

Studies on Peptides. LVII.<sup>1,2)</sup> Synthesis of the Octadecapeptide corresponding to the Entire Amino Acid Sequence of Equine  $\beta$ -Melanocyte-stimulating Hormone

FUSAKO TAMURA, YOSHIKI KISO, HARUAKI YAJIMA,<sup>3a)</sup> AKIRA TANAKA  
and MASUHISA NAKAMURA<sup>3b)</sup>

Faculty of Pharmaceutical Sciences, Kyoto University<sup>3a)</sup> and Shionogi  
Research Laboratory, Shionogi Co., Ltd.<sup>3b)</sup>

(Received April 12, 1975)

The octadecapeptide corresponding to the entire amino acid sequence of equine  $\beta$ -melanocyte-stimulating hormone (MSH) was synthesized by assembling 4 subunits; Z(OMe)-Asp(OBzl)-Glu(OBzl)-Gly-OH (I), Z(OMe)-Pro-Tyr-Lys(Z)-Met-NH<sub>2</sub> (II), Z(OMe)-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-OH (III), Z(OMe)-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub> (IV), followed by deblocking of all protecting groups with hydrogen fluoride. The *in vivo* MSH activity of synthetic equine  $\beta$ -MSH was found equivalent to that of synthetic human  $\beta$ -MSH (*in vitro* activity,  $6.2 \times 10^9$  MSH unit/g).

Structure of a  $\beta$ -melanotropic principle from the intermediate lobe of equine pituitary was determined by Dixon and Li<sup>4)</sup> in 1961. However its total synthesis has remained to be accomplished up to date. This  $\beta$ -melanocyte-stimulating hormone ( $\beta$ -MSH) is composed with 18 amino acids in a straight chain, but structurally different from those of other mammalian species so far known in a respect that the Arg residue locates at position 16, whereas in others the Pro residue occupies at this position.

This paper describes the synthesis of the octadecapeptide which covers the entire amino acid sequence of equine  $\beta$ -MSH. Synthetic scheme we employed (Fig. 1) is essentially the same as those described in the recent total synthesis of porcine  $\beta$ -MSH.<sup>5)</sup> Amino acids bearing protecting groups removable by hydrogen fluoride<sup>6)</sup> were employed; *i.e.*, Asp(OBzl), Glu(OBzl), Arg(Tos) and Lys(Z). Available two fragments for the synthesis of porcine  $\beta$ -MSH, *i.e.*, I (sequence 1-3) and III (sequence 8-13), were utilized for our present purpose. The tetrapeptide unit (II), Z(OMe)-Pro-Tyr-Lys(Z)-Met-NH<sub>2</sub> (sequence 4-7) was prepared by an alternate route and the c-terminal pentapeptide (IV) (sequence 14-18) was newly synthesized in a protected form, Z(OMe)-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub>.

For the synthesis of the protected pentapeptide ester (IV), Z(OMe)-Lys(Z)-Asp(OBzl)<sub>2</sub><sup>5)</sup> was treated with trifluoroacetic acid (TFA) and the resulting TFA salt, after neutralization with potassium carbonate, was extracted with ice-cold ethyl acetate and submitted to the coupling reaction with Z(OMe)-Arg(Tos)-OH by means of *N*-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline (IIDQ).<sup>7)</sup> This newly introduced reagent gave the desired protected

1) Part LVI: N. Fujii and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 2446 (1975).

2) Amino acid, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission on Biochemistry Nomenclature: *Biochemistry*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z(OMe) = *p*-methoxybenzyloxycarbonyl, Z = benzyloxycarbonyl, Tos = tosyl, OBzl = benzyl ester, OQCl = 5-chloro-8-quinolyl ester.

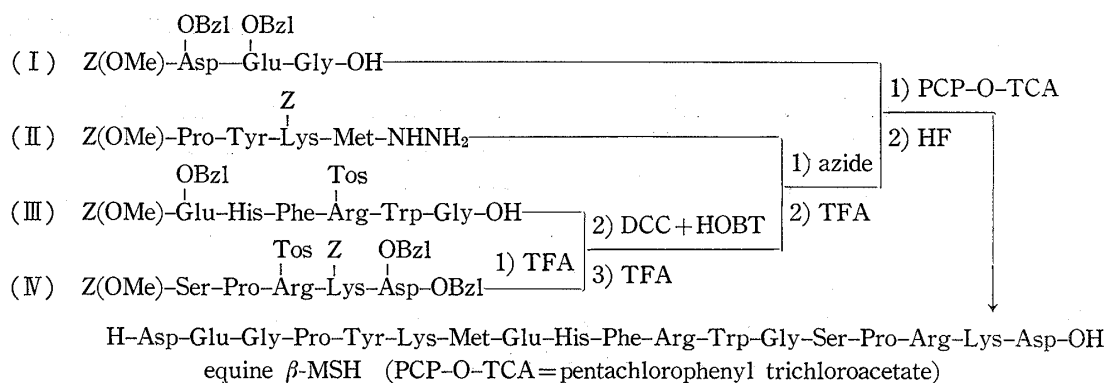
3) Location: a) Sakyo-ku, Kyoto; b) Sagisu, Fukushima, Osaka.

4) J.S. Dixon and C.H. Li, *Gen. Comp. Endocrinol.*, **1**, 161 (1961).

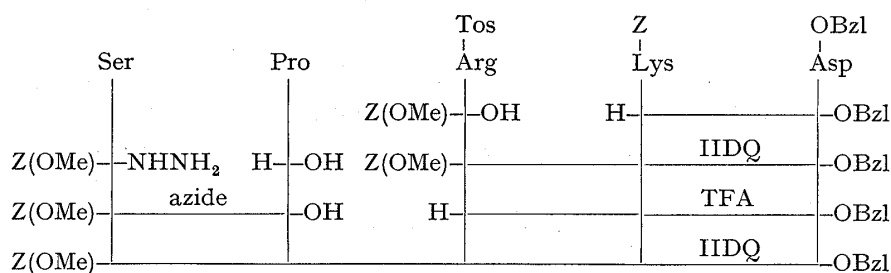
5) H. Watanabe, H. Ogawa and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 375 (1975).

6) S. Sakakibara, Y. Shimonishi, Y. Kishida, H. Okada and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).

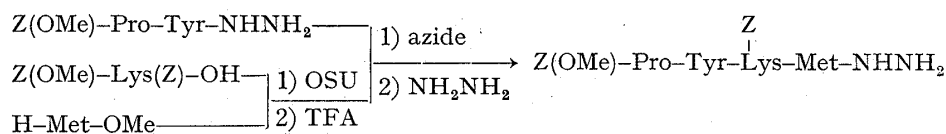
7) Y. Kiso and H. Yajima, *Chem. Commun.*, **1972**, 942; Y. Kiso, Y. Kai and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **21**, 2507 (1973).

Fig. 1. Synthetic Route to Equine  $\beta$ -Melanocyte-stimulating Hormone

tripeptide ester,  $\text{Z(OMe)-Arg(Tos)-Lys(Z)-Asp(Obzl)}_2$ , in 70% yield. This coupling reagent was further applied to condense the TFA treated sample of the above tripeptide ester with  $\text{Z(OMe)-Ser-Pro-OH}$ , which was obtained by the modified azide condensation<sup>8)</sup> of  $\text{Z(OMe)-Ser-NHNH}_2$  with the triethylammonium salt of  $\text{H-Pro-OH}$  as shown in Fig. 2. The desired protected pentapeptide ester (IV) was obtained in analytically pure form in 86% yield.

Fig. 2. Synthetic Scheme of the C-terminal protected Pentapeptide Ester.  
 $\text{Z(OMe)-(equine } \beta\text{-MSH 14-18)-OBzl}$ 

To prepare the protected tetrapeptide hydrazide (II),  $\text{Z(OMe)-Pro-Tyr-Lys(Z)-Met-OMe}$  was directly prepared by the modified azide condensation of  $\text{Z(OMe)-Pro-Tyr-NHNH}_2$  and the TFA treated sample of  $\text{Z(OMe)-Lys(Z)-Met-OMe}$ , instead of  $\text{Z(OMe)-Lys(Z)-Met-OH}$  previously employed (Fig. 3). The resulting protected tetrapeptide ester was converted to II as stated previously.

Fig. 3. Alternate Synthetic Scheme of the protected Tetrapeptide Hydrazide,  
 $\text{Z(OMe)-(equine } \beta\text{-MSH 4-7)-NHNH}_2$ 

Next, the dicyclohexylcarbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBT) procedure<sup>9)</sup> was applied to condense  $\text{Z(OMe)-Glu(Obzl)-His-Phe-Arg(Tos)-Trp-Gly-OH}$  (III)<sup>5)</sup> with the TFA treated sample of IV. Rink and Riniker<sup>10)</sup> mentioned that this condition has a tendency to give a side reaction, if the imidazole function of His residue was not in a protected form. In order to secure the removal of the dicyclohexylamidino moiety, presumably attached, even partly, at the His residue, the crude product was treated with methanol containing acetic acid and further purified by column chromatography on silica using the solvent system of chloroform-methanol and water (8:3:1). Thin-layer chromatographically and analytically

8) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).9) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).10) H. Rink and B. Riniker, *Helv. Chim. Acta*, **57**, 831 (1974).

pure form of the protected undecapeptide ester, Z(OMe)-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub>, was thus achieved.

Next, anisole containing 2% mercaptoethanol<sup>11)</sup> was employed as a scavenger during the TFA treatment of the above protected undecapeptide ester. As mentioned previously, this care prevented the destruction of the Trp residue immensely. The deblocked peptide ester was then condensed with Z(OMe)-Pro-Tyr-Lys(Z)-Met-NHNH<sub>2</sub> by the azide procedure to give the protected pentadecapeptide ester, Z(OMe)-Pro-Tyr-Lys(Z)-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub>. After similar TFA treatment, the deprotected pentadecapeptide ester was submitted to the final condensation with Z(OMe)-Asp(OBzl)-Glu(OBzl)-Gly-OH<sup>5)</sup> by pentachlorophenyl trichloroacetate (PCP-O-TCA)<sup>12)</sup> as performed previously. Column chromatographic purification was very much effective throughout these successive fragment condensation reactions to isolate desired products. Purity of the protected octadecapeptide ester, Z(OMe)-Asp(OBzl)-Glu(OBzl)-Gly-Pro-Tyr-Lys(Z)-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub> thus synthesized, was confirmed by three criteria; thin-layer chromatography, hydrolysis by 3*N* toluenesulfonic acid<sup>13)</sup> and elemental analysis.

All of protecting groups of the above protected octadecapeptide ester was cleaved by treatment with hydrogen fluoride according to Sakakibara, *et al.*<sup>6)</sup> In addition to anisole, Met and Trp were used as scavengers. The deblocked peptide was immediately converted to the corresponding acetate by Amberlite IR-4B (acetate form) and then incubated with dithiothreitol<sup>14)</sup> to reduce Met-sulfoxide possibly formed during the above treatments. Most of scavengers were then removed by Sephadex G-10 and the crude product was purified by column chromatography on CM-Sephadex. Gradient elution with 0.1*M* ammonium acetate at pH 6.9 was employed to elute the desired compound. Examination of the eluates by ultraviolet spectrum (UV) absorbancy at 280 mμ revealed the presence of a single peak with a slight tailing portion. Ammonium acetate was then removed from the desired fractions by Sephadex G-10 and finally repeated lyophilization. The isolated product exhibited a sharp single spot on thin-layer chromatography and its homogeneity was assessed by elemental analysis and hydrolysis by 3*N* toluenesulfonic acid. Complete digestion of the synthetic peptide was possible by aminopeptidase (AP-M).<sup>15)</sup> Recovery of Trp, as well as His, was satisfactory. Thus enzymatic assessment of the L-configuration of constituent amino acids in our synthetic octadecapeptide was thus achieved.

The *in vivo* bioassay of the synthetic equine β-MSH was conducted according to Nakamura, *et al.*<sup>16)</sup> using frogs *Xenopus laevis* D. The relative potency of the synthetic peptide was 1.04 times to that of our synthetic human β-MSH (*in vitro* activity, 6.2 × 10<sup>9</sup> MSH U/g). It was found that synthetic equine β-MSH is active as human β-MSH but possesses only one-third of the activity of synthetic porcine β-MSH.<sup>5)</sup>

### Experimental

Thin-layer chromatography was performed on silica (Kieselgel G, Merck). *R<sub>f</sub>* values refer to the following solvent systems: *R<sub>f1</sub>* CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (8:3:1), *R<sub>f2</sub>* *n*-BuOH-AcOH-pyridine-H<sub>2</sub>O (4:1:1:2).

- 11) R.G. Marshall, *Adv. Exp. Med.*, **2**, 48 (1969); H. Yajima, H. Kawatani and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1333 (1970).
- 12) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.* (Tokyo), **16**, 929 (1968).
- 13) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **216**, 2842 (1971).
- 14) D. Yamashiro and C.H. Li, *J. Am. Chem. Soc.*, **95**, 1310 (1973).
- 15) G. Pfeleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963); K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966); E.C. Jorgensen, G.C. Windridge and W. Patton, *J. Med. Chem.*, **12**, 733 (1969); AP-M (Rohm & Haas Co. P.R.F. 191226) was purchased from Protein Research Foundation (Osaka).
- 16) M. Nakamura, A. Tanaka, M. Hirata and S. Inouye, *Endocrinol. Japon.*, **19**, 383 (1972).

**Z(OMe)-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub>**—Z(OMe)-Lys(Z)-Asp(OBzl)<sub>2</sub> (3.0 g) was treated with TFA (7 ml) in the presence of anisole (1.5 ml) in an ice-bath for 30 min. The excess TFA was evaporated and the residue was treated with 5% K<sub>2</sub>CO<sub>3</sub> under cooling with ice. The resulting precipitate was extracted with AcOEt, which was dried over Na<sub>2</sub>SO<sub>4</sub> and then filtered. To this filtrate, Z(OMe)-Arg(Tos)-OH (2.46 g) in THF (20 ml) was combined. The mixture, after further addition of IIDQ (1.82 g), was stirred at room temperature overnight. The solvent was evaporated and the residue was treated with ether. The resulting gelatinous mass was washed batchwisely with 5% Na<sub>2</sub>CO<sub>3</sub>, 5% citric acid and H<sub>2</sub>O and recrystallized from AcOEt and ether; yield 3.42 g (70%), mp 97–99°,  $[\alpha]_D^{25} -7.9^\circ$  ( $c=1.1$ , DMF).  $R_f$  0.92. *Anal.* Calcd. for C<sub>54</sub>H<sub>63</sub>O<sub>13</sub>N<sub>7</sub>S: C, 61.75; H, 6.04; N, 9.33. Found: C, 61.49; H, 6.04; N, 9.31.

**Z(OMe)-Ser-Pro-OH**—Under cooling with ice-NaCl, isoamylnitrite (3.5 ml) was added to a solution of Z(OMe)-Ser-NHNH<sub>2</sub> (7.08 g) in dimethyl formamide (DMF) (80 ml) and 3.58 N HCl-DMF (14 ml). The solution was stirred at this temperature for 5 min, when the hydrazine test became negative. This solution containing the azide was neutralized with Et<sub>3</sub>N (10.5 ml) and then combined with a solution of H-Pro-OH (5.76 g) in H<sub>2</sub>O (30 ml) containing Et<sub>3</sub>N (7.0 ml). The mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residual oil was dissolved in 3% NH<sub>4</sub>OH, which was washed with AcOEt. The aqueous phase was then acidified with 10% citric acid. The resulting precipitate was extracted with AcOEt, which was washed with H<sub>2</sub>O-NaCl, dried over Na<sub>2</sub>SO<sub>4</sub> and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 5.85 g (64%), mp 126–129°,  $[\alpha]_D^{25} -20.8^\circ$  ( $c=1.0$ , DMF).  $R_f$  0.70. *Anal.* Calcd. for C<sub>17</sub>H<sub>22</sub>O<sub>7</sub>N<sub>2</sub>: C, 55.73; H, 6.05; N, 7.65. Found: C, 55.46; H, 5.96; N, 7.57.

**Z(OMe)-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub> (IV)**—Z(OMe)-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub> (3.15 g) was treated with TFA (6 ml) as usual in the presence of anisole (1.6 ml) in an ice-bath for 60 min. The excess TFA was evaporated and the residue was treated with ice-cold 5% K<sub>2</sub>CO<sub>3</sub> and the resulting precipitate was extracted with AcOEt, which was dried over Na<sub>2</sub>SO<sub>4</sub> and then filtered. This filtrate was combined with a solution of Z(OMe)-Ser-Pro-OH (2.19 g) in THF (50 ml). IIDQ (2.73 g) was added and the mixture was stirred at room temperature overnight. The solvent was evaporated. The oily residue was extracted with AcOEt, which was washed with 5% citric acid, 5% NaHCO<sub>3</sub> and H<sub>2</sub>O-NaCl, dried over Na<sub>2</sub>SO<sub>4</sub> and then evaporated. The residue was recrystallized twice from THF and ether; yield 3.18 g (86%), mp 94–96°,  $[\alpha]_D^{25} -24.5^\circ$  ( $c=0.9$ , DMF).  $R_f$  0.78. Amino acid ratios in a hydrolysate by 3 N Tos-OH: Ser 1.14, Pro 0.99, Arg(Tos) 0.89, Lys 0.87, Asp 1.00 (average recovery 87%). *Anal.* Calcd. for C<sub>62</sub>H<sub>75</sub>O<sub>16</sub>N<sub>9</sub>S·H<sub>2</sub>O: C, 59.45; H, 6.19; N, 10.06. Found: C, 59.62; H, 5.97; N, 9.98.

**Z(OMe)-Lys(Z)-OSU**—The title active ester was prepared according to Anderson, *et al.*<sup>17</sup>; yield 91%. mp 103–105°. *Anal.* Calcd. for C<sub>27</sub>H<sub>31</sub>O<sub>9</sub>N<sub>3</sub>: C, 59.88; H, 5.77; N, 7.76. Found: C, 59.75; H, 5.92; N, 7.71.

**Z(OMe)-Lys(Z)-Met-OMe**—Z(OMe)-Lys(Z)-OSU (10.83 g) was added to a solution of H-Met-OMe (prepared from 4.0 g of the hydrochloride with 2.8 ml of Et<sub>3</sub>N) in DMF (50 ml) and the mixture was stirred at room temperature overnight. The solvent was evaporated and the resulting oily residue was extracted with AcOEt, which was washed with 5% citric acid, 5% Na<sub>2</sub>CO<sub>3</sub> and H<sub>2</sub>O-NaCl, dried over Na<sub>2</sub>SO<sub>4</sub> and then evaporated. The solid residue was recrystallized from AcOEt; yield 8.25 g (70%), mp 121–123°,  $[\alpha]_D^{25} -11.4^\circ$  ( $c=1.0$ , DMF).  $R_f$  0.95. *Anal.* Calcd. for C<sub>29</sub>H<sub>39</sub>O<sub>8</sub>N<sub>3</sub>S: C, 59.06; H, 6.66; N, 7.12. Found: C, 59.48; H, 6.58; N, 7.38.

**Z(OMe)-Pro-Tyr-Lys(Z)-Met-OMe**—Z(OMe)-Lys(Z)-Met-OMe (4.1 g) was treated with TFA (11 ml) in the presence of anisole (3.3 ml) in an ice-bath for 30 min. The excess TFA was evaporated *in vacuo* and the residue was basified with an ice-cold saturated solution of K<sub>2</sub>CO<sub>3</sub>. The resulting oily precipitate was extracted with AcOEt, which was washed with H<sub>2</sub>O-NaCl, dried over Na<sub>2</sub>SO<sub>4</sub> and then filtered. To this ice-cold filtrate, the azide (prepared from 3.77 g of Z(OMe)-Pro-Tyr-NHNH<sub>2</sub> and 4.4 ml of 3.78 N HCl-DMF, 1.1 ml of isoamylnitrite and 2.3 ml of Et<sub>3</sub>N) in DMF (40 ml) was added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was extracted with AcOEt, which was washed with 5% citric acid and H<sub>2</sub>O-NaCl, dried over Na<sub>2</sub>SO<sub>4</sub> and then evaporated. The residue was treated with ether and the resulting solid was recrystallized from MeOH and ether; yield 4.65 g (80%), mp 114–115°,  $[\alpha]_D^{25} -37.9^\circ$  ( $c=0.9$ , DMF). (lit.<sup>9</sup>) mp 114–115°,  $[\alpha]_D^{25} -37.7^\circ$  in DMF). *Anal.* Calcd. for C<sub>43</sub>H<sub>55</sub>O<sub>11</sub>N<sub>5</sub>S: C, 60.76; H, 6.52; N, 8.24. Found: C, 61.01; H, 6.34; N, 8.22.

**Z(OMe)-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub>**—Z(OMe)-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub> (0.49 g) was treated as usual with TFA (2 ml) in the presence of anisole (0.3 ml) in an ice-bath for 60 min. The excess TFA was evaporated *in vacuo* and the residue was treated ice-cold 5% K<sub>2</sub>CO<sub>3</sub> as stated above. The resulting precipitate was extracted with AcOEt, which was dried over Na<sub>2</sub>SO<sub>4</sub> and then filtered. The filtrate was combined with a solution of Z(OMe)-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-OH (0.50 g). HOBT (0.07 g) and DCC (0.10 g) were successively added and the mixture was stirred at room temperature for 48 hr. DC-urea formed during the reaction was removed by filtration, the filtrate was condensed *in vacuo*. The fine powder formed by addition of ether was dissolved in DMF-MeOH-2 N AcOH (5:4:1) and the solution, after incubation at 65° for 4 hr, was filtered. The residue was

17) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

again dissolved in a small amount of the solvent consisting of  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (8:3:1) and the solution was applied to a column of silica ( $3 \times 10$  cm), which was eluted with the same solvent system. Fractions containing the substance of  $R_f$  0.70 were combined and the solvent was evaporated. The residue was treated with  $\text{H}_2\text{O}$  and the resulting fine powder was precipitated from DMF with AcOEt; yield 0.73 g (79%), mp  $105-108^\circ$ ,  $[\alpha]_D^{25} -20.8^\circ$  ( $c=0.9$ , DMF). Amino acid ratios in a hydrolysate by 3 N Tos-OH: Glu 1.11, His 0.93, Phe 1.03, Arg(Tos) 1.91, Trp 0.73, Gly 1.00, Ser 1.13, Pro 1.03, Lys 1.07, Asp 1.19 (average recovery 97%). *Anal.* Calcd. for  $\text{C}_{115}\text{H}_{135}\text{O}_{23}\text{N}_{21}\text{S}_2 \cdot 2\text{H}_2\text{O}$ : C, 59.33; H, 6.01; N, 12.63. Found: C, 59.64; H, 6.01; N, 12.15.

**Z(OMe)-Pro-Tyr-Lys(Z)-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub>**—The above protected undecapeptide ester (0.69 g) was treated as usual with TFA (1.4 ml) in the presence of anisole (0.4 ml) in an ice-bath for 60 min, when dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (5 ml) containing  $\text{Et}_3\text{N}$  (0.12 ml). This ice-cold solution was then combined with a solution of the azide (prepared from 0.31 g of Z(OMe)-Pro-Tyr-Lys(Z)-Met-NHNH<sub>2</sub> with 0.2 ml of 3.78 N HCl-DMF, 0.05 ml of isoamyl nitrite and 0.12 ml of  $\text{Et}_3\text{N}$  in DMF (10 ml). The mixture was stirred at  $4^\circ$  for 48 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was dissolved in a small amount of the solvent consisting of  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (8:3:1). The solution was applied to a column of silica ( $2.8 \times 10$  cm), which was eluted with the same solvent system. Fractions containing the substance of  $R_f$  0.60 were combined and the solvent was evaporated. The residue was treated with  $\text{H}_2\text{O}$  and the resulting powder was precipitated from MeOH with AcOEt; yield 0.55 g (63%), mp  $169-172^\circ$ ,  $[\alpha]_D^{25} -28.0^\circ$  ( $c=1.0$ , DMF). Amino acid ratios in a hydrolysate by 3 N Tos-OH: Pro 2.01, Tyr 1.02, Lys 1.84, Met 0.82, Glu 1.13, His 0.95, Phe 1.08, Arg(Tos) 1.95, Trp 0.74, Gly 1.00, Ser 0.96, Asp 1.07 (average recovery 95%). *Anal.* Calcd. for  $\text{C}_{148}\text{H}_{178}\text{O}_{33}\text{N}_{26}\text{S}_3 \cdot \text{H}_2\text{O}$ : C, 59.98; H, 6.12; N, 12.29. Found: C, 59.73; H, 6.19; N, 12.25.

**Z(OMe)-Asp(OBzl)-Glu(OBzl)-Gly-Pro-Tyr-Lys(Z)-Met-Glu(OBzl)-His-Phe-Arg(Tos)-Trp-Gly-Ser-Pro-Arg(Tos)-Lys(Z)-Asp(OBzl)<sub>2</sub>**—The above protected pentadecapeptide ester (0.41 g) was treated with TFA (1 ml) in the presence of anisole (0.8 ml) in an ice-bath for 60 min. The excess TFA was evaporated *in vacuo* and 3.15 N HCl-DMF (0.1 ml) was added. Addition of dry ether afforded a fine powder, which was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (10 ml) containing  $\text{Et}_3\text{N}$  (0.06 ml). To this solution, the pentachlorophenyl ester (prepared as described previously from 0.19 g of Z(OMe)-Asp(OBzl)-Glu(OBzl)-Gly-OH, 0.12 g of PCP-O-TCA, and 0.04 ml of  $\text{Et}_3\text{N}$  in DMF (2 ml) was combined and the mixture was stirred at room temperature for 48 hr. The solution was then condensed *in vacuo*. The residue was treated with AcOEt and the resulting powder was dissolved in a small amount of the solvent consisting of  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (8:3:1) as stated above. The solution was applied to a column of silica ( $2.8 \times 10$  cm), which was eluted with the same solvent system. Fractions containing the substance of  $R_f$  0.73 were combined and the solvent was evaporated. The residue was washed with  $\text{H}_2\text{O}$  and then precipitated from MeOH with AcOEt; yield 0.30 g (62%), mp  $157-160^\circ$ ,  $[\alpha]_D^{25} -23.9^\circ$  ( $c=0.9$ , DMF).  $R_f$  0.70. Amino acid ratios in a hydrolysate by 3 N Tos-OH: Asp 2.32, Glu 2.45, Gly 2.00, Pro 1.82, Tyr 0.84, Lys 1.96, Met 0.74, His 0.90, Phe 0.93, Arg(Tos) 1.96, Trp 0.82, Ser 0.76 (average recovery 99%). *Anal.* Calcd. for  $\text{C}_{173}\text{H}_{203}\text{O}_{40}\text{N}_{29}\text{S}_3 \cdot 3\text{H}_2\text{O}$ : C, 59.69; H, 6.11; N, 11.67. Found: C, 59.58; H, 5.84; N, 11.55.

**H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Arg-Lys-Asp-OH**—The above protected octadecapeptide ester (240 mg) was treated with hydrogen fluoride (approximately 10 ml) in the presence of anisole (1 ml), Met (100 mg) and Trp (100 mg) in an ice-bath for 60 min. The excess hydrogen fluoride was removed by evaporation and the residue was treated immediately with Amberlite IR-4B (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. The residue was dissolved in 3% AcOH (50 ml) containing dithiothreitol (100 ml) and the solution was incubated at  $60^\circ$  for 4 hr. This solution was then applied to a column of Sephadex G-10  $1.7 \times 45$  cm), which was eluted with 3% AcOH. Individual fractions (5 ml each) were collected and absorbancy at 280 m $\mu$  was determined. Eluates corresponding to the front peak (tube No. 12-24) were combined and the solvent was removed by lyophilization to give a fluffy powder. This was then dissolved in a small amount of  $\text{H}_2\text{O}$  and the solution was applied to a column of CM-Sephadex ( $2.2 \times 3$  cm), which was eluted first with  $\text{H}_2\text{O}$  (100 ml) and then 0.1 M ammonium acetate buffer (pH 6.9) through a mixing flask containing  $\text{H}_2\text{O}$  (200 ml). Individual fractions (5 ml each) were collected and absorbancy at 280 m $\mu$  was determined. A single peak was detected in the gradient eluates. Fractions of this peak (tube No. 67-88) were combined and the solution was condensed to the one-third of the original volume. This solution was applied to a column of Sephadex G-10 ( $3 \times 88$  cm), which was eluted with 3% AcOH. The desired fractions were collected as described above and the solvent was removed by lyophilization to give a white fluffy powder; yield 84 mg (50%),  $[\alpha]_D^{25} -58.7^\circ$  ( $c=0.7$ , 3% AcOH).  $R_f$  0.13. Amino acid ratios in a hydrolysate by 3 N Tos-OH: Asp 1.98, Glu 2.39, Gly 2.00, Pro 1.88, Tyr 1.19, Lys 1.79, Met 0.84, His 0.84, Phe 1.23, Arg 1.94, Trp 0.82, Ser 0.82 (average recover 90%). Amino acid ratios in a AP-M digest: Asp 2.09; Glu 1.98, Gly 1.71, Pro 2.17, Tyr 0.64, Lys 2.05; Met 0.74, His 1.00, Phe 1.00, Arg 2.15, Trp 0.90, Ser 1.10 (average recovery 88%). *Anal.* Calcd. for  $\text{C}_{99}\text{H}_{143}\text{O}_{29}\text{N}_{29}\text{S} \cdot 4 \text{CH}_3\text{COOH} \cdot 4 \text{H}_2\text{O}$ : C, 50.44; H, 6.60; N, 15.94. Found: C, 50.28; H, 6.09; N, 16.43.