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Studies on Peptides. XXIII.[1-3] Total Synthesis of Monkey β-Melanocyte-stimulating Hormone

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Total synthesis of monkey β-melanocyte-stimulating hormone was described. The synthesis involved in the coupling reaction of N^{α}-*t*-butoxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine with prolyltyrosylarginylmethionylglutamylhistidylphenylalanyl-arginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid by the N-hydroxysuccinimide ester method and subsequent removal of all of the protecting groups from the product, *i.e.*, *t*-butyl and *t*-butoxycarbonyl groups by trifluoroacetic acid and the formyl group by hydrazine acetate. Synthetic monkey β-MSH exhibited 6×10^{9} and 2.5×10^{10} MSH U/g.

The occurrence of two melanocyte-stimulating hormones (α-and β-MSH) both capable of stimulating pigment cells in the same mammalian pituitary gland is a mistery in endocrinology. Physiological evaluation of these two hormones, other than the melanotropic activity, is currently under investigation in various laboratories. The monkey pituitary is also known to possess such two hormones.[5] One of which, α-MSH is identical with that of the other animals so far examined. This peptide hormone was synthesized independently by Boissonnas, *et al.*[6] and Schwyzer, *et al.*[7] Recently we have also succeeded in preparing this hormone by the method different from those authors.[8]

The 2nd principle from monkey pituitary gland, β-MSH, was isolated in a pure form by Lee, *et al.*[5] in 1961 and the entire amino acid sequence was elucidated by the same authors. It was found that monkey β-MSH (I) was consisted of octadecapeptide as shown in Fig. 1, which was identical with that of human β-MSH,[9] if tetrapeptide, alanylglutamyllysyllysyl unit, was eliminated from the N-terminus of the latter. We have currently engaged in the synthesis of these two hormones and a portion of which was published.[1,2,10-13] In this paper, we wish to report the synthesis of the peptide which embodies the entire amino acid sequence of β-MSH of monkey origin.

1) Part XXII: H. Yajima, Y. Okada, H. Kawatani and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969).
2) The preliminary communication of this paper has appeared in *J. Am. Chem. Soc.*, **90**, 527 (1968).
3) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration and their abbreviated designations are those recommended by IUPAC-IUB commision for biological nomenclature in July 1965 and July 1966. *Biochemistry*, **5**, 1445, 2485 (1966); **6**, 362 (1967).
4) Location: *Sakyo-ku, Kyoto.*
5) T.H. Lee, A.B. Lerner and V.B.-Janusch, *J. Biol. Chem.*, **236**, 1390 (1961).
6) St. Guttmann and R.A. Boissonnas, *Helv. Chim. Acta*, **42**, 1257 (1959).
7) R. Schwyzer, A. Costopanagiotis and P. Sieber, *Helv. Chim. Acta*, **46**, 870 (1963).
8) H. Yajima, K. Kawasaki, Y. Okada, H. Minami, K. Kubo and I. Yamashita, *Chem. Pharm. Bull.* (Tokyo), **16**, 919 (1968).
9) J.I. Harris, *Nature*, **184**, 167 (1959).
10) H. Yajima, Y. Okada, T. Oshima and S. Lande, *Chem. Pharm. Bull.* (Tokyo), **14**, 707 (1966).
11) H. Yajima, Y. Okada, Y. Kinomura and E. Seto, *Chem. Pharm. Bull.* (Tokyo), **15**, 270 (1967).
12) H. Yajima, Y. Kinomura, T. Oshima and Y. Okada, *Chem. Pharm. Bull.* (Tokyo), **15**, 1922 (1967).
13) H. Yajima, *Gunma Symposia on Endocrinology* (Institute of Endocrinology, Gunma University), **5**, 73 (1968).

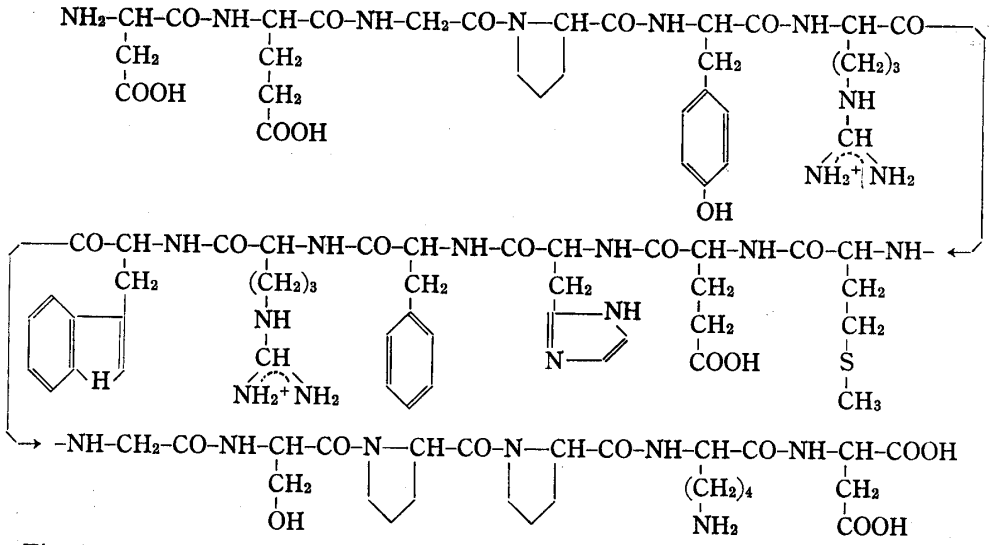

Fig. 1. Amino Acid Sequence of Monkey β-Melanocyte-stimulating Hormone (I)

In the preceding paper,[1] we have described the synthesis of the pentadecapeptide (II) corresponding to position 4 to 18 of monkey β-MSH, prolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid. Synthesis of the tripeptide unit of the N-terminal portion of this hormone, aspartylglutamylglycine, and subsequent coupling reaction of this unit with II followed by removal of all protecting groups will bring us its total synthesis as shown in Fig. 2.

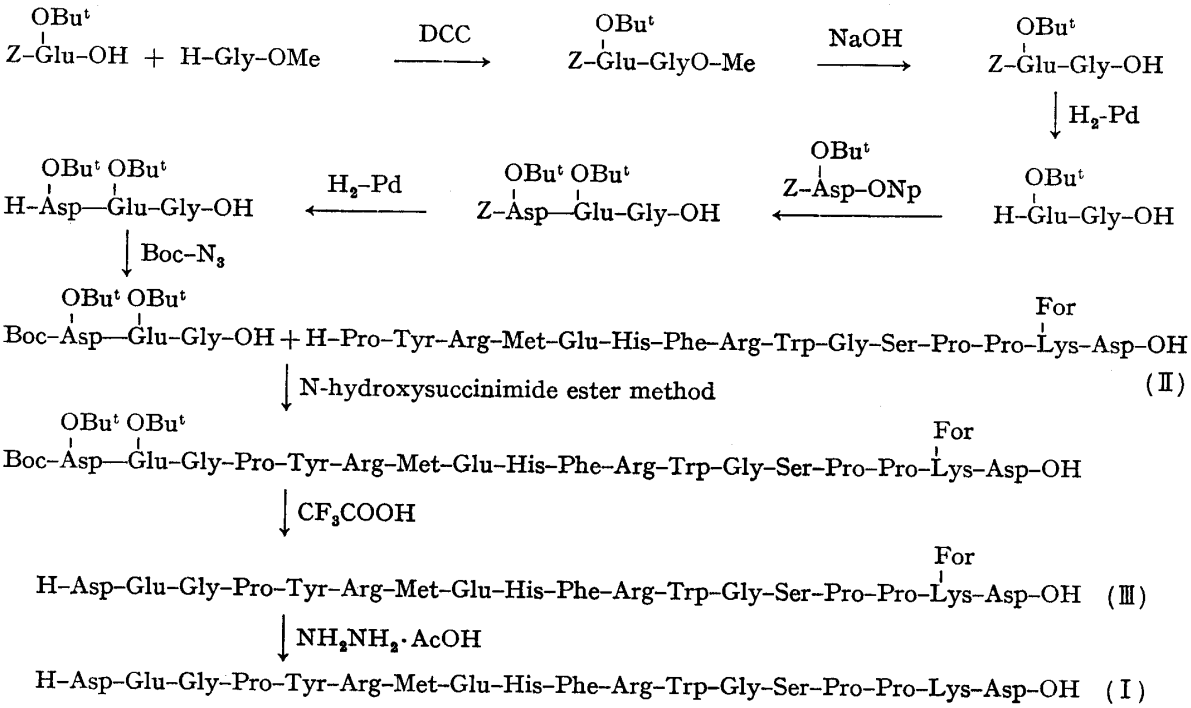

Fig. 2. Final Step in the Synthesis of Monkey β-Melanocyte-stimulating Hormone

For the synthesis of the necessary tripeptide unit, glycine methyl ester was condensed with N^{α}-benzyloxycarbonyl-γ-*t*-butylglutamate[14,15] by either the mixed anhydride[16–18] or the

14) E. Klieger and H. Gibian, *Ann. Chem.* **655**, 195 (1962).
15) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 7 (1962).
16) Th. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951).
17) J.R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).
18) R.A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

dicyclohexylcarbodiimide procedure[19] to give N^{α}-benzyloxycarbonyl-γ-*t*-butylglutamylglycine methyl ester. Homogeneity of this oily product was established by thin-layer chromatography with iodine stain. The terminal carboxyl group of this protected dipeptide was freed from its methyl ester by alkaline saponification at this stage and the resulting N^{α}-benzyloxycarbonyl-γ-*t*-butylglutamylglycine was further characterized as its dicyclohexylamine salt. Hydrogenation of this protected dipeptide gave γ-*t*-butylglutamylglycine as crystalline solid, which was allowed to react with N^{α}-benzyloxycarbonyl-β-*t*-butylaspartate *p*-nitrophenyl ester[20] to give in satisfactory yield the protected tripeptide, N^{α}-benzyloxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine. Column chromatography on silica gel in the solvent system of chloroform was found efficient to isolate the first cup of the crystalline protected tripeptide. Benzyloxycarbonyl group of this protected peptide was removed by catalytic hydrogenation to give crystalline β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine. In order to establish the optical purity, this partially protected tripeptide was treated with trifluoroacetic acid and the resulting free tripeptide, aspartylglutamylglycine was exposed to the action of highly purified leucine aminopeptidase (LAP).[21] It was found that the hydrolysate contained the three amino acids in nearly equi-moles. Thus the definite L-configuration of the constituent amino acids, aspartic acid and glutamic acid, in this tripeptide unit was confirmed. Transpeptidation of aspartyl peptides from α to β in alkaline solution is known[22] and alkaline saponification of the *t*-butyl ester from aspartyl peptides is also known.[23,24] For this reason, we removed the methyl ester of the terminal glycine residue by alkaline at dipeptide stage, *i.e.*, N^{α}-benzyloxycarbonyl-γ-*t*-butylglutamylglycine as mentioned above. With this care, we were able to prepare the partially protected tripeptide, β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine, free from contamination of a β-aspartyl peptide as demonstrated by the complete digestability with the enzyme.

β-*t*-Butylaspartyl-γ-*t*-butylglutamylglycine was converted to the corresponding N^{α}-*t*-butoxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine by *t*-butylazidoformate.[25-27] The carboxyl group of this protected tripeptide of established optical purity was converted to its N-hydroxysuccinimide ester[28] by dicyclohexylcarbodiimide. The excess of the latter reagent was washed with petroleum ether and the resulting active ester was allowed to react with pentadecapeptide (II) under the nitrogen atmosphere for 48 hr until the ninhydrin test of the reaction mixture became negative. The resulting protected octadecapeptide, N^{α}-*t*-butoxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycylprolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid, was precipitated by addition of ethyl acetate and subsequently treated with trifluoroacetic acid to remove the *t*-butoxycarbonyl group from the N-terminal aspartyl residue and the *t*-butyl esters from the aspartyl and glutamyl residues. The deblocked peptide was precipitated with ether. Usually, some contamination of the aspartylglutamylglycine unit was found in this

19) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **74**, 676 (1951).
20) L. Bernardi, G. Bossisio, O. Goffredo and R. de Castiglion, *Experientia*, **20**, 490 (1964).
21) Highly purified LAP was purchased from Sigma Chem. Co., Lot 16B-0201. It has very little prolidase activity. For digestion of proline-containing peptides, partially purified LAP which possesses this activity was prepared according to D.H. Spackman, E.L. Smith and D.M. Brown, *J. Biol. Chem.*, **212**, 244 (1955). When this enzyme preparation was employed, a recovery of Glu is always low. Based on the recovery of the standard sample of Glu (64%), the corrected figure of Glu was given in the experimental section.
22) E. Schröder and K. Lübke, "The Peptide," Academic Press, New York, Vol. 1, 1965, p. 201.
23) F. Chillemi, *Gazz. Chim. Ital.*, **96**, 359 (1966).
24) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel and H. Zuber, *Helv. Chim. Acta*, **46**, 1975 (1963).
25) L.A. Carpino, C.A. Giza and R.A. Carpino, *J. Am. Chem. Soc.*, **81**, 955 (1959).
26) R. Schwyzer, P. Sieber and H. Kappeler, *Helv. Chim. Acta*, **42**, 7622 (1959).
27) H. Yajima and H. Kawatani, *Chem. Pharm. Bull.* (Tokyo), **16**, 182 (1968).
28) G.W. Anderson, J.E.Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

precipitate. This can be easily detected by thin-layer chromatography since it exhibited positive color test only by ninhydrin and not by other color tests such as Ehrlich test. It was found occasionally that, even if we started from the same batch of the sample, the deblocked product exhibited various ninhydrin positive spots on thin-layer chromatography. When this trifluoroacetate salt was dissolved in water and the solution which was slightly acidic was kept on standing overnight at room temperature, a number of the ninhydrin positive spots has diminished and at least two spots were detected. Model experiments indicated that the *t*-butyl ester was cleaved by trifluoroacetic acid in much slower rate than the *t*-butoxycarbonyl group. The result indicated the possibility that incomplete removal of the *t*-butyl group might occurred and this would cause complexity of a reaction product. Our present procedure may be useful when deblocking of these groups was not completed by a single treatment of the reagent.

The resulting product was submitted to purification by column chromatography on carboxymethylcellulose (CM-cellulose).[29] Contaminated tripeptide, aspartylglutamylglycine was washed away from the column with water and the desired compound, aspartylglutamylglycylprolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid, was eluted with ammonium acetate buffers. Homogeneity of the partially protected octadecapeptide (III) thus obtained was established by thin-layer chromatography and by electrophoresis at two different pH values. An amino acid analysis revealed the presence of expected amino acids, except for tryptophan, in ratios predicted by theory. Its nuclear magnetic resonance (NMR) spectra exhibited a characteristic proton signal at τ 1.95 corresponding to the CHO group. The result indicated unequivocally that the ε-amino group of lysine in position 17 in this peptide is still occupied by a substituent, that is, the formyl group.

Deformylation reaction from this partially protected octadecapeptide was performed in essentially the same manner as was demonstrated in the preparation of α-MSH from [11-N$^{\varepsilon}$-formyllysine]-α-MSH.[8] The peptide was treated with hydrazine acetate in a boiling water bath for 3 hr rather than at 37° for 48 hr, since the former condition gave superior results to the latter. Concentration of hydrazine acetate was 5 to 10% and approximately 50 eqi-moles per mole of the peptide was employed. Care was taken to prevent possible oxidation of the methionine residue during this treatment by performing the reaction in the presence of mercaptoethanol. The solution, after lyophilization, was applied to a column of CM-cellulose. Gradient elution was employed to isolate the desired compound using the ammonium acetate buffer. Chromatographic pattern obtained in this purification step was shown in Fig. 3-a. A main peak which possessed a shoulder was detected. This shoulder-portion which consisted of mainly the starting material was removed and the rest was submitted

a) elution pattern of the crude preparation

b) rechromatographic pattern of fraction II obtained in a

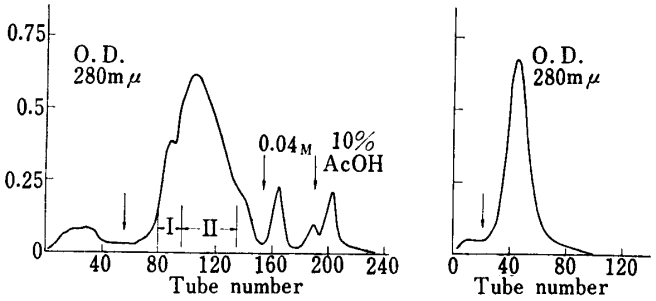

Fig. 3. Purification of Synthetic Monkey β-MSH on CM-cellulose

Arrow indicates where the gradient elution was established with 0.01M pyridine acetate buffer (650 ml) through a mixing chamber of water (300 ml). The column was then eluted with 0.04M pyridine acetate buffer and finally with 10% acetic acid.

Arrow indicates where the gradient elution was established with 0.01M pyridine acetate buffer (450 ml) through a mixing chamber of water (200 ml).

29) E.A. Peterson and H.A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956). Cellex-CM (0.7 eq mole/ g) was purchased from Bio-Rad Co., Richimond, Calif. U.S.A.

to the 2nd chromatography on CM-cellulose. Again gradient elution was performed to obtain a single and symmetrical peak as shown in Fig. 3-b.

The purified compound, corresponding to the entire amino acid sequence of monkey β-MSH was homogeneous on thin-layer chromatography. This exhibited much sharper spot than paper chromatography. The very low *Rf* value was obtained in the solvent system of Partridge[30] and therefore homogeniety of the product was not easily established. However the solvent system of *n*-butanol, pyridine, acetic acid and water (15:10:3:12) gave relatively

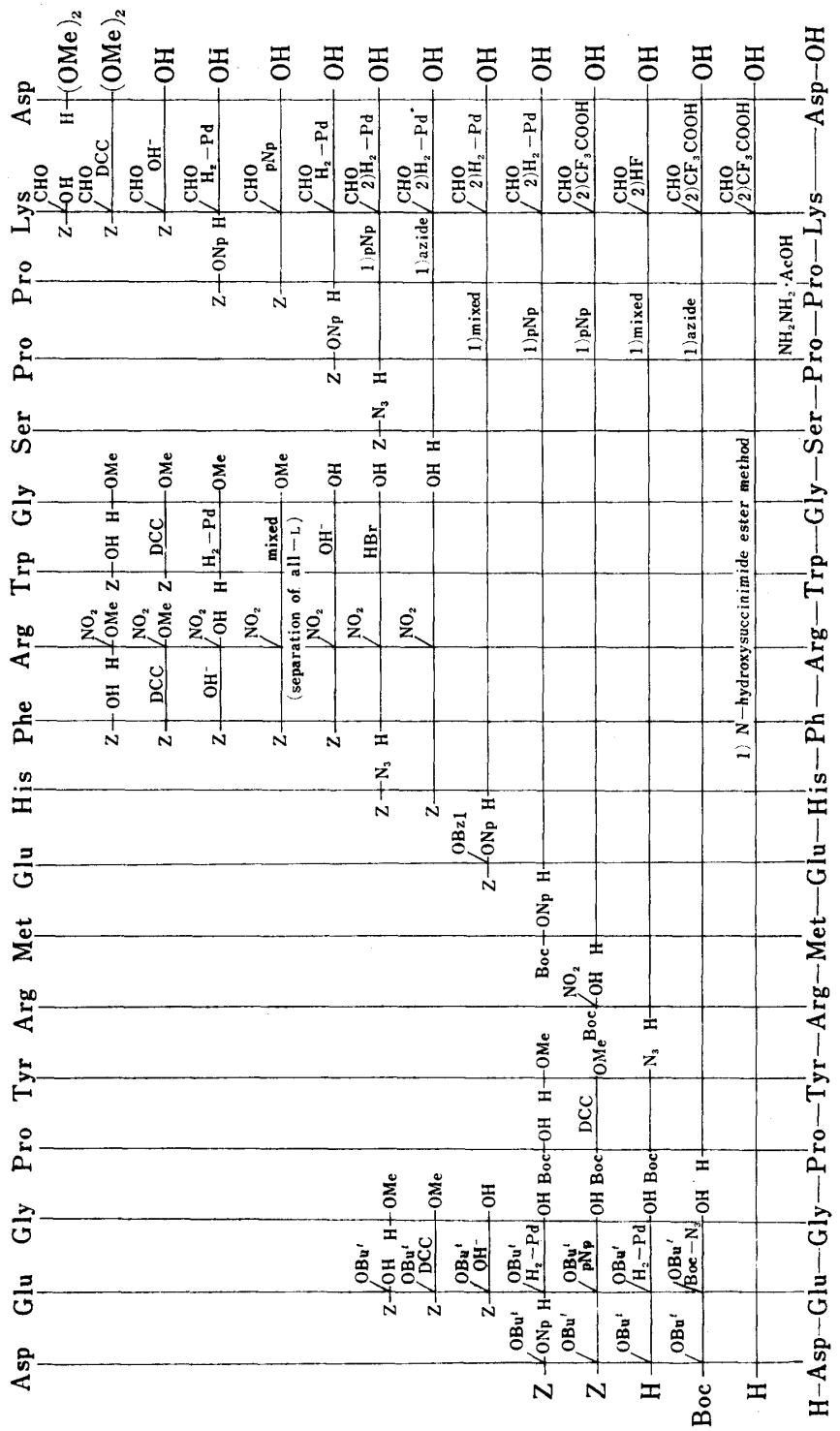

Fig. 4. Entire Synthetic Route to Monkey β-Melanocyte-stimulating Hormone

pNp=*p*-nitrophenyl ester method DCC=dicyclohexylcarbodiimide CHO=N^{ε}-formyl
NO_2=N^{α}-nitro Z=benzyloxycarbonyl Boc=butoxycarbonyl OBu^t=*t*-butyl ester
OBzl=benzyl ester

30) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).

high *Rf* value on thin-layer chromatography and very sharp spot could be detected. In the field of electrophoresis on paper, the product behaved as a single component at two different acidic pH values and exhibited much faster mobility toward the cathode than the starting material. Thus, difference of these two compounds can be detected. Amino acid ratios in an acid hydrolysate of synthetic monkey β-MSH were in close agreement with those predicted by theory. Amino acid recovery in a LAP digest[21] was comparable to those obtained by acid hydrolysis, where lysine instead of N^{ε}-formyllysine was found in the hydrolysate indicating that the formyl group was indeed removed by the above treatment. In its NMR spectra, the proton signal corresponding to CHO at τ 1.95 was absent supporting further the above observation. The experimental results cited above seem to justify the conclusion that our synthetic octadecapeptide possesses a high degree of homogeneity and stereospecificity.

The entire route involved in the total synthesis of monkey β-MSH is summarized in Fig. 4. In order to construct this octadecapeptide, we combined first two pentapeptide subunits of the established optical purity, N^{α}-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycine and serylprolylprolyl-N^{ε}-formyllysylaspartic acid to prepare the partially protected decapeptide, histidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid,[11] where activation of the glycine terminus of the former by the mixed anhydride procedure was employed. Addition of glutamic acid, methionine and arginine residues to the decapeptide was performed stepwisely by either active ester or mixed anhydride method. Further addition of prolyltyrosyl residue to the resulting partially protected tridecapeptide was carried out by the azide procedure.[1] In the final step, addition of tripeptide, aspartylglutamylglycine, to the pentadecapeptide (II), again the glycine residue, free of the asymmetrical center, was selected for the point of combination of the two peptide units. Considering the synthetic methodology which we employed and from the result of the LAP digestion, it can be seen that our synthetic monkey β-MSH possesses well established optical purity.

The synthetic peptides were assayed *in vitro* according to the procedure of Shizume, *et al.*[31] using frog skins from *Rana pipiens*. It was found that the MSH activity of [17-N^{ε}-formyllysine]-monkey-β-MSH (III) was 2.0×10^{9} MSH U/g. The synthetic monkey β-MSH exhibited 2.5×10^{10} MSH U/g and the other batch, 6×10^{9} MSH U/g. It can be judged that these three values are of the same magnitude within the limit of experimental tolerance. Therefore, the formyl group at the N^{ε}-lysine residue at position 17 is neither biologically essential nore contribute to any degree of the MSH potency. Previously, we have observed that the synthetic formylpentadecapeptide, prolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid (II) exhibited the MSH activity of 2×10^{12} U/g.[1] When this value and the activity of synthetic monkey β-MSH are compared, it can be seen that addition of an acidic tripeptide to the pentadecapeptide cause some detrimental effect as far as the melanotropic activity is concerned. We wish to emphasize that such an observation can only be made by the systematic elongation of the amino acid residues of the peptide hormones.

The activity of monkey β-MSH from the natural source was reported as 3 to 5×10^{9} MSH U/g.[5,31] The value we obtained for the synthetic peptide was in close agreement with those values. Structurally related MSH from bovine origin was synthesized by Schwyzer, *et al.*[24] This hormone possesses the lysine residue at position 6 instead of the arginyl residue in monkey β-MSH. This minor difference required quite different synthetic approach from that of Schwyzer, *et al.* as was demonstrated in this paper.

Synthesis of another structurally related MSH from human pituitary gland will be described in the later paper.

31) K. Shizume, A.B. Lerner and T.B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).
32) A.B. Lerner and T.H. Lee, *Vitamins and Hormones*, **20**, 337 (1963).

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[1] of this series. On paper chromatography, Rf_1 values refer to the system of *n*-BuOH, AcOH and H_2O (4:1:5). On thin-layer chromatography (Kiesel gel G, Merck), Rf_2 and Rf_3 values refer to the system of *n*-BuOH, pyridine, AcOH and H_2O (4:1:1:2) and (15:10:3:12) respectively. NMR spectra were measured at 60 Mc on a Varian associate A-60 spectrometer. Electrophoretic mobility of peptides was expressed as a multiple of distance traveled by a Phe marker under identical conditions (their direction was indicated in the blacket). The following abbreviations were used for solvents, dimethylformamide=DMF and tetrahydrofuran=THF.

N^{α}-Benzyloxycarbonyl-γ-*t*-butylglutamylglycine and Its Dicyclohexylamine Salt——N^{α}-Benzyloxycarbonyl-γ-*t*-butylglutamate dicyclohexylamine salt (10.36 g) and methylglycinate hydrochloride (2.51 g) were dissolved in MeOH (110 ml) and the solvent was evaporated. DMF (40 ml) was added and the resulting unsoluble dicyclohexylamine hydrochloride was removed by filtration. To this ice-cooled fitrate, dicyclohexylcarbodiimide (4.96 g) was added portionwise. The mixture was stirred at room temperature overnight. Dicyclohexylurea formed during the reaction was removed by filtration and the filtrate was condensed *in vacuo*. The resulting oily residue was dissolved in AcOEt, which after washing with 10% citric acid, 5% $NaHCO_3$ and H_2O, was dried over Na_2SO_4 and then evaporated to give N^{α}-benzyloxycarbonyl-γ-t-butyl-glutamylglycine methyl ester as an oily product, yield 11.4 g (96%), $[\alpha]_D^{18}$ −14.0° (*c*=1.5, MeOH), Rf_2 0.75, single spot positive to the iodine test.

This protected dipeptide ester was similarly prepared by the mixed anhydride procedure. N^{α}-Benzyloxycarbonyl-γ-*t*-butylglutamate dicyclohexylamine salt (5.18 g) was suspended in dry THF (30 ml) and ethyl chloroformate (0.96 ml) was added under cooling at −10°. After stirring for 15 min, the solution was added to a solution of methyl glycinate hydrochloride (1.88 g) and triethylamine (2.08 ml) in THF (30 ml) and the mixture was stirred in an ice-bath for 2 hr. Purification was carried out as described above; yield 3.80 g (93%).

The oily methyl ester (5.47 g) was dissolved in MeOH (60 ml) and then the solution was treated with 1 N NaOH (11 ml) in an ice-bath for 1 hr. The pH of the solution was adjusted to 6 with AcOH and the solvent was evaporated. The residue was dissolved in ice-cooled 0.1 N NaOH. The aqueous phase was quickly washed with ether and then acidified with ice-cooled 10% citric acid. The resulting precipitate was extracted with AcOEt, which after washing with H_2O, was dried over Na_2SO_4 and then evaporated to give an oily residue. This product, on standing in a refrigerator, turned to a solid, which was recrystallized from ether and *n*-hexane; yield 4.30 g (81%), mp 81—83°, $[\alpha]_D^{28}$ −12.0° (*c*=1.0, MeOH). *Anal.* Calcd. for $C_{19}H_{26}O_7N_2$: C, 57.9; H, 6.6; N, 7.1. Found; C, 57.9; H, 6.7; N, 7.4.

For further characterization, the product (2.25 g) was dissolevd in acetone (10 ml) and dicyclohexylamine (1.2 ml) was added. The crystalline mass formed on standing was recrystallized from MeOH and acetone; yield 2.67 g (81%), mp 147—148°, $[\alpha]_D^{23}$ −52.0° (*c*=1.5, MeOH). *Anal.* Calcd. for $C_{19}H_{26}O_7N_2.C_{12}H_{23}N$: C, 64.7; H, 8.6; N, 7.3. Found: C, 64.4; H, 8.7; N, 7.5.

γ-*t*-Butylglutamylglycine——N^{α}-Benzyloxycarbonyl-γ-*t*-butylglutamylglycine (11.20 g) in MeOH (100 ml) containing AcOH (0.1 ml) was hydrogenated over a Pd catalyst in the usual manner. The solid formed during the hydrogenation was dissolved by addition of H_2O. The catalyst was removed by filtration and the filtrate was evaporated to give the crystalline material which was recrystallized from MeOH; yield 6.77 g (92%), mp 190—192°, $[\alpha]_D^{23}$ +22.8° (*c*=0.9, H_2O). Rf_1 0.57, single ninhydrin positive spot. *Anal.* Calcd. for $C_{11}H_{20}O_5N_2$: C, 50.8; H, 7.8; N, 10.8. Found: C, 50.5; H, 7.9; N, 10.6.

N^{α}-Benzyloxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine and Its Dicyclohexylamine Salt——γ-t-Butylglutamylglycine (12.0 g) was dissolved in a mixture of dioxan (200 ml) and H_2O (80 ml). To this solution, triethylamine (6.4 ml) was added followed by N^{α}-benzyloxycarbonyl-β-*t*-butylaspartate *p*-nitrophenyl ester (24.55 g) and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed successively with 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The oily residue turned to the solid by seeding (see below), it was recrystallized from chloroform and ether; yield 23.8 g (91%), mp 108—110°, $[\alpha]_D^{21}$ −20.2° (*c*=1.1, MeOH). *Anal.* Calcd. for $C_{27}H_{39}O_{10}N_3 \cdot H_2O$: C, 55.6; H, 7.1; N, 7.2. Found: C, 55.6; H, 7.3; N, 7.3.

The first cup of the crystal was obtained by a column chromatography on silica (Mallinckrodt, 100 mesh). The oily product (1.30 g) in chloroform was applied to a column (1.5×18 cm), which was developed with the same solvent. Individual fractions (15 ml each) were collected and the eluates were examined by the iodine test on thin-layer chromatography. In the front eluates (tube 3 and 4) *p*-nitrophenol and unreacted N^{α}-benzyl-oxycarbonyl-β-*t*-butylaspartate *p*-nitorphenyl ester were isolated. The tubes (No. 7 to 15) containing the desired product (Rf_2 0.66) were combined and the solvent was evaporated to give the crystalline solid; yield 0.80 g, (62%), mp 100—105°.

The dicyclohexylamine salt of this protected tripeptide was prepared in the usual manner. It was recrystallized from MeOH and acetone; mp 181—182°, $[\alpha]_D^{24}$ −13.3° (*c*=0.8, MeOH). *Anal.* Calcd. for $C_{27}H_{39}O_{10}N_3 \cdot C_{12}H_{23}N$: C, 62.7; H, 8.3; N, 7.5. Found: C, 62.9; H, 8.6; N, 7.6.

β-*t*-Butylaspartyl-γ-*t*-butylglutamylglycine One and a Half Hydrate——N^{α}-Benzyloxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine (6.00 g) in MeOH (50 ml) containing AcOH (0.9 ml) was hydrogenated over a Pd catalyst in the usual manner. The catalyst was removed by filtration, and the filtrate was evaporated. The residue was dried in an evacuated descicator and recrystallized from MeOH and ether; yield 4.20 g (92%), mp 114—116°, $[\alpha]_D^{23}$ −7.2° (c=0.7, MeOH). Rf_1 0.85, Rf_2 0.82, single ninhydrin positive spot. *Anal.* Calcd. for $C_{19}H_{33}O_8N_3 \cdot 1.5\ H_2O$. C, 49.8; H, 7.9; N, 9.2. Found: C, 49.9; H, 7.6; N, 9.4.

Aspartylglutamylglycine——β-*t*-Butylaspartyl-γ-*t*-butylglutamylglycine (0.80 g) was treated with anhydrous trifluoroacetic acid (2 ml) at room temperature for 1 hr. Dry ether was added to from a solid powder which was collected by decantation, dried *in vacuo*. The product was dissolved in a small amount of H_2O and the solution was neutralized with pyridine (0.5 ml). The solvent was evaporated to a small volume and EtOH was added. The resulting crystalline powder was collected by filtration and recrystallized from H_2O and EtOH; yield 0.41 g (70%), mp 222° (decomp.), $[\alpha]_D^{28}$ −11.1° (c=1.0, H_2O), Rf_1 0.14, single ninhydrin positive spot. Amino acid ratios in an acid hydrolysate $Asp_{0.98}Glu_{1.00}\ Gly_{1.00}$ average recovery 84%, amino acid ratios in a LAP (highly purified) digest $Asp_{1.00}Glu_{0.98}Gly_{1.06}$ average recovery 90%. *Anal.* Calcd. for $C_{11}H_{17}O_8N_3$: C, 41.4; H, 5.3; N, 13.2,. Found: C, 41.1; H, 5.2; N, 13.0.

N^{α}-*t*-Butoxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine Dicyclohexylamine Salt——An ethereal solution (approximately 10 ml) of *t*-butyl azidoformate (prepared from 0.6 g of the corresponding hydrazide) was added to a solution of β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine (1.04 g) and triethylamine (0.7 ml) in DMF (15 ml). The mixture was stirred at 45° for 24 hr and the solvent was evaporated. The residue was dissolved in 5% NH_4OH, which after washing with ether, was acidified with ice-cooled 10% citric acid. The resulting precipitate was extracted with AcOEt, which was washed with H_2O, dried over Na_2SO_4 and then evaporated to give an oily residue; yield 1.14 g (86%). The oily product was converted to its dicyclohexylamine salt in the usual manner. It was recrystallized from acetone and ether; mp 135—139°, $[\alpha]_D^{22}$ −15.3° (c=0.8, MeOH). *Anal.* Calcd. for $C_{24}H_{41}O_{10}N_3 \cdot C_{12}H_{23}N$: C, 60.7; H, 9.0; N, 7.9. Found: C, 60.7; H, 9.3; N, 8.1.

Aspartylglutamylglycylprolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic Acid Diacetate Heptahydrate——To a solution of N^{α}-*t*-butoxycarbonyl-β-*t*-butylaspartyl-γ-*t*-butylglutamylglycine (0.27 g) in AcOEt (10 ml), N-hydroxysuccinimide (0.12 g) and dicyclohexylcarbodiimide (0.10 g) were added and the solution was stirred at 0° for 30 min and then at room temperature overnight. Dicyclohexylurea formed during the reaction was removed by filtration, the filtrate was condensed *in vacuo* and the residue was treated with petroleum ether (bp 30—70°). The resulting semi-solid N-hydroxysuccinimide ester was collected by decantation and then added to a solution of prolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid (0.19 g) in 90% DMF (7 ml) containing triethylamine (0.06 ml). The air of the flask was replaced with the nitrogen gas before use. The solution was stirred at room temperature for 12 hr. During this period, the ninhydrin test of the reaction mixture became completely negative. The solution was evaporated *in vacuo* and the residue was treated with AcOEt. The resulting powder was collected by centrifugation, dried over P_2O_5 *in vacuo* and subsequently treated with anhydrous trifluoroacetic acid (1 ml) at room temperature for 2 hr. Ether (stored over $FeSO_4$) was added to form a white precipitate which was collected by centrifugation, dried over KOH pellets *in vacuo* and then dissolved in H_2O (10 ml). The solution, after on standing overnight, was lyophilized to give a fluffy powder, which exhibited two ninhydrin positive spots on thin-layer chromatography, Rf_3 0.49 and 0.24. The former was also positive to the Ehrlich test. This powder was dissolved in H_2O (400 ml) and the solution, after the pH was adjusted to 3.8 with 5% NH_4OH, was applied to a column of CM-cellulose (2×8 cm), which was eluted first with H_2O (400 ml). Gradient elution was then established by adding 0.01 M, pH 6.9 ammonium acetate buffer (1500 ml) through a mixing flask containing H_2O (300 ml). Individual fractions of 13 ml each were collected and absorbancy at 280 mμ was determined in each fraction. A single peak was detected in the gradient eluate (tube 125 to 210), which were collected and the solvent was removed first by evaporation and finally lyophilization. The residue was repeatedly lyophilized to constant weight; yield 0.17 g (72%), $[\alpha]_D^{30}$ −63.6° (c=0.2, 1 N AcOH); NMR (in D_2O–CD_3COOD) τ: 1.95 (1H, singlet, CHO), 1.35 (1H, singlet, imidazol). Rf_3 0.49, single spot positive to ninhydrin, Pauly, Sakaguchi, methionine and Ehrlich tests. Electrophoretic mobility at pH 3.5 and 6.8 in 0.1 M pyridine acetate buffers (1500 volt, 2 hr using Phe as a marker) was (−) 3.18×Phe and (−) 1.28×Phe respectively. Amino acid ratios in an acid hydrolysate $Asp_{1.96}Glu_{2.20}Gly_{2.05}Pro_{3.10}Tyr_{0.76}Arg_{1.99}Met_{0.93}His_{1.05}Phe_{1.00}Ser_{0.98}Lys_{1.06}$ (average recovery 96%). *Anal.* Calcd. for $C_{99}H_{138}O_{30}N_{28}S \cdot 2CH_3COOH \cdot 7H_2O$: C, 49.9; H, 6.5; N, 15.8. Found: C, 50.1; H, 6.9; N, 15.3.

Aspartylglutamylglycylprolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyllysylaspartic Acid Triacetate Heptahydrate——The pH of an aqueous solution of 10% hydrazine was adjusted to 6 with AcOH. To this solution (1.6 ml), aspartylglutamylglycylprolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid (90 mg) was dissolved and mercaptoethanol (0.7 ml) was added. The air of the flask was replaced by the nitrogen gas. The solution was heated at 85—90° for 3 hr and then the solvent was removed by lyophilization. The residue was dissolved in H_2O (300 ml) and the solution was applied to a column of CM-cellulose (1.2×14 cm), which was eluted with pH 6.9, 0.01M ammonium acetate buffer (650 ml) through a mixing

chamber containing H_2O (300 ml). Individual fractions of 10 ml each were collected and the absorbancy at 280 mμ was determined. A main peak which possessed a shoulder was detected as shown in Figure 3-a. This shoulder (fraction I, tube 80 to 96) was cut off and the desired fraction (fraction II, tube 97 to 135) was collected and the solvent was removed first by evaporation and finally by lyophilization; yield 43 mg (47%). Electrophoretic examination of this product was shown to contain some of the starting material; two spots, mobility −2.5 cm and −5.0 cm (main spot) at pH 6.5, 0.1M pyridine acetate buffer (1500 volt for 2 hr). The highly purified sample was obtained by the 2nd CM-cellulose column chromatography (1.2×5 cm) of the fraction II in the same gradient system as shown in Figure 3-b; yield 27 mg (63%), $[\alpha]_D^{30}$ −50.7° (c=0.2, 1N AcOH). NMR (in D_2O-CD_3COOD) τ: 1.35 (1H singlet, imidazol) and no proton signal at 1.95. Rf_3 0.46, single spot positive to ninhydrin, Pauly, Sakaguchi, methionine and Ehrlich tests. Homogeneous on paper electrophoresis and its mobility at pH 3.5 and 6.8 of 0.1M pyridine acetate buffers (1500 volt, 2 hr using Phe as a marker) was (−) 4.4×Phe and (−) 2.1×Phe respectively. Amino acid ratios in an acid hydrolysate $Asp_{1.96}Glu_{1.97}Gly_{1.94}Pro_{3.02}Tyr_{0.79}Arg_{2.01}Met_{0.76}His_{1.08}Phe_{1.00}Ser_{0.95}Lys_{1.00}$ (average recovery 80%). Amino acid ratios in a LAP (partially purified) digest $Asp_{2.21}Glu_{1.11}Gly_{2.08}Pro_{2.72}Tyr_{1.11}Arg_{1.64}Met_{1.16}His_{1.08}Phe_{1.00}Trp_{0.75}Ser_{0.91}Lys_{0.82}$ (average recovery 89%, corrected Glu 1.73).[21] Amino acid ratios in a LAP (highly purified, and almost free from the prolidase activity, Sigma Chem. Co. Lot 16B-0201) digest $Asp_{1.00}Glu_{1.00}Gly_{0.69}$ other amino acids were negligible). *Anal.* Calcd. for $C_{98}H_{138}O_{29}N_{28}S \cdot 3CH_3COOH \cdot 7H_2O$: C, 49.8; H, 6.6; N, 15.6. Found: C, 50.1; H, 6.5; N, 15.1.

In the first column chromatography, the fraction I (15 mg, 17%) was shown by paper electrophoresis as a mixture of the starting material and the desired compound. When the column was further eluted with 0.04M ammonium acetate and finally with 10% AcOH, very small two peaks were detected in each eluents. Both substances exhibited much faster mobility toward the cathode side than the fraction II, but were not further investigated because of their minor yields.

When the peptide, [17-N^{ε}-formyllysine]-monkey β-MSH, was incubated in 5% hydrazine acetate at 37° for 48 hr in the presence of thioglycolic acid, yield of the desired peptide, after the 2nd purification as stated above, was approximately 10% and recovery of the starting material was approximately 40%.

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