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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.

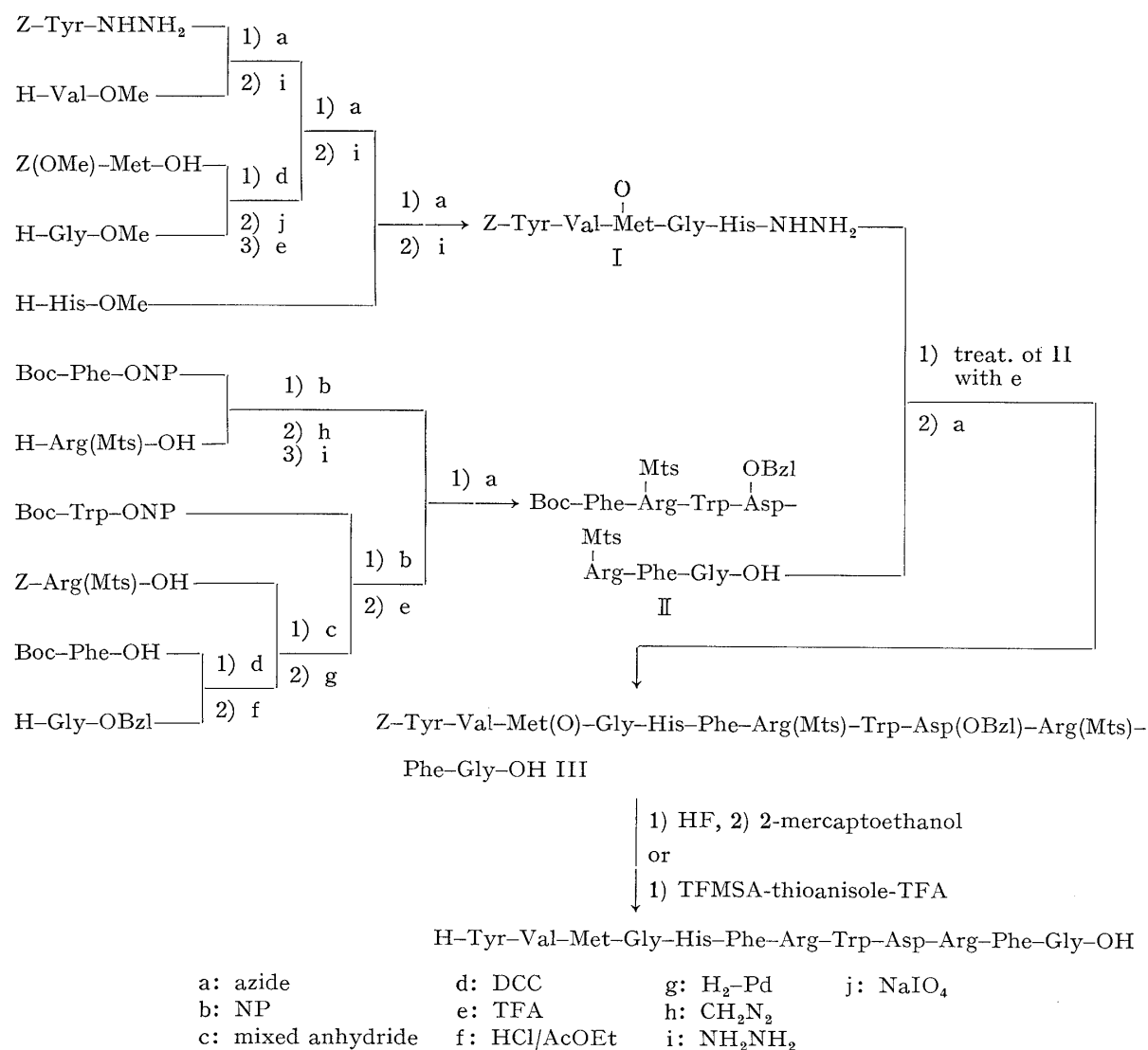
The synthetic peptide exhibited weak melanocyte-stimulating activity, but failed to show any significant steroidogenic and lipolytic activities.

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-NHNH₂ (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.
- 2) Location: a) Yamashina-ku, Kyoto, 607 Japan; b) and d) Sakyo-ku, Kyoto, 606 Japan; c) Fukushima-ku, Osaka, 553, Japan.
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Fig. 1. Synthetic Scheme for Bovine γ -MSH

containing EDT⁸⁾ was employed. Thus, N α -deprotected II was condensed with I by Rudinger's azide procedure⁹⁾ 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 HF¹⁰⁾ 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-mercaptoethanol⁶⁾ 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 hydrolysate¹²⁾ 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 potency¹³⁾ 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 CHCl₃-MeOH (29: 1), *Rf*₂ CHCl₃-MeOH (9: 1), *Rf*₃ CHCl₃-MeOH-AcOH (9: 1: 0.5), *Rf*₄ CHCl₃-MeOH-H₂O (8: 3: 1), *Rf*₅ *n*-BuOH-AcOH-pyridine-H₂O (15: 3: 10: 12), *Rf*₆ *n*-BuOH-AcOH-pyridine-H₂O (4: 1: 1: 2).

Z(OMe)-Met(O)-Gly-OMe—Z(OMe)-Met-Gly-OMe¹⁸⁾ (20.0 g) was oxidized with NaIO₄ (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°, $[\alpha]_D^{25} + 24.5^\circ$ ($c=2.0$, CHCl₃). *Rf*₂ 0.46. *Anal.* Calcd for C₁₇H₂₄N₂O₇S: 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 Et₃N (1.36 ml). Et₃N (0.96 ml) and Z-Tyr-Val-N₃ (prepared from 3.0 g of Z-Tyr-Val-NHNH₂) 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% NaHCO₃ and H₂O-NaCl, then recrystallized from MeOH and AcOEt: yield 2.4 g (54.2%). mp 186–190°, $[\alpha]_D^{25} - 16.9^\circ$ ($c=1.8$, MeOH). *Rf*₃ 0.47. *Anal.* Calcd for C₃₀H₄₀N₄O₉S: 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-NHNH₂—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 H₂O and hot MeOH: yield 1.5 g (75.0%). mp 246–247.5°, $[\alpha]_D^{25} + 5.7^\circ$ ($c=1.2$, DMSO). *Anal.* Calcd for C₂₉H₄₀N₆O₈S: 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-NHNH₂) 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% NaHCO₃ and H₂O-NaCl and then purified by column chromatography on silica (eluent: CHCl₃-MeOH-H₂O=8: 3: 1): yield 1.0 g (58.8%). mp 162–168°, $[\alpha]_D^{25} - 2.7^\circ$ ($c=2.2$, DMF). *Rf*₄ 0.38. *Anal.* Calcd for C₃₆H₄₇N₇O₁₀S·1.5H₂O: 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-NHNH₂ (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 Et₂O: yield 0.8 g (80.0%). mp 202–206°, $[\alpha]_D^{25} + 4.9^\circ$ ($c=1.0$, 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 C₃₅H₄₇N₉O₉S·H₂O: 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-OBzl²⁰ (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 method²¹ 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°, $[\alpha]_D^{25} - 13.2^\circ$ ($c=1.3$, DMF). Rf_2 0.64. *Anal.* Calcd for C₄₁H₄₈N₆O₈S: 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 Et₂O: yield 7.0 g (56.5%). mp 97–100°, $[\alpha]_D^{25} - 19.1^\circ$ ($c=1.9$, MeOH). Rf_3 0.43. *Anal.* Calcd for C₄₆H₅₅N₇O₁₂S: 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: CHCl₃-MeOH-H₂O=90:15:5) and further by precipitation from MeOH with Et₂O to yield a hygroscopic powder: yield 3.4 g (45.4%), $[\alpha]_D^{25} - 11.9^\circ$ ($c=1.4$, MeOH). Rf_3 0.58. *Anal.* Calcd for C₅₃H₆₅N₉O₁₂S·2H₂O: 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: CHCl₃-MeOH=80:1) to afford an amorphous powder: yield 7.2 g (49.7%). $[\alpha]_D^{25} + 0.6^\circ$ ($c=3.5$, CHCl₃). Rf_2 0.62. *Anal.* Calcd for C₃₀H₄₃N₅O₇S·H₂O: C, 56.68; H, 7.13; N, 11.02. Found: C, 56.78; H, 6.74; N, 11.03.

Boc-Phe-Arg(Mts)-NHNH₂—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-Et₂O (2:1) with *n*-hexane: yield 6.0 g (83.3%). mp 130–131°, $[\alpha]_D^{25} - 10.4^\circ$ ($c=2.8$, DMF). Rf_3 0.66. *Anal.* Calcd for C₂₉H₄₃N₇O₆S: 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)-NHNH₂) 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 H₂O-NaCl, dried over Na₂SO₄ and concentrated. The product was purified by column chromatography on silica (eluent: CHCl₃-MeOH-H₂O=90:15:5) and further by precipitation from MeOH-Et₂O (2:1) with *n*-hexane: yield 2.1 g (36.2%). $[\alpha]_D^{25} - 22.1^\circ$ ($c=1.6$, 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 C₇₇H₉₆N₁₄O₁₆S₂·2H₂O: 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°, $[\alpha]_D^{25} - 25.9^\circ$ ($c=0.9$, 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 C₁₀₇H₁₃₁N₂₁O₂₃S₃·2H₂O: 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 H₂O (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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