Synthesis and Biological Activity of β-Melanotropins and Analogues

Simon Lemaire, Donald Yamashiro, A. Jagannadha Rao, and Choh Hao Li*

Hormone Research Laboratory, University of California, San Francisco, California 94143. *Received June 1, 1976*

Synthetic [Arg^8]-, [Gly^{10}]-, and [Phe^{12}]-β_{c1}-MSH and β_p-MSH have been prepared by the solid-phase method. Their lipolytic and melanotropic activities have been compared to each other and with those of previously synthesized β_{c1}-MSH, [formyl-Trp^{12}]-β_{c1}-MSH, and β_b-MSH. Replacement of Gln^8 of β_{c1}-MSH by Arg causes a greater decrease in melanotropic activity than in lipolytic activity. However, when Phe^{10} of β_{c1}-MSH is replaced by Gly, both activities are destroyed. Alteration or replacement of Trp^{12} of β_{c1}-MSH by formyl-Trp or Phe has little effect on the melanotropic activity while the lipolytic activity is greatly decreased. Replacement of Ser^2 of β_b-MSH by Glu induces a threefold increase in melanotropic activity but it does not affect the lipolytic activity.

β-Melanotropins have been isolated from different species and their structures determined.[1] However, relatively little work has been done to examine structure–function relationships in β-MSH.[2] We decided to test the importance of residues in positions 2, 8, 10, and 12 in β_{c1}-MSH (Figure 1)[3] with regard to their melanotropic and lipolytic activities. Results of these studies are described herein.

Experimental Section

Thin-layer chromatography was run on silica gel with the solvent system: 1-butanol–pyridine–acetic acid–water (6.0:6.0:1.2:4.8, v/v). Gel electrophoresis was performed at pH 4.5 in 7% polyacrylamide.[4] Paper electrophoresis was run on Whatman paper No. 3 MM at 400 V for 4 h in the following buffers: pyridine–acetate, pH 3.7, and collidine–acetate, pH 6.9. Amino acid analysis was carried out by the method of Spackman et al.[5] and Trp was determined spectrophotometrically.[6] Chromatography on CMC (1 × 55 cm column) and partition chromatography[7] on Sephadex G-25 (1.9 × 56 cm column) in the solvent system 1-butanol–pyridine–0.1% aqueous acetic acid (5:3:11, v/v) were performed as described.[3] Chloromethylated (0.69 mequiv/g) styrene–1% divinylbenzene was purchased from Bio-Rad Labs. Attachment of Boc-Asp(Bzl)-OH to the resin was carried out as described[3] following the Loffet procedure.[8] A substitution of 0.36 mmol of Asp/g of resin was then obtained.

Protected [Arg^8]-β_{c1}-MSH Polymer. Starting with 0.7 g of N^α-Boc-β-Bzl-Asp resin, synthesis of protected [Arg^8]-β_{c1}-MSH was achieved as described[9] following some modifications[10] of the standard procedure for solid-phase peptide synthesis.[11] The N^α-Boc protecting group was removed by a 15-min treatment with 50% trifluoroacetic acid in CH_2Cl_2. For neutralization, the resin was treated with 5% diisopropylethylamine for 5 min. The coupling reaction was carried out by shaking the resin in the presence of a preformed symmetrical anhydride of *tert*-butyloxycarbonylamino acid[12] (ca. 0.75 mmol of anhydride from 1.55 mmol of *tert*-butyloxycarbonylamino acid and 0.75 mmol of dicyclohexylcarbodiimide). After 30 min of coupling, 0.25 mmol of diisopropylethylamine was added to the mixture and the resin was shaken for another 10 min.[10] The following side-chain protecting groups were employed: tosyl for Arg, benzyl for Ser and Asp, *o*-bromobenzyloxycarbonyl for Lys and Tyr,[13] formyl for Trp,[14] and Boc for His.[15] The yield of the completed octadecapeptide polymer was 1.55 g.

[Arg^8]-β_{c1}-MSH. A sample (0.7 g) of the protected peptide resin was mixed with 15 ml of liquid HF[16,17] containing 2.1 ml of anisole and stirred for 1 h at 0 °C. After evaporation of HF with a stream of nitrogen, the oily residue was washed with two 15-ml portions of ethyl acetate and the peptide was extracted from the resin with three 15-ml portions of 50% acetic acid. The extract was then evaporated to a small volume (2–5 ml) and chromatographed on Sephadex G-10 (2 × 40 cm column) in 0.5 N acetic acid. The single peak (280 nm detection) was collected and lyophilized, yielding 271 mg of material. Chromatography of this material on Sephadex G-25 (2.5 × 136 cm column) in 0.5 N acetic acid also gave a single peak (280 nm detection) with a recovery of 205 mg. For deformylation, a sample (100 mg) was treated[14] with 100 ml of 1 M NH_4HCO_3 at pH 9.0 for 24 h at 25 °C. The reaction was terminated by lyophilization; completion of the reaction was verified by ultraviolet absorption.[18] Chromatography of this material on CMC gave a main peak (280 nm detection) flanked by two small peaks. The main peak was collected and lyophilized to give 80 mg of material. This latter product was then submitted to partition chromatography on Sephadex G-25. A sharp peak (Folin–Lowry detection[19]) was eluted (R_f = 0.2) yielding 61.5 mg (peptide content 85% based on ultraviolet spectrum) of highly purified [Arg^8]-β_{c1}-MSH (ca. 45% yield based on starting N^α-Boc-β-Bzl-Asp resin).

It may be noted that the Boc group of the protected peptide resin was not removed before the HF treatment in spite of the fact that this has been recommended as a means of avoiding *tert*-butylation of the methionine residue.[20] However, procedures for reversing this alkylation were described and one of these is the process of lyophilization in a vessel exposed to ambient temperatures (ca. 25 °C). The [Arg^8]-β_{c1}-MSH was subjected to repeated lyophilization during the purification process.

Amino acid analysis of the purified material gave the values shown in Table I. On gel electrophoresis, the purified material (100-μg sample) gave a single band (detection with Amido-Schwarz dye). Paper electrophoresis at both pH 3.7 and 6.9 gave a single spot (ninhydrin detection) (Table II). On thin-layer chromatography, this material (50 μg) migrated as one spot (ninhydrin detection) (Table II).

[Gly^{10}]- and [Phe^{12}]-β_{c1}-MSH and β_p-MSH. Synthesis and purification of [Gly^{10}]-β_{c1}-MSH, [Phe^{12}]-β_{c1}-MSH, and β_p-MSH were achieved following the same procedures as those already described for the synthesis of [Arg^8]-β_{c1}-MSH. Characterizations of these products were also obtained by amino acid analyses (Table I), partition chromatography on Sephadex G-25 (Table II), thin-layer chromatography (Table II), paper electrophoresis at both pH 3.7 and 6.9 (Table II), and gel electrophoresis.

The melanotropic activity of the synthetic peptides was determined in vitro by the frog skin method.[21,22] Measurement of the lipolytic activity was performed in isolated rabbit fat cells as previously described.[23]

Results

The melanotropic and lipolytic activities of synthetic β-melanotropins and analogues are summarized in Tables III and IV. It may be noted in Table III that replacement of Gln^8 of β_{c1}-MSH by Arg induced a large decrease in its melanotropic activity to only 10.5%.

The ineffectiveness of [Gly^{10}]-β_{c1}-MSH in stimulating frog skin darkening was also observed. However, [formyl-Trp^{12}]-β_{c1}-MSH retained 39.7% of the melanotropic activity of the parent peptide and replacement of Trp^{12} of β_{c1}-MSH by Phe also gave a fairly active compound (28% compared with the activity of the parent peptide). Finally, β_p-MSH was three times more active than β_b-MSH with regard to melanotropic activity as noted previously.[1] The only difference between these two molecules is that residue number 2 of β_b-MSH is Ser instead of Glu for β_p-MSH (Figure 1).

As shown in Table IV, the lipolytic activity of synthetic β_{c1}-MSH was found to be 2.2 times higher than that of β_b-MSH. Replacement of Gln^8 of β_{c1}-MSH by Arg lowered the activity to 34.2% of the original value for β_{c1}-MSH.

β_{c1}-MSH: H-Asp-Gly-Gly-Pro-Tyr-Lys-Met-Gln-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH
1 5 10 15 18

β_p-MSH: H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH

β_b-MSH: H-Asp-Ser-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH

Figure 1. Amino acid sequence of β_{c1}-MSH, β_p-MSH, and β_b-MSH.

Table I. Amino Acid Analyses of Various Synthetic β_{c1}-Melanotropin Analogues and β_p-Melanotropin

	[Arg^8]-β_{c1}-MSH		[Gly^{10}]-β_{c1}-MSH		[Phe^{12}]-β_{c1}-MSH		β_p-MSH	
Amino acid	Calcd	Found	Calcd	Found	Calcd	Found	Calcd	Found
Lys	2	2.2	2	2.1	2	2.1	2	2.1
His	1	1.1	1	1.0	1	1.0	1	1.0
Arg	2	2.0	1	1.1	1	1.0	1	1.0
Asp	2	1.8	2	2.0	2	1.9	2	2.1
Ser	1	0.8	1	0.9	1	0.9	1	0.9
Glu	0	0	1	1.0	1	0.9	2	2.1
Pro	3	3.0	3	3.0	3	3.1	3	2.8
Gly	3	2.8	4	4.0	3	3.0	2	1.9
Met	1	0.9	1	1.0	1	1.0	1	0.9
Tyr	1	1.1	1	1.1	1	0.9	1	1.0
Phe	1	1.0	0	0	2	2.0	1	1.1
Trp	1	1.2	1	1.1	0	0	1	1.1

Table II. R_f Values of Synthetic β-Melanotropins and Analogues in Paper Electrophoresis and Partition and Thin-Layer Chromatography

Melanotropin	Partition chromatography[a]	Thin-layer chromatography[b]		Paper electrophoresis[c]	
		BPAW	BAW	pH 3.7	pH 6.9
β_{c1}-MSH	0.22	0.23	0.15	0.52	0.32
[Arg^8]-β_{c1}-MSH	0.20	0.16	0.14	0.63	0.38
[Gly^{10}]-β_{c1}-MSH	0.11	0.11	0.13	0.55	0.32
[Phe^{12}]-β_{c1}-MSH	0.18	0.20	0.19	0.54	0.32
β_p-MSH	0.15	0.21	0.25	0.42	0.14
β_b-MSH	0.23	0.26	0.20	0.48	0.25

[a] Sephadex G-25; solvent system, 1-butanol-pyridine-0.1% aqueous acetic acid (5:3:11, v/v). [b] Solvent systems: BPAW, 1-butanol-pyridine-acetic acid-water (6.0:6.0:1.2:4.8); BAW, 1-butanol-acetic acid-water (4:3:3). [c] 400 V for 4 h.

Changes in positions 10 and 12 of β_{c1}-MSH appeared to have a more dramatic effect on lipolytic activity than changes in position 8. [Formyl-Trp^{12}]-β_{c1}-MSH retained only 10.2% of the activity of the parent peptide while [Gly^{10}]- and [Phe^{12}]-β_{c1}-MSH had no lipolytic activity. Alteration in position 2 of β-melanotropin did not seem to modify its lipolytic activity, β_b-MSH being as active as β_p-MSH.

Discussion

Alterations in Trp^{12} of β_{c1}-MSH greatly affect its lipolytic activity (Table IV). These results are in good agreement with previous data which indicated that modification or replacement of Trp^9 in ACTH greatly diminished its lipolytic activity in rat fat cells.[24] Our results show that an alteration of Trp^{12} as small as its formylation drops the lipolytic activity of β_{c1}-MSH to 10% of its original value, and we must not exclude the possi-

Table III. Melanotropic Activity of Synthetic β-Melanotropins and Analogues

Melanotropin	Dose, ng	Response[a]	Potency		
			%	95% confidence limit	λ
β_{c1}-MSH	3	32.7 ± 3.6			
	9	63.5 ± 1.1			
[Arg^8]-β_{c1}-MSH	6	17.1 ± 2.8	10.5[b]	5.2-16.5	0.12
	18	24.6 ± 1.7			
[Formyl-Trp^{12}]-β_{c1}-MSH	6	32.8 ± 6.2	39.7[b]	29.4-53.6	0.12
	18	52.4 ± 1.3			
β_{c1}-MSH	6	62 ± 1.7			
	18	71 ± 0.4			
[Gly^{10}]-β_{c1}-MSH	6	5.5 ± 1.7	0.8[b]	0.01-3.8	0.20
	18	7.8 ± 0.8			
[Phe^{12}]-β_{c1}-MSH	6	26.2 ± 3.4	28[b]	6.8-123	0.27
	18	38.2 ± 5.4			
Natural β_b-MSH	2.3	29.1 ± 3.7			
	6.9	44.6 ± 2.0			
β_b-MSH	2.7	30.1 ± 3.7	120.2[c]	81.2-186.2	0.16
	8.1	49.6 ± 1.6			
β_p-MSH	2.6	49.6 ± 3.4	355.6[c]	251.5-604.0	0.12
	7.8	69.8 ± 1.1			

[a] MSH activity expressed as percent decrease in reflectance. Five skins used for each assay. Values in mean ± standard error. [b] Compared with synthetic β_{c1}-MSH. [c] Compared with natural β_b-MSH.

Table IV. Lipolytic Activity of Synthetic Melanotropins and Analogues

Synthetic	Dose, ng	Response,[a] ng	Potency %	Potency 95% confidence limit	Potency λ
β_{c1}-MSH	37	2.94 ± 0.03			
	111	3.60 ± 0.15			
β_b-MSH	37	1.78 ± 0.05	40.2[b]	26.3-55.2	0.09
	111	2.96 ± 0.15			
[Arg[8]]-β_{c1}-MSH	37	1.73 ± 0.10	34.2[b]	21.9-46.7	0.09
	111	3.12 ± 0.15			
[Formyl-Trp[12]]-β_{c1}-MSH	37	1.09 ± 0.23	10.2[b]	0.1-23.5	0.17
	111	2.05 ± 0.19			
[Phe[12]]-β_{c1}-MSH	1100	1.29 ± 0.09	0.006[b]	0-0.034	0.26
	3300	1.63 ± 0.29			
[Gly[10]]-β_{c1}-MSH	1100	0.19 ± 0.04	0.001[b]	0-0.00012	0.16
	3300	0.37 ± 0.05			
Natural β_b-MSH	22	1.38 ± 0.1			
	66	3.76 ± 0.1			
β_b-MSH	22	1.18 ± 0.1	95.5[c]	93.3-125.9	0.03
	66	3.61 ± 0.1			
β_p-MSH	22	1.62 ± 0.1	102.4[c]	85.1-107.2	0.05
	66	3.49 ± 0.1			

[a] Micromole of glycerol production per gram of cells per hour. Determinations in triplicate. Values in mean ± standard error. [b] Compared with β_{c1}-MSH. [c] Compared with natural β_b-MSH.

bility that this low level of activity of [formyl-Trp[12]]-β_{c1}-MSH might be due to some β_{c1}-MSH arising from deformylation during either the purification or the lipolytic assay. Finally, replacement of Trp[12] by Phe completely destroyed the lipolytic activity (Table IV). On the other hand, [Phe[12]]-β_{c1}-MSH retained 28% of the melanotropic activity of the parent peptide (Table III). Thus, the structural requirements of these two activities in the β-melanotropin molecule are different.

The structural requirements for melanotropic activity of β-melanotropins showed other differences from those for lipolytic activity. The melanotropic activity of β_{c1}-MSH was dependent upon the integrity of Gln[8], while the lipolytic activity depended more on that of Trp[12] (Tables III and IV). It may also be noted that changes at position 2 of β-MSH affect the melanotropic activity while the lipolytic activity is untouched. Therefore, the postulate that the melanotropic action of peptides related to ACTH should correlate with their ability to stimulate lipolysis in rabbit fat cells[25] may not be entirely true for β-MSH.

Our findings that [Gly[10]]-β_{c1}-MSH has neither lipolytic nor melanotropic activities pinpoint the importance of Phe[10] of β_{c1}-MSH. With regard to previous results which indicated that replacement of Phe[7] of ACTH-(1-24) by Leu gives a compound with 20% of the steroidogenic activity of the parent peptide,[26] one might speculate that the hydrophobicity of the side chain in this position is involved in its biological activity.

Draper et al.[27,28] have recently synthesized various analogues of ACTH-(4-10) by modification of Glu at position 5 (corresponding to position 8 of β-MSH). They found that replacement of Glu[5] by basic amino acids (Arg or Lys) gives heptapeptides from five to ten times more active than the parent peptide in stimulating lipolysis in rabbit fat cells. Our results demonstrated that replacement of Gln[8] by Arg in β_{c1}-MSH decreases both lipolytic and melanotropic activities (Table III and IV). This discrepancy may be explained by the difference in the length of the molecules tested and suggests that structure–activity studies with only the active part or core of a hormone cannot always be applied to the whole molecule.

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References and Notes

(1) H. Yajima and Y. Kiso, *Pharmacol. Ther., Part B*, 1, 529–543 (1975).
(2) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (1972). Other abbreviations are MSH, melanotropic hormone, melanotropin; β_{c1}-MSH, camel β-melanotropin (structure number 1); β_b-MSH, bovine β-melanotropin; β_p-MSH, porcine β-melanotropin; ACTH, adrenocorticotropic hormone; NPS, 2-nitrophenylsulfenyl; CMC, carboxymethylcellulose.
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Notes

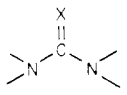
Syntheses and Pharmacological Activity of Substituted Imidazolidinethiones and Thioimidazolines

Harold Kohn,*[1a] Barbara A. Kohn,[1b] Marie Louise Steenberg,[1b] and Joseph P. Buckley*[1b]

Department of Chemistry and College of Pharmacy, University of Houston, Houston, Texas 77004. *Received June 7, 1976*

A series of imidazolidinethiones and thioimidazolines was synthesized and tested for their effects on both forced and spontaneous motor activity as well as for their ability to raise the convulsion threshold. The proton NMR spectra for the thioimidazolines synthesized were unusual in that they showed a sharp singlet for the ring ethylene unit rather than the expected A_2B_2 pattern. The thioimidazolines, **5** and **7**, were the most active CNS depressants and had the highest safety index. Significantly, the isomeric imidazolidinethiones, **8** and **9**, were comparatively much less effective while being considerably more toxic.

In recent years an increasingly larger number of pharmacologically active agents have appeared which contain either the ureido, **1**, or the corresponding isoureido, **2**, moiety.[2] Many of these compounds possess potent



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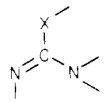

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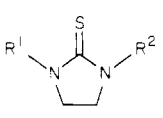
central nervous system (CNS) depressant and anticonvulsant properties.[2] There have been few studies, however, which compare the pharmacological properties of simple compounds which contain the ureido group with those which contain the isoureido group. Two classes of compounds, potentially isomeric to each other and containing one or the other of these functional groups, are the imidazolidinethiones, **3**, and thioimidazolines, **4**. It is worthwhile to note that provided both R^1 and R^2 are either alkyl or aryl groups, tautomerism between **3** and **4** is unlikely and, therefore, the distinction between **3** and **4**



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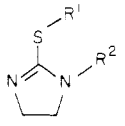

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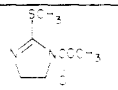
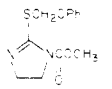
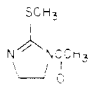
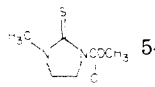
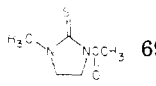
becomes less difficult.[3]

In relation to a current project dealing with the mechanism of biotin catalysis, a series of imidazolidinethiones, **3**, and thioimidazolines, **4**, was synthesized as model substrates.[4] In addition to our mechanistic studies, we felt that it would be desirable to evaluate some of these substrates for biological activity in male Swiss–Webster mice and male Wistar descendant rats. In this communication we report our preliminary findings which indicate that the thioimidazolines, **4**, exhibit more pronounced CNS depressant activity than the corresponding isomeric imidazolidinethiones, **3**.

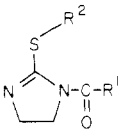
Chemistry. The five compounds, **5–9**, that were initially chosen for pharmacological evaluation are listed in Table I.

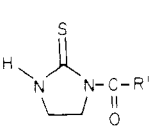
Table I

No.	Substrate	% yield	Mp, °C	ν max[a]	δ[b] $-CH_2CH_2-$
5		61	103.5-105.5	1715	3.92
6		68	153.5-155.5	1715	3.87
7		38	112-113.5	1670	3.97
8		54	111-112.5	1745	3.44-4.23
9		69	81-82	1665	3.40-4.25

[a] C=O infrared absorption of carbomethoxy or acetyl group. [b] ^1H NMR chemical shift value for imidazoline ring protons.

Synthesis of **5–7** was accomplished by alkylation of either *N*-carbomethoxyimidazolidinethione (**10**) or *N*-acetylimidazolidinethione (**11**). *N*-Carbomethoxyimidazolidinethione (**10**) was prepared by the addition of methyl chloroformate to a CH_2Cl_2 solution of imidazolidinethione and pyridine. Subsequent alkylation of **10** with either MeI or α-bromoacetophenone in the presence of Et_3N gave **5** and **6**, respectively. Correspondingly, *N*-





5, $R^1 = OCH_3$; $R^2 = CH_3$
6, $R^1 = OCH_3$; $R^2 = CH_2C(=O)Ph$
7, $R^1 = CH_3$; $R^2 = CH_3$

10, $R^1 = OCH_3$
11, $R^1 = CH_3$

acetyl-2-methylthioimidazoline (**7**) was prepared by al-