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Studies on Peptides. LXXV.[1,2] Synthesis of the Hexadecapeptide corresponding to the Entire Amino Acid Sequence of β-Melanocyte-stimulating Hormone from the Dogfish (*Squalus acanthias*)

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In a conventional manner, the hexadecapeptide corresponding to the entire amino acid sequence of dogfish β-MSH (*Squalus acanthias*) was synthesized using protecting groups removable by hydrogen fluoride. The synthetic peptide exhibited the *in vitro* MSH activity of 1.88×10^9 MSH U/g.

Keywords——dogfish β-MSH (*Scyliorhinus canicula*); dogfish β-MSH (*Squalus acanthias*); deprotection with hydrogen fluoride; 4 M methanesulpfonic acid hydrolysis of Trp-peptides; 5-chloro-8-quinolyl active ester; succinimide formation of Asp–Gly; condensation reaction in the absence of Et_3N salt

Following to the synthesis of β-melanocyte-stimulating hormone (β-MSH) from *Scyliorhinus canicula* (I)[4,5] we wish to report the synthesis of the hexadecapeptide corresponding to the entire amino acid sequence of another dogfish β-MSH (II) from *Squalus acanthias*,[6] the structure of which was determined by Bennet, *et al.*[6] in 1974. These two β-MSHs have structural similarity in a respect of having the common tetrapeptide core, His-Phe-Arg-Trp, like mammalian α and β-MSHs,[7] but are different each other in the chain length and the amino acid composition as well. *Acanthias* β-MSH is composed of 16 amino acids, while *caniculus* β-MSH 18 amino acids as like as mammalian β-MSHs.

Squalus acanthias β-MSH (II)

H-Asp-Gly-Asp-Asp-Tyr-Lys-Phe-Gly-His-Phe-Arg-Trp-Ser-Val-Pro-Leu-OH

Scyliorhinus canicula β-MSH. (I)

H-Asp-Gly-Ile-Asp-Tyr-Lys-Met-Gly-His-Phe-Arg-Trp-Gly-Ala-Pro-Met-Asp-Lys-OH

Fig. 1. Amino Acid sequence of Dogfish β-MSH

Synthetic route to acanthias β-MSH (II) is illustrated in Fig. 2. Synthetic strategies employed here are essentially the same as those employed in the synthesis of *caniculus*

1) Part LXXIV; N. Fujii, T. Sasaki, S. Funakoshi, H. Irie, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 650 (1978).
2) Amino acids, peptides and their derivatives mentioned here are of the L-configuration. Abbreviations used are recommended by IUPAC-IUB Commission of Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Tos=tosyl, Bzl=benzyl, NP=*p*-nitrophenyl, QCl=5-chloro-8-quinolyl, DCC=dicyclohexylcarbodiimide, HOBT=N-hydroxybenzotriazole, TFA=trifluoroacetic acid, DMF=dimethylformamide.
3) Location: *a) Sakyo-ku, Kyoto, 606, Japan*; *b) Fukushima, Osaka, 553, Japan.*
4) H. Yajima, J. Iwai, H. Watanabe, and K. Koyama, *Chem. Pharm. Bull.* (Tokyo), **25**, 2048 (1977).
5) R.M. Love and B.T. Pickering, *Gen. Comp. Endocrinol.*, **24**, 398 (1974).
6) H.P.S. Bennet, C. McMartin, and A.P. Scott., *Biochem. J.*, **141**, 439 (1974).
7) See review article: H. Yajima and Y. Kiso, *Pharm. Therap. B.*, **1**, 529 (1975) and other references therein.

β-MSH.[4] Amino acid derivatives bearing protecting groups removable by hydrogen fluoride[8] were employed. By taking advantage of the suitable location of the Gly at position 2 and 8, the following two fragments, D (position 1—2) and C (3—8) were selected as building blocks. Next, further two units, tripeptide unit B (9—11) and pentapeptide unit A (12—16), were selected, since the Trp residue locates at position 12 and the azide condensation of Trp-containing peptides is not suitable.

The c-terminal pentapeptide (A), Z(OMe)-Trp-Ser-Val-Pro-Leu-OBzl, was synthesized according to the scheme illustrated in Fig. 3. First two protected dipeptides, Z(OMe)-Pro-

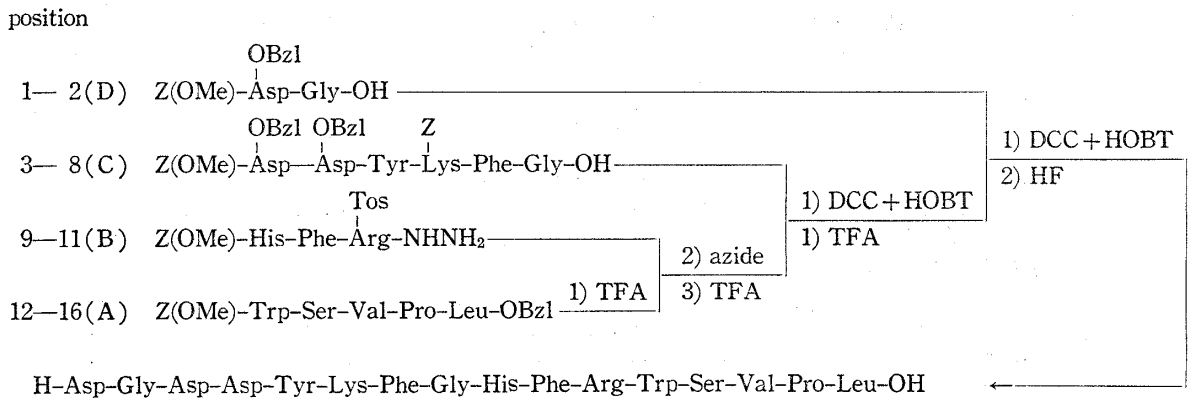

Fig. 2. Synthetic Route to Dogfish β-MSH (*Squalus acanthias*)

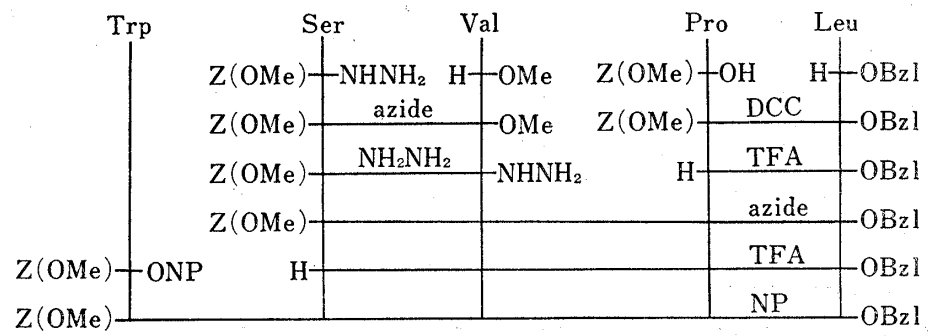

Fig. 3. Synthetic Scheme of the Protected Pentapeptide Ester (A Position 12—16).

Leu-OBzl and Z(OMe)-Ser-Val-OMe, were prepared by the DCC[9] and the azide procedure[10] respectively. The latter dipeptide was converted in the usual manner to the corresponding hydrazide, Z(OMe)-Ser-Val-$NHNH_2$, which was then coupled with H-Pro-Leu-OBzl derived from the former by treatment with TFA.[11] This 2 plus 2 coupling reaction was taken by the reason that previously we observed that the condensation between Z(OMe)-Val-OH and H-Pro-peptides by DCC gave a considerable amount of the acylurea, a side reaction product of DCC.[12] This seems to be due to the steric hindrance of these two amino acids and even in the above azide coupling, the yield was only 31%. The resulting protected tetrapeptide, Z(OMe)-Ser-Val-Pro-Leu-OBzl, after deprotection with TFA, was condensed with Z(OMe)-Trp-OH by the *p*-nitrophenyl ester procedure[13] to give (A). The purity of the product, as

8) S. Sakakibara, Y. Shimonishi, Y. Kishida, H. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
9) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
10) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Soc.*, **26**, 2333 (1961).
11) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
12) Y. Kai, H. Kawatani, H. Yajima, and Z. Itoh, *Chem. Pharm. Bull.* (Tokyo), 23, 2346 (1975).
13) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

well as the other fragments, was assessed by three criteria: thin-layer chromatography, elemental analysis and acid hydrolysis. Especially by the hydrolysis with 4 M methanesulphonic acid (MSA),[14] the content of Trp in (A) was confirmed.

The protected tripeptide hydrazide, Z(OMe)-His-Phe-Arg(Tos)-$NHNH_2$ (B), was prepared starting with an available protected dipeptide, Z-Phe-Arg(Tos)-OMe,[15] used for our previous synthesis of porcine β-MSH. This dipeptide, after removing the Z group with hydrogen bromide in acetic acid, was condensed with Z(OMe)-His-$NHNH_2$[16] by means of the azide procedure to give (B).

Next, the protected hexapeptide, Z(OMe)-Asp(OBzl)-Asp(OBzl)-Tyr-Lys(Z)-Phe-Gly-OH (C) was synthesized as shown in Fig. 4, in a stepwise manner starting with the known protected dipeptide, Z(OMe)-Phe-Gly-OH.[17] The α-amino protecting group, Z(OMe), was removed from each intermediate in the usual TFA treatment and the peptide chain was elongated with the suitable carboxyl activating amino acid derivatives, which are well characterized; Z(OMe)-Lys(Z)-OQCl,[18] Z(OMe)-Tyr-$NHNH_2$,[19] and Z(OMe)-Asp(OBzl)-ONP.[20] Especially, introduction of two Asp(OBzl) groups was performed with care of avoiding the use of excess triethylamine in order to suppress the possible acylation of the Tyr residue.[21]

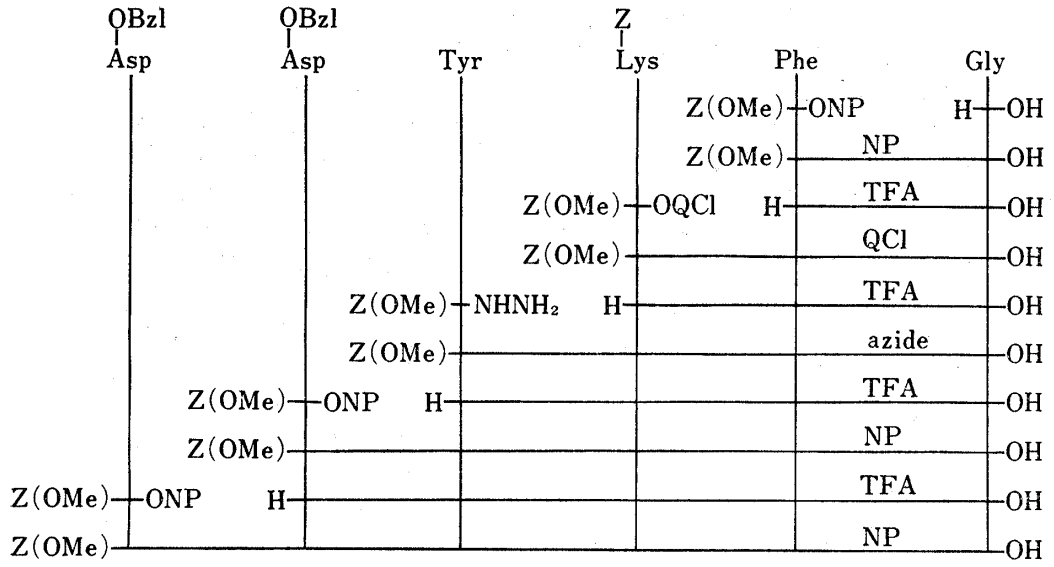

Fig. 4. Synthetic Scheme of the Protected Hexapeptide (C Position 3—8).

The N-terminal dipeptide unit, Z(OMe)-Asp(OBzl)-Gly-OH, is the known derivative.[22] Great tendency of the acid-base catalyzed N-succinimide type side reaction of this particular sequence is known in literatures.[23] Prior to selecting this dipeptide unit, we condensed Z(OMe)-Asp(OBzl)-Gly-OH and H-Gly-NHNH-Troc with DCC in the presence of HOBT[24]

14) R.J. Simpson, M.R. Neuberger, and T.Y. Liu, *J. Biol. Chem.*, **251**, 1936 (1976).
15) H. Watanabe, H. Ogawa, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 375 (1975).
16) H. Yajima, F. Tamura, Y. Kiso, and M. Kurobe, *Chem. Pharm. Bull.* (Tokyo), **21**, 380 (1973).
17) H. Yajima, K. Kitagawa, and T. Segawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 2500 (1973).
18) H.D. Jakubke and A. Voigt, *Chem. Ber.*, **99**, 2944 (1966); H. Yajima, H. Ogawa, H. Watanabe, N. Fujii, M. Kurobe, and S. Miyamoto, *Chem. Pharm. Bull.* (Tokyo), **23**, 371 (1975).
19) N. Fujii and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 2446 (1975).
20) H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **23**, 1061 (1974).
21) J. Ramachandran and C.H. Li, *J. Org. Chem.*, **28**, 173 (1963).
22) H. Yajima and H. Kawatani, *Chem. Pharm. Bull.* (Tokyo), **19**, 1905 (1971).
23) M. Bodanszky and S. Natarajan, *J. Org. Chem.*, **40**, 2495 (1975) and see other references therein.
24) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

and confirmed that the desired protected tripeptide, Z(OMe)-Asp(OBzl)-Gly-Gly-NHNH-Troc,[25] could be obtained as a sole product, when the coupling reaction was performed carefully in a neutral condition as possible, *i.e.*, the TFA treated product of Z(OMe)-Gly-NHNH-Troc was neutralized with 5% sodium bicarbonate and extracted with ethyl acetate, which was then submitted to the coupling reaction. Thus the risk of the excess base, such as triethylamine, can be avoided. *Canicula* β-MSH possesses also this particular dipeptide sequence at the N-terminus and for this synthesis, we introduced Gly and Asp(OBzl) stepwisely. In the present synthesis, we decided to introduce this dipeptide unit alternatively, based on the preliminary experiment stated above.

Four building blocks, A, B, C, and D, were then assembled according to the scheme illustrated in Fig. 2. First, the Trp containing pentapeptide (A) was treated with TFA in the presence of anisole containing 2% ethanedithiol. Some amount of skatole was also added in this deblocking reaction. The use of 2-methylindole during the acid treatment of Trp-peptides was recommended by Wünsch and Wendlberger,[26] especially when *o*-nitrophenyl sulfenyl group was cleaved with hydrochloric acid. As far as examined by thin layer chromatography, the anisole 2% ethanedithiol-skatole system was judged as much more effective to suppress the destruction of the acid sensitive Trp residue rather than the anisole-2% ethanedithiol system, which we previously employed.[4] This deprotecting condition was extended to the latter steps of the present synthesis. The deprotected product of (A) could not be precipitated with ether as fine powders. Therefore the solvent was evaporated and the residue was dissolved in ethyl acetate, which after washing with 5% sodium carbonate, like H-Gly-NHNH-Troc as stated above, was submitted to the coupling reaction with the protected tripeptide unit (B). The azide reaction went smoothly and the product, Z(OMe)-His-Phe-Arg(Tos)-Trp-Ser-Val-Pro-Leu-OBzl, was isolated in fairly good yield.

The protected octapeptide ester obtained above was treated with TFA under conditions stated above and the deprotected peptide, precipitated as a fine powder with ether and 5% sodium carbonate, was submitted to the next coupling reaction with the protected hexapeptide (C) by the DCC plus HOBT procedure. Previously, Rink and Riniker[27] pointed out a side reaction which takes place at the His residue under this coupling condition. Since it is known that this DCC adduct can be removed by the brief treatment with methanol and acetic acid, the product, Z(OMe)-Asp(OBzl)-Asp(OBzl)-Tyr-Lys(Z)-Phe-Gly-His-Phe-Arg(Tos)-Trp-Ser-Val-Pro-Leu-OBzl, was purified by batchwise washing with 5% citric acid and 5% sodium carbonate followed by incubation in methanol containing acetic acid.

The protected tetradecapeptide ester thus obtained was similarly treated with TFA and the deprotected peptide, isolated as a free base as stated above, was submitted to the final condensation reaction with the N-terminal dipeptide unit (D) by means of the DCC plus HOBT procedure. Usually, when the α-protecting group is cleaved with TFA, the resulting TFA salt is neutralized with triethylamine before the coupling reaction. In the present synthesis, every deprotected intermediate, *i.e.*, the TFA salt, is neutralized with 5% sodium carbonate and the either extracted or precipitated free base is washed with water before use. By this procedure, the coupling reaction can be performed nearly in the neutral condition in the absence of the TFA triethylammonium salt. Especially this condition seems to play an important role to establish the Asp-Gly bond by the reason stated above. This coupling reaction seems to have proceeded smoothly, since the product exhibiting a sharp single spot on thin layer chromatography was isolated after purification by batchwise washing and incubation in DMF–methanol containing acetic acid as stated above.

25) F. Tamura, H. Ogawa, N. Fujii, H. Yajima, K. Miyata, M. Nakamura, and A. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **25**, 767 (1977).
26) E. Wünsch and G. Wendlberger, *Chem. Ber.*, **101**, 3659 (1968).
27) H. Rink and B. Riniker, *Helv. Chim. Acta*, **57**, 831 (1974).

The protected hexadecapeptide, Z(OMe)-Asp(OBzl)-Gly-Asp(OBzl)-Asp(OBzl)-Tyr-Lys(Z)-Phe-Gly-His-Phe-Arg(Tos)-Trp-Ser-Val-Pro-Leu-OBzl, thus obtained, was treated with hydrogen fluoride to remove all protecting groups. The reaction was performed in an ice-bath and as cation scavengers, anisole containing 2% ethanedithiol and skatole were added to suppress possible side reactions[28] during this treatment. The deprotected peptide, after conversion to the corresponding acetate by Amberlite CG-4B, was purified by partition column chromatography on Sephadex G-25 according to Yamashiro.[29] This condition was chosen by the reason that the product is less soluble in water and even in dilute acid, presumably due to the presence of three Asp residues.

The product thus obtained exhibited a sharp single spot on thin layer chromatography in two different solvent systems and its 4 N MSA hydrolysate contained the constituent amino acid in ratios predicted by theory. Digestion by aminopeptidase (AP-M)[30] was difficult to perform, because of its less solubility in the buffer at pH 7.7. Thus the Asp-Gly bond in the product could not be established enzymatically. But we think we have a reason to predict that this particular peptide bond is maintained during the synthesis and the deprotection step in the expected α-linkage, since the structurally related *canicula*[4] and camel β-MSHs[25] were previously synthesized in a similar manner employed above and enzymatically examined.

MSH activity was determined *in vitro* using frog skins (*Rana pipiens*) according to Nakamura *et al.*[31] The potency of the synthetic peptide was evaluated as 1.88×10^9 MSH U/g, when synthetic mammalian α-MSH[32] was taken as the standard. This value (relative potency 0.094) is nine times higher than that expected from the literature value (about 1% of that of mammalian α-MSH).[6] This relatively high biological activity seems to support our prediction about the α-Asp-Gly bond in the synthetic peptide mentioned above.

Experimental

General experimental methods employed are essentially the same as described in the Part 62[33] of this series. Thin-layer chromatography (TLC) was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (97:3), Rf_2 $CHCl_3$–MeOH–H_2O (90:10:1), Rf_3 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2).

Z(OMe)-Pro-Leu-OBzl——DCC (24.72 g) was added to a mixture of Z(OMe)–Pro–OH (33.52 g) and H–Leu–OBzl (prepared from 39.35 g of the tosylate with 14 ml of Et_3N) in AcOEt (300 ml) and the solution was stirred at room temperature for 18 hr. The solution was filtered, the filtrate was washed with 10% citric acid, 10% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with *n*-hexane and recrystallized from ether and *n*-hexane; yield 47.29 g (98%), mp 76—77°, $[\alpha]_D^{19}$ −37.7° ($c=0.6$, DMF). Rf_1 0.72. *Anal.* Calcd. for $C_{27}H_{34}N_2O_6$: C, 67.20; H, 7.10; N, 5.81. Found: C, 67.41; H, 7.16; N, 5.93.

Z(OMe)-Ser-Val-OMe——The azide (prepared from 28.33 g of Z(OMe)–Ser–$NHNH_2$ with 98.5 ml of 2.03 N HCl–DMF, 14.1 ml of isoamylnitrite and 28.7 ml of Et_3N) in DMF (40 ml) was combined with a ice-chilled solution of H–Val–OMe (prepared from 18.44 g of the hydrochloride with 28.7 ml of Et_3N) in DMF (100 ml) and the mixture was stirred at 4° for 18 hr. The solvent was evaporated and the residue was extracted with AcOEt, which was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was recrystallized from AcOEt; yield 32.85 g (86%), mp 104—105°, $[\alpha]_D^{23}$ +4.1° ($c=0.7$, DMF). Rf_2 0.51. *Anal.* Calcd. for $C_{18}H_{26}N_2O_7$: C, 56.53; H, 6.85; N, 7.33. Found: C, 56.41; H, 6.93; N, 7.48.

Z(OMe)-Ser-Val-$NHNH_2$——To a solution of Z(OMe)–Ser–Val–OMe (28.68 g) in MeOH (100 ml), 80% hydrazine hydrate (42 ml) was added and the gelatinous mass formed on standing overnight, was collected

28) S. Sano and S. Kawanishi, *J. Am. Chem. Soc.*, **97**, 3480 (1975); R.S. Feinberg and R.B. Merrifield, *ibid.*, **97**, 3485 (1975); R.L. Noble, D. Yamashiro, and C.H. Li., *ibid.*, **98**, 2324 (1976).
29) D. Yamashiro, *Nature* (London), **201**, 76 (1964).
30) G. Pfleiderer and G.P. Celliers, *Biochem. Z.*, **339**, 186 (1963).
31) M. Nakamura, A. Tanaka, M. Hirata, and S. Inouye, *Endocrinol. Japan*, **19**, 383 (1972).
32) H. Yajima, K. Kawasaki, Y. Okada, H. Minami, K. Kubo, and I. Yamashita, *Chem. Pharm. Bull.* (Tokyo), **16**, 919 (1968).
33) H. Ogawa, M. Kubota, and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **24**, 2428 (1976).

by filtration and recrystallized from MeOH; yield 28.60 g (99%), mp 234—235°, $[\alpha]_D^{18}$ −3.6° (c=0.3, DMSO), Rf_3 0.48. *Anal.* Calcd. for $C_{17}H_{26}N_4O_6$: C, 53.59; H, 6.85; N, 14.65. Found: C, 53.43; H, 6.88; N, 14.44.

Z(OMe)-Ser-Val-Pro-Leu-OBzl——Z(OMe)–Pro–Leu–OBzl (24.13 g) was treated with TFA (37 ml) in the presence of anisole (16 ml) in an ice-bath for 30 min and the excess TFA was removed by evaporation *in vacuo*. The oily residue was dissolved in AcOEt, which, after neutralization with Et_3N, was washed with H_2O–NaCl, dried over Na_2SO_4 and then filtered. The filtrate was combined with a solution of the azide (prepared from 19.12 g of Z(OMe)–Ser–Val–$NHNH_2$ with 23.8 ml of 4.21 N HCl–DMF, 6.7 ml of isoamylnitrite and 21 ml of Et_3N) in DMF (200 ml). The mixture was stirred at 4° for 18 hr and the solvent was evaporated. The residue was dissolved in AcOEt. The solution, after washing with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, was dried over Na_2SO_4 and then condensed. The residue was dissolved in a small amount of the solvent consisting of $CHCl_3$–MeOH–H_2O (95 : 4.5 : 0.5) and the solution was applied to a column of silica (5.5×7.5 cm), which was eluted with the same solvent system. Fractions containing the substance of Rf_2 0.58 were combined and the solvent was evaporated. Treatment of the residue with H_2O gave a fine powder, which was recrystallized from AcOEt and n-hexane; yield 10.45 g (31%), mp 55—58°, $[\alpha]_D^{23}$ −30.8° (c=1.0, DMF). Rf_2 0.58. Amino acid ratios in 3 N Tos–OH hydrolysate: Ser 1.00, Val 1.09, Pro 1.00, Leu 0.92 (average recovery 95%). *Anal.* Calcd. for $C_{35}H_{48}N_4O_9$: C, 62.85; H, 7.23; N, 8.38. Found: C, 62.76; H, 7.26; N, 8.48.

Z(OMe)-Trp-Ser-Val-Pro-Leu-OBzl (A)——As stated above, Z(OMe)–Ser–Val–Pro–Leu–OBzl (10.45 g) was treated with TFA (23 ml) in the presence of anisole (8.4 ml) in an ice-bath for 30 min and dry n-hexane was added. The resulting oily precipitate was dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (50 ml) containing Et_3N (4.4 ml). To this solution, Z(OMe)–Trp–ONP (7.65 g) was added and the mixture was stirred at room temperature for 14 hr. The solvent was evaporated and the residue was dissolved in AcOEt. The solution was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and then condensed. The residue was triturated with n-hexane and recrystallized from AcOEt and n-hexane; yield 12.84 g (96%), mp 95—97°, $[\alpha]_D^{18}$ −53.6° (c=0.4, DMF). Rf_2 0.38. Amino acid ratios in 3 N Tos–OH hydrolysate: Trp 0.88, Ser 1.02, Val 1.15, Pro 1.00, Leu 0.97 (average recovery 90%). *Anal.* Calcd. for $C_{46}H_{58}N_6O_{10}$: C, 64.62; H, 6.84; N, 9.83. Found: C, 64.86; H, 6.58; N, 9.55.

Z(OMe)-His-Phe-Arg(Tos)-$NHNH_2$ (B)——Z–Phe–Arg(Tos)–OMe[15] (23.0 g) was treated with 25% HBr–AcOH (92 ml) at room temperature for 60 min and n-hexane was added. The resulting oily precipitate was washed with n-hexane, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (40 ml) containing Et_3N (5.2 ml). To this ice-chilled solution, the azide (prepared from 11.71 g of Z(OMe)–His–$NHNH_2$ with 23.6 ml of 2.98 N HCl–DMF, 4.71 ml of isoamylnitrite and 14.8 ml of Et_3N) in DMF (20 ml) was added. After stirring at 4° for 16 hr, the solution was condensed and the residue was dissolved in n-BuOH and the organic phase was washed with 5% $NaHCO_3$, H_2O–NaCl and H_2O and then evaporated. The residue was treated with ether to give an amorphous powder; yield 15.0 g (51%). Z(OMe)–His–Phe–Arg(Tos)–OMe (13.40 g) thus obtained was dissolved in MeOH (150 ml) and 80% hydrazine hydrate (10.5 ml) was added. After standing at room temperature for 20 hr, the solution was condensed and the residue was treated with H_2O. The resulting powder was recrystallized from EtOH and ether; yield 5.51 g (41%), mp 144—145°, $[\alpha]_D^{24}$ −12.3° (c=0.7, DMF). Rf_3 0.67. Amino acid ratios in an acid hydrolysate: His 0.84, Phe 1.00, Arg 1.04 (average recovery 81%). *Anal.* Calcd. for $C_{37}H_{46}N_{10}O_8S \cdot H_2O$: C, 54.93; H, 5.98; N, 17.32. Found: C, 55.12; H, 5.93; N, 17.30.

Z(OMe)-Lys(Z)-Phe-Gly-OH——Z(OMe)–Phe–Gly–OH[17] (11.59 g) was treated as usual with TFA (22.3 ml) in the presence of anisole (9.7 ml) in an ice-bath for 70 min and dry ether was added. The resulting powder was collected by filtration and then dissolved in DMF (100 ml), to which Et_3N (12.2 ml) and Z(OMe)–Lys(Z)–OQCl (16.52 g) were added. The mixture was stirred at room temperature for 12 hr, the solvent was evaporated and the residue was dissolved in H_2O, which after washing with AcOEt, was acidified with 10% citric acid. The resulting powder was washed batchwisely with H_2O and recrystallized from MeOH and AcOEt; yield 14.50 g (82%), mp 190—193°, $[\alpha]_D^{19}$ −17.1° (c=0.6, DMF). Rf_2 0.25, Rf_3 0.75. *Anal.* Calcd. for $C_{34}H_{40}N_4O_9$: C, 62.95; H, 6.22; N, 8.64. Found: C, 62.70; H, 6.23; N, 8.79.

Z(OMe)-Tyr-Lys(Z)-Phe-Gly-OH——As stated above, Z(OMe)–Lys(Z)–Phe–Gly–OH (7.14 g) was treated with TFA (8.2 ml) in the presence of anisole (3.6 ml) in an ice-bath for 60 min and dry ether was added. The resulting powder was collected by filtration and then dissolved in DMF (20 ml) containing Et_3N (3.1 ml). To this ice-chilled solution, the azide (prepared from 3.59 g of Z(OMe)–Tyr–$NHNH_2$ with 6.71 ml of 2.98 N HCl–DMF, 1.34 ml of isoamylnitrite and 2.8 ml of Et_3N) in DMF (20 ml) was added. The mixture was stirred at 4° for 20 hr. The solvent was evaporated and the residue was dissolved in H_2O (80 ml) with an aid of a small amount of Et_3N. The aqueous phase was washed with ether and then acidified with 10% citric acid. The resulting powder was washed batchwisely with H_2O and recrystallized from MeOH and AcOEt; yield 6.94 g (86%), mp 184—186°, $[\alpha]_D^{19}$ −22.2° (c=0.5, DMF). Rf_3 0.60. *Anal.* Calcd. for $C_{43}H_{49}N_5O_{11}$· C, 63.61; H, 6.08; N, 8.62. Found: C, 63.71; H, 6.20; N, 8.79.

Z(OMe)-Asp(OBzl)-Tyr-Lys(Z)-Phe-Gly-OH——As stated above, Z(OMe)–Tyr–Lys(Z)–Phe–Gly–OH (2.72 g) was treated with TFA (5 ml) in the presence of anisole (1.1 ml) in an ice-bath for 60 min and dry ether was added to form a fine powder, which was collected by filtration and then dissolved in DMF (20 ml). To this solution, Et_3N (1.4 ml) and Z(OMe)–Asp(OBzl)–ONP (2.04 g) were added. The mixture, after

stirring at room temperature for 8 hr, was condensed and the residue was treated with 5% citric acid and AcOEt. The resulting powder was washed batchwisely with 5% citric acid and H_2O and precipitated twice from DMF with AcOEt; yield 2.53 g (74%), mp 196—199°, $[\alpha]_D^{19}$ −23.3° (c=0.6, DMF). Rf_3 0.65. *Anal.* Calcd. for $C_{54}H_{60}N_6O_{14}$: C, 63.77; H, 5.95; N, 8.26. Found: C, 63.49; H, 5.90; N, 8.38.

Z(OMe)-Asp(OBzl)-Asp(OBzl)-Tyr-Lys(Z)-Phe-Gly-OH (C)——As stated above, Z(OMe)–Asp(OBzl)–Tyr–Lys(Z)–Phe–Gly–OH (3.86 g) was treated with TFA (3.0 ml) in the presence of anisole (2.2 ml) and the deprotected peptide was precipitated with ether as powders, which were washed with ether and then dissolved in DMF (20 ml). To this solution, Et_3N (1.06 ml), HOBT (0.58 g) and Z(OMe)–Asp(OBzl)–ONP (2.12 g) were added successively and the mixture was stirred at room temperature for 15 hr, while the pH of the solution was maintained at around 7 as possible with Et_3N. The solvent was evaporated and the residue was treated with 5% citric acid and ether. The resulting powder was washed batchwisely with 5% citric acid and H_2O and then recrystallized from MeOH; yield 2.39 g (52%), mp 192—195°, $[\alpha]_D^{18}$ −18.4° (c=0.4, DMF). Rf_3 0.52. Amino acid ratios in an acid hydrolysate: Asp 2.15, Tyr 0.94, Lys 0.93, Phe 1.00, Gly 1.02 (average recovery 98%). *Anal.* Calcd. for $C_{65}H_{71}N_7O_{17}$: C, 63.87; H, 5.86; N, 8.02. Found: C, 63.78; H, 5.77, N, 8.12.

Z(OMe)-His-Phe-Arg(Tos)-Trp-Ser-Val-Pro-Leu-OBzl——Z(OMe)–Trp–Ser–Val–Pro–Leu–OBzl (0.86 g) was treated with TFA (1.7 ml) in the presence of skatole (0.26 g) and anisole (0.4 ml) containing 2% ethanedithiol in an ice-bath for 60 min. The excess TFA was evaporated *in vacuo* and the residue was dissolved in AcOEt, which was washed with 5% Na_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then filtered. The filtrate was combined with a solution of the azide (prepared from 0.95 g of Z(OMe)–His–Phe–Arg(Tos)–$NHNH_2$ with 0.67 ml of 5.96 N HCl–DMF, 0.18 ml of isoamylnitrite and 0.55 ml of Et_3N) in DMF (5 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with ether. The resulting powder was washed batchwisely with 5% Na_2CO_3 and H_2O and then precipitated from MeOH with AcOEt; yield 0.97 g (67%), mp 146—147°, $[\alpha]_D^{18}$ −35.1° (c=0.5, DMF). Rf_3 0.62. Amino acid ratios in a 4 N MSA hydrolysate: His 0.95, Phe 1.17, Arg+Arg (Tos) 1.09, Trp 0.70, Ser 0.88, Val 1.01, Pro 1.11, Leu 1.00 (average recovery 90%). *Anal.* Calcd. for $C_{74}H_{92}N_{14}O_{15}S\cdot 1/2H_2O$: C, 60.92; H, 6.43; N, 13.44. Found: C, 60.93; H, 6.32; N, 13.07.

Z(OMe)-Asp(OBzl)-Asp(OBzl)-Tyr-Lys(Z)-Phe-Gly-His-Phe-Arg(Tos)-Trp-Ser-Val-Pro-Leu-OBzl——The above protected octapeptide ester (0.66 g) was treated with TFA (1.3 ml) in the presence of skatole (0.13 g) and anisole (0.33 ml) containing 2% ethanedithiol as stated above. The excess TFA was removed by evaporation and the residue was treated with ether and 5% Na_2CO_3. The resulting powder was washed with H_2O, dried over P_2O_5 *in vacuo* for 3 hr and then dissolved in DMF (4 ml), to which Z(OMe)–Asp(OBzl)–Asp(OBzl)–Tyr–Lys(Z)–Phe–Gly–OH (0.50 g), HOBT (68 mg) and DCC (93 mg) were added successively. The mixture was stirred at room temperature for 48 hr and then filtered. The filtrate was condensed and the residue was treated with 5% citric acid and ether. The resulting powder was washed batchwisely with 5% Na_2CO_3, 5% citric acid and H_2O and then incubated in MeOH–AcOH (10 ml—1 ml) at 50° for 3 hr. After filtration, the filtrate was condensed and the treatment of the residue with AcOEt afforded a powder; yield 0.73 g (64%), mp 219—220°, $[\alpha]_D^{18}$ −22.5° (c=0.5, DMF). Rf_3 0.60. Amino acid ratios in a 4 N MSA hydrolysate: Asp 2.19; Tyr 1.00, Lys 1.11, Phe 2.12, Gly 1.13, His 0.90, Arg+Arg (Tos) 0.98, Trp 0.72, Ser 0.90, Val 1.00, Pro 1.04, Leu 0.98 (average recovery 95%). *Anal.* Calcd. for $C_{130}H_{153}N_{21}O_{28}S\cdot 3H_2O$: C, 61.37; H, 6.30; N, 11.56. Found: C, 61.58; H, 6.36; N, 11.53.

Z(OMe)-Asp(OBzl)-Gly-Asp(OBzl)-Asp(OBzl)-Tyr-Lys(Z)-Phe-Gly-His-Phe-Arg(Tos)-Trp-Ser-Val-Pro-Leu-OBzl——The above protected tetradecapeptide ester (0.71 g) was treated with TFA (1.4 ml) in the presence of skatole (75 mg) and anisole (0.35 ml) containing 2% ethanedithiol as stated above and the excess TFA was removed by evaporation. The residue was treated with ether and 5% Na_2CO_3. The resulting powder was washed with H_2O, dried over P_2O_5 for 3 hr and then dissolved in DMF (5 ml), to which Z(OMe)–Asp(OBzl)–Gly–OH (0.19 g), HOBT (52 mg) and DCC (71 mg) were added successively. The mixture was stirred at room temperature for 48 hr and the solution was filtered. The filtrate was condensed *in vacuo* and the residue was treated with 5% citric acid and ether. The resulting powder was washed batchwisely as stated above and then incubated in DMF–MeOH–AcOH (10–5–1 ml each) at 50° for 3 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with MeOH to afford a powder; yield 0.48 g (61%), mp 214—216°, $[\alpha]_D^{22}$ −31.6° (c=0.6, DMF). Rf_3 0.55. Amino acid ratios in a 4 N MSA hydrolysate: Asp 3.05, Gly 2.02, Tyr 1.03, Lys 1.14, Phe 2.01, His 0.87, Arg+Arg (Tos) 1.03, Trp 0.68, Ser 0.85, Val 1.00, Pro 0.94, Leu 0.96 (average recovery 96%). *Anal.* Calcd. for $C_{143}H_{167}N_{23}O_{32}S\cdot 2H_2O$: C, 61.60; H, 6.18; N, 11.56. Found: C, 61.37; H, 6.20; N, 11.85.

H-Asp-Gly-Asp-Asp-Tyr-Lys-Phe-Gly-His-Phe-Arg-Trp-Ser-Val-Pro-Leu-OH——The above protected hexadecapeptide ester (450 mg) was treated with HF (approximately 10 ml) in the presence of anisole (1 ml) containing 2% ethanedithiol and skatole (50 mg) in an ice-bath for 60 min and the excess HF was removed by evaporation *in vacuo* at 0°. Dry ether was added, the resulting powder was collected by filtration, washed with ether and then dissolved in 30% AcOH (40 ml), which was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration, the filtrate was lyophilized to give a powder; yield 320 mg (87%). This powder (180 mg) was dissolved in a small amount of 30% AcOH and the solution was applied to a column of Sephadex G-25 (3.5×112 cm) equilibrated with the upper phase of the

solvent system consisting of *n*-BuOH–AcOH–H_2O (4: 1: 5) and the column was developed with the same solvent. Individual fractions (11 ml each) were collected and absorbancy at 280 mμ was determined. Fractions corresponding to the main peak (tube No. 45—70) were collected and the solvent was removed by evaporation *in vacuo* and the residue dissolved in 30% AcOH (5 ml) was lyophilized to give a fluffy white powder; yield 50 mg (purification step 24%, some tailing portions were discarded). Rf_4 0.72, Rf_5 0.42. $[\alpha]_D^{20}$ −64.7° (c=0.4, 50% AcOH). Amino acid ratios in a 4 N MSA hydrolysate: Asp 3.16, Gly 1.99, Tyr 1.10, Lys 1.00, Phe 2.27, His 0.94, Arg 1.10, Trp 0.65, Ser 0.91, Val 1.10, Pro 1.00, Leu 0.99 (average recovery 87%). *Anal.* Calcd. for $C_{91}H_{123}N_{23}O_{25}\cdot 3CH_3COOH\cdot 5H_2O$: C, 52.73; H, 6.62; N, 14.58. Found: C, 52.86; H, 6.15; N, 14.02.