g), eluting with 3% ether in hexanes (v/v), to yield methyl 2-(4-butoxyphenyl)propionate (1.5 g, 40%).

A solution of this ester (1.5 g, 6.3 mmol) in 2-propanol (20 mL) was added to lithium hydroxide monohydrate (1.5 g) in H_2O (10 mL). After the mixture was heated for 1 h at reflux, the solvent was evaporated and the residue was partitioned between ether and 2 N HCl. The ether layer was dried with $MgSO_4$, and the solvent was evaporated to give 2-(4-butoxyphenyl)propionic acid as a white solid (1.4 g, 98%).

This was converted to **16** by using the method described above for **7** (oil, 52%): ^{1}H NMR (Me_2SO-d_6) δ 0.92 (t, 3 H, J = 8 Hz), 1.24 (d, 3 H, J = 7 Hz), 1.42 (m, 2 H, J = 8 Hz), 1.67 (m, 2 H, J = 8 Hz), 3.06 (s, 3 H), 3.92 (t, 2 H, J = 8 Hz), 4.22 (q, 1 H, J = 7 Hz), 6.83 (d, 2 H, J = 9 Hz), 7.15 (d, 2 H, J = 9 Hz), 9.80 (s, 1 H); MS, m/e 251, 234, 177, 121. Anal. ($C_{14}H_{21}NO_3$) C, H, N.

Acknowledgment. We thank Jennifer Barlow-Donnelly, Dirk Bornemeier, Thomas Clark, Wendy Rach, Ellen Roberts, and Sheryl Steihr for technical assistance.

Registry No. 1, 106359-63-1; **2**, 106359-60-8; **3**, 105847-10-7; **4**, 106359-59-5; **5**, 76790-15-3; **6**, 53648-05-8; **7**, 110330-36-4; **8**, 110319-71-6; **9**, 110319-72-7; **10**, 110319-73-8; **11**, 110319-74-9; **12**, 110319-75-0; **12** (corresponding acid), 95499-72-2; **13**, 110319-76-1; **13** (corresponding acid), 38861-88-0; **14**, 110319-77-2; **15**, 110319-78-3; **16**, 110319-79-4; **17**, 110319-80-7; **18**, 110319-81-8; **19**, 110319-82-9; MeNHOH·HCl, 4229-44-1; 4-*i*-$BuC_6H_4CH_2CO_2Me$, 61566-33-4; i-PrI, 75-30-9; 4-*i*-$BuC_6H_4CH(CO_2Me)Pr$-*i*, 110319-83-0; 4-*i*-$BuC_6H_4CH(CO_2H)Pr$-*i*, 110319-84-1; MeI, 74-88-4; 4-*i*-BuC_6H_4Ac, 38861-78-8; 4-*i*-$BuC_6H_4CO_2H$, 38861-88-0; 4-*i*-$BuC_6H_4CH_2OH$, 110319-85-2; 4-*i*-BuC_6H_4CHO, 40150-98-9; 4-*i*-$BuC_6H_4CH{=}CHCO_2H$, 66734-95-0; 4-$OHC_6H_4CH_2CO_2Me$, 14199-15-6; $Br(CH_2)_3Me$, 109-65-9; 4-$BuOC_6H_4OAc$, 110319-86-3; 4-$BuOC_6H_4CH(Me)CO_2Me$, 110319-87-4; 4-$BuOC_6H_4CH(Me)CO_2H$, 3585-71-5; Ibuprofen, 15687-27-1; malonic acid, 141-82-2; arachidonate-5-lipoxygenase, 80619-02-9.

α-Melanotropin: The Minimal Active Sequence in the Frog Skin Bioassay

Victor J. Hruby,*† Brian C. Wilkes,†,‖ Mac E. Hadley,‡ Fahad Al-Obeidi,† Tomi K. Sawyer,⊥ Douglas J. Staples,⊥ Ann E. deVaux,⊥ Orin Dym,† Ana Maria de L. Castrucci,‡,# Mary F. Hintz,§ John P. Riehm,§ and K. Ranga Rao§

Department of Chemistry, University of Arizona, Tucson, Arizona 85721, Department of Anatomy, University of Arizona, Tucson, Arizona 85721, Department of Biology, University of West Florida, Pensacola, Florida 32514, and Biopolymer Chemistry/Biotechnology, The Upjohn Company, Kalamazoo, Michigan 49001. *Received June 11, 1987*

The minimal sequence required for biological activity of α-MSH (α-melanotropin, α-melanocyte stimulating hormone) was determined in the frog (*Rana pipiens*) skin bioassay. The sequence required to elicit measurable biological activity was the central tetrapeptide sequence, Ac-His-Phe-Arg-Trp-NH_2 (Ac-α-MSH_{6-9}-NH_2), which was about 6 orders of magnitude less potent than the native tridecapeptide. Smaller fragments of this sequence (Ac-His-Phe-NH_2, Ac-Phe-Arg-NH_2, Ac-His-Phe-Arg-NH_2) were devoid of melanotropic activity at concentrations as high as 10^{-4} M. We were unable to demonstrate biological activity for the tetrapeptide, Ac-Phe-Arg-Trp-Gly-NH_2 (Ac-α-MSH_{7-10}-NH_2), and for several carboxy terminal analogues including Ac-Lys-Pro-Val-NH_2 (Ac-α-MSH_{11-13}-NH_2). We prepared a series of fragment analogues of α-MSH in an attempt to determine the contribution of each individual amino acid to the biological activity of the native hormone. The minimal potency of Ac-α-MSH_{6-9}-NH_2 could be enhanced about a factor of 16 by the addition of glycine to the C-terminus, yielding Ac-α-MSH_{6-10}-NH_2 (Ac-His-Phe-Arg-Trp-Gly-NH_2). Addition of glutamic acid to the N-terminus provided the peptide, Ac-α-MSH_{5-10}-NH_2, which was only slightly more potent than Ac-α-MSH_{6-10}-NH_2, indicating that position 5 contributes little to the biological potency of α-MSH in this assay. Addition of methionine to the N-terminus of Ac-α-MSH_{5-10}-NH_2 resulted in the heptapeptide, Ac-α-MSH_{4-10}-NH_2, which was only about 4-fold more potent than Ac-α-MSH_{5-10}-NH_2. Addition of lysine and proline to the C-terminal of the Ac-α-MSH_{4-10}-NH_2 sequence yielded the peptide, Ac-α-MSH_{4-12}-NH_2 with a 360-fold increase in potency relative to Ac-α-MSH_{4-10}-NH_2. This peptide was only about 6-fold less potent than α-MSH. A series of Nle-4-substituted analogues also were prepared. Ac-[Nle^4]-α-MSH_{4-10}-NH_2 was about 4 times more potent than Ac-α-MSH_{4-10}-NH_2. Ac-[Nle^4]-α-MSH_{4-11}-NH_2 also was about 4 times more potent than Ac-α-MSH_{4-10}-NH_2, demonstrating that lysine-11 contributes somewhat to the biological activity of α-MSH on the frog skin melanocyte receptor. However, addition of proline-12 to this fragment, yielding Ac-[Nle^4]-α-MSH_{4-12}-NH_2, resulted in about a 90-fold increase in relative potency of the melanotropin. Addition of the final C-terminal amino acid, valine-13, provided the decapeptide, Ac-[Nle^4]-α-MSH_{4-13}-NH_2, which showed only a small further increase in potency. This analogue was, however, only about 2–3-fold less active than α-MSH. Addition of the N-terminal tripeptide Ac-Ser-Tyr-Ser to yield the tridecapeptide [Nle^4]-α-MSH resulted in an analogue that was 3 times more potent than α-MSH. The importance of the amino acids in the primary structure of α-MSH in contributing to the biological activity of α-MSH in the frog skin bioassay can be summarized as follows: (1) the central tetrapeptide sequence, Ac-His-Phe-Arg-Trp-NH_2, represents the minimum chain length for observable biological activity; (2) the active sequence of α-MSH is contiguous in that no two structurally noncontiguous fragment sequences were found to have biological activity; (3) Met-4, Gly-10, and Pro-12 are important potentiating amino acids and contribute significantly to the biopotency of α-MSH; and (4) Ser-1 and -3, Tyr-2, Glu-5, Lys-11, and Val-13 apparently contribute only minimally to the biological potency of α-MSH at the frog melanocyte skin receptor.

α-Melanotropin (α-melanocyte stimulating hormone, α-MSH),[1] Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, is a tridecapeptide that is synthesized, stored, and secreted by the pars intermedia of

† Department of Chemistry, University of Arizona.
‡ Department of Anatomy, University of Arizona.
§ Department of Biology, University of West Florida.
⊥ Biopolymer Chemistry/Biotechnology, The Upjohn Company.
‖ Present address: Clinical Research Institute of Montreal, Montreal, Quebec, Canada H2W IRZ.
Permanent address: Departmento de Fisiologia, Instituto de Biociencias, Universidade de São Paulo, CP 11176 S.P., 05499 Brazil.

(1) Symbols and abbreviations are in accord with recommendations of the IUPAC-IUB Commision on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). Other abbreviations include: α-MSH, α-melanotropin; Nle, norleucine; TLC, thin-layer chromatography; DCC, dicyclohexylcarbodiimide; HOAc, acetic acid; 2,4-Cl_2-Z, 2,4-dichlorobenzyloxycarbonyl; For, formyl; Tos, tosyl; NH_4OAc, ammonium acetate; OR, optical rotation; NMR, nuclear magnetic resonance. All optically active amino acids are of the L configuration unless otherwise noted.

0022-2623/87/1830-2126$01.50/0

Table I. Relative in Vitro Potencies of α-MSH Peptides in the Frog (*R. pipiens*) Skin Bioassay

no.	peptide	potency relative to α-MSH[a]	no.	peptide	potency relative to α-MSH[a]
I	α-MSH	1.0	X	Ac-α-MSH_{6-10}-NH_2	0.00008
II	[Nle^4]-α-MSH	3.0	XI	Ac-α-MSH_{7-10}-NH_2	0.0
III	Ac-[Nle^4]-α-MSH_{4-13}-NH_2	0.40	XII	Ac-α-MSH_{8-9}-NH_2	0.000005
IV	Ac-[Nle^4]-α-MSH_4-^{12}-NH_2	0.18	XIII	Ac-α-MSH_{6-8}-NH_2	0.0
V	Ac-[Nle^4]-α-MSH_{4-11}-NH_2	0.002	XIV	Ac-α-MSH_{6-7}-NH_2	0.0
VI	Ac-[Nle^4]-α-MSH_{4-10}-NH_2	0.002	XV	Ac-α-MSH_{7-8}-NH_2	0.0
VII	Ac-α-MSH_{4-12}-NH_2	0.18	XVI	H-α-MSH_{7-8}-OH	0.0
VIII	Ac-α-MSH_{4-10}-NH_2	0.0005	XVII	Ac-α-MSH_{11-13}-NH_2	0.0
IX	Ac-α-MSH_{5-10}-NH_2	0.00014	XVIII	Ac-Lys-NH_2	0.0

[a] All potencies are relative to α-MSH, which is taken as 1.0.

```
                1          4          7          10         13
I       Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH2
II      Ac-Ser-Tyr-Ser-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH2
III                Ac-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH2
IV                 Ac-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-NH2
V                  Ac-Nle-Glu-His-Phe-Arg-Trp-Gly-Lys-NH2
VI                 Ac-Nle-Glu-His-Phe-Arg-Trp-Gly-NH2
VII                Ac-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-NH2
VIII               Ac-Met-Glu-His-Phe-Arg-Trp-Gly-NH2
IX                     Ac-Glu-His-Phe-Arg-Trp-Gly-NH2
X                          Ac-His-Phe-Arg-Trp-Gly-NH2
XI                             Ac-Phe-Arg-Trp-Gly-NH2
XII                        Ac-His-Phe-Arg-Trp-NH2
XIII                       Ac-His-Phe-Arg-NH2
XIV                        Ac-His-Phe-NH2
XV                             Ac-Phe-Arg-NH2
XVI                            H-Phe-Arg-OH
XVII                                          Ac-Lys-Pro-Val-NH2
XVIII                                         Ac-Lys-NH2
```

Figure 1. Primary structures of α-melanotropin and analogues.

the vertebrate pituitary.[2,3] This peptide hormone is derived from a precursor protein, proopiomelanocortin, which also contains within its primary sequence other structurally related peptides: γ-MSH, β-MSH, and ACTH. The major systemic physiological role of α-MSH is the control of integumental pigmentation in many vertebrates.[3] Present evidence suggests that α-MSH is the only physiologically relevant melanotropin regulating skin coloration in vertebrates.[3] Other peripheral and neuroregulatory effects of α-MSH have been reported.[4-7] On the basis of the structural homology within the MSH/ACTH family of peptides, the common heptapeptide sequence, Met-Glu-His-Phe-Arg-Trp-Gly, has been suggested to be the important structural determinant for the melanotropic activity of these peptides.

On the basis of a number of previous structure–function studies of α-MSH analogues and fragments with the in vitro frog (*Rana pipiens*) skin bioassay, the active sequence of α-MSH for melanotropic activity was suggested to be Met-Glu-His-Phe-Arg-Trp-Gly.[8] This synthetic fragment

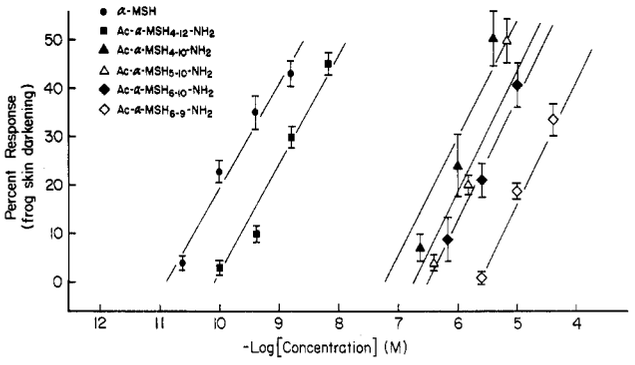

Figure 2. Comparative in vitro dose–response curves of α-MSH (●), Ac-α-MSH_{4-12}-NH_2 (■), Ac-α-MSH_{4-10}-NH_2 (▲), Ac-α-MSH_{5-10}-NH_2 (△), Ac-α-MSH_{6-10}-NH_2 (◆), and Ac-α-MSH_{6-9}-NH_2 (◇) as determined on the frog (*R. pipiens*) skin assay.

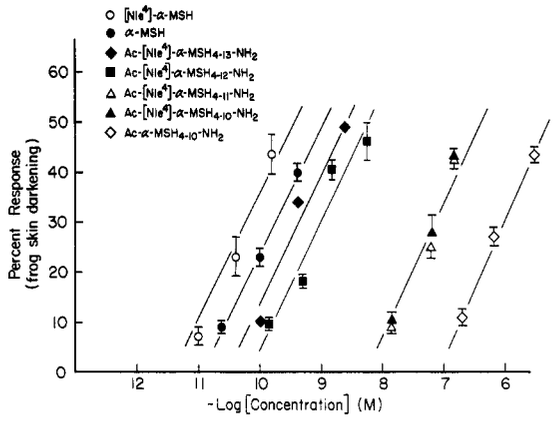

Figure 3. Comparative in vitro dose-response curves of α-MSH (●), [Nle^4]-α-MSH (○), Ac-[Nle^4]-α-MSH_{4-13}-NH_2 (◆), Ac-α-MSH_{4-12}-NH_2 (■), Ac-[Nle_4]-α-MSH_{4-11}-NH_2 (△), Ac-[Nle^4]-α-MSH_{4-10}-NH_2 (▲), and Ac-α-MSH_{4-10}-NH_2 (◇) as determined on the frog (*R. pipiens*) skin assay.

was reported to be a very weak but full agonist in this bioassay. Various workers have prepared and tested a series of α-MSH fragment analogues and reported the smallest fragment of this central sequence with agonist biological activity to be the 6–10 pentapeptide His-Phe-Arg-Trp-Gly,[9,10] the 6–9 tetrapeptide,[11] tripeptides related to the central sequence,[12] and the dipeptide, Phe-Arg.[13-15]

(2) O'Donohue, T. L.; Dorsa, D. M. *Peptides (Fayetteville, N.Y.)* **1982**, *3*, 353–395.
(3) Bagnara, J. T.; Hadley, M. E. *Chromatophores and Color Change: The Comparative Physiology of Animal Pigmentation*; Prentice-Hall: Engelwood Cliffs, N.J., 1973.
(4) Thody, A. J.; Meddis, D.; Schuster, S. *J. Invest. Dermatol.* **1978**, *70*, 328–330.
(5) Beckwith, B. E.; Sandman, C. A. *Neurosci. Biobehav. Rev.* **1978**, *2*, 311–338.
(6) Bohus, B.; *Pharmacology* **1979**, *18*, 113–122.
(7) De Kloot, R.; de Weid, D. *Front. Neuroendocrinol.* **1980**, *6*, 157–199.
(8) Hruby, V. J.; Wilkes, B. C.; Cody, W. L.; Sawyer, T. K.; Hadley, M. E. *Pept. Protein Rev.* **1984**, *3*, 1–64 for a recent review.
(9) Hofmann, K.; Yajima, H. *Recent Prog. Horm. Res.* **1962**, *18*, 41–88.
(10) Pickering, B. T.; Li, C. H. *Biochim. Biophys. Acta* **1962**, *62*, 475–480.
(11) Otsuka, H.; Inouye, K. *Bull. Chem. Soc. Jpn.* **1964**, *37*, 289–290.
(12) Medzihradszky, K.; Medzihradszky-Schweiger, H. *FEBS Lett.* **1976**, *67*, 45–47.

The presence of a second active sequence, Lys-Pro-Val-NH_2 (α-MSH_{11-13}-NH_2), which could independently stimulate melanocytes, has also been reported.[13-15] In addition to this putative second active site, the single amino acid derivative (Ac-Lys-NH_2) was reported to be able to stimulate melanocyte receptors.[13]

We have prepared a number of α-melanotropin peptides and determined their melanotropic activities in the in vitro frog (*R. pipiens*) skin bioassay. We report here on the minimal sequence of α-MSH capable of eliciting minimal, but measurable, melanotropic activity. We also detail the contributions of the individual amino acid residues to the potency of α-MSH.

Results and Discussion

The relative biological potencies of the melanotropin analogues prepared (Figure 1) in this study are summarized in Table I. N-terminal acetylation and C-terminal amidation were introduced into the peptides since the native hormone is protected by these end groups. Norleucine was substituted for methionine-4 in several of these analogues in order to eliminate inherent problems with oxidation of methionine.[16] All analogues were tested for biological activity over 7 orders of magnitude (i.e., 10^{-11}–10^{-4} M) (Figure 2 and 3), and the upper limit of concentration was chosen based on practical limits of solubility, amounts of available peptide, and the biological significance of melanotropin peptides having activity less than $^1/_{10000000}$ (0.000 000 1) that of α-melanotropin (α-MSH).

The Minimal Message Sequence of α-MSH. The following fragment analogues were devoid of any measurable biological activity in the frog skin bioassay: Ac-α-MSH_{7-10}-NH_2 (XI), Ac-α-MSH_{6-8}-NH_2 (XIII), Ac-α-MSH_{6-7}-NH_2 (XIV), Ac-α-MSH_{7-8}-NH_2 (XV), H-α-MSH_{7-8}-OH (XVI), and Ac-Lys-NH_2 (XVIII). The smallest fragment with measurable biological activity was Ac-α-MSH_{6-9}-NH_2 (Ac-His-Phe-Arg-Trp-NH_2) (Figure 2); this peptide was about 200 000 times less potent than α-MSH (0.000 005). The importance of His-6 was demonstrated by the fact that Ac-α-MSH_{7-10}-NH_2 (XI) lacked measurable activity. Trp-9 was also critical for biological activity as was demonstrated by the lack of biological activity of Ac-α-MSH_{6-8}-NH_2 (XIII). It is interesting that the central tetrapeptide fragment, Ac-α-MSH_{6-9}-NH_2 (XII), being the smallest fragment with biological activity, contains three aromatic residues. In this regard, aromatic amino acids are common in the active site of many hormones and are often considered critical for biological activity.[17] Though the 6–9 peptide XII (Figure 1) is the minimum active sequence, we have shown[18] that the entire 6–9 sequence is not needed if the 7–9 sequence is extended in the C-terminal direction since Ac-α-MSH_{7-13}-NH_2 has very weak potency.

Evidence against a Second Message Sequence of α-MSH. It was suggested by Eberle and Schwyzer[13-15] that α-MSH might have two message sequences, the first active sequence centered around the central tetrapeptide His-Phe-Arg-Trp and the second active site centered and around the C-terminal tripeptide Ac-Lys-Pro-Val-NH_2 (XVII). We were unable to confirm[18,19] the reported biological activity of this tripeptide at concentrations as high as 2×10^{-4} M.[18] Nevertheless, when this tripeptide derivative was first synthesized in our laboratory, it was found to be a very weak agonist;[20] however, when this peptide was prepared in another laboratory, which had not previously synthesized potent α-MSH analogues, this tripeptide was found to be inactive. This problem of contamination of trace amounts of biologically active compounds in an apparently inactive fragment has been discussed elsewhere.[18] To avoid this problem in this work, "virgin" columns and stringent conditions were utilized for purification of all of the fragment analogues.

Active-Site Analogues of α-MSH. When the central active site of α-MSH, the tetrapeptide His-Phe-Arg-Trp, was extended by one amino acid, Gly-10, the resulting analogue, Ac-α-MSH_{6-10}-NH_2 (Figure 2), was about 16 times more potent than Ac-α-MSH_{6-9}-NH_2 (0.000 005 relative to α-MSH). Gly-10, therefore, is important for biological potency of α-melanotropin. Addition of Glu-5 to the weakly active pentapeptide, Ac-α-MSH_{6-10}-NH_2, giving Ac-α-MSH_{5-10}-NH_2 (IX), resulted in very little increase in biological activity (Table I). This amino acid did not appear to contribute directly to the biological potency of α-MSH at the frog skin melanocyte receptor.

Ac-α-MSH_{4-10}-NH_2 was about 2 times more potent than Ac-α-MSH_{5-10}-NH,[21] demonstrating the moderate importance of Met-4 for melanotropic activity. A further increase in potency of about 4-fold was achieved by the substitution of norleucine (which is pseudoisosteric to methionine) for Met-4 to provide Ac-[Nle^4]-α-MSH_{4-10}-NH_2 (VI). Addition of Lys-11 to this heptapeptide sequence resulted in the analogue Ac-[Nle^4]-α-MSH_{4-11}-NH_2 (V), which was equipotent to Ac-[Nle^4]-α-MSH_{4-10}-NH_2. These results suggest that Lys-11 does not contribute directly to the biological potency of α-MSH.

A large increase in potency was observed when Ac-[Nle^4]-α-MSH_{4-11}-NH_2 was extended to include the 12-position, Pro-12. In fact, Ac-[Nle^4]-α-MSH_{4-12}-NH_2 was only about 5.5-fold less potent than the native hormone, α-MSH (Figure 3). Therefore, Pro-12 appears to be of major importance to the biological potency of α-MSH in the frog skin bioassay. The α-MSH analogue Ac-[Nle^4]-α-MSH_{4-13}-NH_2 (III), which contained the entire C-terminus, was almost as potent as α-MSH. Valine-13 appears to contribute only moderately to the potency of α-MSH.

The importance of the various amino acid residues in contributing to the biological activity of α-MSH (in the frog skin bioassay) can be summarized as follows: (1) the internal tetrapeptide His-Phe-Arg-Trp sequence is critical for observable biological activity, (2) Met-4, Gly-10, and Pro-12 are important potentiating amino acids and contribute significantly to the potency of α-MSH, and (3) Ser-1 and -3, Tyr-2, Lys-11, and Val-13 contribute little to the biological potency of α-MSH at the frog skin melanotropin receptors.

With the exception of two active-site models (see the introduction and ref 22), these results generally agree with

(13) Eberle, A.; Schwyzer, R. *Helv. Chim. Acta* **1975**, *58*, 1528–1535.
(14) Eberle, A.; Fauchere, J.-L.; Tesser, G. I.; Schwyzer, R. *Helv. Chim. Acta* **1975**, *58*, 2106–2129.
(15) Eberle, A.; Schwyzer, R. *Surface Membrane Receptors*; Bradshaw, R. A., Frazier, W. A., Merrell, R. C., Gotteib, I., Hogue-Anselletti, R. A., Eds; Plenum: New York, **1976**; pp 291–304.
(16) Paldi-Harris, P.; Medzihradszky-Schweiger, H.; Szecsi, J. H.; Medzihradszky, K. *Magy. Kem. Foly.* **1980**, *86*, 514–518.
(17) Sabesan, N. M.; Harper, E. T. *J. Theor. Biol.* **1983**, *83*, 457–467.
(18) Staples, D. J.; Sawyer, T. K.; Hadley, M. E.; Engel, M. H.; deVaux, A. E.; Affholter, J. A.; Darman, P. S.; Cody, W. L.; Wilkes, B. C.; Hruby, V. J. *Peptides: Structure and Function*; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chemical Co.: 1985; pp 691–694.
(19) Hruby, V. J.; Wilkes, B. C.; Cody, W. L.; Sawyer, T. K.; Hadley, M. E. *Pept. Protein Rev.* **1984**, *3*, 1–65.
(20) Sawyer, T. K. Ph.D. Dissertation, University of Arizona, 1981.
(21) Sawyer, T. K.; Hruby, V. J.; Wilkes, B. C.; Draelos, M. T.; Hadley, M. E.; Bergsneider, M. *J. Med. Chem.* **1982**, *25*, 1022–1027.

Table II. Physiochemical Properties of α-MSH Fragment Analogues

	TLC R_f values in various solvent systems					
	A	B	C	D	HPLC K'^a	OR, $[\alpha]^{25}_{546}$ in 10% HOAc
I	0.18	0.67	0.63	0.90	ND	−58.0° (c 0.50)
II	0.24	0.68	0.68	0.80	ND	−60.0° (c 0.25)
III	0.19	0.61	0.60	0.83	ND	−55.4° (c 0.25)
IV	0.08	0.46	0.71	0.47	9.20 (14%)	−54.6° (c 0.23)
V	0.14	0.50	0.56	0.45	15.77 (20%)	−39.1° (c 0.24)
VI	0.30	0.72	0.70	0.86	ND	−39.1° (c 0.25)
VII	0.07	0.46	0.71	0.43	6.68 (18%)	−61.5° (c 0.27)
VIII	0.30	0.61	0.64	0.97	ND	−34.1° (c 0.25)
IX	0.05	0.41	0.72	0.78	12.7 (16%)	−5.97° (c 0.39)
X	0.07	0.13	0.65	0.03	7.54 (14%)	−1.13° (c 0.21)
XI	0.21	0.76	0.82	0.81	ND	−24.0 (c 0.25)
XII	0.02	0.15	0.67	0.03	5.00 (16%)	+16.4° (c 0.17)
XIII	0.02	0.14	0.52	0.05	7.80 (14%)	+4.72° (c 0.72)
XIV	0.07	0.38	0.61	0.56	8.40 (14%)	+17.1° (c 0.45)
XV	0.08	0.37	0.52	0.61	9.10 (14%)	+20.7° (c 0.29)

[a] HPLC K' = (retention time − solvent from)/solvent front. For conditions, see ref 5. Acetonitrile (CH_3CN) concentrations are given in parentheses.

the results of other investigators. The observed relative potencies of these active-site fragment analogues of α-MSH are somewhat different than previously reported values.[13-15] However, the fragment analogues prepared in this study were all N-terminal acetylated and C-terminal amidated, which is unlike many of the fragment analogues reported earlier by other investigators. The presence of only a single active-site region of α-MSH is reasonable, since to our knowledge there is no other example of a peptide hormone that contains two independent active sites capable of stimulating the same biological response at the same receptor. However, at other melanotropin receptors, other peptides related to α-MSH may be biologically active.

Experimental Section

General Methods. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Thin-layer chromatography (TLC) was performed on Silufol plates (Kavalier, Czechoslovakia) 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/HOAc/pyridine/H_2O (5:5:1:4); (D) 2-propanol/25% aqueous ammonia/H_2O (3:1:1). Approximately 100-μg samples were used in each case. Detection was by UV, ninhydrin, and iodine vapor, and single spots were obtained unless otherwise noted. Optical rotations were determined with a Rudolph Research polarimeter (Autopol) at the mercury green line (546 nm). Amino acid analyses[23] were performed with a Beckman 120C amino acid analyzer following hydrolysis for 22 h at 110 °C in 3 M mercaptoethanesulfonic acid. 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), or Chemical Dynamics (South Plainfield, NJ) or were prepared by published procedures. All amino acid derivatives were tested for homogeneity by TLC in solvent systems A, B, and C, melting points, NMR, and the ninhydrin test[24] before use for synthesis. Solvents used for chromatography were redistilled prior to use as previously reported.[25]

General Solid-Phase Methodology. α-MSH and the following α-MSH analogues reported in this paper were synthesized as previously reported: α-MSH;[26] Ac-α-MSH_{4-10}-NH_2, Ac-[Nle^4]-α-MSH_{4-10}-NH_2;[21] Ac-[Nle^4]-α-MSH_{4-11}-NH_2;[27] Ac-α-MSH_{7-10}-NH_2, Ac-α-MSH_{11-13}-NH_2,[18,20] Ac-α-MSH_{9-13}-NH_2;[18] and Ac-[Nle^4]-α-MSH_{4-13}-NH_2.[20] H-Phe-Arg-OH was purchased from Vega Biochemicals and used without further purification.

A *p*-methylbenzhydrylamine (*p*-MBHA) resin was employed as the solid support for these syntheses and was prepared by methods previously reported.[21] Amine substitution levels of 0.22–1.00 mmol/g resin were determined by standard methods.[28] N^{α}-Boc- amino acids and amino acid derivatives were coupled successively to the resin support by methods previously outlined.[20,21] Amino acid side chain functional groups were protected as follows: glutamic acid, γ-benzyl ester; histidine, N^{im}-*p*-tolylsulfonyl; arginine, N^{g}-*p*-tolylsulfonyl; tryptophan, N^{in}-formyl; lysine, N^{ϵ}-2,4-dichlorobenzyloxycarbonyl. After coupling of the final amino acid to the resin, the peptide resin was acetylated with a 6-fold excess of *N*-acetylimidazole for 6–8 h.

In each case, the resulting peptide was cleaved from the solid support with anhydrous HF by one of two procedures. (A) For peptides containing a tryptophan N^{in}-formyl protecting group, the protected peptide resin was treated with anhydrous HF containing 10% anisole and 5% dimercaptoethane for 60 min at 0 °C.[29] (B) For peptides not containing a tryptophan, the peptide resin was treated with anhydrous HF containing 10% anisole for 30 min at 0 °C.[27,30] In either case, the solvents were removed in vacuo, the product was washed with 3 × 30 mL of EtOAc, and the crude product was extracted with 3 × 30 mL of 30% HOAc. The aqueous layer was then lyophilized to yield the crude product.

Each crude peptide was purified by ion-exchange chromatography on a (carboxymethyl)cellulose column (2.0 × 26.0 cm) with a discontinuous gradient of 250 mL of 0.01 M NH_4OAc (pH 4.5) followed by 250 mL each of 0.1 and 0.2 M NH_4OAc (pH 6.8). In each case, the major peak not at the solvent front was isolated and lyophilized, giving a fluffy white powder as the purified product. Detection was at 280 nm for peptides containing a tryptophan residue and at 254 nm for peptides containing phenylalanine as the major chromophore.

The homogeneity of each peptide was examined by TLC in solvent systems A, B, C, and D, optical rotation, amino acid analysis, and reverse-phase high-pressure liquid chromatography.[31]

(22) Eberle, A. N.; de Graan, P. N. E.; Baumann, J. B.; Girard, J.; van Hees, G.; van de Veerdonk, F. C. G. *Pigment Cell 1985: Biological, Molecular and Clinical Aspects of Pigmentation*; Bagnara, J.; Klaus, S. N., Paul, E., Schartl, M., Eds.; University of Tokyo: Tokyo, 1985; pp 191–196.
(23) Spackman, P. H.; Stein, W. H.; Moore, S. *Anal. Chem.* **1958**, *30*, 1190–1206.
(24) Kaiser, E.; Colescott, R. L.; Boissinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.
(25) Hruby, V. J.; Groginsky, C. M. *J. Chromatogr.* **1971**, *68*, 423–428.
(26) Yang, Y. C. S.; Heward, C. B.; Hadley, M. E.; Hruby, V. J. *Int. J. Pept. Protein Res.* **1980**, *15*, 130–138.
(27) Wilkes, B. C.; Sawyer, T. K.; Hruby, V. J.; Hadley, M. E. *Int. J. Pept. Protein Res.* **1983**, *22*, 313–324.
(28) Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248–249.
(29) Matsueda, G. R. *Int. J. Pept. Protein Res.* **1982**, *20*, 26–34.
(30) Wilkes, B. C.; Sawyer, T. K.; Hruby, V. J.; Hadley, M. E. *Int. J. Pept. Protein Res.*, **1984**, *23*, 621–629.
(31) Cody, W. L.; Wilkes, B. C.; Hruby, V. J. *J. Chromatogr.* **1984**, *314*, 313–321.

The analytical data for all of the title compounds are shown in Table II. The results of all amino acid analyses were within ±10% of the theoretical values, except for Ser in compounds I and II (1.72 and 1.75, respectively) and Trp in compound XI (0.87).

Ac-α-MSH$_{4-12}$-NH$_2$ (VII). To 1.3 g of *p*-MBHA resin (0.62 mmol/g, 0.80 mmol total) the following N^{α}-Boc amino acids were coupled: Pro; Lys(2,4-Cl$_2$-Z); Gly; Trp(For); Arg(Tos); Phe; His(Tos); and Glu(Bzl). The resin was dried and weighed (I; 2.50 g). To half of this resin was coupled N^{α}-Boc-Met. After acetylation with *N*-acetylimidazole, the peptide resin was subjected to HF procedure A, giving 419.3 mg, and 100 mg was purified as described. The title product eluted halfway through the 0.1 M NH$_4$OAc (pH 6.8) buffer and was lyophilized, giving 47.3 mg (41.0%). The analytical data are given in Table II.

Ac-[Nle^4]-α-MSH$_{4-12}$-NH$_2$ (IV). To the second half of the above peptide resin (I) was coupled N^{α}-Boc-Nle. After acetylation, the peptide-resin was dried and weighed (1.27 g). The peptide-resin was subjected to HF procedure A, giving 352 mg, and 100 mg was purified as described. The title peptide eluted halfway through the 0.1 M NH$_4$OAc (pH 6.8) buffer and was lyophilized, giving 45.0 mg (32.7%). The analytical data are given in Table II.

Ac-α-MSH$_{5-10}$-NH$_2$ (IX). Starting with 1.0 g of *p*-MBHA resin (0.40 mmol/g, 0.40 mmol total scale), the following N^{α}-Boc amino acids were coupled: Gly, Trp(For), Arg(Tos), Phe, His(Tos), and Glu(Bzl). After acetylation, the resin (1.35 g) was cleaved by the HF procedure B, giving 200 mg of crude product, and 100 mg was purified as described. The title compound eluted early during the 0.1 M NH$_4$OAc (pH 6.8) buffer, giving 43 mg (48%). The analytical data are given in Table II.

Ac-α-MSH$_{6-10}$-NH$_2$ (X). Starting with 1.0 g of *p*-MBHA resin (0.40 mmol/g, 0.40 mmol total scale), the following N^{α}-Boc amino acids were coupled: Gly, Trp(For), Arg(Tos), Phe, and His(Tos). After acetylation, the resin (1.32 g) was cleaved by the HF procedure A, giving 230 mg of crude product. A portion of this (100 mg) was purified as before, and the title compound eluted early during the 0.1 M NH$_4$OAc (pH 6.8) buffer, giving 38 mg (29.5%). The analytical data are given in Table II.

Ac-α-MSH$_{6-9}$-NH$_2$ (XII). Starting with 1.0 g of *p*-MBHA resin (0.4 mmol/g, 0.40 mmol total), the following N^{α}-Boc amino acids were coupled: Trp(For), Arg(Tos), Phe, and His(Tos). Following acetylation, the peptide resin (1.25 g) was subjected to HF procedure A, giving 180 mg crude product, and 100 mg was purified as before. The title compound eluted halfway through the 0.1 M NH$_4$OAc (pH 6.8) buffer, giving 35 mg (33%). The analytical data are given in Table II.

Ac-α-MSH$_{6-8}$-NH$_2$ (XIII). Starting with 1.0 g of *p*-MBHA resin (0.4 mmol/g), the following N^{α}-Boc amino acids were coupled: Arg(Tos), Phe, and His(Tos). After acetylation, the peptide-resin (1.3 g) was subjected to the HF procedure B, giving 102 mg, which was purified as described. The title compound eluted late in the 0.1 M NH$_4$OAc (pH 6.8) buffer, giving 81 mg (41%). The analytical data are given in Table II.

Ac-α-MSH$_{6-7}$-NH$_2$ (XIV). Starting with 1.0 g of *p*-MBHA resin (0.4 mmol/g), the following N^{α}-Boc amino acids were coupled: N^{α}-Boc-Phe and N^{α}-Boc-His(Tos). After acetylation, the peptide-resin (1.0 g) was subjected to HF procedure B, giving 130 mg of crude product, which was purified as before. The title compound eluted late in the 0.01 M NH$_4$OAc (pH 4.5) buffer, giving 35 mg (25.5%). The analytical data are given in Table II.

Ac-α-MSH$_{7-8}$-NH$_2$ (XV). Starting with 1.5 g of *p*-MBHA resin (0.40 mmol/g, 0.60 mmol total). The following N^{α}-Boc amino acids were coupled: N^{α}-Boc-Arg(Tos) and N^{α}-Boc-Phe. After acetylation, the peptide-resin (1.60 g) was subjected to the HF procedure B, giving 100 mg of crude product, which was purified as before. The title compound eluted late in the 0.01 M NH$_4$OAc (pH 4.5) buffer, giving 33 mg (15.0%). The analytical data are given in Table II.

Preparation of Ac-Lys(2,4-Cl$_2$-Z). Lys(2,4-Cl$_2$-Z) (5.0 g) was placed in 20 mL of H$_2$O and heated to reflux. Acetic anhydride (15 mL) was added, and the solution was refluxed for 20 min. The solution was filtered, and the product was allowed to crystallize overnight: starting material recovered 1.3 g; product yield 2.9 g; TLC (A) 0.77, (B) 0.71, (C) 0.67; mp 124–126 °C; NMR (DMSO-d_6) δ 1.1–1.85 (m, 6 H), 1.85 (2, 3 H), 2.8–3.2 (m, 2 H), 4.0–4.4 (t, 1 H), 5.1 (s, 2 H), 7.55–7.8 (m, 3 H).

Preparation of Ac-Lys(2,4-Cl$_2$-Z)-NH$_2$. Ac-Lys(2,4-Cl$_2$-Z) (2.0 g) was placed in 50 mL of CH$_2$Cl$_2$ and cooled to −15 °C. *N*-Methylmorpholine (0.54 g) was added. Isobutyl chloroformate (0.72 g) was added, and the solution was stirred for 3 min. Ammonia gas was then bubbled through the solution for 10 min. The solution was filtered, and the precipitate was dissolved in 20 mL of hot ethanol, filtered, and allowed to crystallize overnight: yield 1.90 g; mp 168–170 °C; TLC (A) 0.15, (B) 0.77.

Preparation of Ac-Lys-NH$_2$ (XVIII). Ac-Lys(2,4-Cl$_2$-Z) (0.50 g) was subjected to HF procedure B. Following the removal of the HF and anisole in vacuo, the product was dissolved in 30 mL of aqueous HOAc. The aqueous layer was extracted with 3 × 30 mL of ethyl ether and then lyophilized. The oily product was recrystallized from methanol and ethyl acetate. The precipitate was taken up in cyclohexane, filtered, and dried: yield 0.34 g; mp 134–138 °C (literature[14] reports an oil); $[\alpha]^{589}_{25}$ −5.3° (*c* 0.47, 1 N HCl). Anal. Calcd for $C_8H_{16}N_3O_2{\cdot}CH_3CO_2H$: C, 48.78; H, 8.13; N, 17.07. Found: C, 48.70; H, 8.26; N, 16.45.

Frog Skin Bioassay. The frog (*R. pipiens*) skin bioassay[32] was utilized to determine the relative potencies of the synthetic melanotropins. This assay measures the amount of light reflected from the surface of the skins in vitro. In response to melanotropic peptides, melanosomes within integumental melanophores migrate from a perinuclear position into the dendritic processes of the pigment cells. This centrifugal organellar dispersion results in a change in color (darkening), which is measured by a Photovolt Reflectometer and is expressed as the percent response compared to the initial (time zero) reflectance value. For α-MSH, subsequent removal of the melanotropin results in a rapid perinuclear (centripetal) reaggregation of melanosomes, leading to a lightening of the skins back to their original (base) value.

Acknowledgment. This research was supported by grants from the U.S. Public Health Service AM-17420 (V.J.H.) and the National Science Foundation (M.E.H.).

(32) Shizume, K.; Lerner, A. B.; Fitzpatrick, J. B. *Endocrinology (Philadelphia)* **1954**, *54*, 553–560.