Synthesis and Structure–Function Studies of Melanocyte Stimulating Hormone Analogues Modified in the 2 and 4(7) Positions: Comparison of Activities on Frog Skin Melanophores and Melanoma Adenylate Cyclase[1]

Victor J. Hruby,* Tomi K. Sawyer, Young C. S. Yang, Marvin D. Bregman,

Department of Chemistry, University of Arizona, Tucson, Arizona 85721

Mac E. Hadley, and Christopher B. Heward

Department of General Biology, University of Arizona, Tucson, Arizona 85721. *Received April 25, 1980*

The synthesis and purification of several analogues of the melanotropins with amino acid substitutions at the tyrosine-2 and methionine-4(7) positions are reported. The compounds synthesized included [4-norleucine]-α-MSH, [7-norleucine]-β_p-MSH, [2–3′,5′-diiodotyrosine]-α-MSH, [2-D-tyrosine]-α-MSH, and [2-phenylalanine,4-norleucine]-α-MSH. The biological activities of these derivatives were measured and compared on normal melanocytes (frog skins) and on transformed melanocytes (mouse melanoma adenylate cyclase), over the entire dose–response range. All compounds tested were full agonists in both assay systems but varied considerably in potency. The relative potencies in the frog skin assay (α-MSH = 1.0) were as follows: [Nle^7]-β_p-MSH (5.2) > [Nle^4]-α-MSH (2.3) > α-MSH (1.0) > [Phe^2,Nle^4]-α-MSH (0.80) > β_p-MSH (0.55) > [I_2-Tyr^2]-α-MSH (0.12) > [D-Tyr^2]-α-MSH (0.04). The relative potencies in the melanoma adenylate cyclase system were [Nle^7]-β_p-MSH (4.2) > β_p-MSH (2.2) > [Nle^4]-α-MSH (2.0) > α-MSH (1.0) ≈ [Phe^2,Nle^4]-α-MSH (0.9) > [I_2-Tyr^2]-α-MSH (0.40) > [D-Tyr^2]-α-MSH (0.20). There appears to be some differences in structural specificity at the melanotropin receptors of the two cell systems.

α-Melanocyte stimulating hormone (α-MSH, α-melanotropin) is a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) which is synthesized and secreted by the pars intermedia of the vertebrate pituitary.[2] This hormone stimulates melanin synthesis and melanosome movements within integumental melanocytes of vertebrates. Besides its affects on other skin chromatophores, it is postulated to play a role in a number of diverse physiological processes.[3]

MSH has a number of effects on mouse melanoma cells grown in tissue culture, including a stimulation of tyrosinase activity, changes in cellular morphology, and growth arrest of the cultured cells.[4,5] These effects appear to be mediated through the second messenger, cyclic 3′,5′-adenosine monophosphate (cAMP).[4] The mechanism by which α-MSH stimulates melanoma cell membrane adenylate cyclase is being investigated using a washed particulate membrane preparation developed in our laboratory.[6,7] We are particularly interested in determining the structural requirements for MSH activity on these cells and comparing these with the structural requirements on normal melanocytes. In addition, we wish to design a radioreceptor assay for MSH using mouse melanoma cell membrane preparations. Ideally, one would like to iodinate the Tyr-2 moiety in α-MSH or the Tyr-5 in β_p-MSH (H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH) for these studies, since it has been reported that iodinated β-MSH binds to melanoma cell MSH receptors.[8,9] However, using conventional methods, we have been unable to obtain *biologically* active α-MSH[10] or β-MSH[11] derivatives, and we have shown by structural investigation that the loss of biological activity was due in part to modification of the Met residues (Met-4 in α-MSH, Met-7 in β-MSH) of these hormones. In addition, the tyrosine residues also were modified under these iodination conditions.

Therefore, we have initiated structure–function studies to obtain an MSH analogue which could be effectively radiolabeled with iodine to high specific radioactivity and high biological activity. We have also investigated the comparative activity of these analogues using the in vitro frog skin bioassay which measures activity of normal melanocytes and in the in vitro melanoma adenylate cyclase assay which measures activity on these transformed cancer cells. We report here on the synthesis and biological activity of a number of α-MSH and β_p-MSH analogues with modifications at the tyrosine and methionine positions of the hormone (Figure 1).

Results and Discussion

According to present evidence, α-MSH contains two message sequences, Phe-Arg and Lys-Pro-Val, each of which can independently stimulate melanocytes.[12] The remaining N-terminal amino acid residues are considered to lack any of the structural components essential for melanotropic activity, but instead only potentiate the biological activity of the C-terminal region of the hormone. It might be expected, therefore, that modifications at the Met-4 and Tyr-2 position of α-MSH [and structurally comparable positions in β-MSH (Figure 1)] would not

(1) All optically active amino acids are of the L variety unless otherwise stated. Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1972)]. Other abbreviations include: α-MSH, α-melanocyte stimulating hormone, α-melanotropin; β_p-MSH, porcine β-melanocyte stimulating hormone, β-melanotropin; [I_2-Tyr^2]-α-MSH, [2–3′,5′-diiodotyrosine]-α-MSH; Nle, norleucine; TLC, thin-layer chromatography; 2,4-Cl_2-Z, [[(2,4-dichlorobenzyl)oxy]-carbonyl]; 2,6-Cl_2-Bzl, 2,6-dichlorobenzyl. Taken in part from the Ph.D Dissertations of Y. C. S. Yang, University of Arizona, 1979, and C. B. Heward, University of Arizona, 1980.

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Table I. Amino Acid Analysis of Melanotropin Analogues[a]

amino acid	melanotropin analogue				
	[Nle^4]-α-MSH	[D-Tyr^2]-α-MSH	[I_2-Tyr^2]-α-MSH	[Phe^2,Nle^4]-α-MSH	[Nle^7]-βp-MSH
Trp	0.90 (1.0)	1.00 (1.0)	0.92 (1.0)	0.90 (1.0)	0.90 (1.0)
Lys	1.00 (1.0)	1.07 (1.0)	1.04 (1.0)	1.04 (1.0)	1.96 (2.0)
His	0.90 (1.0)	0.90 (1.0)	0.90 (1.0)	0.87 (1.0)	0.98 (1.0)
Arg	1.01 (1.0)	0.91 (1.0)	1.05 (1.0)	1.08 (1.0)	1.06 (1.0)
Asp					1.94 (2.0)
Ser	1.72 (2.0)	1.70 (2.0)	1.67 (2.0)	1.82 (2.0)	0.95 (1.0)
Glu	0.99 (1.0)	0.97 (1.0)	1.00 (1.0)	1.08 (1.0)	1.90 (2.0)
Pro	1.07 (1.0)	1.01 (1.0)	1.03 (1.0)	1.06 (1.0)	3.08 (3.0)
Gly	1.06 (1.0)	1.08 (1.0)	1.06 (1.0)	1.06 (1.0)	2.20 (2.0)
Val	1.04 (1.0)	1.10 (1.0)	1.00 (1.0)	1.04 (1.0)	
Met		0.90 (1.0)	0.89 (1.0)		
Nle	0.97 (1.0)			0.96 (1.0)	0.97 (1.0)
Tyr	0.90 (1.0)	0.90 (1.0)	0.98 (1.0)		0.85 (1.0)
Phe	1.03 (1.0)	0.93 (1.0)	1.04 (1.0)	1.95 (2.0)	1.02 (1.0)

[a] Theoretical values in parentheses.

Table II. Analytical Data For Melanotropin Analogues

compd	$[\alpha]^{25}_{546}$ (c, solvent), deg	TLC R_f solv syst A	TLC R_f solv syst B	TLC R_f solv syst C	TLC R_f solv syst D	PE,[a] R_f vs. Arg
[Nle^4]-α-MSH (II)	−46 (0.44, 10% HOAc)	0.20	0.70	0.64		0.62
[D-Tyr^2]-α-MSH (III)	−56 (0.71, 10% HOAc)	0.19	0.67		0.46	0.57
[I_2-Tyr^2]-α-MSH (IV)	−38 (0.40, 10% HOAc)	0.46	0.89	0.64	0.30	0.48
[Phe^2,Nle^4]-α-MSH (V)	−56 (0.48, 10% HOAc)	0.23	0.67		0.65	0.63
[Nle^7]-βp-MSH (VII)	−77 (0.48, 10% HOAc)	0.10	0.20	0.43	0.40	0.47

[a] Paper electrophoresis (PE) was done using pyridine acetate buffer (pH 4.9) at 400 V for 5 h.

radically affect the biological activity of the hormone. However, as previously reported, we have found that the oxidants used in the iodination of α-MSH[11] and β-MSH[10] resulted in a severe loss of biological activity of these hormones. We, therefore, prepared the nearly isosteric norleucine-4 (Nle-4) and Nle-7 analogues of α-MSH and β-MSH, respectively, to examine the sensitivity of these analogues to the oxidative iodination conditions and for structure–function studies.

For the synthesis and purification of [Nle^4]-α-MSH and all other α-MSH analogues reported here, we have used a benzhydrylamine resin[13,14] and solid-phase synthesis procedures similar to those used in our recent synthesis of α-MSH.[15] The synthesis of [Nle^7]-β_p-MSH[1] utilized a normal chloromethylated Merrifield resin and solid-phase methods identical with those used in the synthesis of β-MSH[15] in our laboratory. Purification of all of the analogues was accomplished by gel filtration of Sephadex G-15 and by ion-exchange chromatography on carboxymethylcellulose. The homogeneity of each preparation was checked by amino acid analysis, thin-layer chromatography in a least three solvent systems, and by paper electrophoresis. The details of the analytical results are given under Experimental Section and in Table I and II. The classical frog skin bioassay method of Shizume et al.[16] was used to measure the biological activities of the analogues of normal melanocytes. The potencies of the analogues were examined over the entire dose–response range as previously reported for α-MSH[15] (see Experimental Section).

Table III. Relative Potencies[a] of Melanophore Stimulating Hormones and Related Analogues in the Frog Skin and Melanoma Adenylate Cyclase Assay Systems

compd	assay system: frog skin system	assay system: melanoma adenylate cyclase system
α-MSH	1.00	1.00
β_p-MSH	0.55	2.20
[Nle^4]-α-MSH	2.31	2.00
[Nle^7]-β_p-MSH	5.2	4.20
[I_2-Tyr^2]-α-MSH	0.12	0.40
[Phe^2,Nle^4]-α-MSH	0.82	0.90
[D-Tyr^2]-α-MSH	0.03	0.20

[a] Relative potency = concn of α-MSH at 50% response/concn of compd tested at 50% response.

As previously reported by others,[17] we have found the 4-norleucine analogue of α-MSH to be more active than the parent hormone in the frog skin bioassay system (Table III), having about twice the potency of α-MSH in this standard assay system. β_p-MSH has only about one-half the potency of α-MSH in the frog skin bioassay system as reported by others.[17,18] However, [Nle^7]-β_p-MSH has nearly 10 times the potency of β_p-MSH in this assay system (Table III). This makes [Nle^7]-β_p-MSH the most potent MSH analogue which has been studied thus far on this system. Although we have found that [Nle^4]-α-MSH and, to a lesser extent, [Nle^7]-β_p-MSH are resistant to oxidative inactivation by chloramine-T and sodium metabisulfite,[10,11] these peptides still lost most of their biological activity when NaI was present in the reaction mixture.[10] We have shown[11] that the loss of activity in these latter experiments

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I Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, (α-MSH)
1 2 3 4 5 6 7 8 9 10 11 12 13

II Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, [Nle^4]-α-MSH

III Ac-Ser-D-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, [$D\text{-}Tyr^2$]-α-MSH

IV Ac-Ser-I_2-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, [$I_2\text{-}Tyr^2$]-α-MSH

V Ac-Ser-Phe-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, [Phe^2,Nle^4]-α-MSH

VI H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Lys-Asp-OH, β_p-MSH
17 18

VII H-Asp-Glu-Gly-Pro-Tyr-Lys-Nle-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-

Lys-Asp-OH, [Nle^7]-β_p-MSH

Figure 1. Primary sequences of the melanotropins (α-MSH and β-MSH) and melanotropin analogues referred to in the text. The numbers correspond to amino acid residues in the sequence.

might be due, at least to some extent, by modification of the histidine and tryptophan residues of MSH under the conditions we examined. The above results led us to examine whether changes in the steric, lipophilic, and electronic properties of the tyrosine residue in α-MSH (such as would be produced by iodination) might also be responsible, at least in part, for the loss of biological activity of the peptide.

As indicated above, tyrosine is not in the "active site" of α-MSH[12,18] and its importance in determining the biological potency has not been previously evaluated, except to show that it was not essential to the biological activity of the hormone. Recently, Eberle and Hübscher[19] have shown that [2-3′,5′-diiodotyrosine]-α-MSH has about one-fourth the activity of the native hormone. Using our bioassay,[15] we find that [2–3′,5′-diiodotyrosine]-α-MSH, prepared by total synthesis, possessed about one-eighth the potency of the native hormone. Given the differences in assay conditions and the errors inherent in such biological systems agreement between the two laboratories is satisfactory. These independent studies reveal that iodination of the tyrosine residue per se, while causing a minor reduction in potency in the hormone, cannot account for the severe loss in biological activity by chloramine-T iodination of α-MSH.[11] In this regard, it is interesting that $\alpha\text{-MSH}_{3-13}$, in which the Tyr-2 (and Ser-1) residue has been completely removed from the MSH structure, has about one-seventh the activity of the native hormone.[17] It would appear that the stereoelectronic effects of diiodination of the Tyr-2 residue is about as equally effective as completely removing the tyrosyl residue in reducing the potency of the MSH molecule.

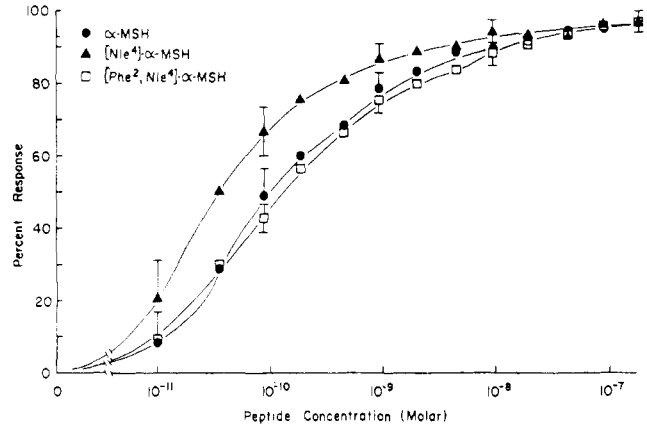

Figure 2. In vitro frog skin bioassay of α-MSH (●), [4-norleucine]-α-MSH (▲), and [2-phenylalanine,4-norleucine]-α-MSH (□). Dose–response curves were obtained by the sequential addition of increasing quantities of each peptide until a maximum response was obtained as described under Experimental Section. Each point represents the mean ± SE darkening response of five frog skins as measured by photoreflectance.

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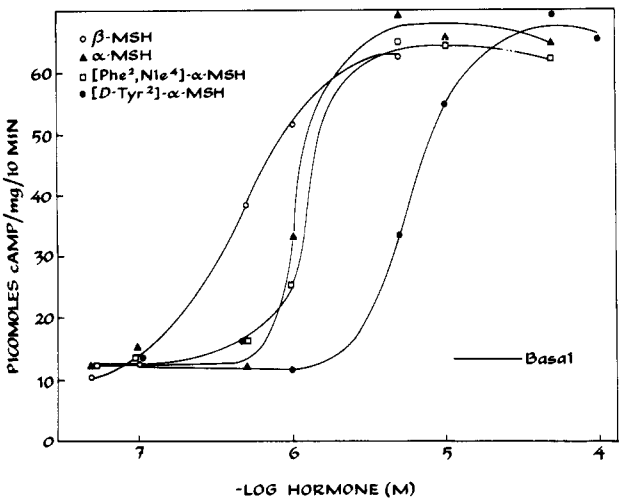

Figure 3. Dose–response of β-MSH (○), α-MSH (▲), [2-phenylalanine,4-norleucine]-α-MSH (□), and [2-D-tyrosine]-α-MSH (●) on melanoma adenylate cyclase. All results are obtained on the basis of 100 μg of protein per assay under standard conditions (see Experimental Section). Each point represents the mean value of triplicate determinations from duplicate experiments and all SE were less than 5%.

To test the importance of the phenol hydroxyl group of tyrosine in the biological activity of α-MSH, we prepared [2-phenylalanine,4-norleucine]-α-MSH. This compound was found to have about 80% the activity of α-MSH in the frog skin bioassay (Figure 2, Table III) and about 30% the activity of [Nle⁴]-α-MSH in this assay system (Table III). Clearly the phenol hydroxyl group of the tyrosine has a significant, though small, effect on the potency of the hormone and, therefore, is of some significance in the binding of the hormone to its receptors. However, this group is not important to the overall activity of the hormone, since [Phe²,Nle⁴]-α-MSH is a full agonist (Figure 2) in the frog skin assay system.

On the other hand, the configuration at the tyrosine-2 position in α-MSH seems to be of considerably greater significance. [2-D-Tyrosine]-α-MSH was prepared and was shown to have only about one-thirtieth the potency of α-MSH in the frog skin assay system (Table III). It is interesting that this configurational change decreases the activity of the hormone further than would have been the case if the Tyr-2 and Ac-Ser-1 residues had simply been left out in the synthesis of the peptide.[5] Presumably in the D configuration the Tyr-2 *side chain group* does not interact properly at the receptor, which leads to a marked decrease in binding for the analogue and, thus, a decrease in potency. However, there is no effect on the overall biological activity, since [D-Tyr²]-α-MSH is a full agonist.

Recently we reported on the preparation of a hormone-sensitive adenylate cyclase preparation from mouse melanoma cells which is suitable for measuring the biological activity of melanotropins.[6,7] The availability of this preparation allows us to compare the structural requirements for activity of MSH and its analogues on normal (frog skins) and transformed (mouse melanoma) cell systems. We, therefore, examined the dose–response curves for α-MSH, β_p-MSH, and all of the analogues reported in this paper. Representative dose–response curves for some analogues are shown in Figure 3, and the relative potencies are shown in Table III where the results are compared with those obtained in the frog skin assay. It is interesting that the concentration range over which β_p-MSH activates the melanoma adenylate cyclase is greater than for α-MSH analogues (Figure 3). At present we have no explanation for this.

It should first be noted that in both the frog skin system *and* the melanoma adenylate cyclase system examined here all the analogues are full agonists; that is, at a suitable concentration they will produce the same maximal response as the native hormones (Figures 2 and 3). It is clear, therefore, that none of the changes in structure made in these analogues are essential to the full biological activity message of the hormone. Rather, the only effects are changes in the potency of the hormone. The significant finding is that the relative potencies of the hormones and analogues are different in the two assay systems. This was immediately seen in comparing the activities of α-MSH and β_p-MSH in the two assay systems. As previously reported by several workers (see ref 17 and 18 for reviews), α-MSH is somewhat more potent than β_p-MSH in the standard frog skin assay system. However, in the melanoma adenylate cyclase system β_p-MSH is about 120% more potent than α-MSH. Substitution of the methionine residue in these hormones by norleucine gives analogues which are significantly more potent than the parent hormones in the frog skin assay system (Table II). This enhanced potency is also seen in the melanoma adenylate cyclase system (Table III), though the effect is not quite as dramatic. More dramatic are the differences of the two assay systems in response to changes in the 2 position in α-MSH. In the case of α-MSH, significant changes in potency were seen in the frog skin assay for each of the analogues reported here. While significant changes in potency are also seen in the melanoma adenylate cyclase assay system, the effects are not as substantial (Table III) and, indeed, [Phe²,Nle⁴]-α-MSH has essentially the same potency as α-MSH in this system. These results suggest that there is some divergence in the structural requirements for biological potency in the normal frog skin and transformed mouse melanocyte cell systems examined here. The precise nature of these differential structural requirements must await further analysis of a larger series of synthetic peptide analogues of melanotropin. However, in view of the biological activities of the various 2-substituted α-MSH analogues in both assay systems used here, it would appear that modification of the Tyr-2 residue by iodination is not of major significance for the substantial loss in biological activity which accompanies chloramine-T iodination of melanotropin.[10,11] Thus, iodination of the norleucine analogues of MSH under suitable conditions should provide a highly potent radiolabeled derivative. These and other studies are in progress in our laboratory.

Experimental Section

General Methods. Nuclear magnetic resonance (NMR) spectra were obtained on Varian T-60 or Bruker WH-90 instruments. The solid-phase synthetic schemes we have developed for α-MSH[15] and its analogues were similar to those we have developed in our laboratory for the synthesis of carboxyamide terminal peptides using benzhydrylamine resins.[13,20] Amino acids were coupled successively to valine-benzhydrylamine resin as their N^{α}-Boc derivatives. The coupling reaction was achieved with a 3-fold excess of Boc amino acid and a 2.4-fold excess of dicyclohexylcarbodiimide. Removal of the N^{α}-Boc protecting groups was effected by treatment of trifluoroacetic acid in dichloromethane. Side chains were protected as follows: serine, *O*-benzyl; tyrosine, *O*-2,6-dichlorobenzyl; glutamic acid, γ-benzyl ester; lysine, N^{ϵ}-[[(2,4-dichlorobenzyl)oxy]carbonyl]; arginine, N^{g}-(*p*-toluenesulfonyl); histidine, N^{im}-(*p*-toluenesulfonyl); tryptophan, N^{i}-formyl.

After the coupling of all amino acid residues to the resin, the amino terminal end of each peptide was acetylated with a threefold excess *N*-acetylimidazole. The finished protected peptides were

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cleaved from the resins and all protecting groups were removed with anhydrous liquid hydrogen fluoride, with the exception of the formyl group on tryptophan. The formylated (For-Trp) peptides were purified by gel filtration on Sephadex G-15 and the final products prepared by deformylation of an aqueous solution of the formylated peptide by addition of 4 N NaOH to a pH of 11.5 for 3 min as previously reported.[15] The deformylation reaction was terminated by the addition of glacial acetic acid to a final pH of 4.5. Chromatography of each peptide was done on carboxymethylcellulose and gave one main peak (280-nm detection). This peak was collected, concentrated, and desalted on Sephadex G-15. Purity of the final products was established by conventional analytical techniques.

The solid-phase syntheses and purification of β_p-MSH and [Nle^7]-β_p-MSH were accomplished by methods very similar to those outlined above except that a standard chloromethylated Merrifield resin was used in the synthesis.[15]

Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on silica gel plates using the following solvent systems: (A) 1-butanol–acetic acid–H_2O (4:1:5, upper phase only); (B) 1-butanol–acetic acid–pyridine–H_2O (15:3:10:12); (C) 1-butanol–pyridine–acetic acid–H_2O (6:6:1.2:4.8); (D) 2-propanol–25% aqueous NH_3–H_2O (3:1:1); (E) 1-pentanol–pyridine–H_2O (7:7:6); (F) 1-butanol–acetic acid–H_2O (2:1:1, upper phase only); (G) pyridine–acetic acid–H_2O (10:6:3). The load size was 50–100 μg and chromatographic lengths were 120–170 mm. Detection was made by ninhydrin, fluorescamine, and iodine. Single spots were obtained in all cases unless otherwise stated. Optical rotation values were measured at the mercury green line (546 nm) using a Perkin/Elmer 241 MC polarimeter. Amino acid analyses were done using a Beckman 120C amino acid analyzer following hydrolysis in 6 N HCl for 24 h at 110 °C.[21] Amino acid analyses for Trp were determined following hydrolysis in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. No corrections were made for destruction of amino acids during hydrolysis. N^{α}-Boc protected amino acids and amino acid derivatives were purchased from Vega Biochemicals or from Peninsula Laboratories. Before use, all amino acid derivatives were tested for homogeneity by thin-layer chromatography in solvent systems A, B, and E and by mixture melting point determinations with authentic samples. Chromatography on Sephadex G-15 and carboxymethylcellulose (Bio-Rad Laboratories) was performed as described herein. Solvents used for gel filtrations, thin-layer chromatography, and other chromatographic methods were purified as previously reported.[22] Boc–valine-benzhydrylamine-resin was prepared as previously described.[15,20] Amino acid analysis showed that the level of Boc-valine substitution was 0.09–0.10 mmol/g of resin.

(*tert*-Butoxycarbonyl)-3,5-diiodotyrosine. A mixture of 3,5-diiodotyrosine (5.0 g, 1.15 mmol) and Boc azide (2.5 mL, 16.6 mmol) was stirred in dimethyl sulfoxide (36 mL) overnight at room temperature. The resulting solution was diluted with cold water (175 mL) and washed with two 100-mL portions of ethyl ether. The aqueous layer was acidified with citric acid with cooling, and the product was extracted with two 100-mL portions of ethyl acetate. The combined ethyl acetate layer was washed with three 50-mL portions of water and dried over anhydrous magnesium sulfate. The drying agent was filtered off, the solvent was removed, and the solid residue was washed with dichloromethane. It was then dissolved in anhydrous ethyl ether and recrystallized in the presence of petroleum ether: yield 3.0 g (50%); mp 188 °C; R_f 0.81 (A), 0.81 (B), 0.83 (E); NMR (Me_2SO, $CDCl_3$), δ 1.47 (s, 9 H, Boc), 3.00 (d, 2 H, β-CH_2), 4.25 (q, 1 H, α-CH), 6.00 (s, 1 H, amide NH), 7.60 (s, 2 H, aromatic), 8.66–9.33 (br, 2 H, carboxyl and hydroxyl). Anal. Calcd for $C_{14}H_{17}NI_2O_5$: C, 31.53; H, 3.22; N, 2.63. Found: C, 31.68; H, 3.18; N, 2.95.

Solid-Phase Synthesis of the Protected Tridecapeptide Benzhydrylamine Resin to [Phe^2,Nle^4]-α-MSH, Ac-Ser(*O*-Bzl)-Phe-Ser(*O*-Bzl)-Nle-Glu(*O*-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Gly-Lys(N^{ϵ}-2,4-Cl_2-Z)-Pro-Val-NH-Resin. The title compound was prepared starting with 2.8 g (0.25 mmol) of the Boc-Val-benzhydrylamine resin. A cycle for the incorporation of the amino acid residue into the growing peptide chain consisted of the following steps: (1) washing with four 22-mL portions of dichloromethane, 1 min/washing; (2) cleavage of the Boc group by addition of 22 mL of 45% trifluoroacetic acid in dichloromethane containing 2% anisole and shaking for 2 min; (3) another addition of 22 mL of 45% trifluoroacetic acid in dichloromethane containing 2% anisole and shaking for 20 min; (4) washing with three 22-mL portions of dichloromethane, 1 min/washing; (5) neutralization by addition of two 22-mL portions of 10% diisopropylethylamine in dichloromethane and shaking for 2 min each; (6) washing four 22-mL portions of dichloromethane, 1 min/washing; (7) addition of 0.75 mmol of Boc amino acid in 10 mL of dichloromethane and 0.60 mmol of dicyclohexylcarbodiimide in 10 mL of dichloromethane and shaking for 1–15 h; (8) washing with three 22-mL portions of dichloromethane, 1 min/washing; (9) washing with three 22-mL portions of ethanol, 1 min/washing; (10) washing with four 22-mL portions of dichloromethane, 1 min/washing. Between steps 1 and 2, a few milligrams of the resin was used in the ninhydrin test.[23] After the coupling cycle of the last Boc-serine residue, the peptide was deblocked, neutralized, and washed as described above, and then the N-terminal amino group was acetylated by the addition of 0.75 mmol of *N*-acetylimidazole in 22 mL of dichloromethane and shaking for 3 h. The resin was washed with four 22-mL portions each of dichloromethane, ethanol, and dichloromethane and then dried in vacuo (3.5 g).

[Phe^2,Nle^4]-α-MSH, Ac-Ser-Phe-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. The dried protected tridecapeptide resin (1.5 g) from above was treated with 15 mL of anhydrous HF in the presence of 2 mL of anisole and 100 mg of Met at 0 °C for 1 h. After evaporation of the HF and anisole in vacuo, the resin was washed with three 30-mL portions of ethyl acetate and extracted with three 30-mL portions each of 30% aqueous acetic acid and H_2O. The combined aqueous extracts were lyophilized to give 127 mg of crude [Phe^2,Nle^4,N^{i}-For-Trp^9]-α-MSH. A 42-mg portion was chromatographed on Sephadex G-15 (2.2 × 83 cm) using 30% aqueous acetic acid as eluent solvent. The major peak (280-nm detection) gave 35 mg of a cream powder after lyophilization. For deformylation, the peptide powder (35 mg) was dissolved in 10 mL of H_2O and the pH was adjusted to 11.5 with 4 N NaOH. After 3 min at pH 11.5, the solution was acidified to pH 4.5 with glacial acetic acid and chromatographed on a carboxymethylcellulose column (2.2 × 25 cm) using a stepwise gradient composed of 25 mL of 0.01 M ammonium acetate (pH 4.5) and then 250 mL each of 0.1, 0.2, and 0.4 M ammonium acetate (pH 6.8). The major peak (280-nm detection) occurred during the 0.1 M ammonium acetate (pH 6.8) elution. It was collected, concentrated, and desalted by gel filtration on Sephadex G-25 (2.2 × 120 cm) in 30% aqueous acid. After lyophilization there was obtained 16.3 mg of pure [Phe^2,Nle^4]-α-MSH. Amino acid analysis results are given in Table I. Optical rotation, TLC, and paper relectrophoresis data are given in Table II.

[Nle^4]-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. The protected tridecapeptide benzhydrylamine resin to the title compound, Ac-Ser(*O*-Bzl)-Tyr(*O*-2,6-Cl_2-Bzl)-Ser(*O*-Bzl)-Nle-Glu(*O*-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Gly-Lys(N^{ϵ}-2,4-Cl_2-Z)-Pro-Val-NH-resin was prepared using the same procedures as discussed above. The peptide was cleaved from the resin, the protecting groups were removed, and purification was accomplished using the same procedures as used on the previous synthesis. The amino acid analyses are given in Table I and the other analytical data are summarized in Table II.

[D-Tyr^2]-α-MSH, Ac-Ser-D-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. The title compound was prepared by the solid-phase method using a benzhydrylamine resin and solid-phase methods the same as those discussed previously, except that the D-tyrosine was added to the growing peptide chain

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without protection of the tyrosyl hydroxyl group using Boc-D-Tyr. The purification procedures were the same as used in the synthesis of α-MSH.[15] The amino acid analysis and other analytical data are given in Tables I and II.

[I_2-Tyr²]-α-MSH, Ac-Ser-3′,5′-I_2-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2. The title compound was prepared by the solid-phase method using a benzhydrylamine resin and solid-phase methods identical with those previously discussed, except that the 3′,5′-diiodotyrosine was added to the growing peptide chain using N^{α}-(*tert*-butoxycarbonyl)-3′,5′-diiodotyrosine. The purification of the analogue was accomplished using the same procedures as used in the synthesis of α-MSH. The amino acid analysis and other analytical data are given in Tables I and II. The data establish that the product is a single homogeneous compound with properties expected for [I_2-Tyr²]-α-MSH and considerably different than α-MSH.[15] In separate experiments we demonstrated that 3′,5′-diiodotyrosine was stable in HF at 0 °C for greater than 1 h with no detectable loss of iodine as determined by thin-layer chromatography in the solvent systems A, B, F, and G. Authentic 3′,5′-diiodotyrosine, 3′-iodotyrosine, and tyrosine were used in all TLC experiments for direct comparison. TLC conditions were such that a 1% conversion to 3′-iodotyrosine or a 1% conversion to tyrosine would have been detectable.

α-MSH was synthesized and purified as previously reported.[15]

β_p-MSH was synthesized and purified as previously reported.[15]

[Nle⁷]-β_p-MSH, H-Asp-Glu-Gly-Pro-Tyr-Lys-Nle-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH, was synthesized using a standard chloromethylated Merrifield resin and procedures essentially identical with those used in the synthesis of β_p-MSH, previously reported.[15] The same side-chain protecting groups as used in the synthesis of α-MSH were utilized and, in addition, the β-carboxyl group of Asp was *O*-benzyl protected. The protected octadecapeptide resin, H-Asp(*O*-Bzl)-Glu(*O*-Bzl)-Gly-Pro-Tyr(*O*-2,6-Cl_2-Bzl)-Lys(N^{ϵ}-2,4-Cl_2-Z)-Nle-Glu(*O*-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Gly-Ser(*O*-Bzl)-Pro-Pro-Lys(N^{ϵ}-2,4-Cl_2-Z)-Asp(*O*-Bzl)-*O*-resin, was prepared starting with 2.60 g of Boc-Asp(*O*-Bzl)-*O*-resin [0.21 mmol of Boc-Asp(*O*-Bzl)/g of resin] and gave 3.5 g of peptide resin. A 1.75-g portion was treated with 15 mL of anhydrous HF containing 2 mL of anisole and 100 mg of methionine at 0 °C for 1 h. The crude peptide was obtained in the usual manner and chromatographed on Sephadex G-15 (2.2 × 120 cm) using 30% aqueous acetic acid as eluent solvent to give 450 mg of crude [Nle⁷,N^{i}-For-Trp¹²]-β_p-MSH after lyophilization. A 100-mg portion was deformylated at pH 11.5 in the usual manner, and the product purified by ion exchange on a carboxymethylcellulose column (2.2 × 25 cm) using a discontinuous gradient of 250 mL of 0.01 M ammonium acetate (pH 4.5), followed by 250 mL each of 0.1, 0.2, and 0.4 M ammonium acetate (pH 6.8). The major peak was collected, lyophilized, and desalted by gel filtration in Sephadex G-15 (2.2 × 120 cm column) using 30% aqueous acetic acid as eluent solvent. [Nle⁷]-β_p-MSH was obtained as a white powder, 64 mg. Amino acid analysis and other analytical data are given in Tables I and II.

Frog Skin Bioassay. The frogs, *Rana berlandieri forrei*, were obtained from Southwest Scientific Supply Co. (Tucson, AZ) and were collected in the vicinity of Sinaloa, Mexico. The frogs were sacrified by decapitation, and the skins from each animal were prepared for photometric reflectance measurements following the methods of Shizume et al.[16] and Wright and Lerner.[24] The lowest concentration of each peptide was added to a group of skins at time zero and the reflectance determined after 15 min. This process was repeated with increasing amounts of each peptide until a maximum response was obtained. Results were then normalized by setting the maximum response at 100% and plotted as the percent response (Figure 2). Each value represents the mean response of five skins, and the standard errors of measurement are shown. The relative potencies correspond to the differences in activity as measured by the concentration of each analogue required to stimulate a half-maximal response relative to α-MSH.

Melanoma Membrane Adenylate Cyclase Assay. The particulate membrane fraction from S-91 melanoma tumors grown in DBA/2J mice was isolated as previously described.[6,10] Adenylate cyclase activity was assayed in a medium containing 1 mM [α-^{32}P]ATP (specific activity 40 to 80 cpm/pmol), 5 mM $MgCl_2$, 1 mM cAMP, 0.5 mM 3-isobutyl-1-methylxanthine, 1 mM dithiothreitol, 20 mM creatine phosphate, 100 U/mL creatine phosphokinase, and 10 μM GTP in 30 mM Tris-HCl, pH 7.5. The assay was initiated by adding 100 μg of membrane protein and terminated[25] after 10 min at 30 °C. [^{32}P]cAMP was isolated, purified, and detected according to the method of Salomon et al.[25] Radiochemicals were purchased from New England Nuclear.

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