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Synthesis of the Pentadecapeptide corresponding to the Entire Amino Acid Sequence of Salmon α-Melanotropin II (α-MSH II)[1]

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The pentadecapeptide corresponding to the entire amino acid sequence of α-MSH II, isolated from salmon pituitary gland, was synthesized by a conventional procedure. The synthetic peptide exhibited *in vitro* melanocyte-stimulating activity of 1.66×10^{10} MSH U/g.

Keywords——new peptide from salmon pituitary gland; salmon α-MSH II; deprotection by trifluoromethanesulfonic acid-TFA; thioanisole-mediated deprotection; melanotropic activity

In 1980, Kawauchi *et al.*[2] isolated, in addition to a deacetylated mammalian type α-melanotropin (α-MSH),[3] a new MSH-like peptide from salmon pituitary gland. This MSH-like peptide is a pentadecapeptide. The amino acid sequence of residues 1 to 12 is identical with that of mammalian α-MSH, but the C-terminal portion (position 13—15) is different. Thus, this new peptide was named salmon α-MSH II.

In this paper, we wish to report the synthesis of the pentadecapeptide corresponding to the entire amino acid sequence of salmon α-MSH II by a conventional procedure.

Three peptide fragments, [1], [2] and [3], were selected as building blocks for construction of the entire amino acid sequence of this new fish hormone, as shown in Fig. 1. Of these, fragments, [2] and [3], are known compounds used for the syntheses of ostrich adrenocorticotropic hormone (ACTH)[4] and α-MSH,[5,6] respectively. Amino acid derivatives bearing protecting groups removable by TFMSA–thioanisole in TFA[7] were employed; *i.e.*, Glu(OBzl),

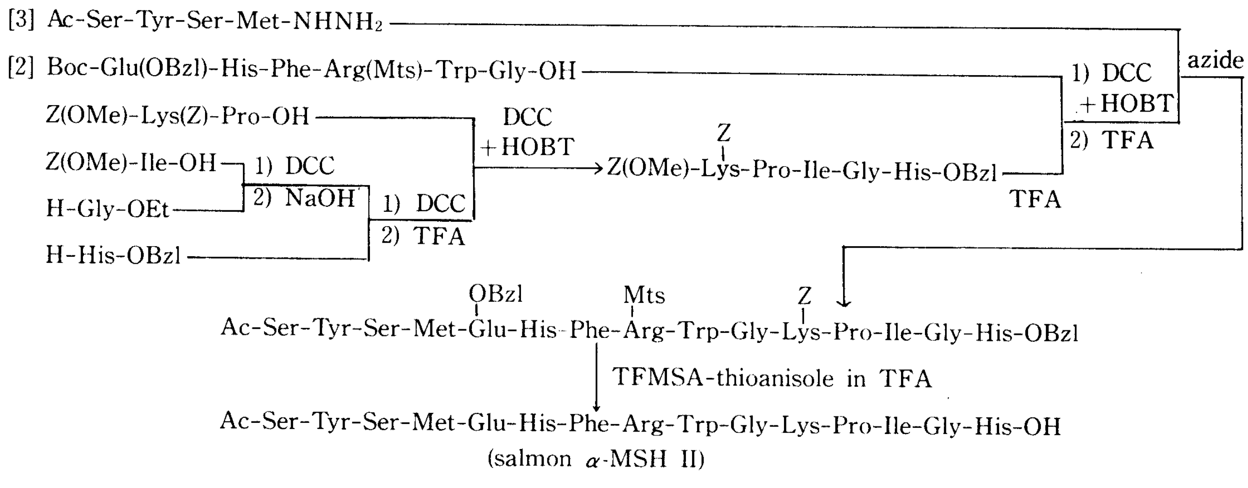

Fig. 1. Synthetic Route to Salmon α-MSH II

Lys(Z) and Arg(Mts).[8]

First, the necessary fragment [1] was synthesized as shown in Fig. 1. Z(OMe)–Ile–Gly–OEt was prepared by the DCC procedure.[9] This, after alkaline saponification, was condensed with H–His–OBzl by DCC to give Z(OMe)–Ile–Gly–His–OBzl. Subsequently, this protected tripeptide, after removal of the Z(OMe) group by TFA, was condensed with Z(OMe)–Lys(Z)–Pro–OH[10] by DCC in the presence of *N*-hydroxybenztriazole (HOBT)[11] to give fragment [1].

Next, fragment [2] was condensed with a TFA-treated sample of fragment [1] by the DCC–HOBT procedure. The resulting protected undecapeptide, Boc–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–Lys(Z)–Pro–Ile–Gly–His–OBzl [4] was purified by column chromatography on silica. Subsequently, the Z(OMe) group was removed from this peptide [4] by TFA; anisole containing 2% ethanedithiol was employed to suppress side reactions at the Trp residue. The deprotected undecapeptide was then condensed with fragment [3] by the azide procedure.[12] The desired protected salmon α-MSH II was purified by column chromatography on silica.

Finally, protected α-MSH II was treated with 1 M TFMSA–thioanisole in TFA in an ice-bath for 2 h to remove all protecting groups. Skatole was used as an additional scavenger. The crude deprotected pentadecapeptide was purified by gel-filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-cellulose. In the latter purification step, 0.04 M ammonium acetate buffer was employed to elute the desired compound. The pentadecapeptide thus purified exhibited a single spot on thin-layer chromatography (TLC) in two different solvent systems and migrated as a single component in 15% polyacrylamide gel electrophoresis (Fig. 2). Further, amino acid ratios in a 6 N HCl hydrolysate and a carboxypeptidase P[13] (CP–P) digest were in good agreement with the theoretical values. It seems noteworthy that CP–P could be effectively used for complete digestion of *N*-blocked salmon α-MSH II. From those results, we conclude that our synthetic α-MSH II has a high degree of purity. Our synthetic peptide, after oxidation with H_2O_2, exhibited an Rf_6 value (0.55, on cellulose plate) and a retention time (25 min, on a μBondapak C_{18} column with isopropanol–10 mM ammonium acetate, pH 4.0) identical with those of natural salmon α-MSH II, which had been stored in a refrigerator for two years. Oxidation of the Met residue in peptides on storage is a well-documented phenomenon.

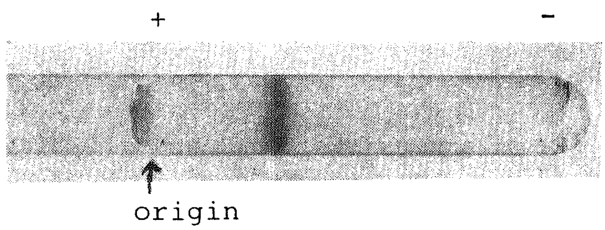

Fig. 2. Disc Electrophoresis of Synthetic Salmon α-MSH II

The *in vitro* melanocyte-stimulating activity[14,15] of our synthetic peptide was judged to be 1.66×10^{10} U/g, when synthetic α-MSH (2.0×10^{10} U/g) was taken as a standard.

Experimental

General experimental methods employed were essentially the same as those described in the previous paper.[4] TLC was performed on silica gel (DC-alurolle Kieselgel 60F 254, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (9: 1); Rf_2 $CHCl_3$–MeOH–AcOH (9: 1: 0.5); Rf_3 $CHCl_3$–MeOH–H_2O (8: 3: 1); Rf_4 MeOH–Me_2CO–H_2O (70: 5: 25); Rf_5 *n*-BuOH–pyridine–AcOH–H_2O (6: 6: 1.2: 4.8); Rf_6 *n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2).

Z(OMe)-Ile-Gly-OEt——DCC (7.0 g) was added to a stirred mixture of Z(OMe)–Ile–OH (10 g) and H–Gly–OEt (prepared from 4.3 g of the hydrochloride) in THF (100 ml). After 18 h, the mixture was filtered, the filtrate was concentrated *in vacuo* and the residue was extracted with AcOEt. The AcOEt layer was washed successively with 10% citric acid, 5% $NaHCO_3$ and NaCl–H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was recrystallized from MeOH; yield 6.0 g (46.5%), mp 155—156°C, $[\alpha]_D^{27}$ −26.7° (*c*=1.1, MeOH), Rf_1 0.87. *Anal.* Calcd for $C_{19}H_{28}N_2O_6$: C, 59.98; H, 7.42; N, 7.36. Found: C, 60.14; H, 7.62; N, 7.53.

Z(OMe)-Ile-Gly-OH——A stirred solution of Z(OMe)–Ile–Gly–OEt (2.5 g) in acetone (20 ml) was treated with 1 N NaOH (7.5 ml). After being stirred for 30 min, the reaction mixture was acidified with 10% citric acid and concentrated *in vacuo*. The residue was recrystallized twice from MeOH; yield 1.5 g (62.5%), mp 162—164°C, $[\alpha]_D^{27}$ −17.4° (c=1.2, MeOH), Rf_1 0.24. *Anal.* Calcd for $C_{17}H_{24}N_2O_6$: C, 57.94; H, 6.86; N, 7.95. Found: C, 57.45; H, 6.67; N, 7.81.

Z(OMe)-Ile-Gly-His-OBzl——DCC (0.88 g) was added to a stirred mixture of Z(OMe)–Ile–Gly–OH (1.5 g) and H–His–OBzl (prepared from 2.5 g of the tosylate) in THF–DMF (3 ml–12 ml). After 18 h, the mixture was filtered, the filtrate was concentrated *in vacuo* and the residue was extracted with AcOEt. The AcOEt layer was washed successively with 10% citric acid, 2% AcOH and NaCl–H_2O, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was recrystallized from MeOH; yield 1.28 g (53.3%), mp 188—190°C, $[\alpha]_D^{27}$ −3.0° (c=1.01, DMF), Rf_1 0.10. *Anal.* Calcd for $C_{30}H_{37}N_5O_7 \cdot H_2O$: C, 60.29; H, 6.58; N, 11.72. Found: C, 59.90; H, 6.64; N, 11.73.

Z(OMe)-Lys(Z)-Pro-Ile-Gly-His-OBzl [1]——Z(OMe)–Ile–Gly–His–OBzl (0.75 g) was treated with TFA–anisole (2.3 ml–0.38 ml) at 0°C for 60 min, then dry ether was added. The resulting powder was collected by filtration, dissolved in 3.2 N HCl/DMF (2 ml) and again precipitated with ether. The resulting oily product was washed with ether, dried over KOH pellets *in vacuo* and dissolved in DMF (5 ml) together with Et_3N (0.36 ml), Z(OMe)–Lys(Z)–Pro–OH[10] (0.7 g) and HOBT (0.18 g). DCC (0.28 g) was added and the mixture was stirred at room temperature for 18 h. After filtration, the filtrate was concentrated. The residue was extracted with AcOEt. The AcOEt layer was washed with 2% AcOH and 5% $NaHCO_3$, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography using $CHCl_3$–MeOH–H_2O (90: 15: 5) as the eluent; yield 0.5 g (42.0%), amorphous powder, $[\alpha]_D^{33}$ −27.2° (c=1.03, DMF), Rf_2 0.20. Amino acid ratios in 6 N HCl hydrolysate: $Lys_{0.99}Pro_{1.10}Ile_{1.00}Gly_{1.00}His_{0.90}$ (recovery of Gly, 90.5%). *Anal.* Calcd for $C_{49}H_{62}N_8O_{11} \cdot H_2O$: C, 61.49; H, 6.74; N, 11.71. Found: C, 61.13; H, 6.56; N, 11.61.

Z(OMe)-Ser-Tyr-OEt——Z(OMe)–Ser–OH (7.4 g) and EEDQ (6.6 g) were added to a solution of H–Tyr–OEt (prepared from 6.5 g of the hydrochloride) in DMF (80 ml). The mixture was stirred at room temperature for 24 h, then the solvent was evaporated off *in vacuo* and the residue was extracted with AcOEt. The AcOEt layer was washed with 10% citric acid, 5% $NaHCO_3$ and NaCl–H_2O, dried over Na_2SO_4 and then concentrated *in vacuo*. The residue was purified by silica gel column chromatography using $CHCl_3$–MeOH (80: 1) as the eluent to give an amorphous powder; yield 6.5 g (52.4%), $[\alpha]_D^{31}$ +6.4° (c=1.87, DMF), Rf_1 0.83. *Anal.* Calcd for $C_{23}H_{28}N_2O_8$: C, 58.84; H, 6.23; N, 5.97. Found: C, 58.60; H, 6.22; N, 5.78.

Z(OMe)-Ser-Tyr-NHNH$_2$——A solution of Z(OMe)–Ser–Tyr–OEt (4.3 g) in MeOH (10 ml) was treated with 80% hydrazine hydrate (4.6 ml) at room temperature for 24 h and ether was added. The resulting precipitate was collected by filtration, washed with ether and recrystallized from DMF and MeOH; yield 3.2 g (74.4%), mp 209—210°C, $[\alpha]_D^{31}$ −0.6° (c=1.7, DMF), Rf_3 0.47. *Anal.* Calcd for $C_{21}H_{26}N_4O_7$: C, 56.50; H, 5.87; N, 12.25. Found: C, 56.22; H, 5.76; N, 12.44.

Z(OMe)-Ser-Tyr-Ser-Met-OMe——Z(OMe)–Ser–Met–OMe[16] (2.0 g) was treated with TFA (4 ml) in the presence of anisole (1 ml) containing 2% ethanedithiol (EDT) at 0°C for 45 min and excess TFA was removed by evaporation at 0°C. The oily residue was washed with n-hexane, dried over KOH pellets *in vacuo* and then dissolved in DMF (5 ml) containing Et_3N (0.66 ml). To this ice-chilled solution, Et_3N (0.66 ml) and the azide (prepared from 2.3 g of Z(OMe)–Ser–Tyr–$NHNH_2$ as usual) in DMF (2 ml) were added and the mixture was stirred at 4°C for 48 h. The solvent was evaporated off and the residue was extracted with AcOEt. The AcOEt layer was washed successively with 10% citric acid, 5% $NaHCO_3$, NaCl–H_2O, dried over Na_2SO_4 and then concentrated. The residue was precipitated from DMF with ether; yield 2.0 g (62.9%), mp 193.5—196.5°C, $[\alpha]_D^{31}$ −12.4° (c=0.97, DMF), Rf_2 0.38. *Anal.* Calcd for $C_{30}H_{40}N_4O_{11}$: C, 54.21; H, 6.07; N, 8.43. Found: C, 54.29; H, 5.97; N, 8.46.

Boc-Glu(OBzl)-His-Phe-Arg(Mts)-Trp-Gly-Lys(Z)-Pro-Ile-Gly-His-OBzl [4]——Z(OMe)–Lys(Z)–Pro–Ile–Gly–His–OBzl (1, 0.5 g) was treated with TFA-anisole (1 ml–0.2 ml) in an ice-bath for 60 min and the N^{α}-deprotected peptide was converted to the hydrochloride as mentioned above. The hydrochloride was dissolved in DMF (5 ml) containing Et_3N (0.15 ml). To this ice-chilled solution, Boc–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–OH (2, 0.63 g), HOBT (0.086 g) and DCC (0.122 g) were added successively and the mixture was stirred at room temperature for 24 h. After filtration, the filtrate was concentrated *in vacuo* and the residue was purified by silica gel column chromatography using $CHCl_3$–MeOH–H_2O (90: 15: 5) as the eluent, followed by reprecipitation from MeOH with ether; yield 0.7 g (70.7%), amorphous powder, $[\alpha]_D^{31}$ −35.4° (c=0.82, DMF), Rf_3 0.86. Amino acid ratios in 6 N HCl hydrolysate: $Glu_{0.89}His_{1.87}Phe_{0.83}Arg_{1.00}$-$Trp_{0.65}Gly_{1.85}Lys_{0.99}Pro_{1.10}Ile_{0.90}$ (recovery of Arg, 89.5%). *Anal.* Calcd for $C_{100}H_{126}N_{20}O_{20}S \cdot 2H_2O$: C, 60.17; H, 6.56; N, 14.03. Found: C, 60.25; H, 6.65; N, 13.95.

Ac-Ser-Tyr-Ser-Met-Glu(OBzl)-His-Phe-Arg(Mts)-Trp-Gly-Lys(Z)-Pro-Ile-Gly-His-OBzl——The above protected undecapeptide [4] (0.5 g) was treated with TFA (0.3 ml) in the presence of anisole (0.2 ml) containing 2% EDT at 0°C for 60 min, then dry ether was added and the resulting powder was dissolved in DMF (5 ml) containing Et_3N (0.075 ml). To this ice-chilled solution, Et_3N (0.037 ml) and the azide (prepared from 0.15 g of Ac–Ser–Tyr–Ser–Met–$NHNH_2$[5,6] as usual) in DMF (2 ml) were added and the mixture was stirred at 4°C for 72 h. After filtration, the filtrate was concentrated and the residue was purified by silica gel column chromatography using $CHCl_3$–MeOH–H_2O (8: 3: 1) as the eluent, followed by reprecipitation from MeOH with ether; yield 0.25 g (41.9%), mp 222°C (dec.), $[\alpha]_D^{25}$ −23.5° (*c*=0.85, DMF). Amino acid ratios in 6 N HCl hydrolysate: $Ser_{1.65}Tyr_{1.01}Met_{0.97}Glu_{0.97}His_{2.04}Phe_{1.00}Arg_{1.00}Trp_{0.50}Gly_{1.86}Lys_{0.99}Pro_{1.08}Ile_{0.97}$ (recovery of Phe, 92.5%). *Anal.* Calcd for $C_{117}H_{148}N_{24}O_{26}S_2 \cdot 3H_2O$: C, 57.96; H, 6.40; N, 13.86. Found: C, 58.02; H, 6.31; N, 13.77.

Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Ile-Gly-His-OH——The above protected pentadecapeptide (100 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of skatole (20 mg) in an ice-bath for 2 h, then dry ether was added and the resulting precipitate was dissolved in H_2O (100 ml). The solution was treated with Amberlite IRA-400 (acetate form, approximately 2 g) for 30 min, then filt ered, and the filtrate was lyophilized. The resulting powder was dissolved in 0.2 N AcOH and the solution was applied to a column of Sephadex G-25 (3.4×65 cm), which was eluted with the same solvent. Individual fractions (7 ml each) were collected and the ultraviolet (UV) absorption at 280 nm was determined. The fractions corresponding to the main peak (tube Nos. 45—54) were collected and the solvent was removed by lyophilization. The residue was dissolved in H_2O (50 ml) and applied to a column of CM-cellulose (2.0×8.0 cm), which was eluted successively with H_2O (100 ml), 0.01 M (150 ml) and 0.04 M ammonium acetate buffer (400 ml). Individual fractions (15 ml each) were collected and monitored by measurement of UV absorption at 280 nm. The fractions corresponding to the main peak present in the 0.04M buffer eluates (tube Nos. 21—24) were combined and the solution was concentrated to approximately 5 ml. Thes solution wa applied to a column of Sephadex G-10 (3.5×40 cm), which was eluted with 0.2 N AcOH. The desired fractions (5 ml each, tube Nos. 17—24) were collected and the solvent was lyophilized to afford a fluffy powder; yield 17.8 mg (17.9%), $[\alpha]_D^{22}$ −58.7° (*c*=0.46, 1 N AcOH), Rf_6 0.24, Rf_5 0.28. Single band in 15% polyacrylamide gel electrophoresis at pH 4.0 (0.35 M *β*-alanine acetate buffer); mobility, 1.9 cm from the origin toward the cathode, after running at 5 mA/tube for 40 min. Amino acid ratıos in 6 N HCl hydrolysate: $Ser_{1.63}Tyr_{0.96}Met_{0.90}Glu_{1.03}$-$His_{1.88}Phe_{1.00}Arg_{1.00}Trp_{0.50}Gly_{1.99}Lys_{0.90}Pro_{1.25}Ile_{0.97}$ (recovery of Phe, 85.3%). Amino acid ratios in CP-P digest: $Ser_{0.84}Tyr_{0.90}Met_{0.92}Glu_{1.05}His_{2.20}Phe_{0.91}Arg_{1.00}Trp_{0.86}Gly_{1.88}$-$Lys_{1.09}Pro_{1.03}Ile_{1.00}$ (recovery of Ile, 87.4%). Trp/Tyr=0.99.[17] *Anal.* Calcd for $C_{84}H_{116}N_{24}O_{20}S$–4AcOH–$9H_2O$: C, 49.86; H, 6.82; N, 15.17. Found: C, 49.83; H, 6.94; N, 15.42.

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References and Notes

1) Amino acids, peptides and their derivatives in this paper are of L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Mts=mesitylene-2-sulfonyl, DCC=dicyclohexylcarbodiimide, EEDQ=*N*-ethyloxycarbonyl-2-ethoxy-1,2-dihydroquinoline, DMF=dimethylformamide, THF=tetrahydrofuran, TFA=trifluoroacetic acid, TFMSA=trifluoromethanesulfonic acid.

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