and saline-injected animals served as controls.[17]

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Synthesis and Biological Activity of Camel and Bovine β-Melanotropins

Simon Lemaire, Donald Yamashiro, and Choh Hao Li*

Hormone Research Laboratory, University of California, San Francisco, California 94143. *Received July 22, 1975*

Two natural occurring melanotropins, camel β_{C2}-MSH and bovine β-MSH, have been synthesized by improved solid-phase procedures. The coupling reaction of *tert*-butyloxycarbonylamino acids was achieved by using their preformed symmetrical anhydrides. The synthetic hormones were purified by gel filtration on Sephadex G-10 and G-25, chromatography on carboxymethylcellulose, and partition chromatography on Sephadex G-25 with final yields of 56 and 35% for β_{C2}-MSH and β_b-MSH, respectively. They were then shown to be identical with their natural hormones in amino acid analysis, paper electrophoresis, disc electrophoresis, thin-layer chromatography, enzymic digests, and bioassays. Bioassay data of these two synthetic melanotropins indicate that the replacement of Ser by Gly in β_b-MSH does not change its melanocyte-stimulating activity.

Recently, the complete amino acid sequences of two β-melanotropins from the camel (*Camelus dromedarius*) have been reported.[1] One of them, β_{C1}-MSH,[2,3] was synthesized[4] using standard procedures for solid-phase peptide synthesis.[5] In this paper, we report the solid-phase synthesis of β_{C2}-MSH with the following primary structure: H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH. Bovine β-MSH, which has already been synthesized by solution methods,[6] differs from β_{C2}-MSH only by its serine residue in position 2.[7] We decided to undertake its solid-phase synthesis in connection with our synthetic studies on ovine β-lipotropin.[8] Furthermore, the β-melanotropins afford a convenient opportunity to test recent improvements in solid-phase techniques.

Esterification of Boc-Asp(OBzl)-OH to the chloromethylated polymer was achieved following some modifications[4] of the Loffet procedure.[9] The resulting Boc-Asp(OBzl)-resin was subjected to the procedure for solid-phase peptide synthesis.[5] Coupling reactions were exclusively achieved by using symmetrical anhydrides of *tert*-butyloxycarbonylamino acids[10] preformed with dicyclohexylcarbodiimide.[11] The advantages of this method in the elimination of side products were recently pointed out.[8] All amino acids were protected on the α-amino position with the Boc group and the following side-chain blocking groups were used: Glu(OBzl), Asp(OBzl), Ser(Bzl), Lys(*o*-Br-Z),[12] Tyr(*o*-Br-Z),[13] Arg(Tos), Trp(HCO),[14] and His(Boc).[15] Removal of the N^{α}-Boc protecting group was carried out by treatment for 15 min with 50% trifluoroacetic acid in CH_2Cl_2.[16]

After completion of the syntheses, the fully protected β_{C2}-MSH resin was treated with 50% trifluoroacetic acid to remove the Boc group, whereas this group was not removed by this procedure in the protected β_b-MSH resin. The advantage in yield gained by this initial removal of the Boc group will be subsequently pointed out for β_{C2}-MSH. Evidence shows that this treatment can be used on methionine-containing peptides to avoid side products.[17]

The two peptides were cleaved from their respective resins and deprotected with liquid HF,[18-20] with the exception of the formyl-protecting group for tryptophan.[21] They were partly purified by gel filtration on Sephadex G-10 and G-25. Preliminary experiments demonstrated that the deformylation of tryptophan can be achieved very rapidly (ca. 3 min) at pH 11.5. The peptides deformylated in this manner were submitted to chromatography on CMC[22] as shown in Figures 1A and 2A. Further purification by partition chromatography[23] on Sephadex G-25 gave the results shown in Figures 1B and 2B. The highly purified synthetic β_{C2}-MSH and β_b-MSH were obtained in overall yields of 56 and 35%, respectively, based on starting Boc-Asp(OBzl)-resin. When the Boc group of the fully protected β_{C2}-MSH resin was not initially removed with the trifluoroacetic acid treatment, β_{C2}-MSH was subsequently isolated in only 32% yield. It may be noted that these yields represent improvements over our previous yields of 25% for β_{C1}-MSH where tryptophan was protected and 10% where this protection was not employed.[4]

The synthetic hormones were homogeneous on thin-layer chromatography and on paper and disc gel elec-

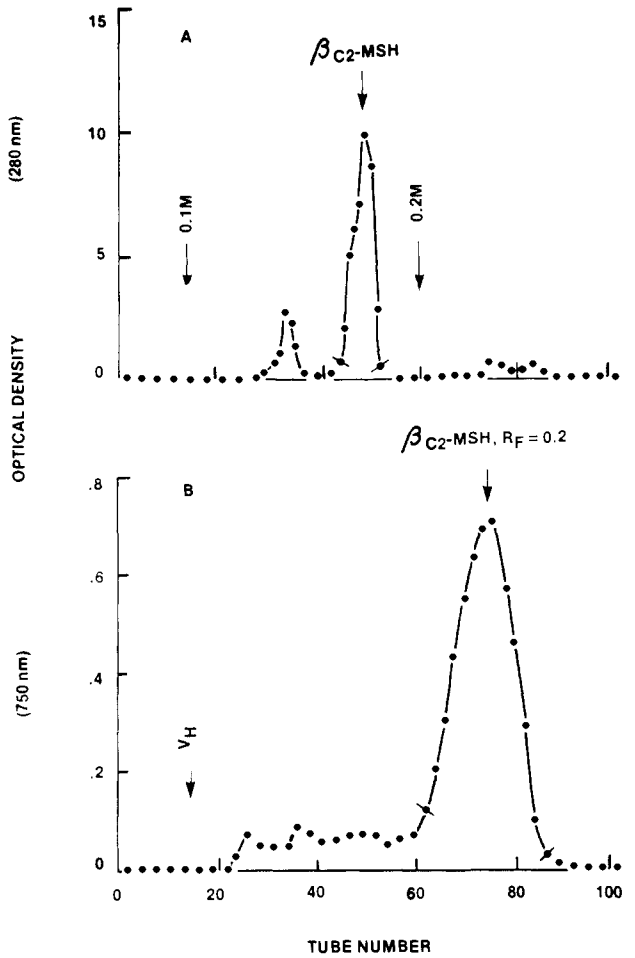

Figure 1. (A) Chromatography of synthetic β_{C_2}-MSH (158 mg) on CMC in NH_4OAc buffers. Fractions of 8.3 ml were collected. (B) Partition chromatography on Sephadex G-25 of synthetic β_{C_2}-MSH (112 mg) obtained from CMC (see A; hold-up volume = 63 ml; 4.2 ml/tube).

Table I. Amino Acid Analyses[a] of Synthetic β_{C_2}-MSH and β_b-MSH

Amino acid	Theoretical		Acid hydrolysis		Enzyme digest	
	Camel[b]	Bovine[c]	Camel	Bovine	Camel	Bovine
Lys	2	2	2.1	1.9	1.3	1.4
His	1	1	1.0	0.9	1.0	1.1
Arg	1	1	1.0	0.9	1.0	1.2
Asp	2	2	2.0	1.9	1.2	1.0
Ser	1	2	0.9	1.8	0.4	1.2
Glu	1	1	1.0	1.0	1.1	0.9
Pro	3	3	2.9	2.9	1.4	2.0
Gly	3	2	2.9	2.0	2.8	1.9
Met	1	1	1.0	1.0	1.0	1.0
Tyr	1	1	1.1	1.0	1.0	1.0
Phe	1	1	1.0	1.0	1.0	1.0
Trp	1	1	1.0[d]	1.0[d]	1.0	1.0

[a] Values in molar ratio. [b] Taken from Li et al.[1] [c] Taken from Geschwind et al.[7] [d] Tryptophan determinations were carried out by the method of T. Y. Liu and Y. H. Chang, *J. Biol. Chem.*, 246, 2842 (1971).

trophoreses; their behavior in these tests was identical with that of the corresponding natural hormones. In addition, paper electrophoresis of tryptic and chymotryptic digests of both synthetic products gave patterns identical with those of the respective natural hormones. Amino acid analyses of acid and enzymic hydrolysates of the synthetic products gave values in agreement with those expected

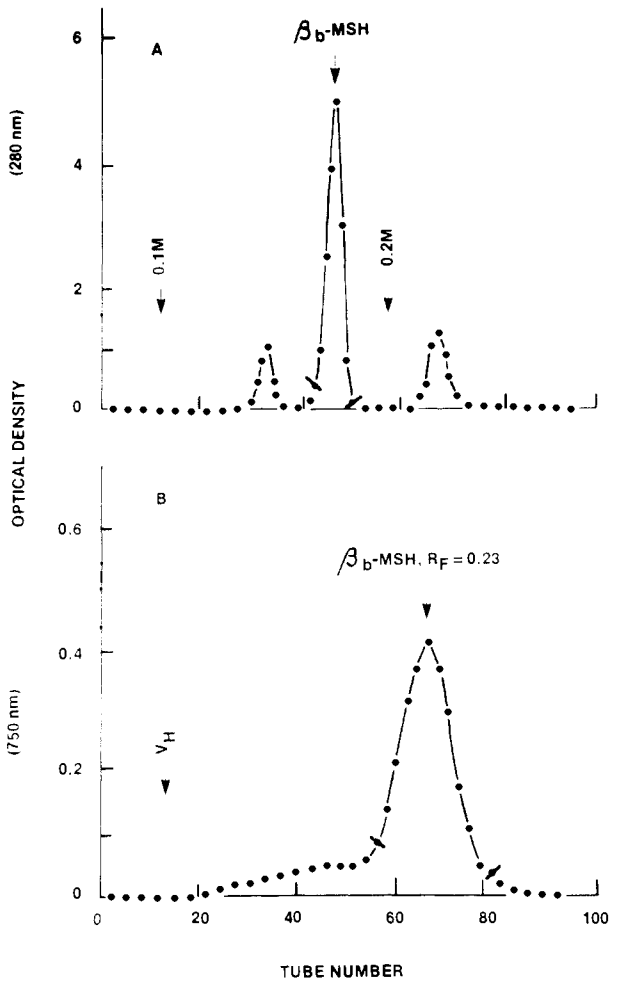

Figure 2. (A) Chromatography of synthetic bovine β-MSH (75 mg) on CMC in NH_4OAc buffers. Fractions of 8.3 ml were collected. (B) Partition chromatography on Sephadex G-25 of synthetic β_b-MSH (41 mg) obtained from CMC (see A; hold-up volume = 63 ml; 4.0 ml/tube).

Table II. Melanocyte Stimulating Activity of Synthetic Camel and Bovine β-Melanotropins

Preparation	Dose, ng	Response[a]	Rel potency %	Rel potency 95% confidence limit	Rel potency λ
Natural β_{C_2}-MSH	1.8	28.4 ± 3.8			
	5.4	53.0 ± 5.5			
Synthetic β_{C_2}-MSH	2.7	36.4 ± 2.9	87.8[b]	54-156	0.19
	8.1	56.5 ± 5.3			
Synthetic β_b-MSH	2.7	41.9 ± 6.8	105.6[b]	58-291	0.24
	8.1	56.2 ± 5.8			
Natural β_b-MSH	2.3	29.1 ± 3.7			
	6.9	44.6 ± 2.0			
Synthetic β_b-MSH	2.7	30.1 ± 3.7	120.2[c]	81-186	0.16
	8.1	49.6 ± 1.6			

[a] MSH activity expressed as percent decrease in reflectance. Four skins were used for each assay. Values in mean ± SE. [b] Compared with the potency of the natural camel β-MSH. [c] Compared with the potency of natural bovine β-MSH.

(Table I). The analyses of the enzymic digests show low values in positions 14–18, but the resistance of this sequence to enzymic attack has previously been noticed.[4,24]

Table II presents the melanotropic potency of the synthetic and natural β_{C2}-MSH and β_b-MSH. It is evident that the activities of the synthetic products are identical

with those exhibited by the natural hormones. It is of interest to note in Table II that the melanotropic activity is nearly identical in βC2-MSH and βb-MSH. Apparently, the replacement of Ser by Gly in βb-MSH does not change its biological activity.

Experimental Section

Thin-layer chromatography was run on silica gel in the following solvent system: 1-butanol–pyridine–acetic acid–water, 6:6:1.2:4.8. Paper electrophoresis, disc electrophoresis, amino acid analysis, chromatography on CMC, and partition chromatography on Sephadex G-25 in the solvent system 1-butanol–pyridine–0.1% aqueous acetic acid (5:3:11) were performed as described previously.[4] Chloromethylated (0.69 mequiv/g) styrene–1% divinylbenzene (200–400 mesh) was obtained from Bio Rad Labs.

N^{α}-*tert*-Butyloxycarbonyl-β-benzyl-L-aspartyl Polymer. The esterification of the COOH-terminal amino acid to the chloromethylated resin was carried out as described[4] following some modifications[12] of the Loffet procedure.[9] A sample of the resulting Boc-Asp(OBzl)-resin was then hydrolyzed in propionic acid–12 M HCl[25] and amino acid analysis gave 0.31 mmol of Asp/g.

Symmetrical Anhydrides of *tert*-Butyloxycarbonylamino Acids. The reaction of *tert*-butyloxycarbonylamino acids with dicyclohexylcarbodiimide[11] was performed as follows. *tert*-Butyloxycarbonylamino acid (1.9 mmol) in 6 ml of CH_2Cl_2 was cooled to 0° and mixed with 1.6 ml of 0.6 M *N,N'*-dicyclohexylcarbodiimide in CH_2Cl_2. After stirring for 20 min at 0 °C, the precipitate of dicyclohexylurea was removed by filtration at 25 °C and washed with 2.4 ml of CH_2Cl_2. The filtrate was used immediately for the coupling reaction. Symmetrical anhydrides of Boc-Trp(HCO)-OH and Boc-Arg(Tos)-OH were prepared in 13 and 17% dimethylformamide in CH_2Cl_2, respectively.

Protected βC2-MSH Resin. Starting with Boc-Asp(OBzl)-resin (1 g, 0.31 mmol), the following cycle was carried out: (1) wash with three 15-ml portions of CH_2Cl_2 (retention volume of the resin for CH_2Cl_2 was 5 ml after filtration); (2) removal of the Boc group with 50% trifluoroacetic acid in CH_2Cl_2 for 15 min; (3) wash with two 15-ml portions of CH_2Cl_2; (4) wash with two 15-ml portions of 50% dioxane in CH_2Cl_2; (5) wash with two 15-ml portions of CH_2Cl_2; (6) 5 min of neutralization with 15 ml of 5% diisopropylethylamine in CH_2Cl_2; (7) wash with six 15-ml portions of CH_2Cl_2; (8) add the solution of preformed symmetrical anhydride of *tert*-butyloxycarbonylamino acid (3 equiv of anhydride from 1.9 mmol of *tert*-butyloxycarbonylamino acid and 0.96 mmol of *N,N'*-dicyclohexylcarbodiimide) and shake for 30 min; (9) add 1.06 ml of 5% diisopropylethylamine in CH_2Cl_2 (0.31 mmol) and shake for another 10 min;[8] (10) wash with three 15-ml portions of CH_2Cl_2; (11) wash with three 15-ml portions of absolute ethanol. This cycle was performed for each of the remaining 17 amino acid residues. The yield of the protected octadecapeptide resin was 2.26 g.

βC2-MSH. A sample of the protected octadecapeptide resin (500 mg) was submitted to the first seven steps of the cycle for the synthesis just described, washed with three 15-ml portions of absolute ethanol, and dried in vacuo over P_2O_5 for 1 h. The resin was then mixed with 1.0 ml of anisole and 15 ml of liquid HF and stirred for 1 h at 0 °C. After the evaporation of HF with a stream of nitrogen, the oily residue was washed with two 15-ml portions of ethyl acetate, and the resin was extracted with three 15-ml portions of 50% acetic acid and filtered off. The combined filtrates were evaporated in vacuo to a small volume (3–5 ml) and submitted to gel filtration on Sephadex G-10 (2 × 25 cm column) in 0.5 N acetic acid. The material in the single peak (280-nm detection) was recovered by lyophilization (190 mg) and submitted to chromatography on Sephadex G-25 (2.5 × 136 cm column) in 0.5 N acetic acid. This chromatograph also gave a single peak, yielding 158 mg. For deformylation of Trp, the material was put into 10 ml of water and 5 ml of 0.1 N NaOH was added, increasing the pH to 11.5. The solution was allowed to stay at this pH for 3 min and then rapidly acidified with glacial acetic acid to pH 4.5. Completion of the reaction was verified by ultraviolet absorption.[26] The solution was then diluted with 15 ml of water and submitted to chromatography on CMC (Figure 1A). Isolation of the material from the main peak (280-nm detection) gave 112 mg. Further purification of this material was achieved by partition chromatography as shown in Figure 1B. Peptide material was detected by the Folin–Lowry reaction.[27] Isolation of the material represented by the major peak (R_f 0.2) gave 89 mg of βC2-MSH [peptide content 91% by ultraviolet absorption spectra; 56% yield based on the starting Boc-Asp(OBzl)-resin].

On thin-layer chromatography, the synthetic and natural[1] hormones (100-μg samples) each gave a single spot (ninhydrin detection) with an R_f value of 0.3. Paper electrophoresis of both synthetic and natural hormones (100-μg samples) gave a single spot with identical R_f values (relative to Lys) of 0.48 at pH 3.7 and 0.26 at pH 6.9. On gel electrophoresis, the synthetic product behaves exactly as the natural hormone, a single band being observed. Samples (0.15 mg) of the synthetic hormone were treated separately with 3 μg each of trypsin and chymotrypsin in 35 μl of Tris buffer (pH 8.5, 0.01 M Mg^{2+}) at 37 °C for 5 h. The natural hormone was treated in the same manner. Paper electrophoresis of the tryptic and chymotryptic digests (10-μl aliquots) was run at pH 3.7 and 6.9, respectively, with ninhydrin used for detection. Amino acid analyses of enzymic (first with trypsin and chymotrypsin and then leucine amino peptidase) and acid hydrolysates of the synthetic material gave the values shown in Table I.

Bovine β-MSH. Starting with 1 g of Boc-Asp(OBz1)-resin (0.31 mmol), synthesis of bovine β-MSH was carried out as with βC2-MSH, yielding 2.31 g of protected octadecapeptide resin. The Boc group was not removed by the trifluoroacetic acid treatment, but the fully protected peptide resin (1-g sample) was taken directly to the HF procedure and worked up as described for βC2-MSH. After the gel filtration on Sephadex G-10, 375 mg of peptide material was isolated. Further gel filtration on Sephadex G-25 gave 305 mg. A portion (75 mg) was subjected to the deformylation procedure previously described and then submitted to chromatography on CMC (Figure 2A). The resulting product (41 mg) was finally purified by the partition chromatography (Figure 2B) to give 27 mg of highly purified synthetic bovine β-MSH [peptide content 90% by ultraviolet absorption spectra; 35% yield based on starting Boc-Asp(OBzl)-resin].

Thin-layer chromatography of the synthetic product (100-μg sample) gave a single spot (R_f 0.34) corresponding to the natural hormone.[7,28] Paper electrophoresis at both pH 3.7 and 6.9 showed a single spot with respective R_f values (relative to Lys) of 0.5 and 0.25, identical with those of the natural hormone. On gel electrophoresis the synthetic product behaved exactly as the natural hormone, a single band being observed. Amino acid analyses of both acid and enzymic hydrolysates of the synthetic product gave values shown in Table I. Paper electrophoresis of tryptic and chymotryptic digests was performed as described in the previous section.

The melanocyte-stimulating activity was determined in vitro by the frog skin assay[29,30] using the four-point design.

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Synthesis and Some Pharmacological Properties of [1-(L-2-Hydroxy-3-mercaptopropanoic acid),4-threonine]oxytocin (Hydroxy[4-Thr]oxytocin), a Peptide with Strikingly High Oxytocic Potency, and of [1-(L-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin (Hydroxy-oxytocin)

Maurice Manning,* John Lowbridge,

Department of Biochemistry, Medical College of Ohio at Toledo, Toledo, Ohio 43614

Jaya Haldar, and Wilbur H. Sawyer

Department of Pharmacology, College of Physicians and Surgeons of Columbia University, New York, New York 10032.
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[1-(L-2-Hydroxy-3-mercaptopropanoic acid),4-threonine]oxytocin (hydroxy[4-Thr]oxytocin) and [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin (hydroxy-oxytocin) were synthesized by a combination of solid phase and classical methods of peptide synthesis. Protected octapeptides were synthesized by the solid-phase method and 1 + 8 couplings in solution were then employed to furnish the required key protected intermediates. Hydroxy[4-Thr]oxytocin has oxytocic potency, as measured in the rat uterus suspended in a Mg^{2+}-free solution, of about 4200 units/mg, eight times the potency of oxytocin, while its antidiuretic potency is approximately equal to that of oxytocin. It thus exhibits a significantly favorable oxytocic–antidiuretic selectivity. Hydroxy-oxytocin has an oxytocic potency of approximately 1300 units/mg, 2.5 times that of oxytocin. Threonine substitution in hydroxy-oxytocin has thus caused a significant enhancement in both oxytocic potency and selectivity. The enhancement in oxytocic potency of these two peptides relative to oxytocin and [4-Thr]oxytocin appears to correlate with their lipophilic characteristics, suggesting a significant role of lipophilicity in the interplay of oxytocin-like peptides with oxytocic receptors.

Among the many synthetic analogs of oxytocin reported to date only four are known in which a single substitution has brought about a significant enhancement of oxytocic potency relative to that of oxytocin. These substitutions and the resulting highly potent analogs are (1) replacement of the amino group at position 1 by (a) hydrogen, deamino-oxytocin[1-3] or (b) a hydroxy group, [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin (hydroxy-oxytocin);[4-6] (2) substitution of threonine for glutamine at position 4, [4-Thr]oxytocin;[7,8] and (3) replacement of the sulfur atom of the cysteine residue at position 1 with a methylene group, [6-1-cystathionine]oxytocin ([carba-1]oxytocin).[9] These modifications also changed the oxytocic–pressor and oxytocic–antidiuretic ratios of the above peptides relative to those of oxytocin. The oxytocic–pressor ratio was enhanced in all peptides with the exception of hydroxy-oxytocin which exhibited a substantially diminished ratio. The antidiuretic potency of hydroxy-oxytocin has not been reported but of the three remaining peptides only [4-Thr]oxytocin exhibited diminished antidiuretic activity. Thus of these four highly potent oxytocic agents, [4-Thr]oxytocin alone possesses the added highly desirable feature of enhanced oxytocic–antidiuretic selectivity (Table I).

Combining two or more of the above four modifications in a single peptide suggested the attractive possibility of further enhancing (a) oxytocic potency and (b) oxytocic–antidiuretic selectivity. Only two such peptides had been reported prior to the present study and their pharmacological evaluation had resulted in surprising and somewhat inconsistent findings.

On the one hand, deamino[4-Thr]oxytocin,[10] which