Comparative Biological Activities of Highly Potent Active-Site Analogues of α-Melanotropin[1]

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The heptapeptide sequence, Met-Glu-His-Phe-Arg-Trp-Gly, found in common within the primary structures of α-melanotropin (α-MSH), β-melanotropin (β-MSH), and adrenal corticotropic hormone (ACTH) may represent the active site mainly responsible for the melanotropic activities of these peptides. In α-MSH, replacement of Met by Nle at position 4 and D-Phe for its L enantiomer at position 7 produced a stereostructural analogue ([Nle^4,D-Phe^7]-α-MSH) with superagonist potency and extraordinarily prolonged biological activity. We have determined the extent to which these chemical modifications affect the melanotropic activity of the heptapeptide-proposed active site of α-MSH (α-MSH$_{4-10}$). Although α-MSH$_{4-10}$ has only about $^1/_{100\,000}$ the potency of α-MSH in the frog skin assay, melanotropic activity was enhanced by (1) acetylation and amidation of the N- and C-terminal residues, respectively; (2) replacement of Met by Nle; and (3) substitution of D-Phe for L-Phe. The final peptide, Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$, possessed one-fifth the potency of α-MSH on the frog (*Rana pipiens*) skin assay and was about *10 times more potent* than α-MSH in the lizard (*Anolis carolinensis*) assay. Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$ was also about *8 times more active* than the native hormone in stimulating mouse melanoma adenylate cyclase. The melanotropic activity of this stereostructural heptapeptide analogue was dramatically prolonged relative to MSH in the *Anolis* skin assay but was less prolonged in the frog skin assay relative to the tridecapeptide analogue, [Nle^4,D-Phe^7]-α-MSH. Interestingly, Ac-[Tyr^4]-α-MSH$_{4-10}$-NH$_2$, which was prepared to provide an analogue that might be radiolabeled, was a partial agonist in the melanoma adenylate cyclase assay. Moreover, although this compound exhibited very low potency in all assays, it exhibited exceptionally prolonged melanotropic activity in the frog skin assay. These results demonstrate that the dramatic changes in potency and prolongation of activity of the native hormone which result from substitution of Met^4 by Nle^4 and Phe^7 by D-Phe are derived primarily, but not exclusively, from these changes within the heptapeptide active site of α-MSH.

α-Melanotropin (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$, α-MSH) is one of several chemically and biologically related peptides which may be derived from a large molecular weight precursor protein, pro-opiomelanocortin,[2,3] in the vertebrate pituitary and hypothalamus (Figure 1). Based on the structural homology in α-MSH, ACTH, β-MSH, and, to a lesser extent, γ-MSH of the common sequence Met-Glu-His-Phe-Arg-Trp-Gly and its apparent stringent conservation throughout evolution, this heptapeptide sequence has been suggested to be the primary structural determinant for the biological activities of each of these peptides.

The regulatory role of α-MSH on vertebrate pigmentation[4,5] was the first described and has been the most intensively studied physiological activity of this peptide hormone. Based on previous structure–function studies[6-9] of α-MSH analogues and fragments using the in vitro frog skin bioassay system, the *primary active site* of α-MSH in this system has been suggested to be Met-Glu-His-Phe-Arg-Trp-Gly. The corresponding synthetic fragment, H-Met-Glu-His-Phe-Arg-Trp-Gly-OH (α-MSH$_{4-10}$), has been reported[6,7] to be a very weak agonist on this in vitro system. To further evaluate the structure–function relationships of this heptapeptide, we have systematically determined the melanotropic activity of a number of α-MSH$_{4-10}$ analogues. We report here the synthesis of several α-MSH$_{4-10}$ analogues, one of which is *more potent than the native hormone* in a number of bioassay systems.

Results and Discussion

The Ac-α-MSH$_{4-10}$-NH$_2$ analogues discussed herein were prepared by solid-phase synthetic methods[10-12] and purified by procedures similar to those used in our previous syntheses of α-melanotropin and analogues.[13-15] The details of the synthetic methods are given under Experimental Section, and the analytical results are given in Table I. The in vitro biological activities of the peptides were determined in both the frog (*Rana pipiens*)[16] and lizard (*Anolis carolinensis*) skin assays[17,18] over the entire dose–response range of the peptides. The results of the

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(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-melanotropin; β_p-MSH, β-melanotropin (porcine); ACTH, adrenal corticotropin hormone; Nle, norleucine; TLC, thin-layer chromatography; 2,4-Cl$_2$-Z, 2,4-dichlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; 2,6-Cl$_2$-Bzl, 2,6-dichlorobenzyl; IR, infrared; *p*-MBHA resin, *p*-methylbenzhydrylamine resin; HOBT, *N*-hydroxybenzotriazole; For, formyl; Tos, tosyl; taken in part from the Senior Honor's Thesis of M. T. Draelos.

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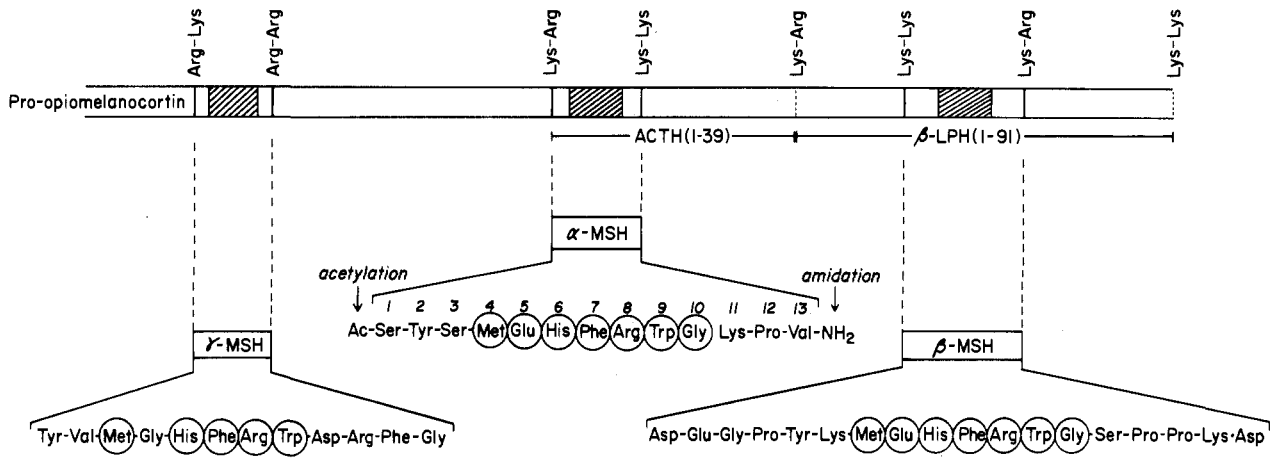

Figure 1. Schematic representation of the known biologically active peptides as derived from pro-opiomelanocortin, which is found in the vertebrate pituitary and hypothalamus.

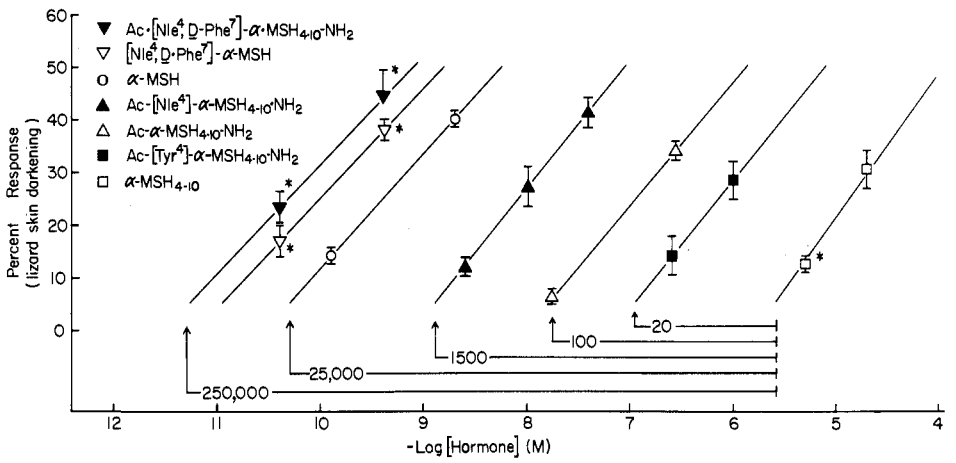

Figure 2. In vitro dose–response of lizard (*Anolis carolinensis*) skins to α-MSH_{4-10} analogues. Each point represents the mean (±SE) percent darkening response of skins (6* or 8 per group).

Table I. Analytical Properties of α-MSH_{4-10} Analogues

peptide	physicochemical properties: TLC R_f in various systems[a] A	B	C	D	$[\alpha]^{25}_{546}$, deg, in 10% HOAc (*c*)
α-MSH	0.18	0.67	0.63	0.90	−58.0 (0.50)
Ac-α-MSH_{4-10}-NH_2	0.30	0.61	0.64	0.97	−34.1 (0.25)
Ac-[Tyr⁴]-α-MSH_{4-10}-NH_2	0.32	0.63	0.65	0.87	−13.3 (0.26)
Ac-[Nle⁴]-α-MSH_{4-10}-NH_2	0.30	0.72	0.70	0.86	−39.1 (0.25)
Ac-[Nle⁴,D-Phe⁷]-α-MSH_{4-10}-NH_2	0.30	0.68	0.69	0.87	−38.9 (0.26)

	amino acid analyses: Ser	Tyr	Met	Nle	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val
α-MSH	1.72	0.92	0.97		1.00	0.90	1.06	0.98	0.90	1.08	1.04	1.08	1.07
α-MSH_{4-10}			0.95		1.00	0.97	0.97	1.03	1.00	0.99			
Ac-α-MSH_{4-10}-NH_2			1.00		0.97	1.02	0.91	1.02	0.90	1.07			
Ac-[Tyr⁴]-α-MSH_{4-10}-NH_2		0.94			0.99	1.06	0.92	1.04	0.93	1.05			
Ac-[Nle⁴]-α-MSH_{4-10}-NH_2				1.01	1.05	0.94	0.91	0.98	0.94	1.08			
Ac-[Nle⁴,D-Phe⁷]-α-MSH_{4-10}-NH_2				1.05	1.02	1.04	0.93	0.98	0.94	1.06			

[a] For solvent systems A–D, see Experimental Section.

latter study are shown in Figure 2, and the relative potencies from both studies are summarized in Table II. In addition, the biological activities of several of the analogues were examined using the mouse melanoma adenylate cyclase assay.[19] The assay results for selected analogues are shown in Figure 3 and comparative potencies in Table II.

In confirmation of previous reports,[6,7] α-MSH_{4-10} (H-Met-Glu-His-Phe-Arg-Trp-Gly-OH) was found to be a very weak agonist with about $^1/_{100\,000}$ the potency of the native hormone (α-MSH) in the frog skin bioassay (Table II).

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Table II. Relative in Vitro Potencies of α-MSH_{4-10} Analogues on Frog (*Rana pipiens*) Skin, Lizard (*Anolis carolinensis*) Skin, and Mouse Melanoma Adenylate Cyclase Assays

peptide	Potency relative to α-MSH[a]		
	frog skin assay	lizard skin assay	mouse melanoma adenylate cyclase assay
α-MSH	1.0	1.0	1.0
α-MSH_{4-10}[b]	0.00001	0.00004	ND[c]
Ac-α-MSH_{4-10}-NH_2	0.0003	0.004	ND
Ac[Tyr^4]-α-MSH_{4-10}-NH_2	0.0002	0.0006	0.001[d]
Ac-[Nle^4]-α-MSH_{4-10}-NH_2	0.002	0.06	0.09
Ac-[Nle^4,D-Phe^7]-α-MSH_{4-10}-NH_2	0.02	10.0	7.7
[Nle^4,D-Phe^7]-α-MSH	60	5.0	26.6

[a] Relative potency = concentration of α-MSH at 50% response/concentration of peptide at 50% response. [b] H-α-MSH_{4-10}-OH was obtained from Peninsula Laboratories (San Carlos, CA). [c] Not determined. [d] Ac-[Tyr^4]-α-MSH_{4-10}-NH_2 was found to be a partial agonist on the melanoma adenylate cyclase assay and possessed about 34% the maximal activity of α-MSH (refer to Figure 3).

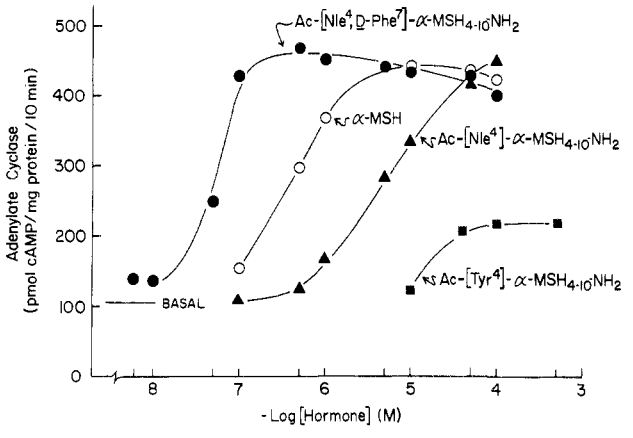

Figure 3. Dose–response of Cloudman S-91 mouse melanoma cell-free adenylate cyclase to α-MSH_{4-10} analogues. All results were determined using 56 μg of protein per assay, and each point represents the mean value of triplicate determinations. Each SE was less than 5%.

However, acetylation of the N terminus (Met-4) *and* amidation of the C terminus (Gly-10) of this heptapeptide to yield Ac-α-MSH_{4-10}-NH_2 resulted in a 30-fold potency increase in stimulating frog skin melanosome dispersion (Table II). A similar potency relationship was observed for these two fragments in the lizard skin bioassay (Table II and Figure 2).

Based on previous reports[7,13] indicating that substitution of Met by Nle in either α-MSH or β_p-MSH (at position 4 or 7, respectively) resulted in increased melanotropic potency, an identical replacement was made in Ac-α-MSH_{4-10}-NH_2. The resultant analogue, Ac-[Nle^4]-α-MSH_{4-10}-NH_2, was 3 times more potent than the Met-4 analogue in the frog skin bioassay and 15 times more potent in the lizard skin bioassay (Table II) and Figure 2). On the mouse melanoma adenylate cyclase assay, Ac-[Nle^4]-α-MSH_{4-10}-NH_2 was about one-eleventh as potent as α-MSH. The relative potency of these two peptides on the lizard skin bioassay was similar (Table II and Figures 2 and 3).

We next explored the possibility of substituting Met by Tyr at position 4 so as to provide a suitable residue for radiolabeling of α-MSH_{4-10} analogues. Relative to Ac-α-MSH_{4-10}-NH_2, the Tyr-4 analogue was found to be slightly less potent on the frog skin and lizard skin bioassays (Table II and Figure 2). However, a potentially important observation emerged from potency and full dose–response studies of this analogue. Ac-[Tyr^4]-α-MSH_{4-10}-NH_2 was found to be only a partial agonist on the melanoma adenylate cyclase system and stimulated the enzyme to only about 35% of the maximal activity of the native hormone (α-MSH, Figure 3). This may provide insight for the development of a melanotropin antagonist (inhibitor).[20,21]

Based on previous reports[14,18] describing the high potency and prolonged melanotropic action of [Nle^4,D-Phe^7]-α-MSH, we investigated the extent to which its altered biological activity might be due primarily to stereoisomeric modifications within the proposed primary active site of α-MSH. As shown in Figures 2 and 3 and summarized in Table II, Ac-[Nle^4,D-Phe^7]-α-MSH_{4-10}-NH_2 was substantially more potent than any of the heptapeptide fragment analogues in all of the assay systems studied. In fact, in both the lizard skin and melanoma adenylate cyclase assays, Ac-[Nle^4,D-Phe^7]-α-MSH_{4-10}-NH_2 was actually about *10 times more active than the native hormone.* This represents approximately a 100-fold increase in potency over the Ac-[Nle^4]-α-MSH_{4-10}-NH_2 analogue and more than a 200 000-fold increase in potency over α-MSH_{4-10} in the lizard skin assay system. A similar potency increase is seen in the frog skin assay. Thus, an α-melanotropin analogue of approximately one-half the molecular weight of α-MSH has been prepared which is more active than the native hormone. These results suggest that it may be possible to synthesize even more potent fragment analogues of α-melanotropin.

A unique characteristic of [Nle^4,D-Phe]-α-MSH was its extraordinarily prolonged activity in vitro and in vivo in the frog and lizard.[14,18] In the lizard skin bioassay, the activity of Ac-[Nle^4,D-Phe^7]-α-MSH_{4-10}-NH_2 was nearly as prolonged as that of the tridecapeptide analogue (Figure 4A). In contrast, the frog skin darkening action of Ac-[Nle^4,D-Phe^7]-α-MSH_{4-10}-NH_2, although somewhat prolonged relative to the native hormone, was rapidly reversed relative to [Nle^4,D-Phe^7]-α-MSH (Figure 4B). These results suggest that in the frog assay, enantiomeric substitution at position 7 is not sufficient to account for the extraordinarily prolonged activity of [Nle^4,D-Phe^7]-α-MSH. Perhaps, there may be cooperative interaction between either the C- or N-terminal residues and the primary active site of the tridecapeptide analogue which is expressed at the frog skin melanotropin receptor site. In the absence of D-Phe-7 replacement, however, both Ac-[Nle^4]-α-MSH_{4-10}-NH_2 and Ac-[Tyr^4]-α-MSH_{4-10}-NH_2 exhibited very marked duration of action on the frog skin bioassay (Figure 5). This was not observed in the lizard skin system, since all L-Phe-7 substituted α-MSH_{4-10} analogues were rapidly reversed (data not shown). Thus, it appears that the differences in prolonged melanosome-stimulating activities observed for the α-MSH_{4-10} analogues were

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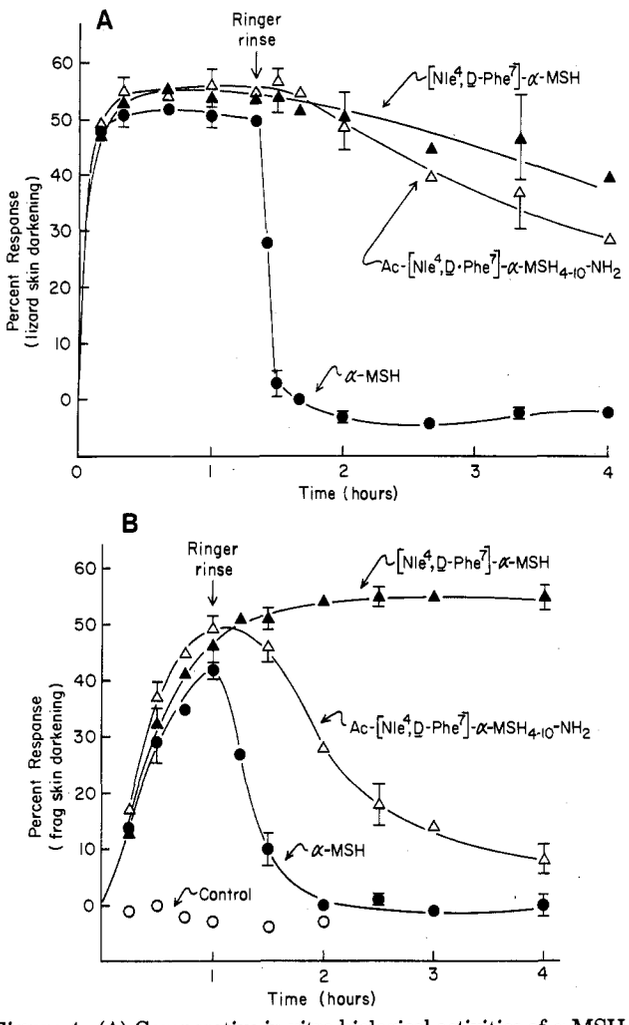

Figure 4. (A) Comparative in vitro biological activities of α-MSH (●, 4×10^{-9} M), Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$ (△, 2×10^{-9} M), and [Nle^4,D-Phe^7]-α-MSH (▲, 2×10^{-9} M) in the lizard (*Anolis*) skin assay. Each melanotropin was used at a concentration to provide an approximately equipotent response. After 75 min, the skins were rinsed repeatedly with fresh Ringer *in the absence* of the melanotropins. Each value represents the mean (±SE) response (darkening) of the skins (7 per group) to the melanotropins. (B) Comparative in vitro biological activities of α-MSH (●, 4×10^{-10} M), Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$ (△, 4×10^{-9} M), and [Nle^4,D-Phe^7]-α-MSH (▲, 4×10^{-11} M) in the frog (*Rana pipiens*) skin assay. Each melanotropin was used at a concentration to provide an approximately equipotent response. After 60 min, the skins were rinsed repeatedly with fresh Ringer *in the absence* of the melanotropins. Each value represents the mean (±SE) response (darkening) of the skins (6 per group) to the melanotropins. A group of control skins (○) was maintained during the first 2 h of the experiment.

species dependent and stereospecific for D-Phe-7 substitution only in the case of the lizard skin bioassay. These results clearly illustrate that potency and prolongation of biological response depend on quite different structural features of the hormone.

In summary, this systematic structure–activity analysis provides the first detailed correlation of stereostructural modification to comparative melanotropic activity within the primary heptapeptide active site of α-melanotropin (Met-Glu-His-Phe-Arg-Trp-Gly). The potency range for the active-site heptapeptide MSH peptides tested was almost five orders of magnitude. Structural modifications at the N- and C-terminal functional groups of Met-4 and Gly-10 positions of H-α-MSH$_{4-10}$-OH resulted in markedly increased (>100-fold) melanotropic potency for the resultant peptide, Ac-[Nle^4]-α-MSH$_{4-10}$-NH$_2$. Furthermore

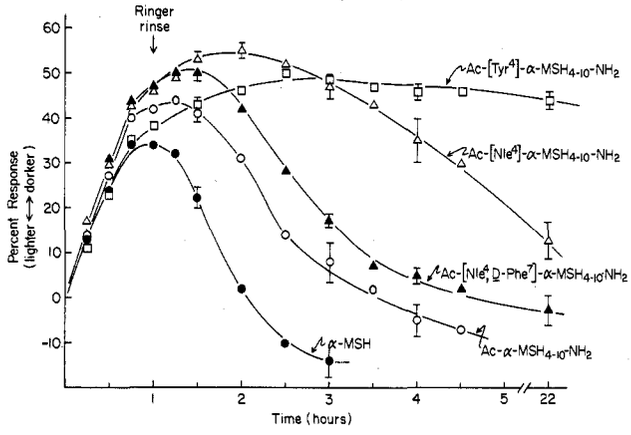

Figure 5. Comparative in vitro biological activities of α-MSH (●, 4×10^{-10} M), Ac-α-MSH$_{4-10}$-NH$_2$ (○, 5×10^{-6} M), Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$ (▲, 4×10^{-9} M), Ac-[Nle^4]-α-MSH$_{4-10}$-NH$_2$ (△, 10^{-6} M), and Ac-[Tyr^4]-α-MSH$_{4-10}$-NH$_2$ (□, 10^{-6} M) in the frog (*Rana pipiens*) skin assay. Each melanotropin was used at a concentration to provide an approximately equipotent response. After 60 min, the skins were rinsed repeatedly in fresh Ringer *in the absence* of the melanotropins. Each value represents the mean (±SE) response (darkening) of the skins (7 per group) to the melanotropins.

substitution of Phe-7 by its D enantiomer to yield Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$ resulted in the most potent α-MSH$_{4-10}$ analogue synthesized to date. In both the lizard skin and melanoma adenylate cyclase assay systems, Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$ is *more active* than the native hormone. Indeed, this fragment analogue is essentially equipotent to the tridecapeptide analogue [Nle^4,D-Phe^7]-α-MSH (Figure 2). However, the fragment analogue clearly does not possess the prolonged activity of [Nle^4,D-Phe^7]-α-MSH in the frog skin assay system (Figure 4B). Obviously, the structural requirements for high potency and prolonged activity are quite different, at least in this assay system.

Based on the biological activities of Ac-[Tyr^4]-α-MSH$_{4-10}$-NH$_2$, it is suggested that further chemical tailoring (perhaps D-Phe-7 substitution) of this fragment might yield a highly potent MSH heptapeptide capable of being radiolabeled for future studies in vitro or in vivo. Overall, the intrinsic potencies of the α-MSH$_{4-10}$ analogues suggest that the lizard skin melanophore system may be functionally more representative of the mammalian melanoma system than the standard frog skin assay system. In addition, the results of the prolongation studies conducted on these heptapeptide analogues indicate notable differences between these distantly related pigment cell receptor systems in recognizing modified structural or stereochemical features of these melanotropins. Work is in progress to delineate further the stereochemical and conformational basis for these observations.

Experimental Section

General Methods. Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on silica gel G plates using the following solvent systems: (A) 1-butanol/HOAc/H$_2$O (4:1:5, upper phase only); (B) 1-butanol/HOAc/pyridine/H$_2$O (15:3:10:12); (C) 1-butanol/pyridine/HOAc/H$_2$O (6:6:1.2:4.8); (D) 2-propanol/25% aqueous NH$_3$/H$_2$O (3:1:1). The load size was 50–100 μg, and chromatographic lengths were 12–18 cm. Detection was made by ninhydrin, fluorescamine, and iodine vapor. Single spots were obtained in all cases unless otherwise noted. Optical rotations were obtained using a Perkin-Elmer 241 MC polarimeter and were measured at the mercury green line (546 nm). Infrared (IR) spectra were obtained using a Perkin Elmer 337 spectrophotometer. Amino acid analyses[22] were ob-

tained using a Beckman 120 C amino acid analyzer following hydrolysis for 22–24 h at 110 °C in one of the following reagents: (1) 6 N HCl containing 0.2% phenol; (2) 3 M mercaptoethanesulfonic acid (MESA);[23] or (3) 4 N methanesulfonic acid (MSA) containing 0.2% 3-(2-aminoethyl)indole.[24] 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 (Tucson, AZ), Peninsula Laboratories (San Carlos, CA), Bachem (Torrance, CA), Chemical Dynamics (South Plainfield, NJ), or Galactica Biochemical (Cleveland, OH). Before use, all amino acid derivatives were tested for homogeneity by thin-layer chromatography in solvent systems A–C by mixture melting point determinations with authentic samples and by the ninhydrin test.[25] Solvents used for gel filtration, thin-layer chromatography, and other chromatographic methods were purified as previously reported.[26]

General Solid-Phase Synthesis Methodology. α-MSH and the α-MSH_{4-10} analogues reported in this paper were synthesized by solid-phase methods similar to those we used for the synthesis of α-MSH[15] but with several modifications. A *p*-methylbenzhydrylamine resin was used, since it was reported[10] to yield higher recoveries of carboxamide-terminal peptides following HF cleavage than the benzhydrylamine resin. The Boc-Gly resin was prepared by methods previously reported[12,15] and provided amino acid substitution levels of 0.20–0.27 mmol/g of resin. N^{α}-Boc-N^{ϵ}-2,4-Cl_2-Z-Lys was prepared as previously reported.[15,27] N^{α}-Boc amino acid derivatives were successively coupled to the Gly-*p*-MBHA resin using a 3-fold excess of each of the Boc amino acid derivative and a 2.4-fold excess of dicyclohexylcarbodiimide, as well as *N*-hydroxybenzotriazole (3-fold excess) as a racemization suppressing additive. Removal of the N^{α}-Boc protecting groups was accomplished by treatment with trifluoroacetic acid in dichloromethane. Side-chain functional groups were protected as follows: tyrosine, *O*-2,6-dichlorobenzyl; glutamic acid, γ-benzyl ester; lysine, N^{ϵ}-2,4-dichlorobenzyloxycarbonyl; arginine, N^{g}-*p*-toluenesulfonyl; histidine, N^{im}-*p*-toluenesulfonyl; tryptophan, N^{i}-formyl. After all of the amino acid residues were coupled to the resin, the amino terminal end of each peptide was acetylated with a 6-fold excess of *N*-acetylimidazole. The finished protected peptides were cleaved from the resin, and all protecting groups (with the exception of the N^{i}-formyl group of Trp) were removed with anhydrous liquid hydrogen fluoride (0 °C, 30–45 min) containing 16% anisole and 0.6% dithioethane. The N^{i}-Trp-For groups were removed by treatment of an aqueous solution of the formylated peptide with 4 N NaOH to pH 11.5 for 3 min as previously reported.[15,28] The deformylation was terminated by the addition of glacial acetic acid to a final pH of 4.5. The α-MSH_{4-10} analogues were purified by ion-exchange chromatography on carboxymethylcellulose (vide infra). Peptide eluents were detected by ultraviolet spectroscopy at 280 nm.

α-Melanotropin (α-MSH). The synthetic peptide hormone was prepared and purified as previously reported.[15]

[Nle^4,D-Phe^7]-α-MSH. The synthetic peptide analogue was prepared and purified as previously reported.[14]

***p*-Methylbenzhydrylamine Resin.** A highly reproducible synthesis of the title compound was obtained by a slight modification of the Friedel–Crafts acylation and Leuckart reductive amination procedures used in our laboratory[12] for the preparation of benzhydrylamine resin. For example, 10 g of beaded polystyrene cross-linked with 1% divinylbenzene (Bio-Beads S-X1, 200–400 mesh; Bio-Rad Laboratories Richmond, CA) in 125 mL of dichloromethane under N_2 was added to 3.33 mL (25 mmol) of *p*-toluoyl chloride. The mixture was cooled to 0 °C, and 2.31 g (17.2 mmol) of $AlCl_3$ was added in approximately three equal portions. The mixture was stirred at 0 °C for 2 h, at 25 °C for 1.5 h, and at reflux for 2.5 h. The mixture was cooled and poured in 400 mL of ice–H_2O, and the resin was filtered off and washed four times with 50-mL portions each of ethanol, 50%g aqueous ethanol, and H_2O. The resin was suspended in 150 mL of water and treated with 25 mL of concentrated HCl with stirring for 1 h. The derivative resin was filtered, washed 3 times with 50 mL of H_2O, and hydrolyzed for 10 min with 50 mL of 0.5 N NaOH with stirring. The resin was filtered off, washed four times with 50-mL portions of H_2O, 50% aqueous ethanol, ethanol, and dichloromethane and then dried in vacuo to give 13.0 g of *p*-methylphenyl ketone resin: IR (KBr) 3100, 1650, 1600 cm^{-1}.

Water (about 210 mL) was distilled off a mixture of 167 mL of 88% aqueous formic acid and 200 mL of concentrated aqueous ammonia in a three-necked flask equipped with thermometer and overhead stirrer until the solution temperature was 150–160 °C. To the hot ammonium formate solution was added 5.14 g of *p*-methylphenyl ketone resin, and the mixture was stirred and maintained at 170 °C (oil bath temperature) for 48 h. The mixture was cooled, and the resin was filtered off and washed four times with 50-mL portions of H_2O, methanol, and dichloromethane. The N-formylated resin was suspended in 80 mL of concentrated HCl–propanoic acid (1:1, v/v) and refluxed for 5 h. The resin was filtered off, washed with four 50-mL portions of H_2O, 50% aqueous ethanol, and dichloromethane, and then neutralized with two 50-mL portions of 10% diisopropylethylamine in dichloromethane. The resin was washed with four 50-mL portions of dichloromethane and dried in vacuo to give 4.6 g of cream-colored *p*-methylbenzhydrylamine resin: IR 3400, 3050, 1650, 1600 cm^{-1}.

Ac-α-MSH_{4-10}-NH_2, Ac-Met-Glu-His-Phe-Arg-Trp-Gly-NH_2. The title compound was prepared starting with 2.50 g of Boc-Gly-*p*-MBHA resin[12,15] (0.49 mmol of Boc-Gly total) using solid-phase methods of peptide synthesis. A cycle for incorporating each amino acid residue into the growing peptide chain consisted of the following: (1) washing with four 25-mL portions of CH_2Cl_2, 1 min/wash; (2) cleavage of the Boc group by addition of 25-mL of 45% trifluoroacetic acid in dichloromethane containing 2% anisole and 1% dithioethane, one treatment for 2 min, a second for 20 min; (3) washing with four 25-mL portions of dichloromethane, 1 min/wash; (4) neutralization by addition of two 25-mL portions of 10% diisopropylethylamine in dichloromethane and shaking for 2 min each; (5) washing with four 25-mL portions of dichloromethane, 1 min/wash; (6) addition of 1.5 mmol of the Boc amino acid derivative in 10 mL of dichloromethane containing 1.5 mmol of *N*-hydroxybenzotriazole (dissolved in a minimum volume of *N*,*N*-dimethylformamide), followed by 1.2 mmol of dicyclohexylcarbodiimide in 12 mL of dichloromethane, followed by 3 mL of dichloromethane wash and shaking for 3.5–12 h; (7) washing with three 25-mL portions of dichloromethane, 1 min/wash; (8) washing with three 25-mL portions of ethanol, 1 min/wash; (9) washing with four 25-mL portions of dichloromethane, 1 min/wash. Between steps 1 and 2, several milligrams of the resin was used in the ninhydrin test[25] to determine completion of coupling. Stepwise coupling of the following N^{α}-Boc amino acids (or derivatives) was performed (in order of addition): N^{α}-Boc-N^{i}-For-Trp, N^{α}-Boc-N^{g}-Tos-Arg, N^{α}-Boc-Phe, N^{α}-Boc-N^{im}-Tos-His, and N^{α}-Boc-γ-Bzl-Glu. The resultant Boc-Glu(γ-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Gly-*p*-MBHA resin (3.63 g, dry weight) was divided into two portions. A 1.41 g (~0.19 mmol) portion of the protected hexapeptide-resin was converted to the protected title peptide resin after coupling N^{α}-Boc-Met. The N^{α}-Boc protecting group was removed, the resin peptide was neutralized as before (steps 1–5), and the protected peptide was N^{α}-acetylated with a 6-fold excess of *N*-acetylimidazole in 15 mL of dichloromethane. The resultant protected peptide resin, Ac-Met-Glu(γ-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Gly-*p*-MBHA resin was dried in vacuo (1.36 g) and treated with 20 mL anhydrous HF in the presence of 4.0 mL of anisole and 135 μL of 1,2-dithioethane for 45 min at 0 °C. After the HF, anisole, and 1,2-dithioethane were evaporated in vacuo, the dried product mixture was washed with three 30-mL portions of ethyl acetate, and the peptide was extracted with three 30-mL portions of 30% acetic acid, 1 N acetic acid, and H_2O. The combined aqueous extracts were lyophilized to give 196.7 mg of

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crude Ac-[N^i-For-Trp^9]-α-MSH$_{4-10}$-NH$_2$.

A portion of the crude peptide (70.2 mg) was dissolved in 3 mL of H_2O, and the pH was adjusted to 11.5 with 4 N NaOH. After 3 min at pH 11.5, the deformylation reaction[28] was terminated as the solution was acidified to pH 4.5 with glacial acetic acid. The solution was chromatographed directly on a carboxymethylcellulose column (2.0 × 20.0 cm) using 250 mL of 0.01 M NH_4OAc (pH 4.5) as the initial eluant, followed by 250 mL each of 0.1, 0.2, and 0.4 M NH_4OAc (pH 6.8). The major peak (280 nm detection) eluted during the 0.1 M NH_4OAc fraction and was lyophilized to give 34.3 mg of white powder (overall yield 46%). The analytical data for the purified compound are given in Table I, and a summary of biological potencies is given in Table II.

Ac-[Tyr^4]-α-MSH$_{4-10}$-NH$_2$, Ac-Tyr-Glu-His-Phe-Arg-Trp-Gly-NH$_2$. From 1.22 g (~0.15 mmol) of Boc-Gly(γ-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Gly-*p*-MBHA resin (prepared above), conversion of the protected heptapeptide to the title peptide was achieved directly after coupling $N^α$-Boc-*O*-2,6-Cl_2-Bzl-Tyr. The coupling reaction was achieved with a 3-fold excess of $N^α$-Boc amino acid derivative, a 2.4-fold excess of DCC, and a 3-fold excess of HOBT following the coupling scheme outline above. Acetylation of the protected heptapeptide-resin was achieved with a 6-fold excess of *N*-acetylimidazole in CH_2Cl_2 (3 h) after deprotection of the $N^α$-Boc group and neutralization of the amino terminus. The resultant Ac-Tyr(*O*-2,6-Cl_2-Bzl)-Glu(*O*-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Gly-*p*-MBHA resin was dried in vacuo (1.49 g) and treated with 22 mL of anhydrous HF in the presence of 4.5 mL of anisole and 150 μL of 1,2-dithioethane for 45 min at 0 °C. After the HF, anisole, and 1,2-dithioethane were evaporated in vacuo, the dried product mixture was washed with three 30-mL portions each of ethyl acetate, and the peptide was extracted with three 30-mL portions each of 30% acetic acid, 1 N acetic acid, and H_2O. The combined aqueous extracts were lyophilized to give 206.2 mg of crude Ac-[Tyr^4,N^i-For-Trp^9]-α-MSH$_{4-10}$-NH$_2$.

A portion of the crude heptapeptide (75.0 mg) was deformylated as for the previous peptide; after acidification to pH 4.5, the solution was directly applied to a carboxymethylcellulose column (1.8 × 15.2 cm) with 250 mL of 0.01 M NH_4OAc at pH 4.5 as the initial eluant solvent. A stepwise gradient of 250 mL each of 0.1, 0.2, and 0.4 M NH_4OAc (pH 6.8) was then applied to the column. The major peak (280 nm detection) eluted during the 0.1 M NH_4OAc (pH 6.8) fraction and was lyophilized to give 35.7 mg of white powder (overall yield 52.7%). The analytical data for the compound are given in Table I, and a summary of the biological potencies is given in Table II.

Ac-[Nle^4]-α-MSH$_{4-10}$-NH$_2$, Ac-Nle-Glu-His-Phe-Arg-Trp-Gly-NH$_2$. From 3.2 g of Boc-Gly-*p*-MBHA resin (0.87 mmol of Boc-Gly total) was prepared 3.66 g of Boc-Arg(N^g-Tos)-Trp(N^i-For)-Gly-*p*-MBHA resin as before. A 1.15-g portion (about 0.27 mm) was converted to the protected heptapeptide resin of the title compound by stepwise coupling of $N^α$-Boc-Phe, $N^α$-Boc-N^{im}-Tos-His, $N^α$-Boc-γ-Bzl-Glu, and $N^α$-Boc-Nle. Each coupling reaction was achieved with a 3-fold excess of $N^α$-Boc amino acid (or derivative), a 2.4-fold excess of DCC, and a 3-fold excess of *N*-hydroxybenzotriazole following the coupling scheme outline above. Acetylation of the protected heptapeptide-resin was achieved with a 6-fold excess of *N*-acetylimidazole in dichloromethane (15 h) after deprotection of the $N^α$-Boc group and subsequent neutralization of the amino terminus. The Ac-Nle-Glu(γ-Bzl)-His(N^{im}-Tos)-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Gly-*p*-MBHA resin was dried in vacuo (1.18 g) and treated with 18 mL of anhydrous HF in the presence of 3.6 mL of anisole and 120 μL of 1,2-dithioethane for 30 min at 0 °C. After the HF, anisole, and 1,2-dithioethane were evaporated in vacuo, the dried product mixture was washed with three 30-mL portions of ethyl acetate, and the peptide was extracted with three 30-mL portions each of 30% acetic acid, 1 N acetic acid, and H_2O. The combined aqueous extracts were lyophilized to give 170.0 mg of crude Ac-[Nle^4,N^i-For-Trp^9]-α-MSH$_{4-10}$-NH$_2$.

A portion of the crude heptapeptide (50.0 mg) was dissolved in 3 mL of H_2O and deformylated as described above. The solution (pH 4.5) was then applied directly to a carboxymethylcellulose column (2.0 × 11.5 cm) with 200 mL of 0.01 M NH_4OAc (pH 4.5) as the initial eluant solvent. A stepwise gradient of 200 mL each of 0.1, 0.2, and 0.4 M NH_4OAc (pH 6.8) was then applied to the column. The major peak (280 nm detection) eluted during the 0.1 M NH_4OAc (pH 6.8) fraction was lyophilized to give 19.9 mg of white powder (overall yield 21.5%). The analytical data for the title compound are given in Table I, and a summary of the biological potencies is given in Table II.

Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$, Ac-Nle-Glu-His-D-Phe-Arg-Trp-Gly-NH$_2$. From 1.22 g (~0.29 mmol) of Boc-Arg(N^g-Tos)-Trp(N^i-For)-Gly-*p*-MBHA resin (prepared above), solid-phase synthesis of the title peptide was achieved directly after stepwise coupling of $N^α$-Boc-D-Phe, $N^α$-Boc-N^{im}-Tos-His, $N^α$-Boc-γ-Bzl-Glu, and $N^α$-Boc-Nle. Each coupling reaction was achieved with a 3-fold excess of $N^α$-Boc amino acid derivative and a 2.4-fold excess of dicyclohexycarbodiimide following the coupling scheme outlined above. In addition, a 3-fold excess of *N*-hydroxylbenzotriazole was used for the DCC-mediated coupling of $N^α$-Boc-N^{im}-Tos-His and $N^α$-Boc-D-Phe. Acetylation of the protected heptapeptide-resin was achieved with a 6-fold excess of *N*-acetylimidazole in dichloromethane (15 h) after deprotection of the $N^α$-Boc group and subsequent neutralization of the amino terminus. The resultant Ac-Nle-Glu(γ-Bzl)-His(N^{im}-Tos)-D-Phe-Arg(N^g-Tos)-Trp(N^i-For)-Gly-*p*-MBHA resin was dried in vacuo (1.30 g) and treated with 20 mL of anhydrous HF in the presence of 4 mL of anisole and 130 μL of 1,2-dithioethane for 30 min at 0 °C. After the HF, anisole, and 1,2-dithioethane were evaporated in vacuo, the dried product mixture was washed with three 30-mL portions of ethyl acetate, and the peptide was subsequently extracted with three 30-mL portions each of 30% HOAc, 1 N HOAc, and H_2O. The combined aqueous extracts were lyophilized to give 155.0 mg of crude Ac-[Nle^4,D-Phe^7,N^i-For-Trp^9]-α-MSH$_{4-10}$-NH$_2$.

A portion of the crude heptapeptide (66.6 mg) was dissolved in 3 mL of H_2O and deformylated as above. The solution (pH 4.5) was then applied directly on a carboxymethylcellulose column (2.0 × 11.5 cm) using 200 mL of 0.01 M NH_4OAc (pH 4.5) as the initial eluant solvent. A stepwise gradient of 200 mL each of 0.1, 0.2, and 0.4 M NH_4OAc (pH 6.8) was then applied to the column. The major peak (280 nm detection) eluted during the 0.1 N NH_4OAc (pH 6.8) fraction and was lyophilized to give 21.8 mg (overall yield 16.1%) of Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-10}$-NH$_2$. The analytical data for the compound are given in Table I, and a summary of the biological potencies is given in Table II.

Frog and Lizard Skin Bioassays. The biological activities of α-melanotropin and α-MSH$_{4-10}$ analogues were determined by their ability to stimulate melanosome dispersion in vitro with the frog and lizard skin bioassays as previously described.[13,18,22] The frogs (*Rana pipiens*) used in these studies were obtained from the Lemberger Co., Germantown, WI, and the lizards (*Anolis carolinensis*) were from the Snake Farm, La Place, LA.

Melanoma Membrane Adenylate Cyclase Assay. The particulate membrane fraction from S-91 mouse melanoma tumors grown in DBA/2J mice was isolated as previously described.[19] The adenylate cyclase activity of these membranes was determined by assay [α-^{32}P]ATP conversion to [^{32}P]cAMP as previously described.[14,19] [^{32}P]cAMP was isolated, purified, and detected according to method of Salomon et al.[29] Radiochemicals were purchased from New England Nuclear Corp.

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