared from 8.64 g of the hydrochloride and 5.6 ml of Et_3N) in DMF(40 ml), Et_3N (5.6 ml) and a solution of azide (prepared from 11.4 g of Z(OMe)–Ser–$NHNH_2$/DMF (10 ml) with 40 ml of 2 N HCl/DMF, 5.87 ml of isoamyl nitrite and 11.2 ml of Et_3N) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and the residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 10% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then evaporated *in vacuo*. The residue was recrystallized from

AcOEt and pet. ether; yield 13.1 g (75.7%), mp 126—127°, $[\alpha]_D^{13}$ −0.7° (c=1.0, MeOH), (lit.[17] mp 118—119°, $[\alpha]_D^{25}$ −8.4° (c=1, MeOH)), Rf_3 0.75. *Anal.* Calcd. for $C_{22}H_{26}N_2O_7$: C, 61.39; H, 6.09; N, 6.51. Found: C, 61.65; H, 6.03; N, 6.32.

Z(OMe)-Ser-Phe-NHNH$_2$——To a solution of Z(OMe)–Ser–Phe–OMe (10 g) in MeOH (50 ml), 80% hydrazine hydrate (5.6 ml) was added and the mixture was kept on standing for 48 hr. The resulting solid was recrystallized from DMF and ether; yield 8.9 g (89.0%), mp 224.5—225°, $[\alpha]_D^{13}$ −1.1° (c=0.44, DMF), Rf_4 0.47. *Anal.* Calcd. for $C_{21}H_{26}N_4O_6$: C, 58.60; H, 6.09; N, 13.02. Found: C, 58.77; H, 6.33; N, 13.25.

Z(OMe)-Ser-Phe-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——The above protected dodecapeptide ester (5.1 g) was treated with TFA (15 ml) in the presence of anisole (2.5 ml) at 0° for 60 min and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and dissolved in DMF (20 ml). To this ice-chilled solution, Et_3N (0.68 ml) and a solution of azide (prepared from 1.6 g of Z(OMe)–Ser–Phe–NHNH$_2$/DMF (5 ml) with 6.0 ml of 1.25 N HCl/DMF, 0.5 ml of isoamyl nitrite and 1.03 ml of Et_3N) were added and the solution, after stirring at 4° for 48 hr, was condensed *in vacuo*. The residue was washed batchwisely with 10% citric acid and H_2O–NaCl. The product was reprecipitated from DMF with MeOH; yield 4.2 g (73.0%), mp 242—246°, $[\alpha]_D^{13}$ −26.0° (c=0.72, DMF), Rf_5 0.35. Amino acid ratios in acid hydrolysate: $Ser_{1.80}Phe_{1.00}Glu_{4.82}Asp_{2.04}Val_{1.00}Met_{0.70}Gly_{1.00}Pro_{0.70}Leu_{1.01}$ (average recovery 97.9%). *Anal.* Calcd. for $C_{117}H_{143}N_{15}O_{31}S$: C, 61.43; H, 6.30; N, 9.18. Found: C, 61.31; H, 6.40; N, 9.33.

Z(OMe)-Asn-Ser-Phe-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl, Z(OMe)-(d-ACTH 25—39)-OBzl (III)——The above protected tetradecapeptide ester (4.55 g) was treated with TFA (10 ml) in the presence of anisole (2.3 ml) at 0° for 90 min and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* and dissolved in DMF (60 ml). To this solution, Et_3N (0.56 ml), Z(OMe)–Asn–ONP (1.0 g) and HOBT (20 mg) were added and the solution, after stirring at room temperature for 72 hr, was condensed *in vacuo*. The residue was washed batchwisely with 10% citric acid, 5% $NaHCO_3$, H_2O–NaCl and MeOH. The product was reprecipitated from DMF with MeOH; yield 3.77 g (79.0%), mp 245.5—247°, $[\alpha]_D^{17}$ −27.6° (c=0.58, DMF), Rf_3 0.37. Amino acid ratios in acid hydrolysate: $Asp_{2.99}Ser_{1.81}Phe_{1.03}Glu_{4.63}Val_{1.01}Met_{0.75}Gly_{1.00}Pro_{0.65}Leu_{1.01}$ (average recovery 78.8%). *Anal.* Calcd. for $C_{121}H_{149}N_{17}O_{33}S$: C, 60.51; H, 6.25; N, 9.91. Found: C, 60.22; H, 6.28; N, 9.89.

Z-Val-Tyr-OMe——The title compound was prepared according to Ramachandran *et al.*;[18] yield 78.0%, mp 149—151°, $[\alpha]_D^{20}$ +12.5° (c=1.53, pyridine), Rf_1 0.56. (Lit.[18] mp 150°, $[\alpha]_D^{25}$ +12.1° (c=1.0, pyridine)). *Anal.* Calcd. for $C_{23}H_{28}N_2O_6$: C, 64.47; H, 6.59; N, 6.54. Found: C, 64.66; H, 6.79; N, 6.34.

Z-Val-Tyr-NHNH$_2$——The title compound was prepared according to Schwarz *et al.*[12]; yield 97.0%, mp 241—243°, $[\alpha]_D^{25}$ −13.0° (c=0.73, DMF), Rf_4 0.60. (Lit.[12] mp 239—241°, $[\alpha]_D^{25}$ −13.7° (c=3.6, DMF)). *Anal.* Calcd. for $C_{22}H_{28}N_4O_5$: C, 61.67; H, 6.59; N, 13.08. Found: C, 61.60; H, 6.31; N, 13.21.

Z-Val-Tyr-Pro-OH——To an ice-chilled suspension of H–Pro–OH (4.0 g) in DMF (60 ml) containing Et_3N (4.9 ml), a solution of azide (prepared from 10 g of Z–Val–Tyr–NHNH$_2$/DMF (30 ml) with 14.5 ml of 3.2 N HCl/DMF, 3.4 ml of isoamyl nitrite and 6.5 ml of Et_3N) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and the residue was dissolved in AcOEt. The solution was washed successively with 1 N HCl and H_2O–NaCl, dried over Na_2SO_4 and then evaporated *in vacuo*. The residue was reprecipitated from AcOEt with pet. ether; yield 9.0 g (76.0%), mp 124—126°, $[\alpha]_D^{19}$ −20.3° (c=1.33, DMF), Rf_4 0.42. *Anal.* Calcd. for $C_{27}H_{32}N_3O_7 \cdot 0.5H_2O$: C, 62.42; H, 6.40; N, 8.09. Found: C, 62.32; H, 6.72; N, 8.20.

H-Val-Tyr-Pro-OH——In the usual manner, Z–Val–Tyr–Pro–OH (7.7 g) in the mixture of MeOH (30 ml) and AcOH (30 ml) was hydrogenated over Pd black in the presence of anisole (3 ml) for 5 hr and then the catalyst was removed by filtration. The filtrate was evaporated *in vacuo*. The residue was recrystallized from H_2O and MeOH; 5.4 g (96.0%), mp 214—216.5°, $[\alpha]_D^{22}$ −29.3° (c=1.15, H_2O), Rf_7 0.66. (Lit.[18] mp 206—208°, $[\alpha]_D^{22}$ −29.0° (c=1.0, H_2O)). *Anal.* Calcd. for $C_{19}H_{27}N_3O_5 \cdot H_2O$: C, 57.71; H, 7.39; N, 10.63. Found: C, 57.85; H, 7.33; N, 10.43.

Z(OMe)-Ile-Lys(Z)-OMe——Z(OMe)–Ile–OH (6.2 g) and DCC (4.7 g) were added to a solution of H–Lys(Z)–OMe (prepared from 7.0 g of the hydrochloride and 2.9 ml of Et_3N) in THF (100 ml) under cooling with ice and the mixture was stirred at room temperature for 20 hr. The solvent was evaporated *in vacuo*. The residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated *in vacuo*. The crude product thus obtained was recrystallized from AcOEt and pet. ether; yield 8.2 g (68.0%), mp 124—127.5°, $[\alpha]_D^{23}$ −5.6° (c=1.57, $CHCl_3$), Rf_1 0.90. *Anal.* Calcd. for $C_{30}H_{41}N_3O_8$: C, 63.03; H, 7.23; N, 7.35. Found: C, 63.20; H, 7.41; N, 7.43.

Z(OMe)-Ile-Lys(Z)-NHNH$_2$——To a solution of Z(OMe)–Ile–Lys(Z)–OMe (8.2 g) in MeOH (60 ml), 80% hydrazine hydrate (1.6 ml) was added and the mixture was kept on standing for 72 hr and then H_2O was added. The resulting solid was recrystallized from MeOH and ether; yield 4.3 g (52.0%), mp 204.5—

17) F. Weygand, W. Steglich, F. Fraunberger, P. Pietta, and J. Schmid, *Chem. Ber.*, **101**, 923 (1968).
18) J. Ramachandran and C.H. Li, *J. Org. Chem.*, **28**, 173 (1963).

206.5°, $[\alpha]_D^{26}$ −4.5° (c=1.14, DMF), Rf_5 0.56. *Anal.* Calcd. for $C_{29}H_{41}N_5O_7$: C, 60.93; H, 7.23; N, 12.25. Found: C, 60.66; H, 7.37; N, 12.18.

Z(OMe)-Ile-Lys(Z)-Val-Tyr-Pro-OH, Z(OMe)-(d-ACTH 20—24)-OH (II)——To a suspension of H–Val–Tyr–Pro–OH (2.69 g) in DMF (40 ml) containing Et_3N (1.9 ml), a solution of azide (prepared from 3.9 g of Z(OMe)–Ile–Lys(Z)–$NHNH_2$/DMF (20 ml) with 6.8 ml of 2 N HCl/DMF, 1.0 ml of isoamyl nitrite and 1.9 ml of Et_3N) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated *in vacuo* and the residue was washed batchwisely with 10% citric acid and H_2O–NaCl. The product was reprecipitated from MeOH with ether; yield 5.3 g (86.0%), mp 204.5—209°, $[\alpha]_D^{30}$ −24.0° (c=1.05, DMF), Rf_4 0.37. Amino acid ratios in acid hydrolysate: $Ile_{0.99}Lys_{0.84}Val_{0.98}Tyr_{0.94}Pro_{1.00}$ (average recovery 89.3%). *Anal.* Calcd. for $C_{48}H_{64}N_6O_{12}$: C, 62.83; H, 7.18; N, 9.06. Found: C, 62.87; H, 7.03; N, 9.16.

Z(OMe)-Ile-Lys(Z)-Val-Tyr-Pro-Asn-Ser-Phe-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——Z(OMe)–(d-ACTH 25—39)–OBzl (3.13 g) was treated with TFA (10 ml) in the presence of anisole (1.5 ml) at 0° for 60 min and dry ether was added. The resulting powder was dissolved in DMF (2 ml). The solution was neutralized with 5% $NaHCO_3$ and then H_2O was added. The resulting powder was dried over P_2O_5 *in vacuo* and dissolved in DMF (25 ml). To this ice-chilled solution, Z(OMe)–(d-ACTH 20—24)–OH (1.21 g), DCC (0.27 g) and HOBT (0.17 g) were added. The solution, after stirring at room temperature for 48 hr, was condensed *in vacuo*. The residue was washed batchwisely with 10% citric acid, 5% $NaHCO_3$, H_2O–NaCl and MeOH. The product thus obtained was reprecipitated from DMF with MeOH; yield 2.68 g (71.2%), mp 239.5—242.5°, $[\alpha]_D^{25}$ −27.5° (c=0.40, DMF), Rf_5 0.11. Amino acid ratios in acid hydrolysate: $Ile_{0.86}Lys_{0.76}Val_{1.89}Tyr_{0.25}Pro_{1.90}Asp_{3.03}Ser_{1.79}Glu_{3.47}Phe_{1.00}Met_{0.67}Gly_{1.00}Leu_{0.99}$ (average recovery 86.6%). *Anal.* Calcd. for $C_{160}H_{207}N_{23}O_{43}S_2 \cdot 2H_2O$: C, 60.57; H, 6.58; N, 10.15. Found: C, 60.31; H, 6.46; N, 10.03.

Z-Arg(MBS)-Pro-OBzl——Under cooling with ice, Z–Arg(MBS)–OH[7] (1.2 g), HOBT (0.36 g) and DCC (0.5 g) were added to a solution of H–Pro–OBzl (prepared from 0.6 g of the hydrochloride and 0.35 ml of Et_3N) in THF (10 ml) and the mixture was stirred at room temperature for 18 hr. After filtration, the filtrate was evaporated *in vacuo*. The oily product thus obtained was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH (20: 1)); yield 1.1 g (65.4%), $[\alpha]_D^{25}$ −41.7° (c=1.06, MeOH), Rf_1 0.48. *Anal.* Calcd. for $C_{33}H_{39}N_5O_8S$: C, 59.53; H, 5.90; N, 10.52. Found: C, 59.74; H, 5.90; N, 10.44.

Z-Arg(MBS)-Pro-OH——Z–Arg(MBS)–Pro–OBzl (1.5 g) in MeOH (20 ml) was treated with 1 N NaOH (4.4 ml) at room temperature for 2 hr. The solvent, after neutralization with 10% citric acid, was evaporated *in vacuo* and the residue was dissolved in 5% $NaHCO_3$. The aqueous phase was washed with AcOEt and then acidified with 10% citric acid. The resulting oily product was extracted with AcOEt. The solution, after washing with H_2O–NaCl, was condensed *in vacuo*. The residue was reprecipitated from $CHCl_3$ with ether; yield 0.83 g (65.0%), mp 104.5—108°, $[\alpha]_D^{25}$ −30.7° (c=1.05, MeOH), Rf_5 0.44. *Anal.* Calcd. for $C_{26}H_{33}N_2O_8S$: C, 54.25; H, 5.78; N, 12.17. Found: C, 54.33; H, 6.07; N, 11.92.

Z-Arg(MBS)-Pro-Ile-Lys(Z)-Val-Tyr-Pro-Asn-Ser-Phe-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu-(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl, Protected CLIP——The above protected eicosapeptide ester (304 mg) was treated with TFA (1 ml) in the presence of anisole (0.2 ml) at 0° for 60 min and dry ether was added. The resulting powder was dissolved in the mixture of DMF (1 ml) and 1.25 N HCl/DMF (0.24 ml) and then dry ether was added. The hydrochloride thus obtained was dried over KOH pellets *in vacuo* and dissolved in DMF (5 ml) and then neutralized with Et_3N (0.013 ml). To this ice-chilled solution, Z–Arg-(MBS)–Pro–OH (72.7 mg), HOBT (18 mg) and DCC (26 mg) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo* and the residue was washed batchwisely with 10% citric acid, 5% $NaHCO_3$, H_2O–NaCl and MeOH. The product was reprecipitated from DMF with MeOH; yield 223 mg (65.5%), mp 233—237°, $[\alpha]_D^{25}$ −32.1° (c=0.7, DMF), Rf_5 0.07. Amino acid ratios in acid hydrolysate: $Asp_{3.01}Ser_{1.81}Glu_{4.81}Pro_{2.91}Gly_{1.00}Val_{1.92}Met_{0.94}Ile_{0.83}Leu_{0.99}Tyr_{0.80}Phe_{1.02}Lys_{0.79}Arg_{0.72}$ (average recovery 94.8%). *Anal.* Calcd. for $C_{177}H_{226}N_{28}O_{45}S_2 \cdot 2H_2O$: C, 59.62; H, 6.50; N, 11.00. Found: C, 59.46; H, 6.49; N, 10.87.

H-Arg-Pro-Ile-Lys-Val-Tyr-Pro-Asn-Ser-Phe-Glu-Asp-Glu-Ser-Val-Glu-Asn-Met-Gly-Pro-Glu-Leu-OH (Dogfish CLIP)——The above protected docosapeptide ester (54 mg) was treated with HF (approximately 3 ml) in the presence of anisole (1 ml) containing 2% ethanedithiol in an ice-bath for 60 min. The excess HF was removed by evaporation *in vacuo* at 0° and the residue was washed with dry ether and then dissolved in H_2O (15 ml). The solution was treated with Amberlite IR-45 (acetate form, approximately 5 g) for 30 min. The resin was removed by filtration and the filtrate, after incubation with dithiothreitol (50 mg) at 45° for 18 hr, was lyophilized. The resulting powder was dissolved in 0.2 N AcOH (2 ml) and the solution was applied to a column of Sephadex G-25 (2.6×75 cm). Individual fractions (10 ml each) eluted with 0.2 N AcOH were collected and absorbancy at 275 nm was determined. The fractions corresponding to the front peak (tube No. 18—23) were combined and the solvent was removed by lyophilization; yield 32.5 mg (deblocking step 76.5%). This powder was dissolved in a small amount of upper phase of the solvent system consisting of *n*-butanol–AcOH–H_2O (4: 1: 5). The solution was applied to a column of Sephadex G-25 (2.6×75 cm) equilibrated previously with the lower phase of the above solvent system. The column was developed with the same upper phase and individual fractions (10 ml each) were collected and absorbancy at 275 nm was determined. The fractions corresponding to the main peak (tube No. 55—60) were collected.

The solvent was removed by evaporation and the residue was lyophilized from 0.2 N AcOH to give fluffy white powder; yield 11.7 mg (27.5%), $[\alpha]_D^{25}$ −96.6° (c=0.15, H_2O), Rf_7 0.21, Rf_8 0.63, Rf_9 0.06. Amino acid ratios in acid hydrolysate: $Asp_{3.10}Ser_{1.87}Glu_{4.00}Pro_{3.12}Gly_{1.00}Val_{1.72}Met_{0.74}Ile_{0.88}Leu_{1.02}Tyr_{0.90}Phe_{0.90}Lys_{1.05}$-$Arg_{1.09}$ (average recovery 92.2%). Amino acid ratios in AP–M digest (peptide 0.6 μmol/AP–M 2U, theory is given in parentheses): $Asp_{0.95(1)}Ser+Asn_{3.10(4\ calcd.\ as\ Ser)}Glu_{3.60(4)}Pro_{2.58(3)}Gly_{1.00(1)}Val_{2.02(2)}Met_{0.86(1)}Ile_{1.05(1)}$-$Leu_{0.98(1)}Tyr_{1.03(1)}Phe_{0.98(1)}Lys_{1.09(1)}Arg_{1.00(1)}$ (average recovery 77.3%). Paper electrophoretic mobility at pH 3.6 (pyridine–AcOH–H_2O=1:10:289, v/v) was 3.4 cm (Lys×0.28) to the cathod and at pH 9.6 (0.1 M ammonium carbonate) was 2.1 cm (Glu×0.21) to the anode. *Anal.* Calcd. for $C_{112}H_{172}N_{28}O_{38}S\cdot 2CH_3COOH\cdot 9H_2O$: C, 49.18; H, 7.04; N, 13.84. Found: C, 49.36; H, 7.01; N, 14.07.

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