Synthesis of the Nonatriacontapeptide corresponding to the Entire Amino Acid Sequence of Dogfish Adrenocorticotropic Hormone (Squalus acantias)\(^{1}\)

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The nonatriacontapeptide corresponding to the entire amino acid sequence of dogfish adrenocorticotropic hormone was synthesized by successive condensations of three peptide fragments; Z-(OMe)-(15—19)-OH, Z-(OMe)-(11—14)-NHNH\(_2\), and Z-(1—10)-OH, with H-(20—38)-OBz, a synthetic intermediate of dogfish corticotropin-like intermediate lobe peptide. The synthetic peptide exhibited the in vivo steroidogenetic activity of 21.3 IU/mg.

**Keywords**—dogfish adrenocorticotropic hormone; deprotection by hydrogen fluoride; 4\(\times\) methanesulfonic acid hydrolysis of Trp-peptides; column chromatographic purification of protected peptides; 4-morpholinyl active ester; in vivo steroidogenetic activity

In 1974, Lowry et al.\(^{3}\) isolated a new adrenocorticotropic hormone (ACTH) from the pars distalis of pituitary of dogfish, *Squalus acantias*, and determined its primary structure (I). The number of amino acid residue of this hormone is identical with that of the known mammalian ACTHs.\(^{4}\) However, on comparison with the amino acid composition of human ACTH, eleven amino acid differences are seen, nine of which are in the 20—39 region of the dogfish ACTH molecule. Thus, the amino acid sequence of N-terminal nonadecapeptide (1—19 region) of this hormone is identical with that of mammalian ACTHs, except at two positions; 13 and 15.

In this paper, we wish to describe the synthesis of the nonatriacontapeptide corresponding to the entire amino acid sequence of the dogfish ACTH (I). For this synthesis, eicosapeptide ester (II), a synthetic intermediate of the dogfish corticotropin-like intermediate lobe peptide (CLIP),\(^{5}\) served as an amino component, with which three fragments, III (position 15—19), IV (position 11—14) and V (position 1—10) were successively condensed as shown in Fig. 1.

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=benzylxoycarbonyl, Z-(OMe)=p-methoxybenzylxocarbonyl, MBS=\(p\)-methoxybenzenesulfonyl, TOS=\(p\)-toluenesulfonyl, OBz=benzyl ester, Omor=4-morpholinyl ester, ONP=\(p\)-nitrophenyl ester, DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, THF=tetrahydrofuran, TFA=trifluoroacetic acid, OCl=5-chloro-8-quinolinyl ester.

2) Location: a) Yamashina-ku, Kyoto, 607, Japan; b) Fukushima-ku, Osaka, 553, Japan; c) Sakyo-ku, Kyoto, 606, Japan.


The α-amino function of intermediates was protected by the TFA-labile Z(OMe) group\(^6\) and the side chain protecting groups adopted to the synthesis of dogfish CLIP\(^8\) were also employed in our present synthesis; i.e., Arg(MBS),\(^7\) Asp(Obzl), Glu(Obzl), and Lys(Z).

First, N-terminal protected decapeptide (V) was prepared by azide coupling\(^8\) of Z–Ser–Tyr–Ser–Met–NHNH\(_2\) (VI)\(^9\) with the TFA treated hexapeptide of Z(OMe)–Glu(Obzl)–His–Phe–Arg(MBS)–Trp–Gly–OH (VII) as shown in Fig. 2. In order to prepare the necessary

\[ \text{Z–Ser–OH} \quad 1) \text{Omor} \quad 2) \text{NH}_2\text{NH}_2 \]
\[ \text{H–Tyr–OMe} \quad 1) \text{azide} \quad 2) \text{NH}_2\text{NH}_2 \]
\[ \text{Z(OMe)–Ser–OH} \quad 1) \text{Omor} \quad 2) \text{TFA} \]
\[ \text{H–Met–OMe} \quad 1) \text{azide} \quad 2) \text{TFA} \]
\[ \text{Z(OMe)–Glu(Obzl)–OH} \quad 1) \text{azide} \quad 2) \text{TFA} \]
\[ \text{Z–Phe–OH} \quad 1) \text{ONP} \quad 2) \text{H}_2\text{–Pd} \]
\[ \text{H–Arg(MBS)–OH} \quad 1) \text{azide} \quad 2) \text{H}_2\text{–Pd} \]
\[ \text{Z–Trp–OH} \quad 1) \text{ONP} \quad 2) \text{H}_2\text{–Pd} \]
\[ \text{H–Gly–OH} \quad 1) \text{ONP} \quad 2) \text{H}_2\text{–Pd} \]

\[ \text{Z–Ser–Tyr–Ser–Met–Glu(Obzl)–His–Phe–Arg(MBS)–Trp–Gly–OH (V)} \]

Fig. 2. Synthetic Route to the Z–(1–10)–OH (V)

fragment VI, Z–Ser–Tyr–NHNH\(_2\)\(^{10}\) was condensed with H–Ser–Met–OMe by the azide procedure\(^8\) and the resulting protected tetrapeptide was converted to the corresponding hydrazide (VI) in the usual manner. In this synthesis, the two intermediates, Z–Ser–

Tyr–OMe\textsuperscript{11}) and Z(OMe)–Ser–Met–OMe, were prepared by our newly introduced 4- morpholinoethyl ester method\textsuperscript{15}) in good yields. Since, without protection of the hydroxyl function, Ser can be incorporated into the peptide chain by this method. VII was synthesized in essentially the same manner as those described in the synthesis of the fragment of porcine \( \beta \)-melanocyte-stimulating hormone by Yajima et al.,\textsuperscript{13}) except that Arg(MBS),\textsuperscript{7}) instead of Arg(Tos), was employed. For the \( \alpha \)-deprotection, the TFA treatment of VII was performed in the presence of anisole containing 2% ethanol, in order to suppress the side reactions on Trp residue.\textsuperscript{14}) The purity of N-terminal protected decapeptide was assessed by thin layer chromatography (TLC), elemental analysis and acid hydrolysis with 4\( \text{N} \) methanesulfonic acid (MSA).\textsuperscript{16}) As shown in Fig. 3, protected tetrapeptide hydrazide, Z(OMe)–Lys(Z)–Pro–Met–Gly–NHNH\textsubscript{2} (IV), was prepared by DCC plus N-hydroxybenzotriazole (HOBT) coupling\textsuperscript{16}) of Z(OMe)–Lys(Z)–Pro–OH\textsuperscript{17}) with the TFA treated sample of Z(OMe)–Met–Gly–OMe followed by treatment of the resulting protected tetrapeptide ester with hydrazine hydrate. The above dipeptide ester was prepared by the 4-morpholinoethyl ester method in excellent yield. Homogeneity of IV was assessed by elemental analysis and TLC.

\[
\begin{align*}
\text{Z} & \quad \text{Lys} \quad \text{Pro} \quad \text{Met} \quad \text{Gly} \\
& \quad \text{Z(OMe)–OQC}^* \quad \text{H} \quad \text{OH} \quad \text{Z(OMe)} \quad \text{OMe} \quad \text{H–} \quad \text{OMe} \\
& \quad \text{Z(OMe)} \quad \text{OH} \quad \text{Z(OMe)} \quad \text{DCC+HOBT} \quad \text{OMe} \quad \text{OMe} \\
& \quad \text{Z(OMe)} \quad \text{NH\textsubscript{2}NH\textsubscript{2}} \quad \text{OMe} \quad \text{NH\textsubscript{2}NH\textsubscript{2}} \\
\end{align*}
\]

Fig. 3. Synthetic Route to the Z(OMe)–(11–14)–NHNH\textsubscript{2} (IV)


The protected pentapeptide, Z(OMe)–Arg(MBS)–Lys(Z)–Arg(MBS)–Arg(MBS)–Pro–OH (III) was prepared by azide coupling of Z(OMe)–Arg(MBS)–Lys(Z)–NHNH\textsubscript{2} with the hydrogenated sample of Z–Arg(MBS)–Arg(MBS)–Pro–OBzI (VIII) as shown in Fig. 4. The former

\[
\begin{align*}
\text{MBS} & \quad \text{Z} \quad \text{Lys} \quad \text{MBS} \quad \text{MBS} \quad \text{MBS} \quad \text{Pro} \\
\text{Arg} & \quad \text{OMe} \quad \text{Z} \quad \text{Arg} \quad \text{Z(OMe)} \quad \text{OH} \quad \text{H–} \quad \text{OBzI} \\
& \quad \text{Z(OMe)} \quad \text{DCC+HOBT} \quad \text{OMe} \quad \text{Z} \quad \text{OH} \quad \text{TFA} \quad \text{OBzI} \\
& \quad \text{Z(OMe)} \quad \text{NH\textsubscript{2}NH\textsubscript{2}} \quad \text{OMe} \quad \text{NH\textsubscript{2}NH\textsubscript{2}} \quad \text{Z} \quad \text{H–} \quad \text{OBzI} \\
& \quad \text{Z(OMe)} \quad \text{azide} \quad \text{H–} \quad \text{OBzI} \quad \text{H\textsubscript{2}–Pd} \quad \text{TFA} \\
\end{align*}
\]

Fig. 4. Synthetic Route to the Z(OMe)–(15–19)–OH (III)

hydrazide was prepared by DCC plus HOBT coupling of Z(OMe)–Arg(MBS)–OH and H–Lys–(Z)–OMe followed by treatment of the resulting protected dipeptide ester with hydrazine hydrate in the usual manner. Protected tripeptide ester (VIII) was prepared as follows. Z(OMe)–Arg(MBS)–OH was condensed with H–Pro–OBzl by the DCC procedure\(^{18}\) to give the corresponding Z(OMe)–Arg(MBS)–Pro–OBzl (IX), which after treatment with TFA, was further condensed with Z–Arg(MBS)–OH by mixed anhydride method\(^{19}\) to give VIII. Homogeneity of the protected pentapeptide (III) was confirmed by elemental analysis, TLC and amino acid analysis.

Assembling of four subunits obtained as mentioned above was then performed according to the scheme illustrated in Fig. 1. The protected eicosapeptide ester, Z(OMe)–Ile–Lys(Z)–Val–Tyr–Pro–Asn–Ser–Phe–Glu(Obz1)–Asp(Obz1)–Glu(Obz1)–Ser–Val–Glu(Obz1)–Asn–Met–Gly–Pro–Glu(Obz1)–Leu–Obz1,\(^{5}\) was treated with TFA in the presence of anisole and the resulting trifluoroacetate was converted to the corresponding hydrochloride, which after neutralization with Et\(_3\)N, was condensed with III by the DCC plus HOBT procedure. The protected pentacosapeptide ester thus obtained, Z(OMe)–Arg(MBS)–Lys(Z)–Arg(MBS)–Arg(MBS)–Pro–Ile–Lys(Z)–Val–Tyr–Pro–Asn–Ser–Phe–Glu(Obz1)–Asp(Obz1)–Glu(Obz1)–Ser–Val–Glu(Obz1)–Asn–Met–Gly–Pro–Glu(Obz1)–Leu–Obz1 (X), was purified by column chromatography on silica using solvent system of CHCl\(_3\)–MeOH–H\(_2\)O (8:3:1). This solvent system was effectively employed also for purifications of other protected intermediates. X was treated with TFA as mentioned above and the resulting pentacosapeptide ester, after neutralization with Et\(_3\)N, was condensed with IV by the azide procedure to give the corresponding Z(OMe)–Lys(Z)–Pro–Met–Gly–Arg(MBS)–Lys(Z)–Arg(MBS)–Arg(MBS)–Pro–Ile–Lys(Z)–Val–Tyr–Pro–Asn–Ser–Phe–Glu(Obz1)–Asp(Obz1)–Glu(Obz1)–Ser–Val–Glu(Obz1)–Asn–Met–Gly–Pro–Glu(Obz1)–Leu–Obz1(XI). Its purification was achieved by column chromatography on silica and the homogeneity was also examined by three criteria as mentioned above.

Finally, XI, after treatment with TFA, conversion to the corresponding hydrochloride and subsequent neutralization with Et\(_3\)N, was condensed with V by the DCC plus HOBT procedure to give the corresponding Z–Ser–Tyr–Ser–Met–Glu(Obz1)–His–Phe–Arg(MBS)–Trp–Gly–Lys(Z)–Pro–Met–Gly–Arg(MBS)–Lys(Z)–Arg(MBS)–Arg(MBS)–Pro–Ile–Lys(Z)–Val–Tyr–Pro–Asn–Ser–Phe–Glu(Obz1)–Asp(Obz1)–Glu(Obz1)–Ser–Val–Glu(Obz1)–Asn–Met–Gly–Pro–Glu(Obz1)–Leu–Obz1 (XII). Rink and Riniker\(^{20}\) pointed out that DCC has a tendency to mask the N\(_{\text{im}}\)-function of His residue during the DCC plus HOBT condensation reaction, the crude product (XII) was therefore heated in a mixture of DMF and methanol containing acetic acid at 65° for 8 hr to remove the masking group of the His residue and then purified by column chromatography on silica as stated above. Homogeneity of XII thus isolated was examined by three criteria; TLC, elemental analysis and acid hydrolysis with 4N MSA.

According to the method developed by Sakakibara et al.,\(^{21}\) all of the protecting groups, i.e. Z, MBS, and BzI were deblocked from XII by hydrogen fluoride in the presence of anisole containing 3% ethanediethiol. The product, after conversion to the corresponding acetate by Amberlite IR-45 (acetate form), was incubated in methanol containing 2N acetic acid at 60° for 14 hr to secure the removal of the dicyclohexylamidino moiety from the N\(_{\text{im}}\) of His residue. Subsequently, the product was incubated with dithiothreitol at 50° for 24 hr to reduce Met-sulfoxide formed possibly during the above deblocking process.\(^{22}\) The crude product thus obtained was passed through a column on Sephadex G-25 and then purified by column chromatography on CM-Sephadex. After the column was eluted with water, gradient elution


was established using 0.3 mM ammonium acetate at pH 6.9. The gradient elutes were examined by measurement of the absorbancy at 280 nm. Ammonium acetate was mostly removed from desired fractions by Sephadex G-25 and finally by repeated lyophilization. The fluffy powder thus obtained exhibited a single spot on TLC in two different solvent systems. The hydrolysate with 4 N MSA contained the constituent amino acids in ratios predicted by theory. Also this Pro-containing nonatriaconopeptide was completely digested by aminopeptidase M (AP-M). In amino acid analysis of the enzymatic hydrolysate, the Asn peak overlapped with that of Ser. From difference of the Asp recovery between acid and enzymatic hydrolysates and that of Ser recovery, the presence of the Asn residue could be confirmed.

The in vivo steroidogenetic activity of our synthetic dogfish ACTH was judged as 21.3 IU/mg, when synthetic human ACTH (98.6 IU/mg) was taken as a standard. It has been reported by Lowry et al. that natural dogfish ACTH has about 15% of the activity of synthetic human ACTH.

**Experimental**

The reactions of Met-containing peptides were performed in N₂ atmosphere. The melting points are uncorrected. Rotations are determined in a Union Automatic Polariometer Model P-101 (cell length: 1 cm). The amino acid composition of the acid and enzymatic hydrolysates were determined with a Hitachi Amino Acid Analyzer, Model KLA-5 according to the method of Moor et al. Solvents were evaporated in vacuo at a bath temperature of 40 to 50°C in a rotary evaporator. Thin-layer chromatography was performed on silica gel (kieselgel G, Merck). Rf values refer to the following solvent systems: Rf, CHCl₃-MeOH (29:1), Rf, CHCl₃-MeOH-H₂O (90:15:5), Rf, CHCl₃-MeOH-H₂O (8:3:1), Rf, CHCl₃-MeOH-ACOH (9:1:0.5), Rf, CHCl₃-MeOH-ACOH (9:1:1), Rf, CHCl₃-MeOH-pyridine-H₂O (8:3:0.05:1), Rf, n-BuOH-ACOH-pyridine-H₂O (15:3:10:12) and Rf, n-BuOH-ACOH-pyridine-H₂O (4:1:1:2).

1) Z(OMe)-Amino Acid 4-Morpholinyl Esters—The title compounds were prepared by DCC coupling of Z(OMe)-amino acid with 4-hydroxymorpholine according to the previous paper.i) 
   i) Z(OMe)-Met-OMe: Yield 68.0%, mp 69–71°C. [α]₂⁰° = 2.0° (c = 3.9, CHCl₃). Rf 0.51. *Anal. Calcd.* for C₁₆H₂₃N₂O₄S: C, 54.26; H, 6.58; N, 7.03. *Found.* C, 54.24; H, 6.48; N, 7.08.

2) Z-Ser-Tyr-OMe—Z-Ser-OMe (1.30 g) was added to a suspension of H-Tyr-OMe-HCl (1.40 g) and sodium acetate (0.5 g) in DMF (10 ml). The reaction mixture was stirred at room temperature for 72 hr. After filtration, the filtrate was evaporated in vacuo to dryness. The residue was extracted with AcOEt. The AcOEt layer was washed successively with 10% citric acid, 5% NaHCO₃ and H₂O-NaCl, dried over Na₂SO₄ and then evaporated in vacuo. The residue was recrystallized from AcOEt and petroleum ether; yield 1.05 g (62.9%), mp 111.5–113°C. [α]₂⁰° = 15.1° (c = 1.9, DMF). (Lit.i) mp 112–113°C, [α]₂⁰° = 13.5° (c = 2.0, DMF). Rf 0.38. *Anal. Calcd.* for C₂₁H₂₃N₂O₅: C, 60.57; H, 5.81; N, 6.73. *Found.* C, 60.32; H, 5.80; N, 6.93.

3) Z(OMe)-Ser-Met-OMe—Z(OMe)-Ser-OMe (1.42 g) was added to a suspension of H-Met-OMe-HCl (1.20 g) and sodium acetate (0.50 g) in DMF (10 ml). The mixture was stirred at room temperature for 72 hr. The title compound was isolated from the reaction mixture as stated above (Exp. 2); yield 1.20 g (72.3%), mp 93.5–95°C. [α]₂⁰° = 18.1° (c = 1.6, MeOH). Rf 0.28. *Anal. Calcd.* for C₁₆H₂₃N₂O₄S: C, 52.16; H, 6.32; N, 6.76. *Found.* C, 52.32; H, 6.54; N, 6.87.

4) Z-Ser-Tyr-Ser-Met-NH₂—Z-Ser-Tyr-Ser-Met-OH was prepared as described by Hofmann et al. using Z(OMe)-Ser-Met-OMe prepared above; yield 56.3%, mp 189.5–192°C. [α]₂⁰° = 30.0° (c = 1.0, MeOH). Rf 0.45. (Lit.i) mp 190°C (dec.) [α]₂⁰° = 39.0° (c = 0.7, MeOH). *Anal. Calcd.* for C₁₆H₂₃N₂O₄S: C, 54.88; H, 6.03; N, 8.83. *Found.* C, 55.13; H, 6.26; N, 8.70. The tetrapeptide ester was converted to the title compound according to Li et al. yield 55.0%, mp 244–245°C. [α]₂⁰° = 12.4° (c = 1.1, DMF). (Lit.i) mp 245–246°C (dec.) [α]₂⁰° = 12.0° (c = 0.5, DMF). Rf 0.40. *Anal. Calcd.* for C₁₆H₂₃N₂O₄S: C, 52.99; H, 6.03; N, 13.24. *Found.* C, 53.27; H, 6.24; N, 13.48.

5) Z-Phe-Arg(MBS)-OH—To a solution of H-Arg(MBS)-OH (6.80 g) in DMF (40 ml) containing Et₃N (2.80 ml), Z-Phe-ONP (8.40 g) and HOBT (5 mg) were added. After the mixture was stirred at room temperature for 72 hr, the solvent was evaporated in vacuo. The residue was dissolved in 5% NaHCO₃ and washed with AcOEt. The aqueous phase was acidified with 1 N HCl and the resulting precipitate was ex-
tracted with AcOEt. The AcOEt layer was washed with 1 N HCl and H₂O–NaCl, dried over Na₂SO₄ and then evaporated in vacuo. The residue was purified by column chromatography on silica using solvent system of CHCl₃–MeOH–H₂O (8: 3: 1); yield 10.1 g (80.8%), amorphous powder, [α]₁₃⁰D = −6.5° (c = 4.6, MeOH). Rf₅ 0.47. Anal. Calcd. for C₁₉H₂₈N₂O₆·3H₂O·H₂O: C, 55.20; H, 5.87; N, 10.75. Found: C, 55.54; H, 5.32; N, 10.62.

6) Z-Phe-Arg(MBS)-OMe—An ethereal solution of diazomethane was excessively added to a solution of Z-Phe-Arg(MBS)-OMe (8.0 g) in MeOH (150 ml). The yellow color persisted for 60 min and a few drops of AcOH were added. The solution was evaporated in vacuo. The residue was purified by column chromatography on silica using CHCl₃: yield 6.0 g (73.3%), amorphous powder, [α]₁₃⁰D = −6.5° (c = 2.8, MeOH). Rf₅ 0.25. Anal. Calcd. for C₁₉H₂₈N₂O₆·3H₂O·H₂O: C, 55.20; H, 5.83; N, 10.95. Found: C, 55.36; H, 6.03; N, 10.67.

7) Z-Phe-Arg(MBS)-NNHNH₂—To a solution of Z-Phe-Arg(MBS)-OMe (6.0 g) in MeOH (20 ml), 80% hydrazine hydrate (2.2 ml) was added. The mixture was kept on standing at room temperature for 48 hr. H₂O was added to the reaction mixture and the resulting precipitate was collected by filtration and then repurified from MeOH with H₂O; yield 5.0 g (83.3%), amorphous powder, [α]₁₃⁰D = −17.1° (c = 1.9, DMF). Rf₅ 0.41. Anal. Calcd. for C₁₉H₂₈N₄O₆·3H₂O·H₂O: C, 56.33; H, 5.83; N, 15.33. Found: C, 56.47; H, 5.95; N, 15.19.

8) Z-Phe-Arg(MBS)-Trp-Gly-OH—To a solution of Z-Phe-Arg(MBS)-NNHNH₂ (5.10 g) in DMF (10 ml), 1.25 N HCl/DMF (13 ml) and isomyl nitrite (1.1 ml) were added under cooling with ice-NaCl and the mixture was stirred until the hydrazine test became negative. The solution, after neutralization with Et₃N (2.21 ml), was combined with a solution of H-Trp-Gly-OH (1.72 g) in 90% aqueous DMF (20 ml) containing Et₃N (1.83 ml) 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 H₂O–NaCl. The crude product was purified by column chromatography on silica using solvent system of CHCl₃–MeOH–H₂O (8: 3: 1) and further repurified from THF–MeOH (1: 3 by vol.) with ether; yield 3.30 g (61.9%), amorphous powder, [α]₁₃⁰D = −11.4° (c = 2.9, DMF). Rf₅ 0.62. Anal. Calcd. for C₁₉H₂₈N₄O₆·3H₂O·H₂O·H₂O: C, 55.96; H, 5.90; N, 12.14. Found: C, 56.19; H, 5.71; N, 12.19.

9) Z-His-Phe-Arg(MBS)-Trp-Gly-OH—In the usual manner, Z-Phe-Arg(MBS)-Trp-Gly-OH (1.70 g) in MeOH (30 ml)–THF (20 ml) containing AcOH (1.0 ml) was hydrogenated over a Pd catalyst. After filtration, the filtrate was evaporated in vacuo. The residue was dissolved in DMF (12 ml) and Et₃N (0.44 ml) was added. To this ice-chilled solution, Et₃N (0.42 ml) and the azide solution (prepared from Z-His—NNHN₄H₂ (0.922 g)/DMF (6 ml) with 0.5 N HCl/DMF (12 ml), isomyl nitrite (0.4 ml) and Et₃N (0.83 ml)) 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 2% AcOH and H₂O. The crude product was repurified from MeOH–THF (3: 1 by vol.) with AcOEt; yield 0.8 g (49.7%), mp 155–157°, [α]₁₃⁰D = −28.9° (c = 1.14, DMF), Rf₅ 0.21. Anal. Calcd. for C₁₉H₂₈N₆O₆·3H₂O·H₂O: C, 57.47; H, 5.61; N, 15.04. Found: C, 57.62; H, 5.69; N, 14.89.

10) Z(OMe)-Glu(Obz)-His-Phe-Arg(MBS)-Trp-Gly-OH—Z-His-Phe-Arg(MBS)-Trp-Gly-OH (0.97 g) in MeOH (40 ml)–THF (20 ml) containing AcOH (0.5 ml) was hydrogenated over a Pd catalyst in the usual manner. After filtration, the filtrate was evaporated in vacuo. The residue was dissolved in DMF (20 ml) and Et₃N (0.13 ml) was added. To this solution, Z(OMe)-Glu(Obz)-ONP (0.60 g), Et₃N (0.26 ml) and HOBT (20 mg) were added and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated in vacuo. The residue was washed batchwisely with 2% AcOH and H₂O. The crude product thus obtained was purified by column chromatography on silica using solvent system of CHCl₃–MeOH–H₂O (8: 3: 1) and further precipitated from MeOH–THF (3: 1 by vol.) with ether; yield 0.47 g (41.6%), amorphous powder, [α]₁₃⁰D = −11.8° (c = 0.7, DMF). Rf₅ 0.24. Amino acid ratios in an acid (4 N HSA) hydrolysate: Glu₁₁His₁₁Phe₁₁Arg₁₁Trp₁₁Gly₁₁ (average recovery 94.4%). Anal. Calcd. for C₉₈H₁₀₈N₁₆O₉·3H₂O: C, 56.87; H, 5.85; N, 12.84. Found: C, 57.01; H, 5.57; N, 12.22.

11) Z-Ser-Tyr-Ser-Met-Glu(Obz)-His-Phe-Arg(MBS)-Trp-Gly-OH—Z(OMe)-Glu(Obz)-His-Phe-Arg(MBS)-Trp-Gly-OH (0.35 g) was treated with TFA (1.5 ml) in the presence of anisole (0.2 ml) containing 2% ethanedithiol at 0° for 60 min and dry ether was added. The resulting precipitate was collected by filtration and dried over KOH pellets in vacuo. The powder was dissolved in DMF (5 ml) and Et₃N (0.078 ml) was added. To this ice-chilled solution, Et₃N (0.039 ml) and the azide solution (prepared from Z-Ser–Tyr–Ser–Met–NNHN₄H₂ (0.26 g)/DMF (5 ml) with 0.5 N HCl/DMF (1.67 ml), isomyl nitrite (0.056 ml) and Et₃N (0.116 ml)) 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 2% AcOH and H₂O. The crude product thus obtained was purified by column chromatography on silica using solvent system of CHCl₃–MeOH–H₂O (8: 3: 1) and further repurified from DMF with AcOEt; yield 291 mg (61.8%), mp 186–190°, [α]₁₃⁰D = −21.3° (c = 0.8, DMF), Rf₅ 0.20. Amino acid ratios in an acid (4 N HSA) hydrolysate: Ser₁₁Trp₁₁Tyr₄Ser₁₁Met₁₁Glu₁₁His₁₁Phe₁₁Arg₁₁Trp₁₁Gly₁₁ (average recovery 89.5%). Anal. Calcd. for C₉₈H₁₀₈N₁₆O₉·3H₂O·2H₂O: C, 56.24; H, 5.83; N, 12.96. Found: C, 55.93; H, 5.98; N, 12.79.

12) Z(OMe)-Met-Gly-OMe—Z(OMe)-Met-Omor (1.40 g) was added to a suspension of H-Gly–OMe–HCl (0.84) and sodium acetate (1.02 g) in dioxane (10 ml). The mixture was stirred at room temperature

for 72 hr. The title compound was isolated from the reaction mixture as stated above (Exp. 2); yield 1.14 g (84.3%), mp 94—95.5°C, [α]_D^20 = -5.0° (c=1.4, CHCl_3). This compound was identical with authentic sample by mixed melting point and TLC.

13) Z(OMe)-Lys(Z)-Pro-Met-Gly-OMe — Z(OMe)-Met-Gly-OMe (3.85 g) was treated with TFA (7 ml) in the presence of anisole (1.9 ml) at 0°C for 40 min. The excess TFA was evaporated at 0°C. The resulting oily product was washed with n-hexane and then dried over KOH pellets in vacuo. The oily product was dissolved in THF (30 ml) and the solution was neutralized with Et_3N (1.4 ml). This ice-chilled solution, a solution of Z(OMe)-Lys(Z)-Pro-OH (5.40 g) in THF (20 ml), HOBt (1.45 g) and DCC (2.06 g) were added and the mixture was stirred at room temperature for 18 hr. After filtration, the filtrate was evaporated in vacuo. The residue was recrystallized from AcOEt and petroleum ether; yield 3.0 g (40.5%), mp 116.5—121°C, [α]_D^20 = -55.9° (c=1.0, CHCl_3). R_f 0.63. Anal. Calcd. for C_22H_31N_4O_12S: C, 58.25; H, 6.82; N, 9.41. Found: C, 58.37; H, 6.87; N, 9.52.

14) Z(OMe)-Lys(Z)-Pro-Met-Gly-NHNH_2 — To a solution of Z(OMe)-Lys(Z)-Pro-Met-Gly-OMe (4.0 g) in MeOH (40 ml), 80% hydrazine hydrate (3.2 ml) was added. The mixture was kept on standing at room temperature for 48 hr and H_2O was added. The resulting precipitate was collected by filtration. The crude product was reprecipitated from dioxane with H_2O; yield 5.0 g (79.5%), mp 90.5—94°C, [α]_D^20 = -93°C (c=1.6, DMF). R_f 0.56. Anal. Calcd. for C_25H_31N_4O_15S: C, 56.31; H, 6.64; N, 13.18. Found: C, 56.49; H, 6.88; N, 13.32.

15) Z(OArg)-Arg(MBS)-Pro-OBzl — A solution of Z(OArg)-Arg(MBS)-OH (4.80 g) in THF (40 ml) and DCC (1.94 g) were added to a solution of H-Pro-OBzl (prepared from the hydrochloride (2.30 g) and Et_3N (1.3 ml) in DMF (5 ml) under cooling with ice. The mixture was stirred at room temperature for 18 hr. After filtration, the filtrate was evaporated in vacuo. The residue was dissolved in AcOEt and the AcOEt layer was washed successively with 10% citric acid, 5% NaHCO_3 and H_2O—NaCl, dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by column chromatography on silica using solvent system of CHCl_3-MeOH (100:1); yield 4.10 g (63.5%), amorphous powder, [α]_D^20 = -34.2° (c=1.6, CHCl_3). R_f 0.44. Anal. Calcd. for C_29H_34N_4O_17S: C, 58.69; H, 5.94; N, 10.07. Found: C, 58.49; H, 5.70; N, 10.04.

16) Z-Arg(MBS)-Arg(MBS)-Pro-OBzl — Z(OArg)-Arg(MBS)-Pro-OBzl (3.50 g) was treated with TFA (7.0 ml) in the presence of anisole (1.0 ml) at 0°C for 60 min and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets in vacuo and then dissolved in DMF (20 ml). The solution was neutralized with Et_3N (0.7 ml). Separately, Et_3N (0.83 ml) and isobutyl chloroformate (0.79 ml) were added to a solution of Z-Arg(MBS)-OH (2.90 g) in DMF (20 ml) at -10°C. After stirring for 15 min, the above amino component was added to this solution and further stirred at room temperature for 18 hr. After filtration, the filtrate was evaporated in vacuo. The residue was extracted with AcOEt and the AcOEt layer 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 residue was purified by column chromatography on silica using solvent system of CHCl_3-MeOH (50:1); yield 3.8 g (76.5%), amorphous powder, [α]_D^20 = -44.2° (c=1.9, CHCl_3). R_f 0.48. Anal. Calcd. for C_33H_36N_4O_18S: C, 56.62; H, 6.16; N, 12.71. Found: C, 55.74; H, 5.50; N, 12.87.

17) Z(OArg)-Arg(MBS)-Lys(Z)-OMe — Z(OArg)-Arg(MBS)-OH (5.10 g), DCC (2.10 g) and HOBt (1.40 g) were added to a solution of H-Lys(Z)-OMe prepared from the hydrochloride (3.30 g) and Et_3N (1.39 ml) in THF (50 ml)—DMF (4 ml) under cooling with ice. The mixture was stirred at room temperature for 18 hr. After filtration, the filtrate was evaporated in vacuo. The residue was extracted with AcOEt and the AcOEt layer 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 residue was purified by column chromatography on silica using solvent system of CHCl_3-MeOH (70:1); yield 5.80 g (74.4%), amorphous powder, [α]_D^20 = -16.8° (c=1.3, CHCl_3). R_f 0.55. Anal. Calcd. for C_40H_56N_6O_19S: C, 56.62; H, 6.16; N, 10.71. Found: C, 56.53; H, 6.28; N, 10.75.

18) Z(OArg)-Arg(MBS)-Lys(Z)-NHNH_2 — To a solution of Z(OArg)-Arg(MBS)-Lys(Z)-OMe (3.20 g) in MeOH (20 ml), 80% hydrazine hydrate (1.0 ml) was added. After standing at room temperature for 48 hr, the solvent was evaporated in vacuo. The residue was reprecipitated from MeOH with H_2O; yield 1.90 g (59.3%), mp 108—115°C. [α]_D^20 = -13.9° (c=1.0, DMF). R_f 0.66. Anal. Calcd. for C_26H_33N_4O_12S: C, 55.09; H, 6.16; N, 14.28. Found: C, 54.94; H, 6.37; N, 14.41.

19) Z(OArg)-Arg(MBS)-Lys(Z)-Arg(MBS)-Pro-OBzl — In the usual manner, Z-Arg(MBS)-Arg(MBS)-Pro-OBzl (1.0 g) in MeOH (20 ml) containing AcOH (5 ml) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration and the filtrate was condensed. The residue was dried over KOH pellets in vacuo and dissolved in DMF (5 ml). From this ice-chilled solution, Et_3N (0.42 ml) and the azide solution (prepared from Z(OArg)-Arg(MBS)-Lys(Z)-NHNH_2 (0.87 g)/DMF (5 ml) with 1.25N HCl/DMF (1.8 ml), isoamyl nitrite (0.16 ml) and Et_3N (0.31 ml) were added) the mixture was stirred at 4°C for 48 hr and the solvent was evaporated in vacuo. The residue was extracted with CHCl_3 and the CHCl_3 layer was washed with 10% citric acid and H_2O—NaCl, dried over Na_2SO_4 and then evaporated in vacuo. The residue was further purified by column chromatography on silica using solvent system of CHCl_3-MeOH—H_2O (8:3:1); yield 0.8 g (52.6%), mp 165—170°C, [α]_D^20 = -9.8° (c=2.2, DMF). R_f 0.17. Amino acid ratios in an acid hydrolysate; Arg 4.09, Lys 0.29, Pro 1.69 (average recovery 98.8%). Anal. Calcd. for C_34H_59N_13O_20S_3.H_2O: C, 51.10; H, 6.08; N, 13.34. Found: C, 51.00; H, 5.95; N, 13.41.
20) Z(OMe)-Arg(MBS)-Lys(Z)-Arg(MBS)-Arg(MBS)-Arg-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, Z(OMe)-(d-ACTH 20-39)-OBzl — Z(OMe)-(d-ACTH 20-39)-OBzl (0.78 g) was treated with TFA (1.5 ml) in the presence of anisole containing 2% ethanoladiol at 0° for 60 min and dry ether was added. The resulting precipitate was collected by filtration and then dissolved in 0.5 N HCl/DMF (1 ml). To this solution, dry ether was added and the resulting piconosapentine ester hydrochloride was collected by filtration and then dried over KOH pellets in vacuo. The powder was dissolved in DMF (5 ml) and Et$_3$N (0.023 ml) was added. To this ice-chilled solution, Z(OMe)-Arg(MBS)-Lys(Z)-Arg(MBS)-Arg(MBS)-Arg-Pro-Oh (0.53 g), HBOT (47 mg) and DCC (72 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% acetic acid, 5% NaHCO$_3$, H$_2$O and MeOH. The crude product thus obtained was purified by column chromatography on silica using solvent system of CHCl$_3$-MeOH-H$_2$O (8 : 3 : 1); yield 0.77 g (68.6%), mp 229-230°, [α]$_D$ = -30.4° (c = 0.6, DMF), R$_f$ 0.31. Amino acid ratios in an acid hydrolysate: Asp$_{0.6}$ Ser$_{0.8}$ Glu$_{0.14}$ Pro$_{0.7}$ Gly$_{0.8}$ Val$_{0.17}$ Me$_{0.6}$ Ile$_{0.8}$ Leu$_{0.7}$ Tyr$_{0.3}$ Lys$_{0.3}$ Arg$_{0.1}$ (average recovery 87.8%). Anal. Calcd. for C$_{218}$H$_{343}$N$_{89}$O$_{89}$S$_{4}$.2H$_2$O: C, 58.04; H, 6.39; N, 11.80. Found: C, 57.35; H, 6.47; N, 11.75.

21) Z(OMe)-Lys(Z)-Pro-Met-Gly-Ary(MBS)-Arg(MBS)-Arg(MBS)-Arg-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, Z(OMe)-(d-ACTH 10-29)-OBzl — The above Z(OMe)-(d-ACTH 15-39)-OBzl (0.46 g) was treated with TFA (2 ml) in the presence of anisole containing 2% ethanoladiol at 0° for 60 min and dry ether was added. The resulting powder was dried over KOH pellets in vacuo. The powder was dissolved in DMF (4 ml) and Et$_3$N (0.014 ml) was added. To this ice-chilled solution, Et$_3$N (0.014 ml) and the azide solution (prepared from Z(OMe)-Lys(Z)-Pro-Met-Gly-NHNH$_3$ (0.12 g)/DMF (3 ml) with 0.5 N HCl/DMF (0.6 ml), isoamyl nitrite (0.02 ml) and Et$_3$N (0.042 ml)) were added and the mixture was stirred at 4° for 72 hr. The solvent was evaporated in vacuo and the residue was washed batchwise with 10% acetic acid, 5% NaHCO$_3$, H$_2$O and MeOH. The crude product thus obtained was purified by column chromatography on silica using CHCl$_3$-MeOH-H$_2$O (8 : 3 : 1); yield 0.41 g (75.9%), mp 222-226°, [α]$_D$ = -33.6° (c = 0.5, DMF), R$_f$ 0.62. Amino acid ratios in an acid hydrolysate: Asp$_{0.3}$ Ser$_{1.8}$ Glu$_{0.6}$ Pro$_{0.6}$ Gly$_{0.8}$ Val$_{0.3}$ Met$_{0.6}$ Ile$_{0.8}$ Leu$_{0.7}$ Tyr$_{0.3}$ Lys$_{0.3}$ Phe$_{0.1}$ Lys$_{0.1}$ Arg$_{0.1}$ (average recovery 97.0%). Anal. Calcd. for C$_{244}$H$_{363}$N$_{90}$O$_{90}$S$_{4}$.2H$_2$O: C, 57.32; H, 6.47; N, 11.78. Found: C, 57.07; H, 6.37; N, 11.53.

22) Z-Ser-Tyr-Ser-Met-Glu(Obzl)-His-Phe-Arg(MBS)-Trp-Gly-Lys(Z)-Pro-Met-Gly-Ary(MBS)-Lys(Z)-Arg(MBS)-Arg(MBS)-Arg-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 Dogfish ACTH — The above Z(OMe)-(d-ACTH 11-39)-OBzl (179 mg) was treated with TFA (1 ml) in the presence of anisole (0.18 ml) at 0° for 60 min and dry ether was added. The resulting powder was converted to the corresponding hydrochloride as stated above. The resulting nonacosapeptide ester hydrochloride was dissolved in DMF (5 ml) and Et$_3$N (0.049 ml of 10% DMF solution) was added. To this ice-chilled solution, Z-Ser-Tyr-Ser-Met-Glu(Obzl)-His-Phe-Arg(MBS)-Trp-Gly-OH (87.4 mg), HBOT (7.0 mg) and DCC (11 mg) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated in vacuo. The residue was washed batchwise with acid and base as stated above and then dissolved in a mixture of DMF-MeOH-2 N AcOH (3 : 4 : 1 by vol., 10 ml). The solution, after heating at 65° for 8 hr, was condensed in vacuo and the residue was purified by column chromatography on silica as stated above. The desired product thus isolated was recrystallized from DMF with MeOH; yield 116 mg (48.7%), mp 220-228°, [α]$_D$ = -19.0° (c = 0.6, DMF), R$_f$ 0.67. Amino acid ratios in an acid (4 x MSA) hydrolysate: Ser$_{0.6}$ Tyr$_{0.8}$ Met$_{0.6}$ Glu$_{0.6}$ His$_{0.8}$ Phe$_{1.2}$ Arg$_{0.1}$ Trp$_{0.4}$ Gly$_{0.6}$ Lys$_{0.6}$ Pro$_{0.6}$ Ile$_{0.8}$ Val$_{0.7}$ Asp$_{0.6}$ Leu$_{0.8}$ (average recovery 89.5%). Anal. Calcd. for C$_{252}$H$_{364}$N$_{93}$O$_{89}$S$_{4}$.8H$_2$O: C, 56.83; H, 6.35; N, 12.37. Found: C, 57.09; H, 6.21; N, 12.05.

23) H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Arg-Trp-Gly-Lys-Met-Gly-Ary-Lys-Met-Alg-Pro-Ile-Lys-Val-Tyr-Pro-Asn-Ser-Phe-Glu-Asp-Ser-Val-Glu-Asn-Met-Gly-Pro-Glu-Leu-OH (Dogfish ACTH) — The above protected nonatriacantapeptide ester (85 mg) was treated with HF (approximately 3 ml) in the presence of anisole containing 2% ethanoladiol (0.8 ml) at -5° for 60 min. The excess HF was removed by evaporation and the residue dissolved in water (10 ml), then was treated with Amberlite IR-45 (acetate form, approximately 4 g) for 30 min. The resin was removed by filtration, the filtrate was lyophilized. The residue was dissolved in MeOH-2 N AcOH (4 : 1 by vol., 20 ml) and heated at 60° for 14 hr. The solvent was evaporated by evaporation and the residue was dissolved in water (20 ml). The solution, after addition of diithiothreitol (30 mg), was incubated at 50° for 24 hr. The solvent was evaporated in vacuo and the residue was dissolved in a small amount of 0.2 N AcOH and then applied to a column of Sephadex G-25 (2.6 x 76 cm), which was eluted with 0.2 N AcOH. Individual fractions (5 ml each) were collected and absorbancy at 280 nm was determined for each fraction. The fractions corresponding to the front peak (tube No. 23-37) were combined and the solvent was removed by lyophilization; yield 41 mg (deblooding step: 68.9%). The product was dissolved in water (30 ml) and the solution was applied to a column of CM-Sephadex C-25 (3.0 x 15 cm), which was eluted first with 0.1 M NaOH (100 ml) and then with 0.3 M ammonium acetate buffer (pH 6.9), through a mixing flask containing washed with 150 ml) Individual fractions (4 ml each) were collected and absorbancy at 280 nm was determined. A main peak present in the gradient elutes (tube No. 71-79) were combined and the solvent was condensed to approximately 5 ml. This solution was then applied to a column of Sephadex G-25 (2.8 x
120 cm), which was eluted with 0.2 N AcOH. The desired fractions (5 ml each, tube No. 69—80) were collected and the solvent was removed by lyophilization to give a fluffy powder; yield 18.4 mg (overall yield: 27.4%), $[\alpha]_D^{25} = -88.0^\circ$ (c=0.13, 0.2 N AcOH), $R_f$ 0.17, $R_f$ 0.28, $R_f$ (cellulose) 0.42.\(^{27}\) Amino acid ratios in an acid (4 N MSA) hydrolysate: Asp$_{3.05}$Ser$_{3.45}$Glu$_{1.10}$Pro$_{2.95}$Gly$_{1.00}$Val$_{1.20}$Met$_{2.57}$Ile$_{0.92}$Leu$_{1.06}$Tyr$_{1.76}$Phe$_{1.82}$Trp$_{0.68}$Lys$_{2.31}$-His$_{0.06}$Arg$_{2.22}$ (average recovery 77.8%). Amino acid ratios in AP-M digest (peptide 0.2 μmol/AP-M 2 U): Asp$_{1.05}(\text{Ser})$ Asn$_{0.10}(\text{Ser})$, Caled. as Ser$_{0.00}$Glu$_{1.76}(\text{Gly})$Pro$_{3.55}(\text{Gly})$Val$_{1.25}(\text{Met})$Met$_{2.07}(\text{Ile})$Leu$_{1.00}(\text{Tyr})$Phe$_{1.82}(\text{Trp})$Lys$_{2.31}(\text{His})$Arg$_{2.22}(\text{Arg})$ (average recovery 82.9%). Number in parentheses are theoretical. Anal. Calcd. for C$_{277}$H$_{312}$N$_{35}$O$_{60}$S$_{3}$·7CH$_{3}$COOH·20H$_2$O: C, 48.55; H, 7.06; N, 15.12. Found: C, 48.40; H, 7.06; N, 15.47.

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27) TLC was performed on cellulose (solvent system: n-BuOH-AcOH-pyridine-H$_2$O=15:3:10:12).