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

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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 adrenocorticotropic-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, adrenocorticotropic (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 ACTH.

MBS
(I) Z-Arg-Pro-OH

Z

(II) Z(OMe)-Ile-Lys-Val-Tyr-Pro-OH

OBzl OBzl OBzl

(III) Z(OMe)-Asn-Ser-Phe-Glu-Asp-Glu-Ser-Val-

OBzl OBzl

Glu-Asn-Met-Gly-Pro-Glu-Leu-OBzl

1) DCC + HOBT

2) HF

1) TFA Treat. of (II) 2) DCC + HOBT 3) TFA

↓

H-Arg-Pro-Ile-Lys-Val-Tyr-Pro-Asn-Ser-Phe-Glu-Asp-

Glu-Ser-Val-Glu-Asn-Met-Gly-Pro-Glu-Leu-OH

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 = benzylxycarbonyl, Z(OMe) = \(p\)-methoxybenzylxycarbonyl, 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.


ACTHs 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.

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 were employed; i.e., Arg(MBS), 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, 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 starting from H-Leu-OBzl. After the TFA treatment of IV, the resulting tripeptide ester was condensed with Z(OMe)-Met-Gly-NNH₂ by modified azide procedure 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 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-NNH₂ was condensed with the triethyl-

![Synthetic Route to the Protected Pentapeptide (II), Z(OMe)-(d-ACTH 20–24)-OH](image)

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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)-NHNH₂ 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 and H-Pro-OBzI 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)-OBzI, 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% NaHCO₃. The deblocked pentadecapeptide ester isolated as a powder was coupled with Z(OMe)-(d-ACTH 20—24)-OH by DCC in the presence of ω-hydroxybenzotriazole (HOBT). The resulting protected eicosapeptide ester, Z(OMe)-Ile-Lys(Z)-Val-Tyr-Pro-Asn-Ser-Phe-Glu(OBzI)-Asp(OBzI)-Glu(OBzI)-Ser-Val-Glu(OBzI)-Asn-Met-Gly-Pro-Glu(OBzI)-Leu-OBzI, abbreviated as Z(OMe)-(d-ACTH 20—39)-OBzI, 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)-OBzI, 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(OBzI)-Asp(OBzI)-Glu(OBzI)-Ser-Val-Glu(OBzI)-Asn-Met-Gly-Pro-Glu(OBzI)-Leu-OBzI (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. 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). 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, 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

15) G. Pfleiderer and P.G. Celliers, Biochem. Z., 339, 186 (1963). AP-M was obtained from Protein Research Foundation (Osaka).
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°C 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, CHCl₃-MeOH (9: 1), RF, CHCl₃-MeOH-MeOH (29: 1), RF, CHCl₃-MeOH-H₂O (90: 15: 5), RF, CHCl₃-MeOH-H₂O (8: 3: 1), RF, CHCl₃-MeOH-MeOH-AcOH (9: 1: 0.5), RF, CHCl₃-MeOH-AcOH (9: 1: 1), RF, n-butanol-AcOH-pyridine-H₂O (15: 10: 12), RF, n-butanol-AcOH-pyridine-H₂O (4: 1: 1: 2) and RF, n-butanol-AcOH-H₂O (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 Et₂N) 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% Na₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ and then evaporated. The residue was recrystallized from AcOEt and pet. ether; yield 23.0 g (75.9%), mp 87—89°C, [α]D²⁰—14.6° (c=1.78, CHCl₃), RF, 0.81. Anal. Calcd. for C₄₄H₄₁N₂O₁₈: 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°C 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, Et₂N (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 2 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% Na₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ and then evaporated. The residue was recrystallized from AcOEt and pet. ether; yield 10.8 g (73.5%), mp 147—148.5°C, [α]D²⁰—57.4° (c=2.16, MeOH), RF, 0.84. Anal. Calcd. for C₄₃H₄₀N₂O₁₉: 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 Et₂N) 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% Na₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ and then evaporated in vacuo. The residue was recrystallized from AcOEt and pet. ether; yield 16.5 g (64.2%), mp 96.5—97.5°C, [α]D²⁰—5.9° (c=1.05, CHCl₃), RF, 0.39. Anal. Calcd. for C₁₇H₂₄N₂O₄S: C, 53.11; H, 6.29; N, 7.29. Found: C, 53.38; H, 6.32; N, 7.35.

Z(OMe)-Met-Gly-NHNH₂—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 H₂O; yield 15.0 g (90.9%), mp 142.5—145°C, [α]D²⁰—7.8° (c=1.95, DMF), RF, 0.58. Anal. Calcd. for C₁₇H₂₃N₃O₅: 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°C 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, Et₂N (4.0 ml) and a solution of azide (prepared from 11.5 g of Z(OMe)-Met—Gly—NHNH₂/DMF (20 ml) with 28.5 ml of 2N HCl/DMF, 4.0 ml of isomyl nitrite and 4.0 ml of Et₂N) were added and the mixture was stirred at 4°C 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% Na₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ and then evaporated in vacuo. The residue was purified by column chromatography on silica (eluent: CHCl₃-MeOH (100: 1)); yield 15.2 g (60.0%), mp 130—132°C, [α]D²⁰—45.8° (c=1.43, DMF), RF, 0.23. Anal. Calcd. for C₄₃H₃₉N₁₂O₁₈S: C, 62.07; H, 6.68; N, 8.17. Found: C, 62.29; H, 6.79; N, 8.17.

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 (25 ml) in the presence of anisole (7.5 ml) at 0°C 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), Et₂N (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 batchwise with 10% citric acid, 10% Na₂CO₃ and H₂O-NaCl. The product was precipitated by column chromatography on silica (eluent: CHCl₃-MeOH (40: 1)); yield 10.2 g (67.0%), mp 200—203°C, [α]D²⁰—42.5° (c=1.04, DMF), RF, 0.66. Anal. Calcd. for C₃₀H₃₄N₁₂O₁₈S: 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°C 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), Et₂N (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 batchwise with 10% citric acid, 10% Na₂CO₃ and H₂O—
NaCl. The product was purified by column chromatography on silica (eluents: CHCl₃–MeOH (30:1)); yield 8.8 g (55.0%) mp 204.5–206.5°, [z]₁₀° −36.4° (c = 0.93, DMF), R₁₀₀₆ 0.64. Anal. Calcd. for C₄₄H₈₂N₁₆O₁₆S: C, 60.87; H, 6.43; N, 0.16. Found: C, 61.17; H, 6.34; N, 0.07.

Z(OMe)-Ser-Val-Ome——Under cooling with ice-NaCl, Et₂N (5.6 ml) and a solution of azide (prepared from 9.0 g of Z(OMe)-Ser-NNHN₃/DMF (20 ml) with 40 ml of 2N HCl/DMF, 5.9 ml of isomyl nitrite and 11.2 ml of Et₂N) were added to a solution of H-Val-Ome (prepared from 6.8 g of the hydrochloride and 5.6 ml of Et₂N) 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\% Na₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ 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°, [z]₁₀° −15.9° (c = 1.17, CHCl₃), R₁₀₀₆ 0.63. Anal. Calcd. for C₁₅H₂₇N₁₅O₁₇: C, 56.54; H, 6.85; N, 7.33. Found: C, 56.81; H, 6.88; N, 7.14.

Z(OMe)-Ser-Val-NNHN₃——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°, [z]₁₀° +11.8° (c = 0.47, DMF), R₁₀₀₆ 0.66. Anal. Calcd. for C₁₇H₃₈N₁₅O₁₇: C, 53.39; H, 6.85; N, 14.65. Found: C, 53.65; H, 7.03; N, 14.57.

Z(OMe)-Ser-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——Z(OMe)-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——The above protected nonapeptide ester (5.0 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, Et₂N (1.8 ml) and a solution of azide (prepared from 3 g of Z(OMe)-Ser-Val-NNHN₃/DMF (13 ml) with 17.3 ml of 0.7 N HCl/DMF, 1.14 ml of isomyl nitrite and 2.2 ml of Et₂N) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated in vacuo and the residue was washed batchwise with 10% citric acid and H₂O-NaCl. The product was purified by column chromatography on silica (eluents: CHCl₃–MeOH–H₂O (100:10:1)); yield 7.0 g (76.0%), mp 225–230°, [z]₁₀° −31.0° (c = 0.86, DMF), R₁₀₀₆ 0.56. Anal. Calcd. for C₉₉H₉₉N₁₉O₁₉S·0.5H₂O: 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 (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 (30 ml). To this solution, Et₂N (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 batchwise with 10% citric acid, 5% NaHCO₃ and H₂O-NaCl. The product was purified by column chromatography on silica (eluents: CHCl₃–MeOH–H₂O (90:15:5)); yield 4.2 g (73.0%), mp 234–239°, [z]₁₀° −29.8° (c = 0.61, DMF), R₁₀₀₆ 0.47. Amino acid ratios in acid hydrolysates: Glu₁₉,Asp₁₀,Ser₉,Se₀,Pro₇,Val₄,Met₉,Sε₉,Gly₁₀,Leu₂,0.8 (average recovery 78.1%). Anal. Calcd. for C₆₀H₇₀N₂₅O₂₅S·0.5H₂O: C, 60.13; H, 6.32; N, 9.41. Found: C, 59.98; H, 6.57; 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 (20 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, Et₂N (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 batchwise with 10% citric acid, 5% NaHCO₃ and H₂O-NaCl. The product was reprecipitated from DMF with MeOH; yield 3.8 g (87.0%), mp 230–235°, [z]₁₀° −28.2° (c = 0.77, DMF), R₁₀₀₆ 0.44. Amino acid ratios in acid hydrolysates: Asp₁₉,Glu₁₉,Se₀,Pro₁₀,Sε₀,Val₄,Met₉,Sε₉,Gly₁₀,Leu₂,0.8 (average recovery 90.4%). Anal. Calcd. for C₁₉₉H₁₉₉N₁₉O₁₉: C, 60.90; H, 6.38; N, 9.16. Found: C, 60.68; H, 6.32; N, 8.88.

Z(OMe)-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Val-Glu(OBzl)-Asn-Met-Gly-Pro-Glu(OBzl)-Leu-OBzl——The above protected undecaepptide ester (4.1 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 dissolved in DMF (40 ml). To this solution, Et₂N (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 batchwise with 10% citric acid, 5% NaHCO₃ and H₂O-NaCl. The product was reprecipitated from DMF with MeOH; yield 3.7 g (82.0%), mp 232–235°, [z]₁₀° −30.3° (c = 0.66, DMF), R₁₀₀₆ 0.53. Amino acid ratios in acid hydrolysates: Glu₁₉,Asp₁₀,Sε₀,Val₄,Met₉,Sε₀,Gly₁₀,Pro₁₀,Leu₂,0.8 (average recovery 97.0%). Anal. Calcd. for C₁₉₉H₁₉₉N₁₉O₁₉S·0.5H₂O: 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 Et₂N) in DMF (40 ml), Et₂N (5.6 ml) and a solution of azide (prepared from 11.4 g of Z(OMe)-Ser-NNHN₃/DMF (10 ml) with 40 ml of 2N HCl/DMF, 5.87 ml of isomyl nitrite and 11.2 ml of Et₂N) 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\% Na₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ and then evaporated in vacuo. The residue was recrystallized from
AcOEt and pet. ether; yield 13.1 g (75.7%), mp 126—127°C, [α]D = —0.7° (c=1.0, MeOH), (lit.17) mp 118—119°, [α]D = —8.4° (c=1, MeOH), Rf = 0.75. Anal. Calcd. for C24H38N4O4: C, 61.39; H, 6.09; N, 6.51. Found: C, 61.65; H, 6.03; N, 6.32.

Z(OMe)-Ser-Phe-NHNH₂—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—225°C, [α]D = —1.1° (c=0.44, DMF), Rf = 0.47. Anal. Calcd. for C19H34N4O4: C, 65.80; H, 6.09; N, 10.12. Found: C, 65.77; H, 6.33; N, 10.25.

Z(OMe)-Ser-Glu (OBzl)-Asp (OBz) -Glu (OBz)-Ser-Val-Glu (OBz)-Asn-Met-Gly-Pro-Glu (OBz)-Leu-OBz.—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, Et₃N (0.68 ml) and a solution of azide (prepared from 1.6 g of Z(Ome)-Ser-Phe-NHNH₂/DMF (5 ml) with 6.0 ml of 1.25 n HCl/DMF, 0.5 ml of isomyl nitrite and 1.03 ml of Et₃N) were added and the solution, after stirring at 4° for 48 hr, was condensed in vacuo. The residue was washed batchwise with 10% citric acid and H₂O-NaCl. The product was recrystallized from DMF with MeOH; yield 4.2 g (73.0%), mp 242—246°C, [α]D = —26.0° (c=0.72, DMF), Rf = 0.35. Amino acid ratios in acid hydrolysate: Ser, 9%; Phe, 1%; Glu, 41%; Asp, 8%; Val, 8%; Met, 7%; Gly, 10%; Pro, 8%; Leu, 9% (average recovery 97.9%). Anal. Calcd. for C₁₁₁H₁₄₁N₂₁₃O₁₅S: C, 61.43; H, 6.30; N, 9.18. Found: C, 61.31; H, 6.40; N, 9.33.

Z(Ome)-Asn-Ser-Glu (OBz)-Asp (OBz) -Glu (OBz)-Ser-Val-Glu (OBz)-Asn-Met-Gly-Pro-Glu (OBz)-Leu-OBz, Z(Ome)-(d-ACHT 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, Et₃N (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 batchwise with 10% citric acid, 5% NaHCO₃, H₂O-NaCl and MeOH. The product was recrystallized from DMF with MeOH; yield 3.77 g (79.0%), mp 245—247°C, [α]D = —26.0° (c=0.58, DMF), Rf = 0.37. Amino acid ratios in acid hydrolysate: Asp, 27%; Ser, 14%; Phe, 1%; Glu, 41%; Val, 8%; Met, 7%; Gly, 10%; Pro, 8%; Leu, 9% (average recovery 78.8%). Anal. Calcd. for C₁₁₁H₁₄₁N₂₁₃O₁₅S: 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°, [α]D = +12.5° (c=1.53, pyridine), Rf = 0.56. (Lit.18) mp 150°, [α]D = +12.1° (c=1.0, pyridine). Anal. Calcd. for C₁₉H₂₅N₃O₅: C, 64.47; H, 6.59; N, 6.54. Found: C, 64.66; H, 6.79; N, 6.34.

Z-Val-Tyr-NHNH₂—The title compound was prepared according to Schwarz et al.;19 yield 97.0%, mp 241—243°C, [α]D = —13.0° (c=0.73, DMF), Rf = 0.60. (Lit.19) mp 239—241°C, [α]D = —13.7° (c=3.6, DMF). Anal. Calcd. for C₁₉H₂₅N₃O₅: C, 61.67; H, 6.59; N, 13.08. Found: C, 61.60; H, 6.31; N, 13.21.

Z-Val-Pro-OH—To an ice-chilled suspension of H-Pro-OH (4.0 g) in DMF (60 ml) containing Et₃N (4.9 ml), a solution of azide (prepared from 10 g of Z-Val-Tyr-NHNH₂/DMF (30 ml) with 15.4 ml of 3.2 n HCl/DMF, 3.4 ml of isomyl nitrite and 6.5 ml of Et₃N) 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 H₂O-NaCl, dried over Na₂SO₄ and then evaporated in vacuo. The residue was recrystallized from AcOEt with pet. ether; yield 9.0 g (76.0%), mp 124—129°, [α]D = —20.3° (c=1.35, DMF), Rf = 0.42. Anal. Calcd. for C₁₉H₂₅N₃O₅·0.5H₂O: C, 62.42; H, 6.40; N, 8.09. Found: C, 62.42; H, 6.72; N, 8.20.

H-Val-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 H₂O and MeOH; 5.4 g (96.0%), mp 214—216.5°C, [α]D = —29.8° (c=1.15, H₂O), Rf = 0.66. (Lit.20) mp 206—208°C, [α]D = —29.0° (c=1.0, H₂O). Anal. Calcd. for C₁₉H₂₅N₃O₅·H₂O: 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-OMe (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 EtOH) in THF (100 ml) under cooling with ice and the mixture was stirred at room temperature for 20 hr. The solution was evaporated in vacuo. The residue was dissolved in AcOEt. The solution was washed successively with 10% citric acid, 5% NaH₂CO₃ and H₂O-NaCl, dried over Na₂SO₄ and then evaporated in vacuo. The crude product thus obtained was recrystallized from AcOEt and pet. ether; yield 8.2 g (68.0%), mp 121—127.5°C, [α]D = —5.6° (c=1.57, CHCl₃), Rf = 0.90. Anal. Calcd. for C₁₉H₂₅N₃O₅·H₂O: C, 63.03; H, 7.23; N, 7.35. Found: C, 63.20; H, 7.41; N, 7.43.

Z(OMe)-Ile-Lys(Z)-NHNH₂—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 H₂O was added. The resulting solid was recrystallized from MeOH and ether; yield 4.3 g (52.0%), mp 204.5°—


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,N (1.9 ml), a solution of azide (prepared from 3.9 g of Z(OMe)-Ile-Lys(Z)-NNHH2/DMF (20 ml) with 6.8 ml of 2 N HCl/DMF, 1.0 ml of isoamyl nitrite and 1.9 ml of Et,N) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated in vacuo and the residue was washed batchwise with 10% citric acid and H2O-NaCl. The product was precipitated from MeOH with ether; yield 5.3 g (86.0%), mp 204.5-209°, [x]D -24.0° (c=1.05, DMF), Rf0.37. Amino acid ratios in acid hydrolysate: Ile9.0, Lys9.1, Val9.6, Tyr9.4, Pro1.06 (average recovery 90.3%). Anal. Calcd. for C26H42N4O6: 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(Obz)-Asp(Obz)-Glu(Obz)-Ser-Val-Glu(Obz)-Asn-Met-Gly-Pro-Glu(Obz)-Leu-Obz—Z(OMe)-(d-ACTH 25-39)-Obz (3.13 g) was treated with TFA (10 ml) in the presence of anisole (1.5 ml) at 6° for 60 min and dry ether was added. The resulting powder was dissolved in DMF (2 ml). The solution was neutralized with 5% NaHCO3, and then H2O was added. The resulting powder was dried over P2O5 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 batchwise with 10% citric acid, 5% NaHCO3, H2O-NaCl and MeOH. The product thus obtained was precipitated from DMF with MeOH; yield 2.68 g (71.5%), mp 239.5-242.5°, [x]D -27.5° (c=0.40, DMF), Rf 0.11. Amino acid ratios in acid hydrolysate: Ile9.3, Lys7.1, Val9.0, Tyr7.0, Asp9.0, Ser7.7, Gly2.5, Phe1.0, MeOe9.5, Gly1.0, Leu9.0 (average recovery 86.8%). Anal. Calcd. for C41H67O12N12S2: H2O: C, 60.57; H, 6.58; N, 10.15. Found: C, 60.31; H, 6.46; N, 10.03.

Z-Arg(MBS)-Pro-Obz—Under cooling with ice, Z-Arg(MBS)-OH7 (1.2 g), HOBT (0.36 g) and DCC (0.5 g) were added to a solution of Z-Arg(Obz) (prepared from 0.6 g of the hydrochloride and 0.35 ml of Et2N 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: CHCl3-MeOH (20:1)); yield 1.1 g (65.4%), [x]D -41.7° (c=1.06, MeOH), Rf 0.48. Anal. Calcd. for C55H82N8O2S: C, 59.53; H, 5.90; N, 10.52. Found: C, 59.74; H, 5.99; N, 10.44.

Z-Arg(MBS)-Pro-Oh—Z-Arg(MBS)-Pro-Obz (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% NaHCO3. 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 H2O-NaCl, was condensed in vacuo. The residue was precipitated from CHCl3 with ether; yield 0.83 g (65.0%), mp 104.5-108°, [x]D -30.7° (c=1.05, MeOH), Rf 0.44. Anal. Calcd. for C56H83N8O2S: 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(Obz)-Asp(Obz)-Glu(Obz)-Ser-Val-Glu(Obz)-Asn-Met-Gly-Pro-Glu(Obz)-Leu-Obz, 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 Et2N (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 batchwise with 10% citric acid, 5% NaHCO3, H2O-NaCl and MeOH. The product was precipitated from DMF with MeOH; yield 223 mg (65.5%), mp 233-237°, [x]D -32.1° (c=0.7, DMF), Rf 0.07. Amino acid ratios in acid hydrolysate: Asp1.0, Ser1.0, Glu1.0, Pro1.0, Gly1.0, Val1.0, Met0.5, lle0.4, Leu0.9, Tyr0.6, Phe1.0, Ile0.5, Asp0.7, Arg0.7 (average recovery 94.8%). Anal. Calcd. for C77H123N14O23S3: 2H2O: 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 H2O (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 diethithirol (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 x 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-H2O (4:1:5). The solution was applied to a column of Sephadex G-25 (2.6 x 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%), $\left[\alpha\right]_D^20 -96.6^\circ (c=0.15, H_2O), R_f 0.21, R_f 0.63, R_f 0.06$. Amino acid ratios in acid hydrolysate: Asp$_{2.16}$Ser$_{1.37}$Glu$_{4.66}$Pro$_{2.13}$Gly$_{1.00}$Val$_{1.72}$Met$_{0.71}$Ile$_{0.93}$Leu$_{1.02}$Tyr$_{0.30}$Phe$_{0.96}$Lys$_{1.65}$Arg$_{1.09}$ (average recovery 92.2%). Amino acid ratios in AP-M digest (peptide 0.6 $\mu$mol/AP-M 2U, theory is given in parentheses): Asp$_{0.26(1)}$Ser$_{+}$Asn$_{2.26(4)}$ (calcd. as Ser) Glu$_{2.40(5)}$Pro$_{2.38(5)}$Gly$_{1.38(5)}$Val$_{2.05(5)}$Met$_{0.95(5)}$Ile$_{0.35(5)}$Leu$_{0.05(5)}$Tyr$_{0.05(5)}$Phe$_{0.94(5)}$Lys$_{0.95(5)}$Arg$_{0.05(5)}$ (average recovery 77.3%). Paper electrophoretic mobility at pH 3.6 (pyridine-AcOH-H$_2$O = 1:10:289, v/v) was 3.4 cm (Lys $\times$ 0.28) to the cathode and at pH 9.6 (0.1 M ammonium carbonate) was 2.1 cm (Glu $\times$ 0.21) to the anode. *Anal. Calcd.* for C$_{141}$H$_{172}$N$_{35}$O$_{36}$S·2CH$_3$COOH·9H$_2$O: C, 49.18; H, 7.04; N, 13.84. Found: C, 49.36; H, 7.01; N, 14.07.

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