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Synthesis of the Dodecapeptide Designated as Bovine γ-Melanotropin (γ-MSH)[1]

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The dodecapeptide corresponding to γ-melanotropin, a newly found amino acid sequence in bovine corticotropin-β-lipotropin precursor protein, was synthesized in a conventional manner.

The synthetic peptide exhibited weak melanocyte-stimulating activity, but failed to show any significant steroidogenic and lipolytic activities.

Keywords——corticotropin-β-lipotropin precursor protein; synthesis of bovine γ-melanotropin; a core tetrapeptide, H-His–Phe–Arg–Trp–OH; melanocyte-stimulating activity; deprotection with trifluoromethanesulfonic acid-thioanisole-trifluoroacetic acid; reduction of Met(O) by thioanisole during deprotection

In 1979, Nakanishi *et al.*[3] determined the nucleotide sequence of a cloned cDNA insert encoding corticotropin-β-lipotropin (ACTH-β-LPH) precursor m-RNA isolated from the intermediate lobe of bovine pituitary, and pointed out the presence of a third melanotropin fragment (a dodecapeptide termed γ-MSH) in the region outside the ACTH-β-LPH portion of the precursor protein. Subsequently, Guillemin *et al.*[4] reported solid phase syntheses of γ-MSH and its derivatives. We wish to report our synthetic data, obtained by conventional methods.

For this synthesis, Arg(Mts)[5] and Asp(OBzl) were employed. The former protecting group in particular was demonstrated to be smoothly removed by treatment with HF, MSA or TFMSA. In addition, Met(O)[6] was adopted to prevent partial oxidation at the sulfur atom during the synthesis. Thus, two fragments, Z–Tyr–Val–Met(O)–Gly–His–$NHNH_2$ (I, position 1—5) and Boc–Phe–Arg(Mts)–Trp–Asp(OBzl)–Arg(Mts)–Phe–Gly–OH (II, position 6—12), were synthesized by known amide forming reactions as shown in Fig. 1. In order to suppress the side reaction at the Trp residue during the N^{α}-deprotection with TFA,[7] anisole

1) The amino acids (except glycine) employed in this work were of the L-configuration. Boc=*tert*-butoxycarbonyl, Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Mts=mesitylene-2-sulfonyl, Bzl=benzyl, ONP=*p*-nitrophenyl ester, EDT=ethanedithiol, DMF=dimethylformamide, DMSO=dimethylsulfoxide, THF=tetrahydrofuran, TFA=trifluoroacetic acid, MSA=methanesulfonic acid, TFMSA=trifluoromethanesulfonic acid.

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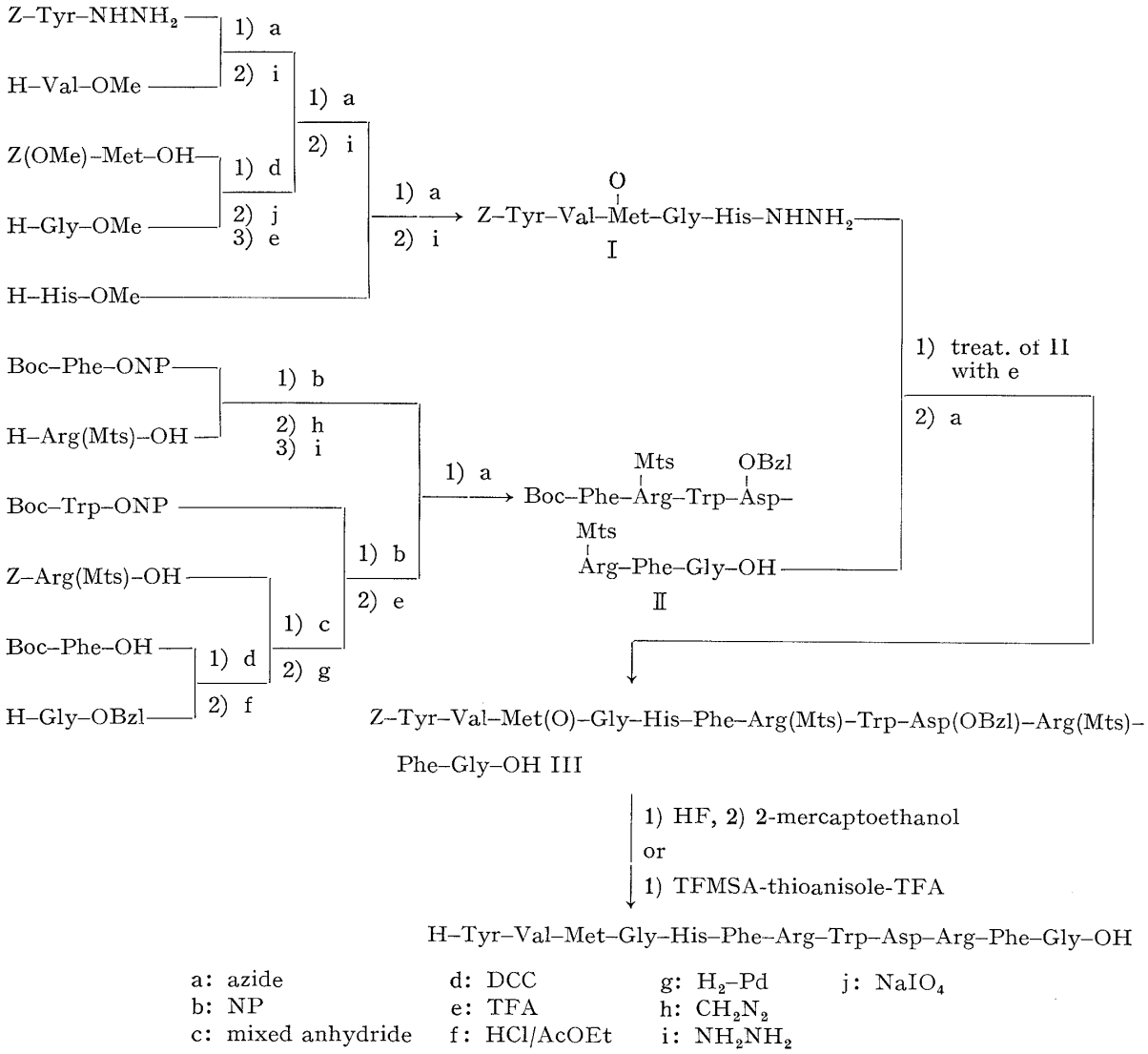

Fig. 1. Synthetic Scheme for Bovine γ-MSH

containing EDT[8] was employed. Thus, N^{α}-deprotected II was condensed with I by Rudinger's azide procedure[9] to give the protected γ-MSH, Z–Tyr–Val–Met(O)–Gly–His–Phe–Arg(Mts)–Trp–Asp(OBzl)–Arg(Mts)–Phe–Gly–OH (III), from which all protecting groups were removed by one of two alternative procedures.

First, the protected dodecapeptide (III) was treated with HF[10] in the presence of anisole containing 2% EDT in an ice-bath for 60 min. The protected product, after conversion to the corresponding acetate by treatment with Amberlite CG-400 (acetate form), was purified by column chromatography on CM-cellulose and then incubated with 2-mercaptoethanol[6] to reduce the Met(O) residue. After gel-filtration on Sephadex G-10, the desired peptide was obtained in 36% yield.

Alternatively, III was treated with 1 M TFMSA-thioanisole in TFA.[11] It seems interesting that as far as TLC examination is concerned, the Met(O) residue was quantitatively

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reduced to Met under this acidic condition with thioanisole. The product was obtained in 20% yield, after purification by column chromatography on CM-cellulose. Thus, the particular reduction step for Met(O) was eliminated. The peptides obtained by the two methods exhibited identical *Rf* values on TLC in two different solvent systems. The purity of the synthetic γ-MSH was confirmed by amino acid analysis in a 4 N MSA hydrolysate[12] and by elemental analysis.

As stated above, we found that the Met(O) residue was quantitatively reduced to Met in the TFMSA-thioanisole-TFA system. Thus, this reagent system seems to be a useful deprotecting tool for the synthesis of peptides containing Met, though some improvement in the yield would be desirable. At present, we presume that a side reaction at Trp may be responsible for lowering the yield in our case.

The biological activities of our synthetic γ-MSH were evaluated. *In vivo* melanocyte-stimulating potency[13] was estimated to be 7.14×10^6 U/g. This value is approximately 1/2800 of that of α-MSH (2×10^{10} U/g) on a weight basis. The peptide showed negligible steroidogenic potency,[14,15] or lipolytic activity.[16] Guillemin *et al.*[4] reported that the *in vitro* melanocyte-stimulating potency of synthetic γ-MSH was approximately 1/7100 of that of α-MSH. Further evaluation of this peptide is in progress.

Experimental

General experimental methods employed were essentially the same as those described in the previous paper.[17] Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (29: 1), Rf_2 $CHCl_3$–MeOH (9: 1), Rf_3 $CHCl_3$–MeOH–AcOH (9: 1: 0.5), Rf_4 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (15: 3: 10: 12), Rf_6 *n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2).

Z(OMe)-Met(O)-Gly-OMe——Z(OMe)–Met–Gly–OMe[18] (20.0 g) was oxidized with $NaIO_4$ (12.2 g) according to the method of Yajima *et al.*[19] The crude product was recrystallized from AcOEt and pet·ether: yield 11.0 g (52.9%), mp 121—125.5°, $[\alpha]_D^{15}$ +24.5° (c=2.0, $CHCl_3$). Rf_2 0.46. *Anal.* Calcd for $C_{17}H_{24}N_2O_7S$: C, 50.99; H, 6.04; N, 7.00. Found: C, 50.83; H, 6.01; N, 6.92.

Z-Tyr-Val-Met(O)-Gly-OMe——Z(OMe)–Met(O)–Gly–OMe (2.8 g) was treated with TFA-anisole (5.6 ml—1.4 ml) in an ice-bath for 60 min, then TFA was removed by evaporation. The oily residue was washed with *n*-hexane, dried over KOH pellets *in vacuo*, and dissolved in DMF (10 ml) containing Et_3N (1.36 ml). Et_3N (0.96 ml) and Z–Tyr–Val–N_3 (prepared from 3.0 g of Z–Tyr–Val–$NHNH_2$) in DMF (10 ml) were added to the above ice-chilled solution and the mixture was stirred at 4° for 72 hr. The solvent was evaporated off *in vacuo* and the residue was washed with 10% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, then recrystallized from MeOH and AcOEt: yield 2.4 g (54.2%). mp 186—190°, $[\alpha]_D^{17}$ −16.9° (c=1.8, MeOH). Rf_3 0.47. *Anal.* Calcd for $C_{30}H_{40}N_4O_9S$: C, 56.95; H, 6.37; N, 8.85. Found: C, 56.95; H, 6.26; N, 8.80.

Z-Tyr-Val-Met(O)-Gly-NHNH$_2$——In the usual manner, Z–Tyr–Val–Met(O)–Gly–OMe (2.0 g) was treated with 80% hydrazine hydrate (1.6 ml) at room temperature overnight. The crude product was washed successively with H_2O and hot MeOH: yield 1.5 g (75.0%). mp 246—247.5°, $[\alpha]_D^{33}$ +5.7° (c=1.2, DMSO). *Anal.* Calcd for $C_{29}H_{40}N_6O_8S$: C, 55.05; H, 6.37; N, 13.28. Found: C, 55.07; H, 6.29; N, 13.14.

Z-Tyr-Val-Met(O)-Gly-His-OMe——The azide (prepared from 1.5 g of Z–Tyr–Val–Met(O)–Gly–$NHNH_2$) was allowed to react with H–His–OMe (prepared from 0.63 g of H–His–OMe·HCl) as usual at 4° for 48 hr. The crude product was washed with 2% AcOH, 5% $NaHCO_3$ and H_2O–NaCl and then purified by column chromatography on silica (eluent: $CHCl_3$–MeOH–H_2O=8: 3: 1): yield 1.0 g (58.8%). mp 162—168°, $[\alpha]_D^{29}$ −2.7° (c=2.2, DMF). Rf_4 0.38. *Anal.* Calcd for $C_{36}H_{47}N_7O_{10}S\cdot1.5H_2O$: C, 54.26; H, 6.32; N, 12.30. Found: C, 54.24; H, 6.25; N, 12.22.

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Z-Tyr-Val-Met(O)-Gly-His-NHNH$_2$ (I)——In the usual manner, Z–Tyr–Val–Met(O)–Gly–His–OMe (1.0 g) was treated with 80% hydrazine hydrate (0.3 ml) overnight. The crude product was reprecipitated from DMSO–DMF with Et_2O: yield 0.8 g (80.0%). mp 202—206°, $[\alpha]_D^{31}$ +4.9° (c=1.0, DMSO). Rf_4 0.15. Amino acid ratios in 4 N MSA hydrolysate: $Tyr_{1.01}Val_{1.00}Met(O)_{0.88}Gly_{1.14}His_{1.16}$ (recovery of Val, 100.2%). *Anal.* Calcd for $C_{35}H_{47}N_9O_9S\cdot H_2O$: C, 53.36; H, 6.27; N, 16.00. Found: C, 53.34; H, 6.29; N, 15.76.

Z-Arg(Mts)-Phe-Gly-OBzl——Boc–Phe–Gly–OBzl[20] (10.0 g) was treated with TFA-anisole (25 ml—5 ml). The resulting oily product isolated as described above was condensed with Z–Arg(Mts)–OH (12.5 g) in THF (15 ml) by the mixed anhydride method[21] using isobutyl chloroformate (3.36 ml). The crude product was precipitated from MeOH–THF (1: 1) with n-hexane: yield 14.0 g (75.3%). mp 175—176.5°, $[\alpha]_D^{26}$ −13.2° (c=1.3, DMF). Rf_2 0.64. *Anal.* Calcd for $C_{41}H_{48}N_6O_8S$: C, 62.74; H, 6.16; N, 10.71. Found: C, 62.71; H, 6.19; N, 10.85.

Z(OMe)-Asp(OBzl)-Arg(Mts)-Phe-Gly-OH——In the usual manner, Z–Arg(Mts)–Phe–Gly–OBzl (10.5 g) dissolved in MeOH–THF–AcOH (2: 2: 1, 200 ml) was hydrogenated over a Pd catalyst for 7 hr. The N^{α}-deprotected peptide in DMF (50 ml) was allowed to react with Z(OMe)–Asp(OBzl)–ONP (7.2 g) as usual for 24 hr. The crude product, after washing with 10% citric acid, was reprecipitated from AcOEt with Et_2O: yield 7.0 g (56.5%). mp 97—100°, $[\alpha]_D^{15}$ −19.1° (c=1.9, MeOH). Rf_3 0.43. *Anal.* Calcd for $C_{46}H_{55}N_7O_{12}S$: C, 59.41; H, 5.96; N, 10.54. Found: C, 59.11; H, 6.13; N, 10.49.

Boc-Trp-Asp(OBzl)-Arg(Mts)-Phe-Gly-OH——Z(OMe)–Asp(OBzl)–Arg(Mts)–Phe–Gly–OH (5.8 g) was treated with TFA-anisole (11 ml—3 ml) as described above, and the N^{α}-deprotected tetrapeptide dissolved in DMF (30 ml) was allowed to condense with Boc–Trp–ONP (2.6 g) for 24 hr. The crude product was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH–H_2O=90: 15: 5) and further by precipitation from MeOH with Et_2O to yield a hygroscopic powder: yield 3.4 g (45.4%), $[\alpha]_D^{17}$ −11.9° (c=1.4, MeOH). Rf_3 0.58. *Anal.* Calcd for $C_{53}H_{65}N_9O_{12}S\cdot 2H_2O$: C, 58.50; H, 6.39; N, 11.58. Found: C, 58.42; H, 6.13; N, 11.50.

Boc-Phe-Arg(Mts)-OMe——Boc–Phe–ONP (10.0 g) in DMF (30 ml) was allowed to condense with H–Arg(Mts)–OH (8.4 g) for 24 hr, and the crude product (Rf_3 0.50) was treated with an ethereal solution of diazomethane as usual. The resulting ester was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH=80: 1) to afford an amorphous powder: yield 7.2 g (49.7%). $[\alpha]_D^{33}$ +0.6° (c=3.5, $CHCl_3$). Rf_2 0.62. *Anal.* Calcd for $C_{30}H_{43}N_5O_7S\cdot H_2O$: C, 56.68; H, 7.13; N, 11.02. Found: C, 56.78; H, 6.74; N, 11.03.

Boc-Phe-Arg(Mts)-NHNH$_2$——In the usual manner, Boc–Phe–Arg(Mts)–OMe (7.2 g) was treated with 80% hydrazine hydrate (2.9 ml) overnight. The product was reprecipitated from EtOH–Et_2O (2: 1) with n-hexane: yield 6.0 g (83.3%). mp 130—131°, $[\alpha]_D^{23}$ −10.4° (c=2.8, DMF). Rf_3 0.66. *Anal.* Calcd for $C_{29}H_{43}N_7O_6S$: C, 56.38; H, 7.02; N, 15.87. Found: C, 56.57; H, 7.06; N, 15.70.

Boc-Phe-Arg(Mts)-Trp-Asp(OBzl)-Arg(Mts)-Phe-Gly-OH (II)——Boc–Trp–Asp(OBzl)–Arg(Mts)–Phe–Gly–OH (4.0 g) was treated with TFA (12 ml) in the presence of anisole (4 ml) containing 2% EDT in an ice-bath for 60 min, then dry ether was added. The resulting powder was dissolved in DMF (10 ml) and allowed to react with the azide (prepared from 2.48 g of Boc–Phe–Arg(Mts)–NHNH$_2$) at 4° for 48 hr. After removal of the solvent, the crude product was extracted with AcOEt. The AcOEt layer was washed with 10% citric acid and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The product was purified by column chromatography on silica (eluent: $CHCl_3$–MeOH–H_2O=90: 15: 5) and further by precipitation from MeOH–Et_2O (2: 1) with n-hexane: yield 2.1 g (36.2%). $[\alpha]_D^{28}$ −22.1° (c=1.6, DMF). Rf_3 0.54. Amino acid ratios in 4 N MSA hydrolysate: $Phe_{2.16}Arg_{2.19}Trp_{0.82}Asp_{1.02}Gly_{1.00}$ (recovery of Gly, 77.4%). *Anal.* Calcd for $C_{77}H_{96}N_{14}O_{16}S_2\cdot 2H_2O$: C, 58.76; H, 6.40; N, 12.46. Found: C, 58.61; H, 6.25; N, 12.27.

Z-Tyr-Val-Met(O)-Gly-His-Phe-Arg(Mts)-Trp-Asp(OBzl)-Arg(Mts)-Phe-Gly-OH (III)——The Boc group was removed from II (1.5 g) by treatment with TFA (4.5 ml) in the presence of anisole (0.8 ml) containing 2% EDT, and the N^{α}-deprotected peptide isolated as described above was allowed to react with the azide (prepared from 1.13 g of I) at 4° for 72 hr. The solvent was removed by evaporation and the residue was triturated with ether. The resulting powder was washed with 2% AcOH and hot MeOH and then precipitated from DMF with MeOH: yield 1.2 g (55.3%). mp 219—222°, $[\alpha]_D^{28}$ −25.9° (c=0.9, DMF). Rf_4 0.35. Amino acid ratios in 4 N MSA hydrolysate: $Tyr_{0.89}Val_{0.85}Met+Met(O)_{1.00}Gly_{2.00}His_{1.01}Phe_{1.91}Arg_{1.93}Trp_{0.85}Asp_{1.01}$ (recovery of Gly, 76.4%). *Anal.* Calcd for $C_{107}H_{131}N_{21}O_{23}S_3\cdot 2H_2O$: C, 58.11; H, 6.15; N, 13.30. Found: C, 58.05; H, 6.36; N, 13.59.

H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH (bovine γ-MSH)——a) The above protected dodecapeptide (III, 210 mg) was treated with HF in the presence of anisole (0.6 ml) containing 2% EDT in an ice-bath for 60 min, then HF was removed by evaporation. The residue was dissolved in H_2O (20 ml) and treated with Amberlite CG-400 (acetate form) for 30 min. After filtration, the filtrate was lyophilized. The crude product was dissolved in 3% AcOH and the solution was applied to a column of Sephadex G-25 (3.3×60 cm), which was eluted with the same solvent. Individual fractions (10 ml each) were collected

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