# Cyclic Melanotropins. 5.[1] Importance of the C-Terminal Tripeptide (Lys-Pro-Val)

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In previous work we reported that [Cys[4],Cys[10]]-α-MSH (II) and Ac-[Cys[4],Cys[10]]-α-$MSH_{4-13}$-$NH_2$ (III) were superpotent melanotropins.[2,3] Ac-[Cys[4],Cys[10]]-α-$MSH_{4-10}$-$NH_2$ (VI), which constitutes the cyclic analogue of the putative active site sequence -Met[4]-Glu[5]-His[6]-Phe[7]-Arg[8]-Trp[9]-Gly[10]- of α-MSH, was much less active. In the present investigation the contribution of the Lys[11] and Pro[12] residues of the C-terminal carboxamide tripeptide -Lys[11]-Pro[12]-Val[13]-$NH_2$ to the potency of Cys[4],Cys[10] containing cyclic melanotropins was studied. Ac-[Cys[4],Cys[10]]-α-$MSH_{4-11}$-$NH_2$ (V) was less potent than α-MSH in the frog and lizard skin bioassays and the mouse S-91 (Cloudman) melanoma adenylate cyclase assay but more potent than Ac-[Cys[4],Cys[10]]-α-$MSH_{4-10}$-$NH_2$ in the three assays studied. Ac-[Cys[4],Cys[10]]-α-$MSH_{4-12}$-$NH_2$ (IV) was considerably more potent than the cyclic 4–11 melanotropin and was, in fact, equipotent or even slightly more potent than [Cys[4],Cys[10]]-α-MSH and Ac-[Cys[4],Cys[10]]-α-$MSH_{4-13}$-$NH_2$ over the linear portion of the dose–response in all three bioassays. These results demonstrate that Lys[11] and Pro[12] but to a lesser extent Val[13] of the C-terminal tripeptide sequence contributes to the potency of the cyclic melanotropins. The further substitution of a D-Phe[7] for the L-Phe[7] residue into the cyclic 4–12 analogue resulted in a highly potent compound Ac-[Cys[4],D-Phe[7],Cys[10]]-α-$MSH_{4-12}$-$NH_2$ (VII) that exhibited highly prolonged biological activity.

α-Melanocyte stimulating hormone (α-melanotropin, α-MSH) is a linear tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-$NH_2$), which along with other melanotropins, is derived from propiomelanocortin within cells of the pars intermedia and the brain.[4] α-MSH is primarily recognized for its ability to stimulate melanosome dispersion within integumental melanophores,[5] but it also appears to participate in a number of other physiological processes.[6,7]

The central heptapeptide sequences, -Met[4]-Glu[5]-His[6]-Phe[7]-Arg[8]-Trp[9]-Gly[10]-, is common to three melanotropins (α-MSH, β-MSH, and ACTH) and is believed to comprise the primary active site (message sequence) of these peptides.[8] In addition to this active-site sequence, the acetylated C-terminal carboxamide tripeptide, Ac-Lys[11]-Pro[12]-Val[13]-$NH_2$, has been reported to be an additional message sequence with inherent melanotropic activity.[9,10] In any case, it is clear from structure–function studies of α-MSH fragments and related analogues that some component of the C-terminal tripeptide sequence is essential for high melanotropic potency of α-MSH.[8,11-13]

It has recently been proposed by Sawyer et al.[2] that the presence of some type of reverse-turn conformation at the center of the active site (-His[6]-Phe[7]-Arg[8]-Trp[9]-) of α-MSH is important to biological activity. In order to further investigate this claim, we have synthesized a number of cyclic analogues of α-MSH via a pseudoisosteric substitution of disulfide-linked Cys[4],Cys[10] for Met[4] and Gly[10]. Two of these cyclic melanotropins, [Cys[4],Cys[10]]-α-MSH and Ac-[Cys[4],Cys[10]]-α-$MSH_{4-13}$-$NH_2$, proved to be highly potent agonists, whereas Ac-[Cys[4],Cys[10]]-α-$MSH_{4-10}$-$NH_2$ (Figure 1) was considerably less active than α-MSH.

In order to determine the contribution of the individual amino acids (Lys[11], Pro[12], Val[13]) of the C-terminal tripeptide sequence of α-MSH to the superpotent activities of the cyclic melanotropins, we have synthesized Ac-[Cys[4],Cys[10]]-α-$MSH_{4-11}$-$NH_2$ and Ac-[Cys[4],Cys[10]]-α-$MSH_{4-12}$-$NH_2$. The results from this study are outlined in this report.

**Table I.** Relative in Vitro Potencies of Cyclic α-MSH Analogues in the Frog (*Rana pipiens*) and Lizard (*Anolis carolinensis*) Skin Bioassays

| peptide | rel potency to α-MSH[a] | | |
|---|---|---|---|
| | frog skin | lizard skin | adenylate cyclase |
| I, α-MSH | 1.0 | 1.0 | 1.0 |
| II, [Cys[4],Cys[10]]-α-MSH | 10.0[b] | 2.0 | 3.0 |
| III, Ac-[Cys[4],Cys[10]]-α-$MSH_{4-13}$-$NH_2$ | 30.0[b] | 0.6 | 1.1 |
| IV, Ac-[Cys[4],Cys[10]]-α-$MSH_{4-12}$-$NH_2$ | 10.0[b] | 1.5 | 1.4 |
| V, Ac-[Cys[4],Cys[10]]-α-$MSH_{4-11}$-$NH_2$ | 0.16 | 0.07 | 0.16 |
| VI, Ac-[Cys[4],Cys[10]]-α-$MSH_{4-10}$-$NH_2$ | 0.06 | 0.003 | 0.016 |
| VII, Ac-[Cys[4],D-Phe[7],Cys[10]]-α-$MSH_{4-12}$-$NH_2$ | 20. | 6.0 | ND[c] |
| VIII, Ac-α-$MSH_{4-10}$-$NH_2$ | 0.0007 | 0.03 | 0.03 |

[a] Values derived from parallel dose–response curves. [b] Potencies calculated from minimum effective doses are generally higher than those reported here. [c] ND = not determined.

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I. Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-$NH_2$ — α-MSH

II. Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-10}NH_2$

III. Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-11}NH_2$

IV. Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-12}NH_2$

V. Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-13}NH_2$

VI. Ac-Ser-Tyr-Ser-[Cys⁴,Cys¹⁰]-α-$MSH_{4-10}NH_2$

**Figure 1.** Primary structures of the melanotropins studied.

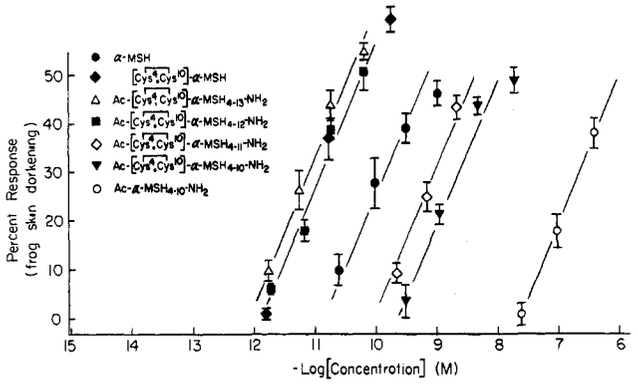


**Figure 2.** Comparative in vitro dose–response curves of a number of cyclic [Cys⁴,Cys¹⁰]melanotropins as determined by the frog (*Rana pipiens*) skin bioassay. Each value represents the mean ± SE, response (darkening) of the skins ($n$ = 6 or more in all experiments) to the melanotropins at the concentrations indicated.

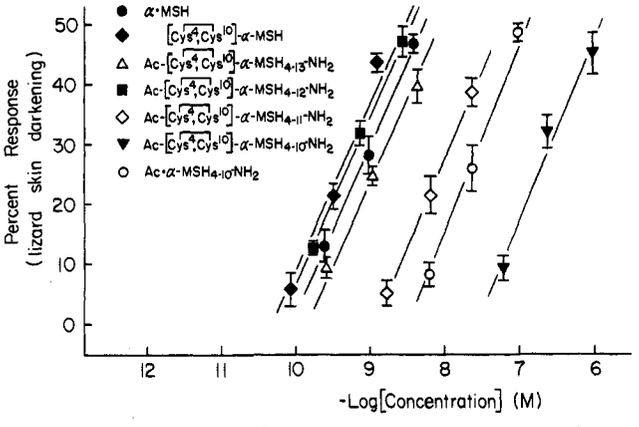


**Figure 3.** As in Figure 2. Lizard (*Anolis carolinensis*) skin bioassay.

## Results

The melanotropic activities of Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-11}$-$NH_2$ (V) and Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-12}$-$NH_2$ (IV) are compared to α-MSH (I), Ac-α-$MSH_{4-10}$-$NH_2$ (VIII) and the other disulfide-bridged cyclic melanotropins (See Figure 1 and Table I) in the lizard (*Anolis carolinensis*) (Figure 2) and frog (*Rana pipiens*) (Figure 3) skin bioassays over the linear portion of the dose–response curve, as well as in the S-91 mouse melanoma adenylate cyclase assay (Figure 4). Previously, we discussed the biological activities of other related cyclic compounds.[2,3] In particular, we reported Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-13}$-$NH_2$ (III) and

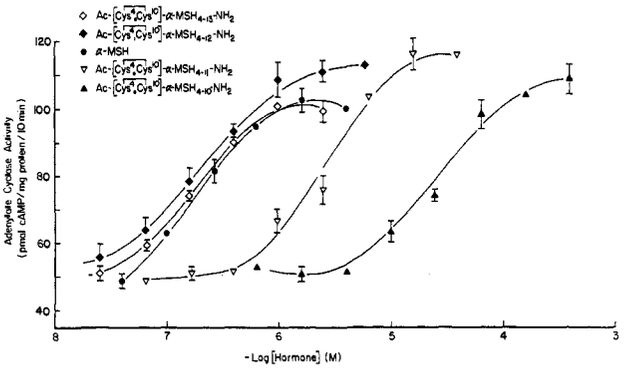


**Figure 4.** Comparative in vitro dose–response curves of a number of cyclic [Cys⁴,Cys¹⁰]melanotropins as determined by the S-91 mouse melanoma adenylate cyclase bioassay.

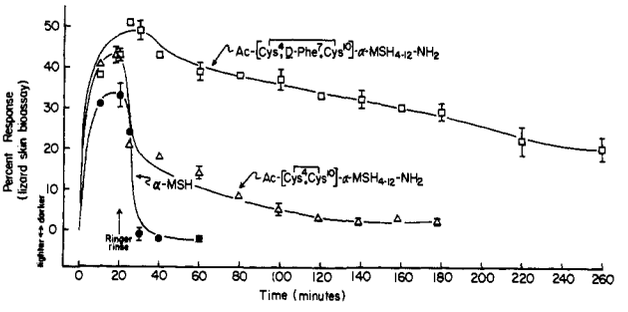


**Figure 5.** In vitro demonstration of the prolonged activity (skin darkening) of Ac-[Cys⁴,D-Phe⁷,Cys¹⁰]-α-$MSH_{4-12}$-$NH_2$ (□) compared to Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-12}$-$NH_2$ (△) and to α-MSH (●). Each value represents the mean ± SE, response (lizard skin darkening) of the skins ($n$ = 6) to the melanotropins at the concentrations noted.

[Cys⁴,Cys¹⁰]-α-MSH (II) to be superpotent agonists[2,3] while Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-10}$-$NH_2$ (VI) was only a weak agonist (on all three assay systems).[3] With only one exception (in the frog and lizard skin bioassays), the cyclic melanotropins were always more potent than their linear counterparts. The exception was that, although Ac-[Cys,⁴Cys¹⁰]-α-$MSH_{4-10}$-$NH_2$ was more active than Ac-α-$MSH_{4-10}$-$NH_2$ on the frog skin assay, this analogue was less active than the linear 4–10 on lizard skin melanophores (Figures 2, 3).[3]

The cyclic analogue Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-11}$-$NH_2$ was more potent than the cyclic 4–10 compound in the frog and lizard skin bioassays (Figures 2, 3). The cyclic 4–12 analogue Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-12}$-$NH_2$ was found to be at least equipotent to Ac-[Cys⁴,Cys¹⁰]-α-$MSH_{4-13}$-$NH_2$ and [Cys⁴,Cys¹⁰]-α-MSH in both assays (Figures 2, 3). All of these compounds, the 4–12, 4–13, and 1–13 cyclic melanotropins, were considerably more active than α-MSH on the frog skin bioassay (Figure 2), being greater than 10 times more potent than α-MSH over the linear portion of the dose–response curve. They were also equipotent or slightly more potent than α-MSH on the lizard skin (Figure 3) and the adenylate cyclase (Figure 4) bioassays.

We previously reported[13–15] that [Nle⁴,D-Phe⁷]-containing melanotropins were superpotent agonists possessing

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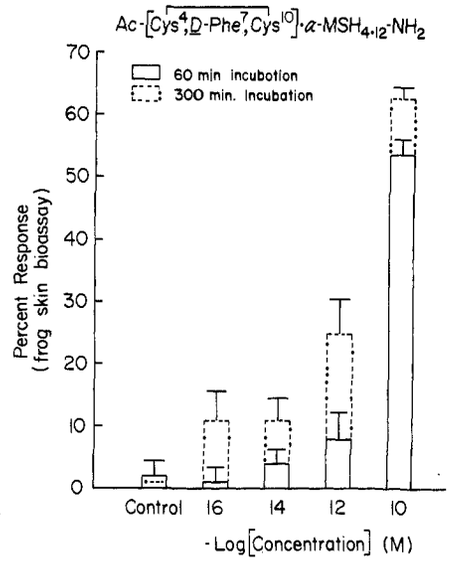


**Figure 6.** In vitro demonstration of the potencies obtained when stock solutions of the melanotropins were serially diluted and skin darkening measured at different times.

in most cases extremely prolonged biological activities, particularly on frog skin melanophores. The 1–13, 4–13, and 4–12 [Cys$^4$,Cys$^{10}$]-containing cyclic melanotropins do not exhibit such prolonged biological activity in the frog skin bioassay, but the incorporation of D-Phe$^7$ into the cyclic 4–12 melanotropin resulted in a potent agonist VII (Table I) with prolonged activity in the lizard (Figure 5) and frog (not shown) bioassays. This prolonged activity is essentially identical with that previously reported for the linear analogue [Nle$^4$,D-Phe$^7$]-$\alpha$-MSH. The relative potencies reported in the three bioassays were determined from parallel dose–response curves.

As previously reported,[2,11,16] the minimal effective doses that were reported for the 1–13 and 4–13 cyclic melanotropins on the frog skin bioassay are quite variable and are greatly influenced by experimental technique. We have now carefully investigated this problem and report the results of our findings here. The potency values presented in Table I for [Cys$^4$,Cys$^{10}$]-$\alpha$-MSH and Ac-[Cys$^4$,Cys$^{10}$]-$\alpha$-MSH$_{4-13}$-NH$_2$ are those derived by examining the activity of these peptides over the linear portion of the dose–response curve. These values are lower than those previously reported as minimal effective dose potencies. When the peptides are measured over the linear portion of the dose–response curve, we now find that 1–13, 4–13, and 4–12 cyclic melanotropin analogues are always 10–100 times more active than $\alpha$-MSH in the frog skin bioassay using our standard assay conditions. These compounds are superagonists, but we believe the potencies reported here more nearly correspond to an accurate measure of the relative potencies to their interaction with the melanotropin receptor on the frog skin (Table I). Nonetheless, it is interesting to note that [Cys$^4$,Cys$^{10}$]-$\alpha$-MSH, and the corresponding 4–13 analogue (not shown), as well as Ac-[Cys$^4$,D-Phe$^7$,Cys$^{10}$]-$\alpha$-MSH$_{4-12}$-NH$_2$ (Figure 6) still displays some activity even at $10^{-14}$ M, especially with prolonged assay time. Thus these analogues possess minimum effective doses at least 1000 times greater than that of $\alpha$-MSH. Such effects are not seen with the superpotent [Nle$^4$,D-Phe$^7$]-$\alpha$-MSH analogues.[14,15]

## Discussion

The results clearly demonstrate the importance of the C-terminal tripeptide (-Lys$^{11}$-Pro$^{12}$-Val$^{13}$-) to the biological potency of the cyclic melanotropins. In all three bioassays, sequential addition of Lys-11 and Pro-12 residues markedly enhances the biological activity. Incorporation of the 13th residue (Val) results in no increase of potency, and in two cases (the lizard skin and S-91 mouse melanoma adenylate cyclase assays) actually causes a slight loss of potency. The importance of valine is therefore unclear, but it could be speculated that valine is a remnant of propiomelanocortin (a protein precursor of $\alpha$-MSH) biosynthesis, but further analysis at this time would only be speculation.

Previously, we have reported on the relative unimportance of the N-terminal tripeptide (-Ser$^1$-Tyr$^2$-Ser$^3$-)[11] to the overall biological activity of the native hormone, and this is again demonstrated here in all three bioassays. Previously reported data[3] along with the results now presented suggest that the minimal sequence required for the superpotency of the cyclic melanotropins is the 4–12 sequence. Interestingly, when Phe$^7$ is replaced by D-Phe$^7$ in the cyclic 4–12 analogue, a substitution that would be expected[17,18] to further stabilize any reverse-turn conformation already stabilized by cyclization, the compound VII showed potencies slightly higher than the non-D-Phe$^7$-containing analogue IV (Table I). However, a new property, highly prolonged activity, was observed (Figure 5). This prolonged activity is not seen in any of the other cyclic analogues discussed here.

In comparing the action of cyclic melanotropins on different species of melanophores, it can be seen that the frog and lizard melanophores differ considerably in their ability to recognize the cyclic structure of melanotropins. For example, on frog skin melanophores the cyclic melanotropins studied are considerably more potent than their linear counterparts. However, in the lizard skin the 4–10 linear compound (Ac-$\alpha$-MSH$_{4-10}$-NH$_2$) is 10 times more potent than Ac-[Cys$^4$,Cys$^{10}$]-$\alpha$-MSH$_{4-10}$-NH$_2$. As documented previously for linear melanotropins, mouse S-91 melanoma cell receptors appear very similar to the lizard, *Anolis carolinensis*, skin receptor. For example, as determined from the parallel dose response curves (Table I), the cyclic 4–10 heptapeptide was also less active than its linear counterpart on the adenylate cyclase and on the lizard skin systems.

In conclusion, it appears that position 13 in $\alpha$-MSH is unimportant for full biological activity of the cyclic compounds. As hypothesized previously,[2,3] we feel that the high potency of cyclic melanotropins is related to a conformational restraint imposed by the disulfide bridge which may favor the bioactive conformation. It is also clear that the 4–12 sequence is the minimal sequence required for superpotency.

## 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 with the following solvent systems: (A) 1-butanol/HOAc/$H_2O$ (4:1:5 upper phase only), (B) 1-butanol/HOAc/pyridine/$H_2O$ (15:3:10:12), (C) 1-butanol/pyridine/HOAc/$H_2O$ (5:5:1:4). Detection was by iodine vapors and ninhydrin. Single spots were obtained unless otherwise noted. Amino acid analyses were obtained with a Beckman 120C amino acid analyzer following hydrolysis for 48 h at 110 °C with 4 M methanesulfonic acid

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**Table II.** Analytical Data for Cyclic Melanotropins

| compd | amino acid analysis | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | half-Cys | Glu | His | Phe | Arg | Trp | Lys | Pro | Val |
| III | 1.94 | 1.00 | 0.93 | 0.98 | 1.04 | 1.02 | 1.00 | 0.06 | 1.06 |
| IV | 1.99 | 1.06 | 0.98 | 0.94 | 1.01 | 0.95 | 1.02 | 0.97 | |
| V | 1.99 | 1.05 | 0.94 | 0.96 | 1.04 | 1.04 | 0.97 | | |
| VI | 1.80 | 1.03 | 1.04 | 0.97 | 1.05 | 0.92 | | | |
| VII | 1.90 | 1.05 | 1.03 | 1.01 | 1.02 | 0.93 | 1.02 | 1.05 | |

| | TLC[a] | | | | | |
|---|---|---|---|---|---|---|
| | A | B | C | HPLC, $k'$[b] | MS, $MH^+$ | optical rotations, $[\alpha]^{25}_{546}$ in 10% HOAc, deg |
| III | 0.13 | 0.42 | 0.52 | 2.75 | 1344 | −63.9 |
| IV | 0.13 | 0.45 | 0.14 | 1.89 | 1245 | −61.2 |
| V | 0.07 | 0.41 | 0.11 | 1.73 | 1148 | −25.6 |
| VI | 0.18 | 0.60 | 0.60 | 2.80 | 1020 | −20.0 |
| VIII | 0.02 | 0.48 | 0.47 | 1.41 | ND[c] | ND |

[a] A = 1-butanol/HOAc/$H_2O$ (4:1:5 upper phase only); B = *sec*-butanol/HOAc/pyridine/$H_2O$ (15:3:10:12); C = 1-butanol/pyridine/HOAc/$H_2O$ (5:5:1:4). [b] $k'$ values are determined on an Altech C18 (5 μm, 25 × 0.46 cm) column, 70% ± 0.5% TFA:30% $CH_3CN$. [c] ND = not determined.

containing 0.2% 3-(2-aminoethyl)indole and subsequent neutralization with 3.5 N NaOH. No corrections were made for destruction of amino acids during hydrolysis. Fast atom bombardment mass spectra were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas. Optical rotations were obtained on a Rudolph Research Autopol III polarimeter at the mercury green line (546 nm).

$N^{\alpha}$-Boc-protected amino acids and amino acid derivatives were purchased from Vega Biochemicals (Tucson, AZ), Peninsula Laboratories (San Carlos, CA), and Bachem (Torrance, CA) or were prepared by using published procedures. Before use, all amino acid derivatives were tested for purity by TLC in solvent systems A, B, and C, melting point, and the ninhydrin test.[19] Solvents used for gel filtration, TLC, and ion-exchange chromatography were purified by redistillation before use. The *p*-methylbenzhydrylamine resin (*p*-MBHA) (1% divinylbenzene cross-linked polystyrene) was prepared by previously reported methods with an amine substitution of 0.62 mmol/g.[15]

**Solid-Phase Peptide Synthesis of Melanotropins.** The α-MSH analogues reported were synthesized by solid-phase methods similar to those used previously for the synthesis of analogues of α-MSH. α-MSH, [$\overline{Cys^4,Cys^{10}}$]-α-MSH, Ac-[$\overline{Cys^4,Cys^{10}}$]-α-$MSH_{4-10}$-$NH_2$, Ac-[$\overline{Cys^4,Cys^{10}}$]-α-$MSH_{4-13}$-$NH_2$ were prepared and purified as reported.[2,3] $N^{\alpha}$-Boc-protected amino acid derivatives were successively coupled to a *p*-methylbenzhydrylamine resin with a 3-fold excess of the Boc-protected amino acid derivative, a 3-fold excess of *N*-hydroxybenzotriazole (HOBt), and a 2.4-fold excess of dicyclohexylcarbodiimide (DCC). Cleavage of the $N^{\alpha}$-Boc protecting group was performed by treatment with 45% trifluoroacetic acid containing 2% anisole in dichloromethane. Side-chain functionalities were protected as follows: arginine, $N^{g}$-tosyl; glutamic acid, γ-*O*-benzyl ester; lysine, $N^{\epsilon}$-2,4-dichlorobenzyloxycarbonyl; tryptophan, $N^{in}$-formyl; histidine, $N^{im}$-tosyl; and cysteine, *S*-4-methylbenzyl. It has been reported that HOBt removes the tosyl protecting group from histidine under the conditions used for synthesis;[20] therefore HOBt was not used in the presence of histidine in the peptide fragments.

A cycle for the incorporation of each amino acid residue into the growing peptide chain consisted of the following: (1) washing with $CH_2Cl_2$ (4 × 25 mL, 1 min/wash), (2) cleaving the $N^{\alpha}$-Boc group with 25 mL of 45% trifluoroacetic acid in dichloromethane containing 2% anisole (one treatment for 2 min, a second for 20 min), (3) washing with $CH_2Cl_2$ (4 × 25 mL, 1 min/wash), (4) neutralizing with 10% diisopropylethylamine in $CH_2Cl_2$ (2 × 25 mL, 2 min/wash), (5) washing with $CH_2Cl_2$ (3 × 25 mL, 1 min/wash), (6) adding the Boc-protected amino acid derivative in 20 mL of $CH_2Cl_2$, followed by HOBt (dissolved in a minimal amount of anhydrous DMF), followed by DCC and shaking for 3.5–12 h, (7) washing with $CH_2Cl_2$ (3 × 25 mL, 1 min/wash), (8) washing with EtOH (3 × 25 mL, 1 min/wash). Between steps 1 and 2 and steps 6 and 7, several milligrams of the resin were removed and used in a ninhydrin test[19] to determine the progress of the coupling. After coupling all of the amino acid residues to the resin, the amino terminal of each peptide was acetylated with a 6-fold excess of *N*-acetylimidazole in $CH_2Cl_2$. The finished protected peptides were cleaved from the resin, and all protecting groups were removed with anhydrous liquid HF (0 °C for 50–60 min) containing 10% anisole and 5% 1,2-dithioethane.[21] Cyclization was carried out in dilute solution via oxidation with potassium ferricyanide as reported in the literature.[2]

**Ac-[$\overline{Cys^4,Cys^{10}}$]-α-$MSH_{4-11}$-$NH_2$ (V).** Starting with 2.00 g of $N^{\alpha}$-Boc-Lys(2,4-$Cl_2$-Z)-*p*-MBHA resin (0.60 mmol of $N^{\alpha}$-Boc-Lys-(2,4-$Cl_2$-Z), the protected peptide resin corresponding to the title peptide was obtained after stepwise coupling of the following $N^{\alpha}$-Boc-protected amino acids (in order of addition): $N^{\alpha}$-Boc-*S*-4-MeBzl-Cys, $N^{\alpha}$-Boc-$N^{in}$-For-Trp, $N^{\alpha}$-Boc-$N^{g}$-Tos-Arg, $N^{\alpha}$-Boc-Phe, $N^{\alpha}$-Boc-$N^{im}$-Tos-His, $N^{\alpha}$-Boc-γ-*O*-Bzl-Glu, and $N^{\alpha}$-Boc-*S*-4-MeBzl-Cys. After coupling the last amino acid and removing the $N^{\alpha}$-Boc group, the peptide was acetylated by using a 6-fold excess of *N*-acetylimidazole in 25 mL of $CH_2Cl_2$. The resultant, Ac-Cys(*S*-4-MeBzl)-Glu(*O*-γ-Bzl)-His($N^{im}$-Tos)-Phe-Arg($N^{g}$-Tos)-Trp($N^{in}$-For)-Cys(*S*-4-MeBzl)-Lys($N^{\epsilon}$-2,4-$Cl_2$-Z)-*p*-MBHA resin was dried in vacuo. A portion (1.00 g) of the protected peptide was cleaved from the resin along with all the protecting groups by treatment with anhydrous HF (13.0 mL), anisole (1.4 mL), and 1,2-dithioethane (0.70 mL) (50 min, 0 °C). After evaporation of the HF, anisole, and 1,2-dithioethane in vacuo, the dried product was washed with EtOAc (3 × 30 mL) and extracted with 30% HOAc (3 × 30 mL), 10% HOAc (3 × 30 mL), and distilled water (3 × 30 mL), successively, under a stream of argon. The combined aqueous extracts were lyophilized to give 212.5 mg of Ac-[$Cys(H)^4$,$Cys(H)^{10}$]-α-$MSH_{4-11}$-$NH_2$. The fully deprotected peptide was diluted with 600 mL of distilled deareated water, adjusting the pH to 8.5 with 10% $NH_4OH$, and was subjected to oxidative cyclization with 60 mL (100% excess) of 0.01 N $K_3Fe(CN)_6$. After 30 min at room temperature, the reaction was terminated by the addition of 10% AcOH until a pH of 5.0 was reached. Excess ferro- and ferricyanide ions were removed by the addition of Rexyn 203 ($Cl^-$ form) anion-exchange resin. After stirring for 30 min and subsequent gravity filtration, the solution was lyophilized to give 301.0 mg of the crude peptide fragment. The crude peptide was dissolved in a minimal amount of 30% acetic acid and eluted on a Sephadex G-15 column with 30% AcOH. The only peak (280-nm detection) was found immediately after the void volume and it was lyophilized. The desalted peptide (63.9 mg) was dissolved in 3 mL of 0.01 N $NH_4OAc$, pH 4.5, and chromatographed on (carboxymethyl)-cellulose column (2.0 × 18.0 cm) with a discontinuous gradient (250 mL each) of 0.01 (pH 4.5), 0.1, 0.2, and 0.4 N $NH_4OAc$ (pH 6.8). The major peak (280-nm detection) eluted during the 0.1 N $NH_4OAc$ (pH 6.8) buffer and was lyophilized to give 9.38 mg

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of a white powder. Analytical data are found in Table II.

**Ac-[Cys$^4$,Cys$^{10}$]-α-MSH$_{4-12}$-NH$_2$ (IV).** Starting with 2.25 g of $N^{\alpha}$-Boc-Pro-*p*-MBHA resin (0.62 mmol/g of $N^{\alpha}$-Boc-Pro), the title peptide was obtained after stepwise coupling of the following $N^{\alpha}$-Boc protected amino acids (in order of addition): $N^{\alpha}$-Boc-$N^{\epsilon}$-2,4-Cl$_2$-Z-Lys, $N^{\alpha}$-Boc-*S*-4-MeBzl-Cys, $N^{\alpha}$-Boc-$N^{in}$-For-Trp, $N^{\alpha}$-Boc-$N^{g}$-Tos-Arg, $N^{\alpha}$-Boc-Phe, $N^{\alpha}$-Boc-$N^{im}$-Tos-His, $N^{\alpha}$-Boc-γ-Bzl-Glu, and $N^{\alpha}$-Boc-*S*-4-MeBzl-Cys. Acetylation of the N terminus of the protected peptide was performed with a 6-fold excess of *N*-acetylimidazole in 25 mL of $CH_2Cl_2$. The resultant Ac-Cys(*S*-4-MeBzl)-Glu(*O*-γ-Bzl)-His($N^{im}$-Tos)-Phe-Arg($N^{g}$-Tos)-Trp($N^{i}$-For)-Cys(*S*-4-MeBzl)-Lys($N^{\epsilon}$-2,4-Cl$_2$-Z)-Pro-*p*-MBHA resin (2.85 g) was dried in vacuo. A portion of this material (1.00 g) was treated with anhydrous HF (20.0 mL) in the presence of dithioethane (1:1 mL) and anisole (2.1 mL) (55 min, 0 °C). Following evaporation of the HF, anisole, and 1,2-dithioethane in vacuo, the product was washed under a stream of argon with EtOAc (3 × 30 mL) to remove the residual 1,2-dithioethane and anisole. It was then extracted with three 30-mL portions of 30% HOAc, 10% HOAc, and distilled water, successively. The combined extracts were lyophilized to give Ac-[Cys(H)$^4$,Cys(H)$^{10}$]-α-MSH$_{4-12}$-NH$_2$. The crude peptide was diluted with 1200 mL of distilled $H_2O$ and the pH was adjusted to 8.7 with 10% $NH_4OH$. Oxidative cyclization was achieved by stirring with 0.01 N $K_3Fe(CN)_6$ (90.0 mL, 100% excess) for 1 h at room temperature. The reaction was quenched by the addition of 30% HOAc to adjust the final pH to 4.5. Excess ferro- and ferricyanide ions were removed by addition of Bio-Rad AG3-X4A anion-exchange resin ($Cl^-$ form). Filtration followed by lyophilization of the aqueous peptide gave 551.4 mg of the crude peptide material. A portion of the material (152.5 mg) was dissolved in a minimal amount of 30% HOAc and chromatographed on a Sephadex G-15 column with 30% HOAc as the elutant. The only peak appeared immediately after the void volume (280-nm detection) and was lyophilized to give 70.24 mg of the crude peptide. This material was then dissolved in 3 mL of 0.01 N $NH_4OAc$ (pH 4.5) and chromatographed on a (carboxymethyl)cellulose column with the conditions used in the previous synthesis. The major peak eluted during the 0.1 N $NH_4OAc$ (pH 6.8) buffer and was lyophilized to give 30.24 mg of a white powder. Analytical data are found in Table II.

**Ac-[Cys$^4$,D-Phe$^7$,Cys$^{10}$]-α-MSH$_{4-12}$-NH$_2$ (VII).** Starting with 1.00 g of $N^{\alpha}$-Boc-Pro-*p*-MBHA resin (0.62 mmol/g of $N^{\alpha}$-Boc-Pro), the title peptide was synthesized by the stepwise coupling of the following $N^{\alpha}$-Boc-protected amino acids (in order of addition): $N^{\alpha}$-Boc-$N^{\epsilon}$-2,4-Cl$_2$-Z-Lys, $N^{\alpha}$-Boc-*S*-4-MeBzl-Cys, $N^{\alpha}$-Boc-$N^{in}$-For-Trp, $N^{\alpha}$-Boc-$N^{g}$-Tos-Arg, $N^{\alpha}$-Boc-D-Phe, $N^{\alpha}$-Boc-$N^{im}$-Tos-His, $N^{\alpha}$-Boc-γ-Bzl-Glu, and $N^{\alpha}$-Boc-*S*-4-MeBzl-Cys. Acetylation of the N terminus of the protected peptide was performed with a 6-fold excess of *N*-acetylimidazole in 25 mL of $CH_2Cl_2$. The resultant Ac-Cys(S-4-MeBzl)-Glu(*O*-γ-Bzl)-His($N^{im}$-Tos)-D-Phe-Arg($N^{g}$-Tos)-Trp($N^{in}$-For)-Cys(*S*-4-MeBzl)-Lys($N^{\epsilon}$-2,4-Cl$_2$-Z)-Pro-*p*-MBHA resin (1.84 g) was dried in vacuo. A portion of this material (0.90 g) was treated with anhydrous HF (13.0 mL), anisole (1.4 mL), and 1,2-dithioethane (0.7 mL) at 0 °C for 60 min. The HF, anisole, and 1,2-dithioethane were removed in vacuo, and the residue was washed with EtOAc (3 × 60 mL). It was then extracted with 30% HOAc (3 × 60 mL), 10% HOAc (3 × 60 mL), and distilled water (3 × 60 mL). The combined extracts were lyophilized to give Ac-[Cys(H)$^4$,Cys(H)$^{10}$]-α-MSH$_{4-12}$-NH$_2$ as a white powder. The crude peptide wad diluted with 1200 mL of $H_2O$, adjusting the pH was to 8.4 with 50% $NH_4OH$, and cyclized with the addition of 74 mL of 0.01 N $K_3Fe(CN)_6$. It was allowed to stir for 1 h, at which time the reaction was quenched by acidifying to pH 4.6 with glacial HOAc. The excess ferro- and ferricyanide ions were removed with 12 mL of packed Bio-Rad AG3-X4A anion-exchange resin ($Cl^-$ form). The resulting peptide after lyophilization was dissolved in a minimal amount of 30% HOAc and chromatographed on a Sephadex G-15 column with 30% HOAc as the elutant. The only peak detected, directly after the void volume, was lyophilized to give 96.52 mg of a white powder. The powder was dissolved in a minimal amount of 0.01 N $NH_4OAc$ (pH 4.5) and chromatographed on a (carboxymethyl)cellulose column with the conditions used in the synthesis of compound V. The title compound eluted during the 0.1 N $NH_4OAc$ (pH 6.8) buffer and was lyophilized to give 27.15 mg of peptide. Analytical data are found in Table II.

**Frog and Lizard Skin Bioassays.** The biological activities of α-MSH and the cyclic analogues were determined by their ability to stimulate melanosome dispersion in vitro in the frog and lizard bioassays as previously described.[22-24] All the solutions were prepared via serial dilutions from a stock solution ($10^{-4}$ M). The frogs (*Rana pipiens*) used in these studies were obtained from Kons Scientific, Germantown, WI, and the lizards (*Anolis carolinensis*) were from the Snake Farm, La Place, LA.

**Melanoma Adenylate Cyclase Assay.** The particulate membrane fraction form S-91 mouse melanoma tumors grown in DBA/20 mice was isolated as previously reported.[25] The adenylate cyclase activity of these membranes was determined by assay ($\alpha$-$^{32}$P)ATP conversion to ($^{32}$P)cAMP as previously described.[14,25] ($^{32}$P)cAMP was isolated, purified, and detected according to the method of Salomon et al.[26] Radiochemicals were purchased from ICN Chemical and Radioisotope Division, Irvine, CA.

**Acknowledgment.** This research was supported by grants from the U.S. Public Health Service AM 17420 and the National Science Foundation. Dr. A. M. Castrucci is supported by a fellowship of the Conselho Nacional de Desenvolvimento Tecnológico e Científico of Brasil, Grant 200 430/82. We thank the referees for useful comments.

**Registry No.** I, 581-05-5; II, 81854-62-8; III, 83897-18-1; IV, 90858-01-8; V, 90858-02-9; VI, 83877-16-1; VII, 90899-28-8; VIII, 82219-24-7; adenylcyclase, 9012-42-4.

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