[Chem. Pharm. Bull. 32(8)2963—2970(1984)]

Synthesis of the Heptadecapeptide Corresponding to the Entire Amino Acid Sequence of Salmon Melanin-Concentrating Hormone (MCH)[1)]

KENJI OKAMOTO,*[,a] KOICHI YASUMURA,[a] KAZUYOSHI FUJITANI,[a] YOSHIAKI KISO,*[,a] HIROSHI KAWAUCHI,[b] ICHIRO KAWAZOE,[b] and HARUAKI YAJIMA[c]

Kyoto Pharmaceutical University,[a] Yamashina-ku, Kyoto 607, Japan, School of Fisheries Science, Kitasato University,[b] Sanriku-cho, Kesen-gun, Iwate 022-01, Japan, and Faculty of Pharmaceutical Sciences, Kyoto University,[c] Sakyo-ku, Kyoto 606, Japan

(Received November 10, 1983)

The heptadecapeptide corresponding to the entire amino acid sequence of melanin-concentrating hormone (MCH), isolated from chum salmon pituitaries, was synthesized by successive condensation of four peptide fragments and the N-terminal amino acid, *i.e.*, Boc–(14—17)–OBzl, Boc–(9—13)–OH, Boc–(5—8)–$NHNH_2$, Boc–(2—4)–$NHNH_2$ and Boc–Asp(OBzl)–OH, followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole in trifluoroacetic acid (TFA) and disulfide formation through the S-sulfonate. When melanin-concentrating activity was measured on a tilapia scale, the minimum effective concentration of the synthetic peptide was equivalent to that of natural salmon MCH (1 nM).

Keywords——chum salmon pituitary hormone; melanin-concentrating hormone; trifluoromethanesulfonic acid deprotection; thioanisole-mediated deprotection; S-sulfonate disulfide formation

In 1982, Kawauchi *et al.*[2)] isolated from chum salmon a new pituitary hormone which antagonizes α-melanocyte-stimulating hormone (MSH) and causes melanin concentration in fish scale melanophores. This peptide is a heptadecapeptide with one disulfide bridge and was named salmon melanin-concentrating hormone (MCH).

In this paper, we wish to report the synthesis of this salmon MCH in a conventional manner. Four peptide fragments, [I] (positions 14—17), [II] (positions 9—13), [III] (positions 5—8) and [IV] (positions 2—4), were selected as building blocks for construction of the hexadecapeptide segment of salmon MCH and the N-terminal Asp residue was introduced at the final step of the synthesis as shown in Fig. 1. Amino acid derivatives bearing protecting groups removable by trifluoromethanesulfonic acid–thioanisole in TFA[3)] were employed; *i.e.*, Asp(OBzl), Thr(Bzl), Cys(MBzl), Glu(OBzl), and Arg(Mds).[4)] The α-amino function of intermediates was temporarily protected with the TFA–labile Boc group. During acidolysis of the N^{α}-Boc group with TFA, 3,5-dimethylanisole (DMA)[5)] was employed as a cation scavenger to trap the alkyl cation more effectively than anisole.

First, fragment [I] was synthesized according to the scheme shown in Fig. 2. Boc–Glu(OBzl)–Val–OBzl was prepared by the DCC plus HOBt condensation[6)] of Boc–Glu(OBzl)–OH and H–Val–OBzl. After removal of the N^{α}-protecting group by TFA treatment, Boc–Trp–OH and Boc–Cys(MBzl)–OH were successively introduced onto the above protected dipeptide ester by the *p*-nitrophenyl ester procedure[7)] to afford Boc–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl [I]. In the above active ester reactions, HOBt was used in order to accelerate each reaction.[8)] After incorporation of the Trp residue into the chain,

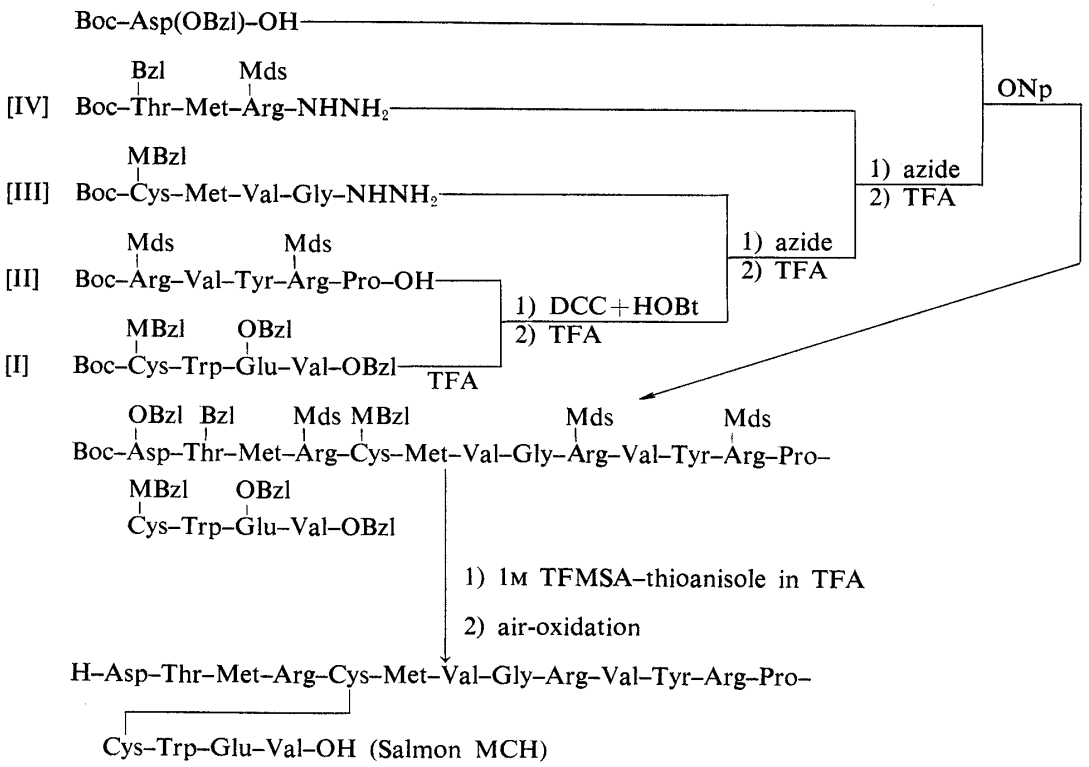

Fig. 1. Synthetic Route to Salmon Melanin-Concentrating Hormone

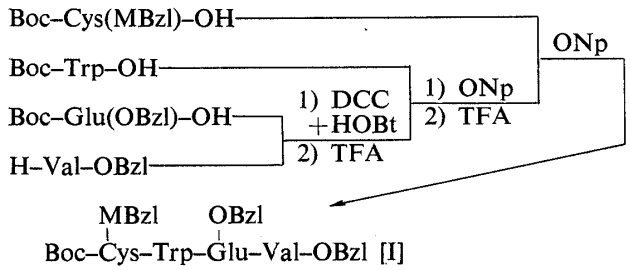

Fig. 2. Synthetic Scheme for the Protected Tetrapeptide Ester [I]

the N^{α}-Boc group was removed with TFA in the presence of DMA containing 2% ethanedithiol (EDT).[9] The above combined scavenger system was more effective in suppressing the side reaction at the Trp residue than the use of anisole containing 2% EDT (it is also more effective at Met, when Met is present).

Next, fragment [II] was synthesized according to the scheme illustrated in Fig. 3. Boc–Arg(Mds)–Val–$NHNH_2$ was prepared by the DCC plus HOBt condensation of Boc–Arg(Mds)–OH and H–Val–OBzl, followed by hydrazinolysis. This dipeptide hydrazide was condensed with H–Tyr–OMe by the azide procedure[10] to give Boc–Arg(Mds)–Val–Tyr–OMe, which was then converted to the corresponding hydrazide as usual. Boc–Arg(Mds)–Val–Tyr–$NHNH_2$ thus obtained was next condensed *via* the azide with H–Arg(Mds)–Pro–OH, which was prepared by the DCC plus HOBt condensation of Z–Arg(Mds)–OH and H–Pro–OBzl followed by hydrogenation over a Pd catalyst. The product [II] was purified by column chromatography on silica.

The synthetic scheme for fragment [III] is shown in Fig. 4. The two necessary dipeptide fragments, Boc–Cys(MBzl)–Met–OMe and Boc–Val–Gly–OEt,[11] were prepared by the DCC plus HOBt condensation of the respective acyl components and amino components as usual. The former, after being converted to the corresponding hydrazide as usual, was coupled with

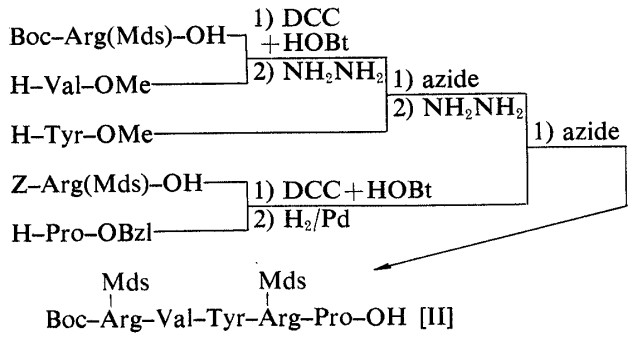

Fig. 3. Synthetic Scheme for the Protected Pentapeptide [II]

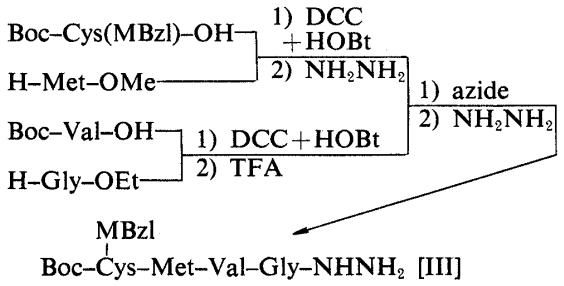

Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide [III]

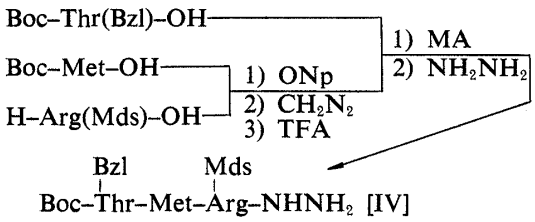

Fig. 5. Synthetic Scheme for the Protected Tripeptide Hydrazide [IV]

the TFA-treated sample of the latter dipeptide by the azide procedure to give Boc–Cys(MBzl)–Met–Val–Gly–OMe, which was smoothly converted to fragment [III] by the usual hydrazinolysis.

Fragment [IV] was synthesized according to the scheme shown in Fig. 5. Boc–Met–Arg(Mds)–OMe was prepared by the *p*-nitrophenyl ester condensation of Boc–Met–OH and H–Arg(Mds)–OH in the presence of HOBt, followed by esterification with diazomethane. The possibility was pointed out by Takaya *et al.* that the HOBt-mediated active ester condensation with an amino acid with a free carboxyl group might give a certain amount of the L–D isomer.[12] High performance liquid chromatographic (HPLC) examination of a sample of Boc–Met–Arg(Mds)–OH deprotected by TFA–thioanisole showed the presence of a very small amount of the L–D isomer (1.0%). Next, this dipeptide, after TFA treatment, was condensed with Boc–Thr(Bzl)–OH by the mixed anhydride procedure[13] and the resulting tripeptide ester was converted to [IV] by the usual hydrazinolysis as stated above. The purity of every fragment, [I], [II], [III], and [IV] thus obtained, was ascertained by thin-layer chromatography (TLC) and elemental analysis.

The four fragments were then assembled according to the route illustrated in Fig. 1. Prior to each condensation reaction, the Boc group was removed from each intermediate by treatment with TFA in the presence of DMA containing 2% EDT as described above. The coupling reaction between fragment [I] and [II] was carried out by the DCC plus HOBt procedure to give Boc–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl. This, after purification by column chromatography on silica, was condensed with fragment [III] by the azide procedure to give Boc–Cys(MBzl)–Met–Val–Gly–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl. Again, column chromatography on silica was required to obtain a homogeneous product. The azide procedure was further employed to introduce fragment [IV] onto the above tridecapeptide. Purification of the resulting protected hexadecapeptide ester, Boc–Thr(Bzl)–Met–Arg(Mds)–Cys(MBzl)–Met–Val–Gly–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl, could

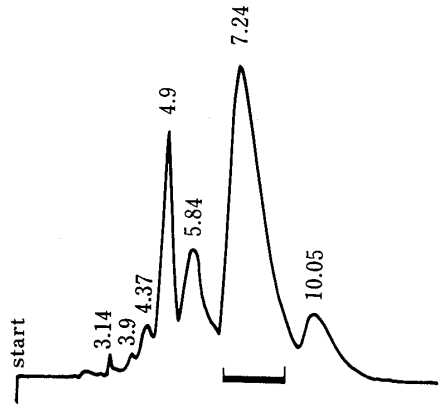

Fig. 6. Purification of Synthetic Salmon MCH by HPLC

HPLC was carried out on a reverse phase column (Zorbax ODS, 4.6 × 250 mm) with 0.02 N TEAP–CH_3CN (80 : 20). The material (1 mg for each run) was loaded and the effluent was monitored by measuring the absorption at 280 nm. The flow rate was 1.0 ml/min.

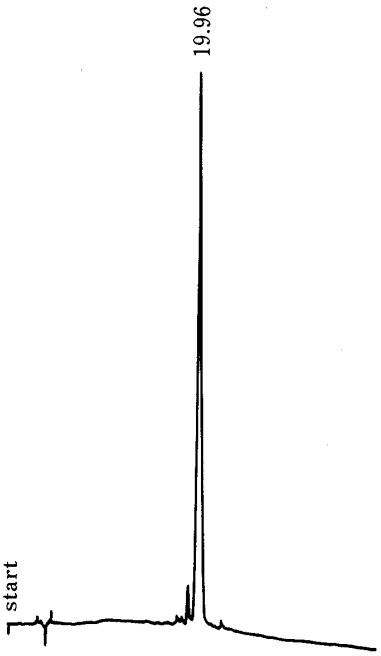

Fig. 7. HPLC of a Mixture of Synthetic Salmon MCH and Natural MCH

Sample: 20 μg each.
Column: Finepak sil C_{18} (4.6 × 250 mm).
Mobile phase: 20—60% CH_3CN (0.1% TFA) linear gradient (40 min).
Flow rate: 1 ml/min.
Detection: 210 nm.

be achieved by simple precipitation from DMF with AcOEt–ether. Finally, the above protected hexadecapeptide ester was condensed with Boc–Asp(OBzl)–OH by the *p*-nitrophenyl ester procedure to give Boc–Asp(OBzl)–Thr(Bzl)–Met–Arg(Mds)–Cys(MBzl)–Met–Val–Gly–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl (protected salmon MCH). The purities of the intermediates and the protected salmon MCH were assessed by three criteria; TLC, elemental analysis and amino acid analysis after 6 N HCl hydrolysis.

Protected salmon MCH was treated with 1 M TFMSA–thioanisole in TFA in an ice-bath for 2 h to remove all the protecting groups. *m*-Cresol[14] was used as an additional scavenger. The crude deprotected heptadecapeptide was converted to the corresponding S-sulfonate[15] in the presence of 8 M guanidine hydrochloride. The sulfonate, after isolation by dialysis against distilled water followed by gel-filtration on Sephadex G-15, was reduced with 2-mercaptoethanol at room temperature for 24 h. The reduced product was applied to a column of Sephadex G-15 and the desired eluates were then diluted with water (400 ml). The pH of this solution was adjusted to 6.9 with 1 M NH_4OH and the solution was kept standing at room temperature for 3 d to establish the intramolecular disulfide bridge. The progress of the air-oxidation was monitored with Ellman's reagent.[16] The oxidized product was first purified by gel-filtration on Sephadex G-25 and then by HPLC (Fig. 6). The homogeneity of the synthetic salmon MCH was further assessed by TLC, elemental analysis, and amino acid analyses after 6 N HCl hydrolysis and aminopeptidase M digestion.

Our synthetic peptide showed a retention time identical with that of natural salmon MCH on HPLC (19.96 min; Fig. 7) and contracted melanin in the melanophores of a tilapia scale with the same minimum effective concentration as that of natural salmon MCH (1 nM).[17]

Experimental

General experimental methods employed were essentially the same as those described in the previous paper.[18] Unless otherwise mentioned, products were purified by either one of the following procedures. A: The residue was dissolved in AcOEt; the AcOEt layer was washed successively with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and then concentrated *in vacuo*. B: The solid residue was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O, then dried *in vacuo* over P_2O_5. TLC was performed on silica gel (CD-aluroll Kieselgel 60F 254, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (29 : 1), Rf_2 $CHCl_3$–MeOH (15 : 1), Rf_3 $CHCl_3$–MeOH (9 : 1), Rf_4 $CHCl_3$–MeOH–AcOH (9 : 1 : 0.5), Rf_5 $CHCl_3$–MeOH–H_2O (8 : 3 : 1), Rf_6 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2). Aminopeptidase M was purchased from Nakarai Chemicals Co., Ltd. (Merck, Lot. No. 1181832). HPLC was conducted on a Shimadzu LC-4A liquid chromatograph.

Boc–Glu(OBzl)–Val–OBzl——Boc–Glu(OBzl)–OH (22.0 g), HOBt (8.9 g) and DCC (13.5 g) were successively added to a solution of H–Val–OBzl (prepared from 25.9 g of the tosylate) in THF (200 ml) under cooling with ice and the mixture was stirred at room temperature for 18 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure A, followed by recrystallization from AcOEt and pet. ether; yield 23.0 g (67.1%), mp 87.0—88.5 °C, $[\alpha]_D^{32}$ −28.0° (*c* = 1.8, MeOH), Rf_1 0.85. *Anal.* Calcd for $C_{29}H_{38}N_2O_7$: C, 66.14; H, 7.23; N, 5.32. Found: C, 66.22; H, 7.33; N, 5.42.

Boc–Trp–Glu(OBzl)–Val–OBzl——Boc–Glu(OBzl)–Val–OBzl (10.0 g) was treated with TFA–DMA (20 ml–5 ml) in an ice-bath for 40 min and excess TFA was evaporated off *in vacuo*. The oily product was washed with *n*-hexane, dried *in vacuo* over KOH pellets and then dissolved in DMF (30 ml) containing Et_3N (2.7 ml). To this solution, Et_3N (2.7 ml), Boc–Trp–ONp (8.1 g) and HOBt (20 mg) were successively added and the mixture was stirred at room temperature for 20 h. The solution was concentrated *in vacuo* and the residue was purified by procedure A, followed by recrystallization from AcOEt and pet. ether; yield 12.0 g (88.9%), mp 130.5—132.0 °C, $[\alpha]_D^{31}$ −30.6° (*c* = 1.5, MeOH), Rf_2 0.10. *Anal.* Calcd for $C_{40}H_{48}N_4O_8$: C, 67.40; H, 6.79; N, 7.86. Found: C, 67.54; H, 6.62; N, 7.89.

Boc–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl [I]——Boc–Trp–Glu(OBzl)–Val–OBzl (5.0 g) was treated with TFA–DMA containing EDT (12.0 ml–2.5 ml) in an ice-bath for 40 min and dry ether–*n*-hexane (1 : 10) was added. The resulting precipitate was collected by filtration, dried *in vacuo* over KOH pellets and then dissolved in DMF (20 ml) containing Et_3N (1.0 ml). To this solution, Et_3N (1.0 ml), Boc–Cys(MBzl)–ONp (3.3 g) and HOBt (20 mg) were added and the mixture was stirred at room temperature for 48 h. The solvent was evaporated off *in vacuo* and the residue was purified by procedure A, followed by column chromatography on silica using $CHCl_3$ as an eluent; yield 5.8 g (88.3%), amorphous powder, $[\alpha]_D^{31}$ −32.6° (*c* = 1.8, MeOH), Rf_2 0.07. *Anal.* Calcd for $C_{51}H_{61}N_5O_{10}S$: C, 65.43; H, 6.57; N, 7.48. Found: C, 65.71; H, 6.66; N, 7.18.

Z–Arg(Mds)–Pro–OBzl——Z–Arg(Mds)–OH (11.2 g), HOBt (2.7 g) and DCC (4.2 g) were successively added to a solution of H–Pro–OBzl (prepared from 4.8 g of the hydrochloride) in THF (50 ml) under cooling with ice and the mixture was stirred at room temperature for 18 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure A, followed by column chromatography on silica using $CHCCl_3$–MeOH (80 : 1) as an eluent; yield 9.1 g (65.7%), amorphous powder, $[\alpha]_D^{13}$ −36.8° (*c* = 1.3, MeOH), Rf_1 0.16. *Anal.* Calcd for $C_{35}H_{43}N_5O_8S$: C, 59.81; H, 6.31; N, 9.96. Found: C, 59.80; H, 6.15; N, 10.07.

Boc–Arg(Mds)–Val–OMe——Boc–Arg(Mds)–OH[19] (8.0 g), HOBt (2.3 g) and DCC (3.5 g) were successively added to a solution of H–Val–OMe (prepared from 2.8 g of the hydrochloride) in DMF (30 ml) under cooling with ice and the mixture was stirred at room temperature for 48 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure A, followed by reprecipitation from AcOEt with pet. ether; yield 9.0 g (90.9%), amorphous powder, $[\alpha]_D^{13}$ −11.6° (*c* = 0.7, MeOH), Rf_1 0.16. *Anal.* Calcd for $C_{26}H_{43}N_5O_8S$: C, 53.32; H, 7.40; N, 11.96. Found: C, 53.57; H, 7.63; N, 11.88.

Boc–Arg(Mds)–Val–NHNH$_2$——Boc–Arg(Mds)–Val–OMe (10.0 g) in MeOH (50 ml) was treated with 80% hydrazine hydrate (8.6 ml) at room temperature for 72 h. The solvent was evaporated off *in vacuo* and the residue was treated with H_2O to afford a powder, which was thoroughly washed with water and dried *in vacuo*; yield 9.0 g (90.0%), $[\alpha]_D^{10}$ −4.0° (*c* = 2.5, DMF), Rf_4 0.45. *Anal.* Calcd for $C_{25}H_{43}N_7O_7S$: C, 51.27; H, 7.40; N, 16.74. Found: C, 51.54; H, 7.57; N, 16.43.

Boc–Arg(Mds)–Val–Tyr–OMe——The azide (prepared from 2.9 g of Boc–Arg(Mds)–Val–NHNH$_2$) in DMF (5 ml) and Et_3N (0.7 ml) were added to an ice-chilled solution of H–Tyr–OMe (prepared from 1.2 g of the hydrochloride) in DMF (2 ml) and the mixture was stirred at 4 °C for 48 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure A, followed by column chromatography on silica using $CHCl_3$–MeOH (40 : 1) as an eluent; yield 2.4 g (64.0%), amorphous powder, $[\alpha]_D^{13}$ −15.1° (*c* = 0.5, MeOH), Rf_3 0.40. *Anal.* Calcd for $C_{35}H_{52}N_6O_{10}S$: C, 56.13; H, 7.00; N, 11.22. Found: C, 56.23; H, 6.89; N, 11.08.

Boc–Arg(Mds)–Val–Tyr–NHNH$_2$——Boc–Arg(Mds)–Val–Tyr–OMe (5.3 g) in MeOH (10 ml) was treated with 80% hydrazine hydrate (2 ml) at room temperature for 24 h and ether was added. The resulting precipitate was collected by filtration, washed with water and then dried *in vacuo*; yield 3.5 g (66.0%), mp 171—173 °C, $[\alpha]_D^{11}$ −6.0° (*c* = 0.5, DMF), Rf_4 0.15. *Anal.* Calcd for $C_{34}H_{52}N_8O_9S \cdot 1/2H_2O$: C, 53.88; H, 7.05; N, 14.78. Found: C, 53.89; H, 7.00; N, 14.93.

Boc–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–OH [II]——In the usual manner, Z–Arg(Mds)–Pro–OBzl (4.9 g) in a mixture of MeOH–DMF–AcOH (50 ml–10 ml–3 ml) was hydrogenated over a Pd catalyst for 8 h. The catalyst was then removed by filtration. The filtrate was concentrated *in vacuo* and the residue was dissolved in DMF (10 ml) containing Et_3N (1.0 ml). To this ice-chilled solution, the azide (prepared from 4.0 g of Boc–Arg(Mds)–Val–Tyr–$NHNH_2$) in DMF (3 ml) and Et_3N (0.7 ml) were added and the mixture was stirred at 4 °C for 48 h. After filtration, the filtrate was concentrated *in vacuo*. The residue was purified by column chromatography on silica using $CHCl_3$–MeOH–H_2O (90 : 15 : 5) as an eluent, followed by reprecipitation from $CHCl_3$–MeOH (1 : 1) with ether; yield 4.0 g (63.6%), amorphous powder, $[\alpha]_D^{11}$ −18.2° (c=1.6, DMF), Rf_4 0.31. *Anal.* Calcd for $C_{54}H_{79}N_{11}O_{15}S_2 \cdot 3H_2O$: C, 52.31; H, 6.91; N, 12.42. Found: C, 52.60; H, 6.79; N, 12.59.

Boc–Cys(MBzl)–Met–OMe——Boc–Cys(MBzl)–OH (8.5 g), HOBt (3.4 g) and DCC (5.2 g) were successively added to a solution of H–Met–OMe (prepared from 5.1 g of the hydrochloride) in THF (100 ml) under cooling with ice and the mixture was stirred at room temperature for 18 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure A, followed by recrystallization from AcOEt and pet. ether; yield 10.0 g (82.0%), mp 103.5—104.5 °C, $[\alpha]_D^{13}$ −41.0° (c=0.8, MeOH), Rf_1 0.67. *Anal.* Calcd for $C_{22}H_{34}N_2O_6S_2$: C, 54.30; H, 7.04; N, 5.76. Found: C, 54.84; H, 7.23; N, 5.96.

Boc–Cys(MBzl)–Met–$NHNH_2$——Boc–Cys(MBzl)–Met–OMe (10.0 g) in MeOH (100 ml) was treated with 80% hydrazine hydrate (5.0 ml) at room temperature for 48 h and the solvent was evaporated off *in vacuo*. The resulting solid was washed with ether and water, and then dried *in vacuo* over P_2O_5; yield 9.0 g (90.0%), mp 125—127 °C, $[\alpha]_D^{12}$ −22.8° (c=1.5, DMF), Rf_3 0.59. *Anal.* Calcd for $C_{21}H_{34}N_4O_5S_2$: C, 51.83; H, 7.04; N, 11.51. Found: C, 51.67; H, 7.07; N, 11.63.

Boc–Cys(MBzl)–Met–Val–Gly–OEt——Boc–Val–Gly–OEt[11] (2.0 g) was treated with TFA–DMA (4 ml–1 ml) in an ice-bath for 40 min and excess TFA was evaporated off *in vacuo*. The resulting oily product, after being washed with *n*-hexane, was dried *in vacuo* over KOH pellets and dissolved in DMF (5 ml) containing Et_3N (1.4 ml). To this ice-chilled solution, the azide (prepared from 5.3 g of Boc–Cys(MBzl)–Met–$NHNH_2$) in DMF (5 ml) and Et_3N (1.4 ml) were added and the mixture was stirred at 4 °C for 48 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure B, followed by recrystallization from MeOH and ether; 4.2 g (58.1%), mp 159—161 °C, $[\alpha]_D^{11}$ −25.6° (c=1.8, DMF), Rf_1 0.30. *Anal.* Calcd for $C_{30}H_{48}N_4O_8S_2$: C, 54.86; H, 7.37; N, 8.53. Found: C, 54.58; H, 7.37; N, 8.45.

Boc–Cys(MBzl)–Met–Val–Gly–$NHNH_2$——Boc–Cys(MBzl)–Met–Val–Gly–OEt (1.9 g) in MeOH–DMF (5 ml–5 ml) was treated with 80% hydrazine hydrate (1 ml) at room temperature for 24 h and ether was added. The resulting precipitate was collected by filtration, washed with ether and reprecipitated from MeOH with ether; yield 1.3 g (69.9%), mp 207—208 °C, $[\alpha]_D^{11}$ −19.2° (c=0.8, DMF), Rf_5 0.67. *Anal.* Calcd for $C_{28}H_{46}N_6O_7S_2$: C, 52.09; H, 7.25; N, 13.13. Found: C, 52.00; H, 7.22; N, 12.93.

Boc–Met–Arg(Mds)–OH——Boc–Met–ONp (5.0 g) and HOBt (20 mg) were added to a solution of H–Arg(Mds)–OH (5.0 g) in DMF (30 ml) containing Et_3N (3.4 ml) and the mixture was stirred at room temperature for 48 h. The solvent was evaporated off *in vacuo* and the residue was purified by column chromatography on silica using $CHCl_3$–MeOH (60 : 1) as an eluent; yield 5.6 g (69.3%), amorphous powder, $[\alpha]_D^{13}$ −5.4° (c=0.7, MeOH), Rf_4 0.63. HPLC examination of this deprotected peptide by TFA–thioanisole (50 °C, 2 h) revealed the presence of 1.0% L–D isomer [conditions: column, Zorbax ODS (4.6 × 250 mm); eluent, 0.1 M H_3PO_4–K_2HPO_4 buffer (pH 3.5)–CH_3CN (94 : 6, v/v); flow rate, 0.5 ml/min; detection, 210 nm. Retention time; L–L. 5.52 min; L–D, 6.95 min]. *Anal.* Calcd for $C_{25}H_{41}N_5O_8S_2 \cdot 1/2H_2O$: C, 49.00; H, 6.91; N, 11.43. Found: C, 48.71; H, 6.67; N, 11.20.

Boc–Met–Arg(Mds)–OMe——An ethereal solution of diazomethane was added to an ice-chilled solution of Boc–Met–Arg(Mds)–OH (5.0 g) in MeOH (5 ml). The yellow color persisted for 2 h and a few drops of AcOH were added. The solvent was evaporated off *in vacuo* and the product was purified by column chromatography on silica using $CHCl_3$–MeOH (100 : 1) as an eluent; yield 4.0 g (78.1%), amorphous powder, $[\alpha]_D^{22}$ −10.4° (c=6.9, MeOH), Rf_2 0.40. *Anal.* Calcd for $C_{26}H_{43}N_5O_8S_2$: C, 50.55; H, 7.02; N, 11.34. Found: C, 50.58; H, 7.15; N, 11.35.

Boc–Thr(Bzl)–Met–Arg(Mds)–OMe——Boc–Met–Arg(Mds)–OMe (3.3 g) was treated with TFA–DMA containing 2% EDT (6 ml–1.5 ml) in an ice-bath for 30 min and the excess TFA was evaporated off *in vacuo*. The oily residue, after being washed with *n*-hexane, was dried *in vacuo* over KOH pellets and then dissolved in THF (20 ml) containing Et_3N (0.7 ml). To this ice-chilled solution, the mixed anhydride (prepared from 1.6 g of Boc–Thr(OBzl)–OH) in THF (20 ml) was added and the mixture was stirred in an ice-bath for 5 h. The solution was concentrated *in vacuo* and the residue was purified by procedure A, followed by column chromatography on silica using $CHCl_3$–MeOH (150 : 1) as an eluent; yield 2.5 g (58.4%), amorphous powder, $[\alpha]_D^{22}$ −9.6° (c=0.7, MeOH), Rf_2 0.83. *Anal.* Calcd for $C_{37}H_{56}N_6O_{10}S_2 \cdot 1/2H_2O$: C, 54.33; H, 7.02; N, 10.27. Found: C, 54.16; H, 6.99; N, 10.36.

Boc–Thr(Bzl)–Met–Arg(Mds)–$NHNH_2$ [IV]——Boc–Thr(Bzl)–Met–Arg(Mds)–OMe (0.8 g) in MeOH (5 ml) was treated with 80% hydrazine hydrate (0.5 ml) at room temperature for 48 h and the solvent was evaporated off *in vacuo*. The residue was washed with ether and water, and reprecipitated from MeOH with ether; yield 0.5 g (62.5%), amorphous powder, $[\alpha]_D^{31}$ −6.3° (c=0.8, MeOH), Rf_3 0.30. *Anal.* Calcd for $C_{36}H_{56}N_8O_9S_2$: C, 53.45; H, 6.98; N, 13.85. Found: C, 53.39; H, 7.06; N, 13.58.

Boc–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl——Boc–Cys(MBzl)–Trp–

Glu(OBzl)–Val–OBzl (2.8 g) was treated with TFA–DMA containing 2% EDT (9 ml–1.5 ml) in an ice-bath for 40 min and *n*-hexane–ether (10 : 1) was added. The resulting powder was collected by filtration, dried *in vacuo* over KOH pellets and then dissolved in DMF (5 ml) containing Et_3N (0.2 ml). DCC (0.6 g) and HOBt (0.4 g) were added to a solution of Boc–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–OH (II, 3.6 g) in DMF (5 ml) and the mixture, after being stirred at 0 °C for 2 h, was added to the above solution containing the amino component. After being stirred at room temperature for 18 h, the solution was filtered. The filtrate was concentrated *in vacuo* and the residue was purified by procedure B, followed by column chromatography on silica using $CHCl_3$–MeOH (30 : 1) as an eluent; yield 3.0 g (49.9%), amorphous powder, $[\alpha]_D^{31}$ −29.4° (c = 1.8, DMF), Rf_3 0.29. Amino acid ratios in 6 N HCl hydrolysate: Arg 1.91, Val 1.91, Tyr 0.90, Pro 0.94, Trp 0.65, Glu 1.00, 1/2Cys 0.95 (recovery of Glu, 85.0%). *Anal.* Calcd for $C_{100}H_{130}N_{16}O_{22}S_3 \cdot H_2O$: C, 59.39; H, 6.58; N, 11.08. Found: C, 59.40; H, 6.56; N, 11.17.

Boc–Cys(MBzl)–Met–Val–Gly–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl——The above protected nonapeptide ester (1.5 g) was treated with TFA–DMA containing 2% EDT (4 ml–0.8 ml) in an ice-bath for 60 min and dry ether was added. The resulting precipitate was collected by filtration, dried *in vacuo* over KOH pellets and then dissolved in DMF (5 ml) containing Et_3N (0.1 ml). The azide (prepared from 0.5 g of Boc–Cys(MBzl)–Met–Val–Gly–$NHNH_2$, III) and Et_3N (0.1 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure B, followed by column chromatography on silica using $CHCl_3$–MeOH (20 : 1) as an eluent; yield 1.2 g (63.2%), amorphous powder, $[\alpha]_D^{28}$ −30.5° (c = 1.7, DMF), Rf_3 0.31. Amino acid ratios in 6 N HCl hydrolysate: Met 0.90, Val 2.91, Gly 0.90, Arg 2.00, Try 0.93, Trp 0.59, Glu 1.00, 1/2Cys 1.83, Pro 0.95 (recovery of Glu, 82.2%). *Anal.* Calcd for $C_{123}H_{164}N_{20}O_{27}S_5 \cdot H_2O$: C, 58.32; H, 6.61; N, 11.06. Found: C, 58.16; H, 6.64; N, 11.11.

Boc–Thr(Bzl)–Met–Arg(Mds)–Bys(MBzl)–Met–Val–Gly–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl——The above protected tridecapeptide ester (0.5 g) was treated with TFA–DMA containing 2% EDT (3 ml–0.3 ml) and the N^{α}-deprotected peptide, isolated as described above, was dissolved in DMF (3 ml) containing Et_3N (0.03 ml). The azide (prepared from 0.2 g of Boc–Thr(Bzl)–Met–Arg(Mds)–$NHNH_2$, IV) in DMF (2 ml) and Et_3N (0.03 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 72 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by procedure B, followed by reprecipitation from DMF with AcOEt–ether (1 : 1); yield 0.5 g (72.1%), mp 218—221 °C (dec.), $[\alpha]_D^{28}$ −28.9° (c = 1.1, DMF), Rf_3 0.27. Amino acid ratios in 6 N HCl hydrolysate: Thr 0.94, Arg 2.89, Met 1.81, Val 2.94, Gly 1.03, Tyr 0.91, Pro 0.98, Trp 0.63, Glu 1.00, 1/2Cys 1.86 (recovery of Glu, 89.0%). *Anal.* Calcd for $C_{154}H_{208}N_{26}O_{34}S_7 \cdot H_2O$: C, 57.62; H, 6.59; N, 11.35. Found: C, 57.47; H, 6.50; N, 11.57.

Boc–Asp(OBzl)–Thr(Bzl)–Met–Arg(Mds)–Cys(MBzl)–Met–Val–Gly–Arg(Mds)–Val–Tyr–Arg(Mds)–Pro–Cys(MBzl)–Trp–Glu(OBzl)–Val–OBzl——The above protected hexadecapeptide ester (0.4 g) was treated with TFA–DMA containing 2% EDT (3 ml—0.2 ml) and the N^{α}-deprotected peptide, isolated as described above, was dissolved in DMF (5 ml) containing Et_3N (0.02 ml). Boc–Asp(OBzl)–ONp (40.4 mg), Et_3N (0.02 ml) and HOBt (2 mg) were added successively and the mixture was stirred at room temperature for 48 h. The solvent was evaporated off *in vacuo*. The residue, after being washed with MeOH, was reprecipitated from DMF with AcOEt; yield 0.34 g (79.3%), mp 240—245 °C, $[\alpha]_D^{29}$ −35.6° (c = 2.1, DMF), Rf_3 0.29. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.00, Thr 0.92, Met 1.71, Arg 2.83, Val 2.89, Gly 1.01, Tyr 0.89, Pro 0.98, Trp 0.51, Glu 1.00, 1/2Cys 1.90 (recovery of Glu, 85.1%). *Anal.* Calcd for $C_{165}H_{219}N_{27}O_{37}S_7$: C, 58.34; H, 6.50; N, 11.13. Found: C, 58.34; H, 6.65; N, 11.11.

H–Asp–Thr–Met–Arg–Cys–Met–Val–Gly–Arg–Val–Tyr–Arg–Pro–Cys–Trp–Glu–Val–OH (salmon MCH)——The above protected heptadecapeptide ester (50 mg) was treated with 1 M TFMSA–thioanisole in TFA (1.5 ml) in the presence of *m*-cresol (0.05 ml) in an ice-bath for 2 h, then dry ether was added. The resulting precipitate was dissolved in 8 M guanidine hydrochloride in H_2O (10 ml) and the pH of the solution was adjusted to 8.9 with 5% methylamine in H_2O. To this solution, Na_2SO_3 (130 mg) and $Na_2S_4O_6$ (67 mg) were added. The mixture, after being stirred at room temperature for 24 h under an N_2 atmosphere, was dialyzed in cellulose tubing[20] against distilled water (500 ml) four times (for desalting) and lyophilized. The residue was dissolved in 25% AcOH (2 ml) and the solution was applied to a column of Sephadex G-15 (2.0 × 80 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and the absorption at 280 nm was determined. The desired fractions (tube Nos. 20—29) containing the S-sulfonate were pooled and the solvent was removed by lyophilization to give a white powder. This powder, after incubation in a mixture of H_2O–1 M AcOH–2-mercaptoethanol (5 ml–0.3 ml–0.1 ml) at room temperature for 24 h, was applied to a column of Sephadex G-15 (2.0 × 85 cm), which was eluted with 3% AcOH. Individual fractions (5 ml each) were collected and the absorption at 280 nm was determined. The fractions corresponding to the front peak (tube Nos. 20—30) were combined and diluted with H_2O (400 ml). The pH of the solution was adjusted to 6.9 with 1 M NH_4OH. The solution was kept standing at room temperature for 3 d. Meanwhile, the Ellman test value dropped from 0.04 to a constant value, 0.002. The entire solution was lyophilized and the residue was dissolved in 3% AcOH (2 ml). The solution was applied to a column of Sephadex G-25 (2.0 × 140 cm), which was eluted with 3% AcOH. Individual fractions (5 ml each) were collected and the absorption at 280 nm was determined. The fractions corresponding to the main peak (tube Nos. 43—50) were combined and the solvent was removed by lyophilization to give a white powder (10 mg, 19.9%). The product thus obtained was purified by HPLC using 0.02 N TEAP–CH_3CN

(80:20) as an eluent (Fig. 6), followed by gel-filtration on Sephadex G-10 (1.5 × 36 cm) for desalting: yield 4.0 mg (8.0%), $[\alpha]_D^{30}$ −15.0° (c=0.4, 3% AcOH), Rf_6 0.22. HPLC: retention time, 19.96 min (Fig. 7). Amino acid ratios in 6 N HCl hydrolysate: Asp 1.04, Thr 1.00, Met 1.96, Arg 2.90, 1/2Cys 1.22, Val 2.95, Gly 1.09, Tyr 1.00, Pro 1.02, Trp 0.60, Glu 1.00 (recovery of Glu, 81.0%). Amino acid ratios in AP-M digest: Asp 0.86, Thr 0.91, Met 1.92, Arg 3.02, 1/2Cys 2.00, Val 2.85, Gly 1.00, Tyr 0.93, Pro 0.99, Trp 1.05, Glu 1.00 (recovery of Glu, 65.2%). *Anal.* Calcd for $C_{89}H_{139}N_{28}O_{23}S_4 \cdot 3AcOH : 10H_2O$: C, 46.42; H, 7.01; N, 15.96. Found: C, 46.68; H, 6.74; N, 15.60.

Acknowledgement The authors wish to thank the staff of the Analysis Center of Kyoto University for elemental analysis.

References and Notes

1) Amino acids, peptides and their derivatives in this peper are of L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Boc=*tert*-butyloxycarbonyl, Bzl=benzyl, MBzl=4-methoxybenzyl, Mds=4-methoxy-2,6-dimethylbenzenesulfonyl, DCC=dicyclohexylcarbodiimide, MA= mixed anhydride, ONp=*p*-nitrophenyl ester, HOBt=1-hydroxybenzotriazole, DMF=dimethylformamide, THF=tetrahydrofuran, TFMSA=trifluoromethanesulfonic acid, TFA=trifluoroacetic acid, TEAP=triethylammonium phosphate.
2) H. Kawauchi, I. Kawazoe, M. Tsubokawa, and M. Kishida, "Peptide Chemistry 1982," ed. by S. Sakakibara, Protein Research Foundation, 1983, p. 187; H. Kawauchi, I. Kawazoe, M. Tsubokawa, M. Kishida, and B. I. Baker, *Nature* (London), **305**, 321 (1983).
3) N. Fujii, S. Funakoshi, I. Sasaki, and H. Yajima, *Chem. Pharm. Bull.*, **25**, 3096 (1977); Y. Kiso, K. Ito, S. Nakamura, K. Kitagawa, T. Akita, and H. Moritoki, *Chem. Pharm. Bull.*, **27**, 1472 (1979).
4) M. Fujino, M. Wakimasu, O. Nishimura, and C. Kitada, "Peptide Chemistry 1980," ed. by K. Okawa, Protein Research Foundation, 1981, p. 21; M. Wakimasu, C. Kitada, and M. Fujino, *Chem. Pharm. Bull.*, **29**, 2592 (1981).
5) K. Okamoto, K. Yasumura, K. Fujitani, S. Katakura, K. Akaji, H. Yajima, Y. Nakata, A. Inoue, and T. Segawa, *Chem. Pharm. Bull.*, **32**, 430 (1984).
6) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
7) M. Bodanszky and V. duVineaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
8) W. König and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).
9) J. J. Sharp, A. B. Robinson, and M. D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973).
10) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
11) E. Schnabel, *Justus Liebigs Ann, Chem.*, **688**, 238 (1965).
12) T. Takaya, T. Ohnishi, and S. Sakakibara, "Peptide Chemistry 1978," ed. by N. Izumiya, Protein Research Foundation, 1979, p. 61.
13) J. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).
14) H. Yajima, M. Takeyama, J. Kanaki, and K. Mitani, *J. Chem. Soc., Chem. Commun.*, **1978**, 482; H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 3752 (1978).
15) R. D. Cole, "Methods in Enzymology," Vol. 11, ed. by C. H. W. Hirs, Academic Press, New York, 1967, p. 206.
16) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
17) Fifty percent of the melanin granules of melanophores on a tilapia scale was fully concentrated.
18) K. Yasumura, K. Okamoto, S. Shimamura, M. Nakamura, K. Odaguchi, A. Tanaka, and H. Yajima, *Chem. Pharm. Bull.*, **30**, 866 (1982).
19) M. Fujino, O. Nishimura, M. Wakimasu, and C. Kitada, *J. Chem. Soc., Chem. Commun.*, **1980**, 668.
20) Purchased from Spectrum Medical Industries INC (M. W. cutoff 1000).