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Studies on Peptides. XXVIII.[1,2,3] Synthesis of the Docosapeptide which Embodies the Entire Amino Acid Sequence of β-Melanocyte-stimulating Hormone from Human Pituitary Gland

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Human β-melanocyte-stimulating hormone (MSH), H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH (I), was synthesized by deformylation of [21-N^{ε}-formyllysine]-human-β-MSH (IX) by hydrazine acetate. This formyl derivative (IX) was prepared by coupling reaction of Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-Asp(OBu^t)-Glu(OBu^t)-Gly-pentachlorophenyl ester and H-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH (II) followed by treatment with trifluoroacetic acid. The latter partially protected pentadecapeptide (II) is the synthetic intermediate of monkey β-MSH (*J. Am. Chem. Soc.*, **90**, 527 (1968); *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969)) structurally related to human β-MSH. By column chromatography on silica, the former protected heptapeptide active ester was isolated in pure form from the reaction mixture of dicyclohexylcarbodiimide-pentachlorophenol complex and Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-Asp(OBu^t)-Glu(OBu^t)-Gly-OH, which was prepared by the coupling reaction of Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-$NHNH_2$ and H-Asp(OBu^t)-Glu(OBu^t)-Gly-OH by the azide procedure. The synthetic human β-MSH and the formyl derivative (IX) exhibited the *in vitro* MSH activity of 6.2×10^9 and 3.4×10^9 units/g respectively.

In 1959, Harris[5] elucidated the entire amino acid sequence of β-melanocyte-stimulating hormone (β–MSH) (I) from human pituitary gland as shown in Fig. 1. A minute amount of the starting material served to his structural study. This hormone, as claimed by Harris, was the first peptide hormone to be isolated in pure form from human pituitary and to be characterized in terms of its complete structure.

Species variation of β–MSH from various animals is known. Among those, human β–MSH is structurally related to that of monkey pituitary gland.[6] Some genetic relationship between monkey and human can be seen by comparison of these two structures. Human β–MSH possesses the extra tetrapeptide unit, Ala-Glu-Lys-Lys, at the N-terminal portion of monkey β–MSH which consists of the octadecapeptide. Recently we have completed the synthesis of monkey β–MSH[7] and now extended our synthesis to the docosapeptide which embodied the entire amino acid sequence of β–MSH of human origin.

1) Part XXVII: H. Yajima, H. Kawatani and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1333(1970).
2) The preliminary communication of this investigation has appeared in *Biochim. Biophys. Acta*, **175**, 228 (1969).
3) The amino acids, peptides and their derivatives (except for glycine) mentioned in this communication are of the L-configuration. Their abbreviated designations are those recommended by IUPAC-IUB Commission on Biochemistry Nomenclature in July 1965 and July 1966; *Biochemistry*, **5**, 2485 (1966); **6**, 362 (1967): benzyloxycarbonyl=Z, *tert*-butoxycarbonyl=Boc, *tert*-butyl ester=OBu^t, γ-*tert*-butylglutamate=Glu(OBu^t), β-*tert*-butylaspartate=Asp(OBu^t), N^{ε}-*tert*-butoxycarbonyllysine=Lys(Boc), N^{ε}-formyllysine=Lys(For), *p*-nitrophenyl ester=ONp, pentachlorophenyl ester=OPCP.
4) Location: *Sakyo-ku, Kyoto.*
5) J.I. Harris, *Nature*, **184**, 167 (1959).
6) T.H. Lee, A.B. Lerner and V.B.-Janusch, *J. Biol. Chem.*, **236**, 1390 (1961).
7) H. Yajima, Y. Okada, Y. Kinomura and H. Minami, *J. Am. Chem. Soc.*, **90**, 527 (1968); H. Yajima, Y. Okada, Y. Kinomura, N. Mizokami and H. Kawatani, *Chem. Pharm. Bull.* (Tokyo), **17**, 1237 (1969).

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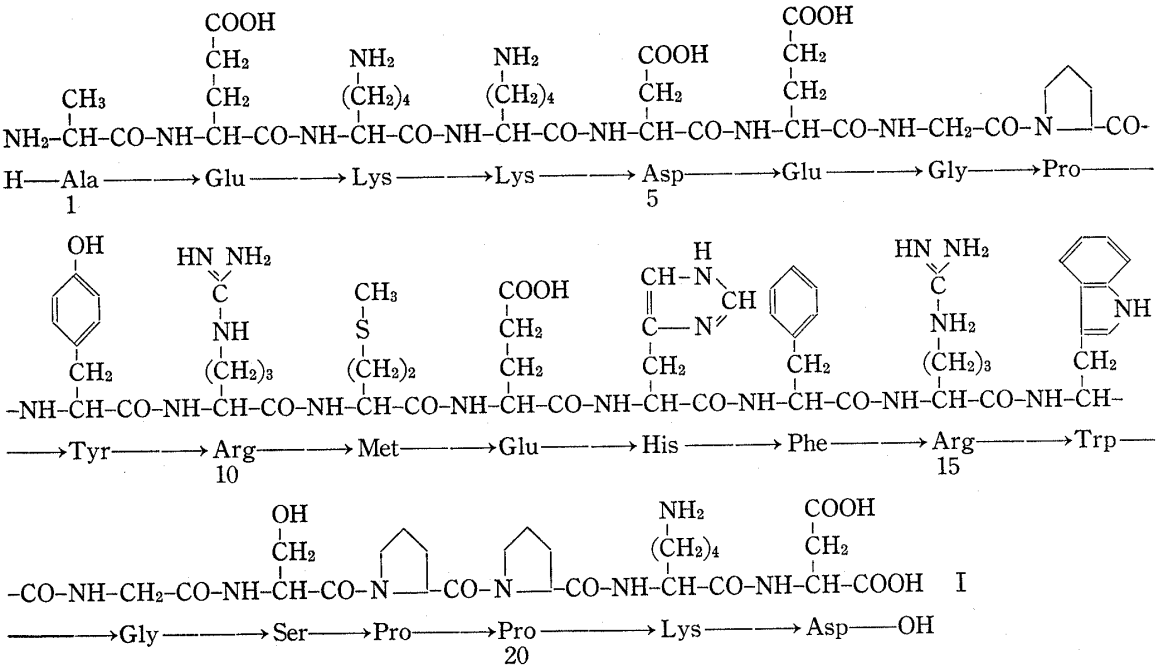
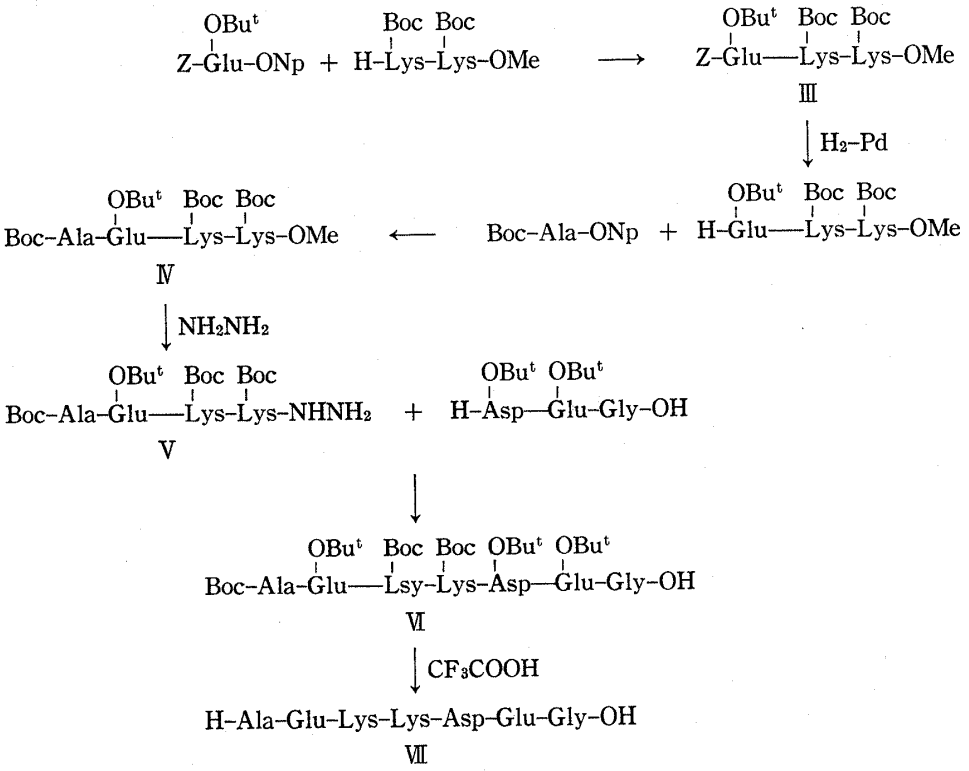


Chart 1. Amino Acid Sequence of Human β-Melanocyte-stimulating Hormone



Z=benzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, OBu^t=*tert*-butyl ester
ONp=*p*-nitrophenyl ester

Chart 2. Synthetic Route to the Protected Heptapeptide corresponding to the N-Terminal Portion of Human β-MSH

Partially protected pentadecapeptide previously described,[8] H-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH (II), served as an amino component at

8) H. Yajima, Y. Okada, H. Kawatani and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969).

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the last stage of the coupling reaction with the heptapeptide unit of the N-terminal portion of human β–MSH, Ala-Glu-Lys-Lys-Asp-Glu-Gly.

The necessary synthetic subunits of this heptapeptide derivative are illustrated in Fig. 2. Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-$NHNH_2$ was prepared in the stepwise manner starting from C-terminal H-Lys(Boc)-OMe. The dipeptide ester, H-Lys(Boc)-Lys(Boc)-OMe was prepared as described by Schwyzer, *et al.*[9] It was condensed with Z-Glu(OBu^t)-OH by the *p*-nitrophenyl ester procedure[10] to give Z-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-OMe (III). This was obtained in a crystalline form after purification by a column chromatography on silica in the solvent system of chloroform and methanol.

The N^{α}-protecting group of this protected tripeptide ester was removed by hydrogenation and the resulting tripeptide ester, H-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-OMe was allowed to react with Boc-Ala-ONp[11] to give Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-OMe (IV) in a crystalline form after purification by column chromatography on silica. Again, a mixture of chloroform and methanol served to elute the desired compound. This protected tetrapeptide ester was converted to the corresponding hydrazide, Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-$NHNH_2$ (V), in the usual manner. The analytically pure hydrazide thus obtained was converted to the corresponding azide by *tert*-butyl nitrite according to the method of Rudinger, *et al.*[12] The resulting azide was allowed to react with H-Asp(OBu^t)-Glu(OBu^t)-Gly-OH[7] to afford the protected heptapeptide, Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-Asp-(OBu^t)-Glu(OBu^t)-Gly-OH (VI) as colorless powder. A column chromatography on silica gel was found efficient to purify the desired product and its purity was established by thin–layer chromatography on silica with the iodine stain. The data of elemental analysis and the result of the amino acid analysis supported the homogeneity of this protected heptapeptide (VI).

In order to establish the optical purity of this synthetic subunit (VI), it was treated with 90% trifluoroacetic acid and the resulting heptapeptide, H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-OH (VII), was examined by acid hydrolysis and by digestion with leucine aminopeptidase (LAP).[13] Both hydrolysates contained every amino acid in ratios predicted by theory. The results cited above appear to demonstrate that every constituent amino acid, except for glycine, in the protected heptapeptide possesses the well defined L-configuration and that the aspartyl and glutamyl residues in the heptapeptide unit were both involved in the α-linkage.

The final coupling reaction of the synthesis of human β–MSH is illustrated in Fig. 3. The protected heptapeptide (VI) of the established optical purity was treated with a complex of pentachlorophenol (PCP) and dicyclohexylcarbodiimide (DCC) according to Kovacs, *et al.*[14] Thus, the carboxyl group of the C-terminal glycine, free from the asymmetric center, was converted to the corresponding pentachlorophenyl ester. The resulting active ester (VIII) was isolated in an analytically pure form by chromatography on silica; a very little side-product, presumably the acylurea was separated. The purified active ester was allowed to react with the pentadecapeptide (II) until the ninhydrin color test of the reaction mixture in dimethylformamide (DMF) became negative.

The crude product was partially purified by a CM-cellulose column in the solvent system of aqueous methanol and acetic acid. The unreacted active ester and liberated pentachlorophenol during the reaction were washed away form the column by 70% methanol prior to the elution of the desired fully protected docosapeptide. The product was treated with anhydrous

9) R. Schwyzer and W. Rittel, *Helv. Chim. Acta*, **44**, 159 (1961).
10) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1956).
11) Ed. Sandrin and R.A. Boissonnas, *Helv. Chim. Acta*, **46**, 1637 (1963).
12) J. Honzl and J. Rudinger, *Collection Czech. Chem. Commun.*, **26**, 2333 (1961).
13) Highly purified LAP was purchased from C.F. Boehringer and Soehne GmbH Mannheim, No. 6238403. Digestion was carried out according to K. Hofmann and H. Yajima, *J. Am. Chem. Soc.*, **83**, 2289 (1961).
14) K. Kovacs, L. Kisfaludy and M.Q. Ceprini, *J. Am. Chem. Soc.*, **89**, 183 (1967).

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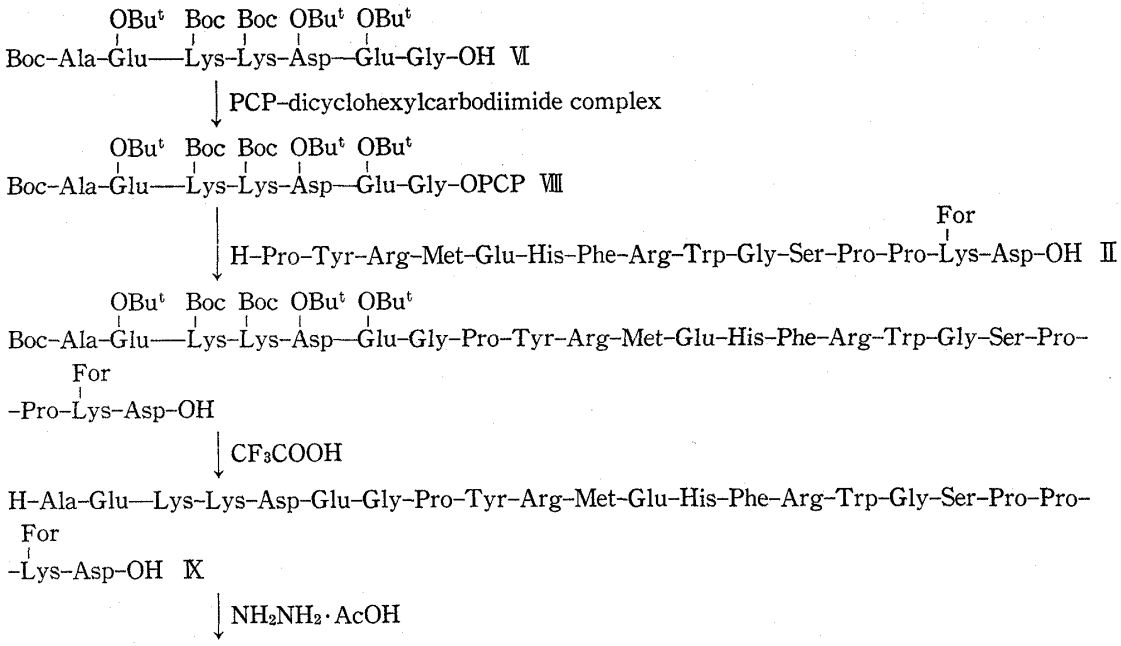

Chart 3. Synthetic Route to Human β-MSH

trifluoroacetic acid to remove the *tert*-butoxycarbonyl groups from alanine and lysine residues and the *tert*-butyl groups from the glutamic acid and aspartic acid residues. In order to prevent possible alkylation by the resulting *tert*-butyl cation,[15] anisole and methionine were added in the reaction mixture. The use of anhydrous trifluoroacetic acid, instead of 90% reagent, prevent the partial hydrolysis of the formyl group attached at the ε-amino function of the lysine residue at position 21 at this stage. The resulting partially protected docosapeptide, H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH (IX), was purified by column chromatography on CM-cellulose using ammonium acetate buffers. The acid hydrolysate of the purified peptide contained every amino acid in ratios predicted by theory except for tryptophan which was destroyed during this treatment. The peptide (IX) behaved as a single component in the field of paper electrophoresis at two different pH values.

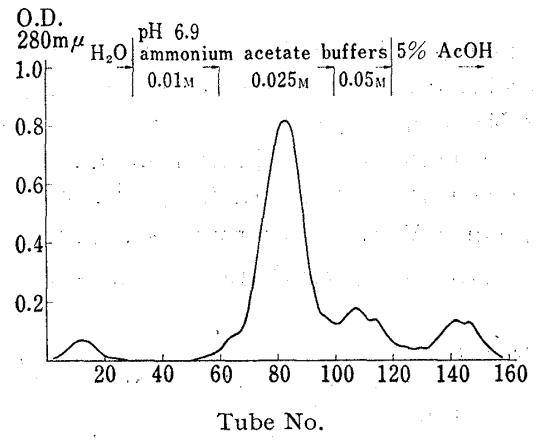

Fig. 1. Purification of Synthetic Human β-MSH by Column Chromatography on CM-cellulose

Removal of the formyl group from (IX) was performed in essentially the same manner as described in our synthesis of monkey β-MSH.[7] The peptide (IX) was dissolved in a solution of hydrazine acetate at pH 6.0 and the solution was heated in a boiling water-bath for 3 hr under nitrogen atmosphere. Mercaptoethanol was used to prevent possible oxidation of the methionine residue during this treatment. The product, after lyophilization, was submitted to purification by column chromatography on CM-cellulose. When the column was developed with ammonium acetate buffers with increasing ionic strength, a fairly well

15) G. Losse, D. Zeidler and T. Grieshaber, *Ann.*, **715**, 196 (1968).

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separated main peak with slight tailing was detected as judged by measuring of the absorbancy of the eluates at 280 mμ as shown in Fig. 4. The content of this main peak was found electrophoretically different from the starting material. It migrated slightly faster to the cathode side than the starting material in pyridinium acetate buffers at the acidic pH value. Thus the difference between the formyl derivative (IX) and the deformylated product could be detected. The material obtained exhibited a single spot on thin-layer chromatography. Amino acid analysis of an acid hydrolysate of the product revealed the presence of the constituent amino acids of human β-MSH (I) in ratios predicted by theory except for tryptophan destroyed by acid. Homogeneity of the final product was thus assessed.

Next, digestion of the product with LAP was performed. Complete hydrolysis of relatively large peptides with this enzyme is known to be difficult, since the highly purified enzyme does not attack the peptide bond attached at the nitrogen atom of proline, *i.e.*, the hydrolysis stops at this stage.[16] In 1963, Pfleiderer and Celliers[17] described the isolation of new aminopeptidase (AP-M) which is not activated by heavy metals and this enzyme is now commercially available. When our synthetic docosapeptide was exposed to the action of this enzyme for 20 hr according to Hofmann, *et al.*[18] the recovery of amino acids, glycine and serine, attached at the amino function of the prolyl residue at positions 8 and 19, was both somewhat low and only a trace of proline was detected. However the peptide chain attached at the carboxyl end of the proline was digested completely. Recent literatures[19] also recorded this unique property of this enzyme. Fairly good recovery of aspartic acid is noteworthy since the result seems to support the view that the aspartyl peptide bond in the synthetic docosapeptide is involved in the α-peptide bond as we have examined in the synthesis of monkey β-MSH[7] after similar treatment of its formyl derivative with hydrazine acetate.

The analytical data cited above appears to justify the conclusion that our synthetic docosapeptide corresponding to the entire amino acid sequence of human β-MSH possesses high degree of homogeneity and stereochemical purity.

Previously we have demonstrated that the N^{ε}-formyl group of lysine could be removed by hydroxylamine hydrochloride in pyridine as well as hydrazine acetate and succeeded in preparing α-MSH from [11-N^{ε}-formyllysine]-α-MSH.[20] Recently Geiger, *et al.*[21] examined in detail the deformylation reaction by these reagents. From these results, it can be seen that the formyl group is fairly well applicable for the synthesis of relatively large lysine-containing peptides.

The MSH activity of the synthetic peptides was determined according to Shizume, *et al.*[22] using frog skins from *Rana nigromaculata*. It was found that the [21-N^{ε}-formyl-lysine]-human-β-MSH (IX) exhibited the *in vitro* activity 3.4×10^9 MSH units/g and the synthetic human β-MSH (I), 6.2×10^9 MSH units/g respectively. These figures can be judged as equivalent and are in close agreement with the value reported in the natural source, *i.e.*, 3.3×10^9 units/g.[23]

Recently Rittel[24] reported the synthesis of human β-MSH, whose route was quite different from ours. The rotation value and the MSH activity of his synthetic peptide were re-

16) J. Meienhofer and C.H. Li, *J. Am. Chem. Soc.*, **84**, 2434(1962).
17) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963); G. Pfleiderer, P.G. Celliers, M. Stanulovic, E.D. Washmuth, H. Determann and G. Braunitzer, *ibid.*, **340**, 552 (1964).
18) K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966). Röhm & Haas (Darmstadt) preparation was used.
19) E.C. Jorgensen, G.C. Windridge and W. Patton, *J. Med. Chem.*, **12**, 733 (1969).
20) H. Yajima, K. Kawasaki, Y. Okada, K. Kubo and I. Yamashita, *Chem. Pharm. Bull.* (Tokyo), **16**, 919 (1968).
21) R. Geiger and W. Siedel, *Chem. Ber.*, **101**, 3386 (1968).
22) K. Shizume, A.B. Lerner and T.A. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).
23) B.T. Pickering and C.H. Li, *Biochim. Biophys. Acta*, **74**, 156 (1963).
24) W. Rittel, "Advances in Experimental Medicine and Biology," Vol. 2, ed. by N. Back, L. Martini and R. Paoletti, Pleum Press, New York, 1968, p. 35.

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corded. Though some difference in the rotation values between his peptide and ours is to be noted, the MSH activity of our synthetic human β-MSH was nearly equivalent to that reported by this author, *i.e.*, 3×10^9—6×10^9 units/g.

Accumulated data indicated that MSH in mammalian pituitary glands of β-type possesses many physiological responses other than the pigmentory effect:[25] for examples, thyrotropin-like activity,[26] release of free fatty acids into serum,[27] decrease of the weight of the testicles of mice,[28] occurence of menstrual bleeding in women with amenorrhoea,[29] influence on neurotransmission,[30] induction of a stretching and yawning reflex in mammals,[31] induction of hyperexcitability,[32] inhibitory action of extinction of conditioned avoidance behavior in rat,[33] increase the heart rate in the dog,[34] stimulation of an aqueous flare response in rabbit eyes,[35] adaptation to darkness[36] and so on. Precise physiological response of β-MSH in human being has not been established. It has been shown that both white and nigro secrete β-MSH of the same element.[25] Secretion of two principles capable to stimulate the pigment cell in urine has been suggested.[37] However, α-MSH, a most potent pigmentory principle in mammalian pituitary glands, is known to be present in human pituitary but has as yet not been well characterized.[38] In addition, chemical elucidation of hypothalamic MSH-releasing factor[39,40] and its inhibitory factor[40] is waited. There seems much works have to be done in evaluating the physiological effect of β-MSH in human subject. Synthetic route to human β-MSH opened here may serve to clarify this mysterious physiological principle which exists in human pituitary gland.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[8] of this series. Thin-layer chromatography was performed on silica gel (Kieselgel G. Merck). *Rf* values refer to the following solvent systems; Rf_1: *n*-BuOH–AcOH–H_2O (5:1:4), Rf_2: AcOEt–MeOH (3:1), Rf_3: *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_4: *n*-BuOH–AcOH–pyridine–H_2O (15:3:10:12).

Z-Lys(Boc)-Lys(Boc)-OMe——The title compound was prepared according to the method of Schwyzer, *et al.*[9] The product was recrystallized from ether and petroleum ether (bp 35—70°), mp 97—99°, $[\alpha]_D^{20}$ −5.5° (c=2.1, acetone), (lit.[9] mp 78—84°, $[\alpha]_D$ −5.3° in acetone). *Anal.* Calcd. for $C_{31}H_{50}O_9N_4$: C, 59.8; H, 8.1; N, 9.0. Found: C, 59.7; H, 8.3; N, 8.9.

Z-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-OMe (III)——Z-Lys(Boc)-Lys(Boc)-OMe (5.0 g) in MeOH (200 ml) containing AcOH (2.5 ml) was hydrogenated over a Pd catalyst in the usual manner until the evolution of CO_2 ceased. The catalyst was removed by filtration and the filtrate was evaporated at 35° under the reduced

25) A.B. Lerner and T.H. Lee, *Vitamines and Hormones*, **20**, 337 (1963); S. Lande and A.B. Lerner, *Pharmacol. Review*, **19**, 1 (1967).
26) G. Cehovic, *Compt Rend Soc. Biol.*, **254**, 1872 (1962); C.Y. Bowers, T.W. Redding and A.V. Schally, *Endocrinology*, **74**, 559 (1964).
27) S.L. Steelman and W.W. Smith, *Acta Endocrinol.*, **33**, 67 (1960); A. Tanaka, D.T. Pickering and C.H. Li, *Arch. Biochem. Biophys.*, **99**, 294 (1962); D. Rudman, S.J. Brown and M.F. Malkin, *Endocrinology*, **72**, 527 (1963).
28) G. Cehovic, *Compt Rend Soc. Biol.*, **159**, 1491 (1965).
29) A.B. Kastin, *The Lancet*, **1968**, 1007.
30) W.A. Krivoy, M. Lande and D.C. Kroeger, *Ann. N.Y. Acad. Sci.*, **104**, 312 (1963).
31) W. Ferrari, *Ann. N.Y. Acad. Sci.*, **104**, 330 (1963); G.L. Gessa and W. Ferrari, *Adv. Expl. Med. Biol.*, **2**, 190 (1968).
32) A. Sakamoto, *Nature*, **211**, 1370 (1966).
33) D. De Wied, *Proc. Soc. Expl. Biol. Med.*, **122**, 28 (1966); B. Bohno and D. De Wied, *Science*, **153**, 318 (1966).
34) O. Krayer, E.B. Astwood, D.R. Ward and M.H. Alper, *Proc. Natl. Akad. Sci.*, **47**, 1227 (1961).
35) K.D. Aas, *Endocrinology*, **74**, 255 (1964).
36) T. Hanaoka, *Japan J. Physiol.*, **2**, 9 (1951); **3**, 219 (1953).
37) B. Dahlberg, *Ann. N.Y. Acad. Sci.*, **100**, 631 (1963).
38) K. Abe, D.P. Island, G.W. Liddle, N. Fleischer and W.E. Nicholson, *J. Clin. Endocr. Metab.*, **27**, 46 (1967).
39) A.P.S. Dhariwal and S.M. McCann, *Proc. Soc. Exp. Biol. Med.*, **121**, 995 (1966).
40) S. Taleisnik and M.E. Tomatis, *Am. J. Physiol.*, **212**, 157 (1967).

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pressure. The oily residue (Rf_1 0.94) was dissolved in DMF (50 ml) and Z-Glu(OBu^t)-ONp[41] (5.50 g) was added. The solution was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed successively with a saturated solution of $NaHCO_3$, 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The oily residue was turned to solid from ether by seeding (see below). It was recrystallized twice from ether; yield 4.32 g (67%), mp 154—156°, $[\alpha]_D^{25}$ −37.0° (c=0.3, MeOH). *Anal.* Calcd. for $C_{40}H_{65}O_{12}N_5$: C, 59.5; H, 8.1; N, 8.7. Found: C, 59.3; H, 8.0; N, 8.8.

The first cup of the crystalline product was obtained as follow. The oily product prepared from 1.0 g of the protected dipeptide ester was dissolved in $CHCl_3$ and the solution was applied to a column of silica (2×15 cm), which was eluted with $CHCl_3$ and then with a mixture of $CHCl_3$ and MeOH (9:1, v/v). *p*-Nitrophenol and unreacted Z-Glu(OBu^t)-ONp were found in the $CHCl_3$ eluate. A solid material obtained in the $CHCl_3$–MeOH eluate was recrystallized from petroleum ether; yield 1.0 g (78%), mp 150—155°.

Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-OMe (IV)——Z-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-OMe (III) (0.50 g) in MeOH (40 ml) containing AcOH (0.2 ml) was hydrogenated over a Pd catalyst in the usual manner. After filtration, the filtrate was evaporated *in vacuo*. The oily residue (Rf_1 0.96) was dissolved in DMF (10 ml) and Boc-Ala-ONp[11] (0.40 g) was added. The solution was stirred at room temperatur for 24 hr and then filtered. The filtrate was evaporated and the residue was taken up into AcOEt, which was washed successively with a saturated solution of $NaHCO_3$, 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated to give an oily residue. This crude product in $CHCl_3$ was applied to a column of silica (1×10 cm), which was developed first with $CHCl_3$ and then with a mixture of $CHCl_3$–MeOH (9:1, v/v). The solid compound obtained in the latter eluate was recrystallized from ether and petroleum ether; yield 0.48 g (93%), mp 91—95°, $[\alpha]_D^{20}$ −30.9° (c=1.0, MeOH); amino acid ratios in an acid hydrolysate $Ala_{1.00}Glu_{1.00}Lys_{1.99}$ (average recovery 99.6%). *Anal.* Calcd. for $C_{40}H_{72}O_{13}N_6$: C, 56.9; H, 8.6; N, 10.0. Found: C, 56.8; H, 8.8; N, 10.1.

Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-NHNH$_2$ (V)——Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-OMe (IV) (4.0 g) was dissolved in MeOH (100 ml). To this solution, 80% hydrazine hydrate (3 ml) was added and the solution was kept on standing at room temperature for 48 hr. The solvent was evaporated and the residue was treated with ether to form, after standing in a refrigerator overnight, a gelatinous mass, which was recrystallized from MeOH and ether; yield 3.6 g (90%), mp 165—167°, $[\alpha]_D^{20}$ −25.0° (c=1.1, MeOH). *Anal.* Calcd. for $C_{39}H_{72}O_{12}N_8$: C, 55.4; H, 8.6; N, 13.3. Found: C, 55.7; H, 8.8; N, 13.0.

Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-Asp(OBu^t)-Glu(OBu^t)-Gly-OH (VI)——A solution of Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-NHNH$_2$ (V) (1.0 g) in DMF (13 ml) was chilled in an ice–bath. To this solution, 1.5N HCl in DMF (1.6 ml) was added followed by *tert*-butyl nitrite (0.16 ml). The mixture was stirred for 5 min and then 1M triethylamine in DMF (1.3 ml) was added. This solution containing the above azide was combined with a solution of H-Asp(OBu^t)-Glu(OBu^t)-Gly-OH (0.50 g) in DMF (13 ml) containing 1M triethylamine in DMF (1.2 ml). After stirring at 4° for 48 hr, the solution was condensed and the residue was triturated with an ice-cold solution of 10% citric acid and the resulting powder was collected by filtration, washed with H_2O and dried over P_2O_5. This crude product was dissolved in MeOH and after filtration, the solvent was evaporated to give an oily residue, which was dissolved in $CHCl_3$ (120 ml). The solution was applied to a column of silica (1.5×15 cm), which was eluted with the same solvent (200 ml) and then with a mixture of $CHCl_3$–MeOH (97:3 v/v, 400 ml), in which the rearrangement product of the tetrapeptide azide was emerged. The column was then eluted with a mixture of $CHCl_3$–MeOH (90:10, v/v, 400 ml) to yield the desired compound. The location of which was established by thin-layer chromatography with the iodine stain, Rf_2 0.62. The contents of these tubes were combined and the solvent was evaporated. The residue was treated with H_2O to form colorless powder; yield 1.02 g (70%), mp 229—231°, $[\alpha]_D^{23}$ −36.0° (c=0.5, MeOH). Amino acid ratios in an acid hydrolysate $Ala_{1.00}Glu_{2.04}Lys_{2.04}Asp_{0.99}Gly_{1.05}$ (average recovery 100%). *Anal.* Calcd. for $C_{58}H_{101}O_{20}N_9$: C, 56.0; H, 8.2; N, 10.1. Found: C, 56.2; H, 8.4; N, 9.9.

H-Ala-Glu-Lys-Lys-Asp-Gly-Gly-OH (VII)——The protected heptapeptide, Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-Asp(OBu^t)-Glu(OBu^t)-Gly-OH (VI) (0.20 g) was dissolved in 90% trifluoroacetic acid (1 ml). After 1 hr, ether was added and the resulting powder was collected by centrifugation, washed with ether and then dissolved in H_2O. The aqueous solution was kept overnight at room temperature and then lyophilized. The fluffy powder thus obtained was again dissolved in H_2O (100 ml) and the pH of the solution was adjusted to 5 with pyridine. This solution was applied to a column of CM-cellulose (1.5×20 cm), which was eluted with H_2O. The eluent was collected in 12 ml aliquots at a rate of 1 ml per min. The fractions (tube No. 15 to 33) which contained the ninhydrin positive substance were collected and the solvent was evaporated. The residue was lyophilized to give colorless fluffy powder; yield 73 mg (53%), $[\alpha]_D^{19}$ −51.0° (c=0.5, H_2O), Rf_1 0.03, Rf_3 0.04, single spot positive to ninhydrin. Amino acid ratios in an acid hydrolysate $Ala_{1.00}Glu_{2.16}Lys_{2.01}Asp_{1.01}Gly_{1.00}$ (average recovery 100%), amino acid ratios in a LAP digest $Ala_{1.00}Glu_{1.90}Lys_{2.07}Asp_{0.92}Gly_{0.95}$ (average recovery 95%). *Anal.* Calcd. for $C_{31}H_{53}O_{14}N_9 \cdot 5H_2O$: C, 43.0; H, 7.3; N, 14.6. Found: C, 43.3; H, 7.3; N, 14.5.

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Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-Asp(OBu^t)-Glu(OBu^t)-Gly-OPCP (VIII)——To an ice cooled solution of the protected heptapeptide (VI) (0.13 g) in DMF (3 ml), the pentachlorophenol-DCC complex (0.10 g) prepared according to Kovacs, *et al.*[14] was added and the solution was stirred at room temperature for 16 hr. Dicyclohexylurea formed during the reaction was removed by filtration and the solvent was evaporated. The residue, after washing with petroleum ether, was dissolved in $CHCl_3$ (7 ml). The solution was applied to a column of silica gel (2×10 cm), which was first developed with the same solvent (200 ml) to elute the pentachlorophenol and a side reaction product, presumably the acylurea. Next the column was eluted with $CHCl_3$ containing 3% MeOH (100 ml). The eluates were combined and the solvent was evaporated. The residue was treated with H_2O to give powder, yield 0.11 g (73%), $[\alpha]_D^{28}$ −12.3° (c=0.5, MeOH), $[\alpha]_D^{29}$ −2.0° (c=0.5, $CHCl_3$), IR 1780 cm^{-1} (–COO–C_6Cl_5). *Anal.* Calcd. for $C_{64}H_{100}O_{20}N_9Cl_5$: C, 51.5; H, 6.8; N, 8.4. Found: C, 51.3; H, 6.6; N, 8.3.

H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Try-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH, [21-N$^\varepsilon$-formyllysine]-human-β-MSH (IX)——Boc-Ala-Glu(OBu^t)-Lys(Boc)-Lys(Boc)-Asp(OBu^t)-Glu(OBu^t)-Gly-OPCP (VIII) (100 mg) was added to a solution of H-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys(For)-Asp-OH (II) (100 mg) in 90% aqueous DMF (4 ml) containing 1M triethylamine in DMF (0.2 ml). The solution was stirred at room temperature for 24 hr, when an additional active ester (50 mg) was added. Totally 72 hr's stirring was required until the ninhydrin color test of the starting material became completely negative. A new spot of Rf_3 0.77 was detected by the Ehrlich stain. The solvent was evaporated *in vacuo* and the residue was dissolved in 70% aqueous MeOH (100 ml). The solution was applied to a column of CM-cellulose (1.5×15 cm), which was eluted with 70% MeOH (300 ml) and then with 70% MeOH containing 2% AcOH (400 ml). Individual fractions, 10 ml each, were collected and absorbancy at 280 mμ was determined. Fraction I in 70% MeOH eluate gave no Ehrlich test. Fraction II in the 70% MeOH–2% AcOH eluate gave the Ehrlich–positive material (140 mg) of Rf_3 0.66 with a trace impurity of Rf_3 0.46. The fraction II was dissolved in trifluoroacetic acid (1.5 ml) in the presence of anisole (0.14 ml) and methionine (15 mg) for 3 hr at room temperature. The product was precipitated by addition of ether (peroxide free), washed with the same solvent, and dried over KOH pellets *in vacuo*. It was dissolved in H_2O, which after standing for 3 hr, was lyophilized. The fluffy powder thus obtained was dissolved in H_2O (200 ml) and the solution, after adjusting the pH to 5 with NH_4OH, was applied to a column of CM-cellulose (1×20 cm), which was eluted with the following pH 6.9 ammonium acetate buffers; 0.01M (600 ml), and 0.03M (300 ml). Individual fractions, 10 ml each, were collected and absorbancy at 280 mμ was determined in each fraction. A peak present in the 0.01M eluate was detected and the contents of these tubes (59 to 100) were combined. The solvent was evaporated and the residue was lyophilized to give fluffy powder; yield 71 mg (45%), $[\alpha]_D^{29}$ −75.9° (c=0.2, 10% AcOH). Rf_3 0.25. Electrophoretical mobility in 0.1M pyridinium acetate buffers at pH 6.2 and 3.5 was −9.8 and −4.8 cm respectively under the condition of 1000 V for 2 hr. Amino acid ratios in an acid hydrolysate $Ala_{1.00}Glu_{3.00}Lys_{3.41}Asp_{2.17}Gly_{2.00}Pro_{3.25}Tyr_{1.00}$-$Arg_{2.08}Met_{0.92}His_{1.17}Phe_{1.17}Ser_{1.08}$ (average recovery 96%).

H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH (I)——[21-N$^\varepsilon$-Formyllysine]-human-β-MSH (IX) (70 mg) was dissolved in 10% hydrazine acetate (3 ml). The pH of the solution was adjusted to 6.0 with AcOH prior to the reaction. Mercaptoethanol (0.03 ml) was added to the solution and the air in the flask was replaced by nitrogen gas. The solution was heated in a boiling water bath for 3 hr and then repeatedly lyophilized. The residue was dissolved in H_2O (150 ml) and the solution was applied to a column of CM-cellulose (1.5×20 cm) which was eluted with the following pH 6.9 ammonium acetate buffers; 0.01M (300 ml), 0.025M (400 ml) and 0.05M (200 ml). Individual fractions (10 ml) were collected and the absorbancy at 280 mμ was determined in each fraction. The contents of the main peak present in the 0.025M eluate (tube 69 to 115) were combined and the solvent was evaporated and the residue was repeatedly lyophilized to remove the last trace of ammonium acetate. Fluffy powder was obtained; yield 55 mg (70%), $[\alpha]_D^{29}$ −76.4° (c=0.5, 10% AcOH), (lit.[24] −99.8° in 0.1M AcOH). Rf_3 0.25, Rf_4 0.25. Mobility on paper electrophoresis in 0.1M pyridinium acetate buffers at pH 6.2 and 3.5 were −11.1 and −6.0 cm respectively under the condition of 1000 V for 2 hr. Amino acid ratios in an acid hydrolysate $Ala_{1.00}Glu_{3.24}Lys_{2.81}Asp_{2.20}Gly_{2.00}Pro_{2.89}Tyr_{0.87}Arg_{2.15}Met_{1.04}His_{1.03}Phe_{1.12}Ser_{0.95}$ (average recovery 90%). Amino acid ratios in a AP-M digest $Ala_{1.00}Glu_{2.98}Lys_{3.16}Asp_{1.78}Gly_{1.25}Pro_{0.31}Tyr_{0.92}Arg_{1.78}Met_{0.93}$-$His_{1.02}Phe_{1.00}Trp_{0.93}Ser_{0.41}$. *Anal.* Calcd. for $C_{118}H_{174}O_{35}N_{34}S \cdot 5CH_3COOH \cdot 14H_2O$: C, 47.7; H, 6.5; N, 14.9. Found: C, 47.4; H, 6.7; N, 15.1.

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