clinical application of this amine as a cerebral blood flow indicator has recently been demonstrated.[12,13] The mechanism of brain localization for these monoamines may be similar to that of diamines **12–16**. The major advantage of the phenolic diamines **12–16** is the simple labeling procedure that could be used in any nuclear medicine clinic.

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Registry No. **2**, 824-42-0; **3**, 83816-53-9; **4**, 83816-54-0; **5**, 66232-33-5; **6**, 24623-65-2; **7**, 83816-55-1; **8**, 83816-56-2; **9**, 83816-57-3; **10**, 83816-58-4; 11, 83816-59-5; **12**, 83816-60-8; **12**·2HCl, 83816-67-5; [^{123}I]**12**, 83816-61-9; [^{123}I]**12**, 83816-62-0; **13**, 83816-63-1; **13**·2HCl, 83816-68-6; **14**, 83816-64-2; **14**·2HCl, 83816-69-7; **15**, 83816-65-3; **15**·2HCl, 83816-70-0; **16**, 83816-66-4; **16**·2HCl, 83816-71-1; *o*-methylphenol, 95-48-7; *o*-propylphenol, 644-35-9; *o*-(1-methylpropyl)phenol, 89-72-5; *o*-cyclohexylphenol, 119-42-6; *o*-(1,1-dimethylethyl)phenol, 88-18-6.

(12) D. E. Kuhl, J. R. Barrio, S. C. Huang, C. Selin, R. F. Ackermann, J. J. Lear, J. L. Wu, and T. H. Lin, *J. Nucl. Med.*, **23**, 196 (1982).
(13) T. C. Hill, B. L. Holman, R. Lovett, D. H. O'Leary, D. Front, P. Magistretti, R. E. Zimerman, S. Moore, M. E. Clouse, J. L. Wu, T. H. Lin, and R. M. Baldwin, *J. Nucl. Med.*, **23**, 191 (1982).

Structure–Activity Studies of Highly Potent Cyclic [Cys^4,Cys^{10}]Melanotropin Analogues[1]

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It has been proposed[14] that a β turn or other chain-reversal structure involving the residues His-Phe-Arg-Trp in α-melanocyte stimulating hormone (α-MSH) contributes to the bioactive conformation of this peptide hormone. This proposal is supported by the observation that [Cys^4,Cys^{10}]-α-MSH exhibits superagonist ($\geq$10 000 α-MSH) activity in the frog skin bioassay and is about 30 times more potent in the lizard skin bioassay. Studies on the possible role of a reverse turn in the biological activities of [Cys^4,Cys^{10}]-α-MSH have been extended with the synthesis of Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-10}$-NH$_2$ and Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$. The cyclic 4–10-heptapeptide was found to be less active than α-MSH in both the frog and lizard skin bioassays, but much more potent (100 times) than its linear congener Ac-α-MSH$_{4-10}$-NH$_2$ in the frog. With the cyclic 4–13-decapeptide, superagonist potency (equipotent to the cyclic tridecapeptide) was observed on the frog skin, and the analogue was equipotent to α-MSH in the lizard skin assay. These results support the suggestion that a cyclic reverse-turn conformation in α-MSH plays a significant role in the hormone–receptor interaction. However, the reduced potency observed with Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-10}$-NH$_2$ and the superagonist activity with Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ suggest that the C-terminal tripeptide sequence Lys-Pro-Val-NH$_2$ (or some component thereof) in combination with a reverse turn in the cyclic moiety are both important for the superagonist activity of the latter cyclic analogue and of [Cys^4,Cys^{10}]-α-MSH.

The linear tridecapeptide α-melanocyte stimulating hormone (α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$) is synthesized and secreted by the pars intermedia of the vertebrate pituitary.[2] This peptide hormone has been implicated in many important physiological functions, including integumental melanogenesis,[3] neural functioning related to facilitated memory and attention and other behavioral paradigms,[4-7] and fetal development.[8] Previous structure–function studies on α-melanotropin analogues and fragments using the in vivo frog skin bioassay system[9-12] have resulted in the proposal that the primary active site of α-MSH consists of the seven residues Met-Glu-His-Phe-Arg-Trp-Gly. Other studies[13] have suggested an additional active sequence containing the carboxamide terminal tripeptide Lys-Pro-Val-NH$_2$.

Recently, Sawyer et al.[14] proposed that a β turn or other peptide chain-reversal conformation within the central active site (His-Phe-Arg-Trp) of α-MSH may be important in the biologically active conformation of the hormone. The cyclic disulfide tridecapeptide analogue [Cys^4,Cys^{10}]-α-MSH (Figure 1) was then synthesized in order to evaluate the effect of covalently stabilizing a reverse turn conformation in α-MSH involving Met-Glu-His-Phe-Arg-Trp-Gly. This compound was found to possess superagonist potency and prolonged in vitro activity in stimulating melanosome dispersion within frog

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(1) 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, α-melanocyte stimulating hormone; Nle, norleucine; 2,4-Cl$_2$-Z, 2,4-dichlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; 2,6-Cl$_2$-Bzl, 2,6-dichlorobenzyl; *p*-MBHA resin, *p*-methylbenzhydrylamine resin; HOBT, *N*-hydroxybenzotriazole. Taken in part from the Ph.D. Dissertation of T. K. Sawyer, Department of Chemistry, University of Arizona, 1981.
(2) M. E. Hadley and J. T. Bagnara, *Am. Zool.*, 15(Suppl. 1), 81 (1975).
(3) M. E. Hadley, C. B. Heward, V. J. Hruby, T. K. Sawyer, and Y. C. S. Yang, *Ciba Found. Symp.*, **no. 81**, 242–261 (1981).
(4) T. L. O'Donohue and D. M. Jacobowitz, in "Polypeptide Hormones", R. F. Beers and E. G. Bassett, Eds., Raven Press, New York, 1980, pp 203–222.
(5) B. E. Beckwith and C. A. Sandman, *Neurosci. Biobehav. Res.*, **2**, 311 (1978).
(6) B. Bohus, *Pharmacology*, **18**, 113 (1979).
(7) R. de Kloet and D. de Wied, *Front. Neuroendocrinol.*, **6**, 157 (1980).
(8) F. J. H. Tilders, D. F. Swaab, and Tj. B. van Wimersma Greidanus, Eds., "Frontiers of Hormone Research", Vol. 4, S. Karger, Basel, 1977.
(9) K. Hoffman, *Hand. Physiol., Sect. 7: Endocrinol.*, **2**, 29 (1974).
(10) K. Medzihradszky in "Recent Developments in the Chemistry of Natural Carbon Compounds", R. Bogner, R. Bruckner, and Cs. Szantay, Eds., Publishing House of the Hungarian Academy of Science, Budapest, 1976, pp 203–219.

0022-2623/83/1826-0125$01.50/0

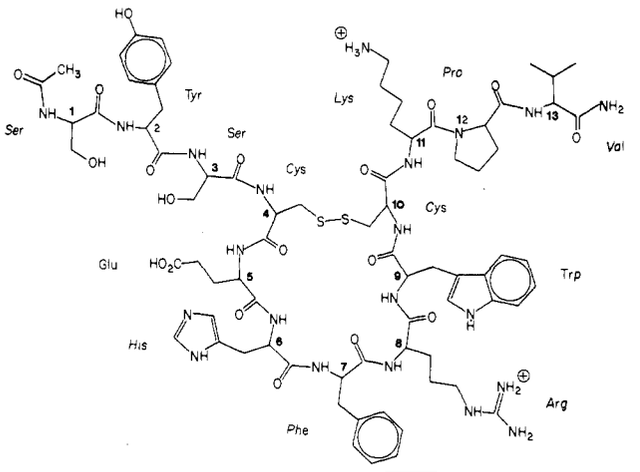

Figure 1. Molecular structure of [Cys^4,Cys^{10}]·α·MSH.

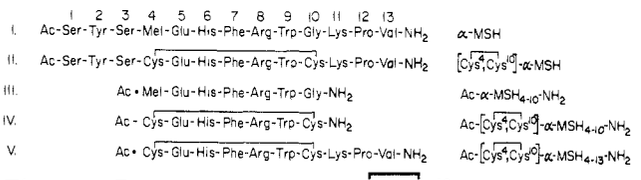

Figure 2. Primary structures of [Cys^4,Cys^{10}]·cyclic melanotropins compared to α·MSH.

skin melanophores.[14] In order to study more thoroughly the role of this proposed reverse turn on the biological activity of α·MSH, we have synthesized two additional cyclic analogues of α·MSH: Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-10}$·NH$_2$ and Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-13}$·NH$_2$ (Figure 2).

Results and Discussion

The synthesis and purification of the cyclic α·MSH analogues were accomplished by methods similar to those utilized in our previous syntheses of α·MSH and analogues.[14-17] The analogues were purified by ion·exchange on carboxymethylcellulose, followed by gel filtration on Sephadex G·25. The homogeneity of each peptide was assessed by amino acid analysis and thin·layer chromatography (TLC) in four solvent systems (see Experimental Section for details).

Biological activities of the synthetic peptides were examined in vitro in the frog (*Rana pipiens*) and lizard (*Anolis carolinensis*) bioassays as described previously.[15,17] In the frog skin assay, Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-10}$·NH$_2$ is one·tenth as potent as α·MSH but 100 times more potent than its linear congener, Ac·α·MSH$_{4-10}$·NH$_2$ (Figure 3a, Table I). This potency increase is similar to that seen with the cyclic tridecapeptide [Cys^4,Cys^{10}]·α·MSH and α·MSH[14] and suggests that stabilization of a reverse·turn conformation involving the sequence His·Phe·Arg·Trp by covalent cyclization greatly enhances potency but is not the only structural feature that can enhance potency in α·MSH. Interestingly, Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-10}$·NH$_2$ does not exhibit prolongation of melanosome dispersion (data not shown) as seen with the cyclic tridecapeptide.[14] It, therefore, appears that other structural features separate from (or in addition to) a reverse turn are also important for prolongation of melanotropic activity.

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
α·MSH	1.0	1.0
Ac·α·MSH$_{4-10}$·NH$_2$	0.0006	0.02
Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-10}$·NH$_2$	0.07	0.003
Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-13}$·NH$_2$	~10 000	1.6
Ac·[Cys^4,Cys^{10}]·α·MSH	~10 000	32

[a] Relative potency = concentration of α·MSH at 50% response/concentration of peptide at 50% response.

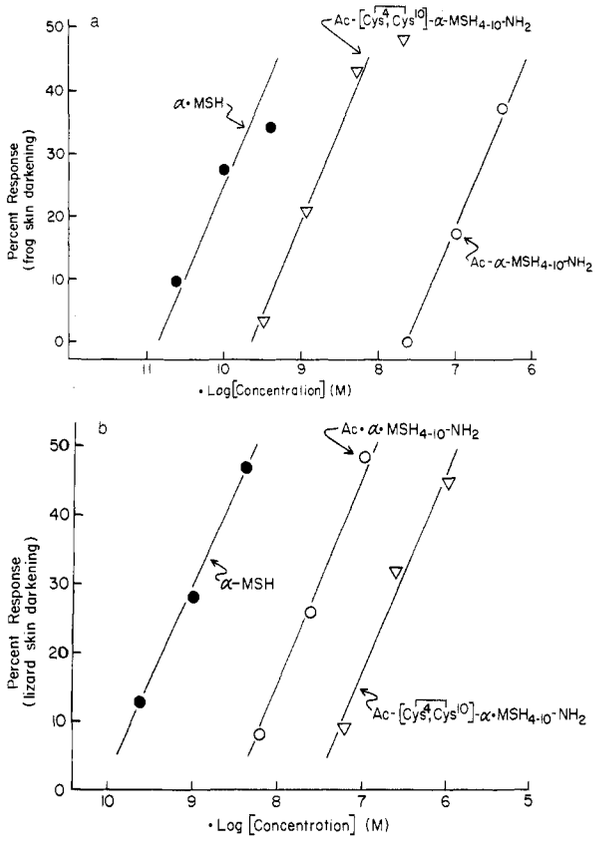

Figure 3. Comparative in vitro dose–response curves of Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-10}$·NH$_2$ (▽), Ac·α·MSH$_{4-10}$·NH$_2$ (○), and α·MSH (●) as determined in the (a) frog (*R. pipiens*) and (b) lizard (*A. carolinensis*) skin bioassays. Each value represents the mean ± SE darkening response of the skins (a, 10 per group; b, 6 per group) to the melanotropins at the concentrations indicated.

In the lizard skin assay, Ac·[Cys^4,Cys^{10}]·α·MSH$_{4-10}$·NH$_2$ was found to be less active than α·MSH *and also* less active than the linear peptide Ac·α·MSH$_{4-10}$·NH$_2$ (Figure 3). This result contrasts dramatically from that of the frog skin assay where the cyclic 4–10·peptide was consid·

(11) A. Eberle in "Cellular Receptors for Hormones and Neurotransmitters", D. Schulster and A. Levitzki, Eds., Wiley, New York, 1980, pp 219–231.
(12) R. Schwyzer, *Ann. N.Y. Acad. Sci.*, **297**, 3 (1977).
(13) A Eberle and R. Schwyzer, *Helv. Chim. Acta*, **58**, 1528 (1979).
(14) T. K. Sawyer, V. J. Hruby, P. S. Darmon, and M. E. Hadley, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 1751 (1982).
(15) V. J. Hruby, T. K. Sawyer, Y. C. S. Yang, M. D. Bregman, M. E. Hadley, and C. B. Heward, *J. Med. Chem.*, **23**, 1432 (1980).
(16) T. K. Sawyer, P. J. Sanfilippo, V. J. Hruby, M. H. Engel, C. B. Heward, J. B. Burnett, and M. E. Hadley, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 5754 (1980).
(17) Y. C. S. Yang, C. B. Heward, M. E. Hadley, and V. J. Hruby, *Int. J. Pept. Protein Res.*, **15**, 130 (1980).

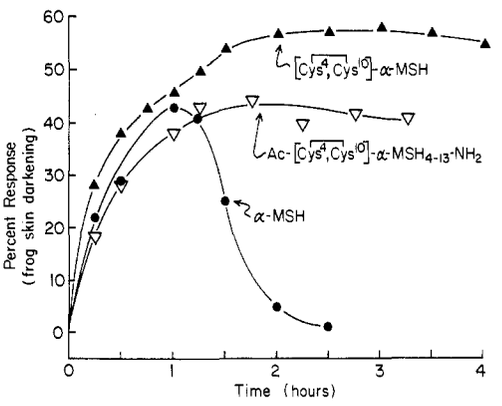

Figure 4. In vitro demonstration of the relatively prolonged activity of [Cys⁴, Cys¹⁰]·α·MSH (▲), Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-13}·NH_2 (▽), and α·MSH (●). Each value represents the mean ± SE response (darkening or lightening) of frog (*R. pipiens*) skins (7 per group) in the presence or absence of the melanotropins.

erably more active than the linear 4–10 fragment. Thus, introduction of a cyclic structure does not appear to provide an optimum hormone–receptor interaction on lizard melanophores. The profound difference in response of frog and lizard skin melanophores to cyclic melanotropin analogues and other analogues[18] clearly suggests that the melanophore receptors of these two species differ considerably relative to the specific structural and/or conformational requirements of melanotropins for receptor interaction.

The low potency relative to α·MSH and the absence of a prolongation effect with Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-10}·NH_2 suggest that this peptide lacks some feature present within the first three (Ac-Ser·Tyr-Ser) or last three (Lys·Pro·Val·NH_2) or both groups of residues that is responsible for the superagonist and prolonged activity of [Cys⁴,Cys¹⁰]-α-MSH. Based on previous structure–function studies of α·MSH analogues and fragments,[13,18] it was anticipated that addition of the C·terminal tripeptide Lys·Pro·Val·NH_2 to the cyclic heptapeptide, Ac·[Cys⁴,Cys¹⁰]·α-MSH_{4-10}·NH_2, might produce an increase in potency as well as a prolongation of melanotropin activity. We, therefore, synthesized Ac·[Cys⁴,Cys¹⁰]·α-MSH_{4-13}·NH_2 and obtained an exceptionally large increase in potency on frog melanophores comparable to the cyclic tridecapeptide (Table I). In addition, Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-13}·NH_2 exhibited prolonged activity comparable to the cyclic tridecapeptide after the solution containing the peptide was replaced with fresh Ringer solution (in the absence of the melanotropin) (Figure 4). In the lizard skin assay, Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-13}·NH_2 was equipotent to α·MSH; this represented a 1000·fold increase in potency over Ac·[Cys⁴,Cys¹⁰]-α·MSH_{4-10}·NH_2 (Table I). These data suggest that a receptor site for the C·terminal tripeptide segment or the tripeptide terminal segment, alone, favorably affects the conformational and dynamic properties of the semirigid ring (Cys-Glu·His·Phe·Arg·Trp-Cys) to produce a molecule that more readily attains the receptor·bound or bioactive conformation.

Separate from the superagonist activity of [Cys⁴,Cys¹⁰]·α·MSH and Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-13}·NH_2 is the phenomenon of prolongation of melanosome·dispersing activity by these compounds in the frog skin bioassay (Figure 4). The prolonged activity exhibited by these peptides and by [Nle⁴,D·Phe⁷]·α·MSH[16] suggests the possibility of either an "irreversible" hormone–receptor interaction or an "irreversible" activation of the melanotropin–receptor–adenylate cyclase complex in the amphibian melanophore. The prolongation effect of these compounds appears to be a function of the covalent combination of the two putative active sites (Met·Glu·His·Phe·Arg·Trp·Gly and Lys·Pro·Val·NH_2) and the presence of a reverse turn involving the residues His·Phe·Arg·Trp. This is supported by the fact that the linear analogue [Nle⁴]·α·MSH does not possess prolonged activity, whereas [Nle⁴,D·Phe⁷]·α·MSH does;[15,16] it is also supported by the lack of prolonged activity by Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-10}·NH_2. The introduction of D·amino acid residues into the $i + 1$ or $i + 2$ positions of β turns should stabilize this reverse turn.[19,20] However, the in vitro prolongation exhibited by [Cys⁴,Cys¹⁰]·α·MSH and Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-13}·NH_2 is significantly less than that observed for [Nle⁴,D·Phe⁷]·α·MSH.[16] Since the potencies of the cyclic analogues are much greater than [Nle⁴,D·Phe⁷]·α·MSH, it appears that prolongation and potency have separate structural and perhaps conformational requirements.

In summary, we have provided evidence that covalent cyclization that would stabilize the proposed reverse turn[14] in α·MSH cannot fully account for the exceptional potency on frog skin melanophores of [Cys⁴,Cys¹⁰]·α·MSH. However, the results do suggest that since the cyclic heptapeptide Ac·[Cys⁴,Cys¹⁰]·α·MSH_{4-10}·NH_2 has much greater potency than its linear analogue Ac·α·MSH_{4-10}·NH_2, the conformational and dynamic restrictions imposed by cyclization, e.g., a reverse turn, do significantly contribute to the biologically active conformation of α·MSH. An exceptional increase in potency was obtained in both bioassays when the terminal tripeptide Lys·Pro·Val·NH_2 was appended onto the cyclic heptapeptide. The resulting cyclic decapeptide possessed all of the characteristics of [Cys⁴,Cys¹⁰]-α·MSH, suggesting that an intramolecular interaction occurs between the 4–10 and 11–13 sequences, which produces a more active conformation, and/or that the terminal tripeptide possesses a strong additional site for interaction with the receptor.

Such cyclic α·MSH analogues, being semirigid in nature, should be amenable to conformational analysis and provide further insight into the bioactive conformation of α·MSH. These analogues may also provide a model for the design of an antagonist to α·MSH. High affinity and long·acting melanotropins could possibly act as carriers for antitumor drugs in the treatment of malignant melanoma or they may be radiolabeled to provide a probe for detection of melanoma in vivo. Studies related to these problems are currently in progress in our laboratory.

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/

(18) T. K. Sawyer, V. J. Hruby, B. C. Wilkes, M. J. Draelos, M. E. Hadley, and M. Bergsneider, *J. Med. Chem.*, **25**, 1022 (1982).

(19) C. M. Venkatachalam, *Biopolymers*, **6**, 1425 (1968).

(20) R. Chandrasekaran, A. V. Lakshminarayanan, U. V. Pandya, and G. N. Ramachandran, *Biochim. Biophys. Acta*, **303**, 14 (1973).

pyridine/H_2O (15:3:10:12); (c) 1-butanol/pyridine/HOAc/H_2O (6:6:1.2:4.8); (d) 2-propanol/25% aqueous NH_3/H_2O (3:1:1). The load size was approximately 50–100 μg, and chromatographic lengths were 12–18 cm. Detection was by iodine vapors and ninhydrin. Single spots were obtained unless otherwise noted. Amino acid analyses[21] were obtained with a Beckman 120C amino acid analyzer following hydrolysis for 22 h at 110 °C with 4 M methansulfonic acid containing 0.2% 3-(2-aminoethyl)indole.[22] 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. Paper electrophoresis was performed on a Gelman Instrument Co. unit at 4 °C in pyridine–acetate buffer (pH 5.3, 400 V, 5 h). Each peptide was run along with α-MSH as a standard. Detection was by chlorination and 1% KI/starch spray. Migration toward the cathode of each peptide is reported in centimeters from the origin.

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) and were of the L configuration. Before use, all amino acid derivatives were tested for homogeneity by TLC and a ninhydrin test.[23] Solvents used for gel filtration, TLC, and other chromatographic methods were purified as previously reported.[24] The *p*-methylbenzhydrylamine resin (0.44 mg/g, 1% divinylbenzene cross-linked polystyrene) was obtained from United States Biochemical Corp. (Cleveland, OH).

Solid-Phase Peptide Synthesis of Melanotropins. The α-MSH analogues reported here were synthesized by solid-phase methods similar to those used previously for the synthesis of α-MSH.[17] α-MSH, Ac-α-MSH_{4-10}-NH_2, and [Cys^4,Cys^{10}]-α-MSH were prepared and purified as reported previously.[14,17,18] N^{α}-Boc-protected amino acid derivatives were successively coupled to a substituted *p*-methylbenzhydrylamine resin with a 3-fold excess of Boc-protected amino acid derivative and a 2.4-fold excess of dicyclohexylcarbodiimide (DCC). *N*-Hydroxybenzotriazole (HOBT, 3-fold excess) was also added to suppress racemization.[25] Removal of the N^{α}-Boc protecting groups was achieved 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; and cysteine, *S*-3,4-dimethylbenzyl. It has been reported[26] that HOBT removes the Tos protecting group from His under the conditions described below; however, no significant synthetic problems were detected for the compounds prepared in this report.

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) cleaving the Boc group by adding 25-mL of 45% trifluoroacetic acid in dichloromethane containing 2% anisole, one treatment for 2 min, a second for 20 min; (3) washing with four 25-mL portions of CH_2Cl_2, 1 min/wash; (4) neutralizing by adding two 25-mL portions of 10% diisopropylethylamine in CH_2Cl_2 and shaking for 2 min each; (5) washing with four 25-mL portions of CH_2Cl_2, 1 min/wash; (6) adding Boc-protected amino acid derivative in 10 mL of CH_2Cl_2 containing HOBT (dissolved in a minimum volume of DMF), followed by DCC in 12 mL of CH_2Cl_2, followed by 3 mL of CH_2Cl_2 wash and then shaking for 3.5–12 h; (7) washing with three 25-mL portions of EtOH, 1 min/wash; (8) washing with four 25-mL portions of CH_2Cl_2, 1 min/wash. Between steps 1 and 2, several milligrams of resin was removed and used in a ninhydrin test[23] to determine completion of coupling.

After coupling all of the amino acid residues 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 (except the formyl group of Trp) were removed with anhydrous liquid HF (0 °C, 45–60 min) containing 10% anisole. The N^{i}-formyl group was removed by treatment of an aqueous solution of formylated peptide with 4 N NaOH to pH 11.5 for 3 min as previously reported.[17,27] The deformylation was terminated by addition of glacial acetic acid. Cyclization was carried out in dilute solution via oxidation with potassium ferricyanide as reported previously.[14]

Ac-[Cys^4,Cys^{10}]-α-MSH_{4-10}-NH_2. Starting with 3.67 g of Boc-Cys(*S*-3,4-Me_2Bzl)-*p*-MBHA resin [0.36 mmol of Boc-Cys(*S*-3,4-Me_2Bzl) total] the protected peptide resin to the title peptide was obtained after stepwise coupling of the following N^{α}-Boc-protected amino acids (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, N^{α}-Boc-γ-Bzl-Glu, and N^{α}-Boc-(*S*-3,4-Me_2Bzl)-Cys. After coupling the last amino acid and removing the N^{α}-Boc group, we acetylated the protected peptide using a 6-fold excess of *N*-acetylimidazole in CH_2Cl_2. The resultant Ac-Cys(*S*-3,4-Me_2Bzl)-Glu(*O*-Bzl)-His-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Cys(*S*-3,4-Me_2Bzl)-*p*-MBHA resin was dried in vacuo (4.21 g). A portion (2.0 g) of the protected peptide was cleaved from the resin, and all protecting groups (except the N^{i}-formyl group of Trp) were removed by treatment with anhydrous HF (30 mL) containing 10% (v/v) anisole (60 min, 0 °C). After evaporation of the HF and anisole in vacuo, the dried product was washed, under a stream of N_2, with three 30-mL portions of EtOAc and extracted with three 60-mL portions of 30% HOAc. The combined extracts were lyophilized to give 211.1 mg of Ac-[half-Cys(SH)^4,N^{i}-For-Trp^9,half-Cys(SH)^{10}]-α-MSH_{4-10}-NH_2. The free disulfhydryl peptide was then deformylated under an atmosphere of N_2 by the addition of 4 N NaOH until a pH of 11.5 was reached. After 3 min, we terminated the reaction by lowering the pH to 8.5 with 0.1 N acetic acid. The fully deprotected peptide was diluted with 300 mL of 0.1 N NH_4OAc, pH 8.5, and subjected to oxidative cyclization with 41 mL of 0.01 N $K_3Fe(CN)_6$. After 30 min at 25 °C, the reaction was terminated by the addition of 10% AcOH until a final pH of 5.0 was reached. Excess ferro- and ferricyanide ions were removed by addition of Rexyn 203 (Cl^- form). After filtration to remove the Rexyn 203 resin, the solution was lyophilized to give 557 mg of salty peptide material. A portion of the crude peptide (189.5 mg) was dissolved in 3 mL of 0.1 N NH_4OAc, pH 4.5, and chromatographed on carboxymethylcellulose (2.0 × 20.0 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) fraction was lyophilized to give 9.38 mg of white powder. The ion exchange was repeated on the remaining crude peptide to give a total of 15 mg. The purified peptide gave single uniform spots on TLC: R_f 0.18 (A), 0.60 (B), 0.60 (C), and 0.69 (D). Amino acid analysis gave the following molar ratios: Glu, 1.03; His, 1.04; Phe, 0.97; Arg, 1.05; Trp, 0.92; half-Cys, 1.80. Paper electrophoresis (centimeters from origin): 9.1; α-MSH, 11.5. FAB mass spectrum: MH^+ calcd, 1020; found, 1020.

Ac-[Cys^4,Cys^{10}]-α-MSH_{4-13}-NH_2. Starting with 3.00 g of Boc-protected valine-*p*-methylbenzhydrylamine resin (0.9 mmol of Boc-Val total) the title peptide was obtained after stepwise coupling of the following N^{α}-Boc-protected amino acids (in order of addition): N^{α}-Boc-Pro, N^{α}-Boc-N^{ϵ}-2,4-Cl_2-Z-Lys, N^{α}-Boc-*S*-(3,4-Me_2Bzl)Cys, 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, N^{α}-Boc-γ-Bzl-Glu, and N^{α}-Boc-*S*-(3,4-Me_2Bzl)Cys. Acetylation of the protected decapeptide was achieved with a 6-fold excess of *N*-acetylimidazole in CH_2Cl_2 following deprotection of the N^{α}-Boc group and neutralization of the amino terminus. The resultant Ac-Cys(*S*-3,4-Me_2Bzl)-Glu(γ-Bzl)-His-Phe-Arg(N^{g}-Tos)-Trp(N^{i}-For)-Cys(*S*-3,4-Me_2Bzl)-Lys(N^{ϵ}-2,4-Cl_2-Z)-Pro-Val-*p*-MBHA resin was dried in

(21) D. J. Spackman, W. J. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
(22) R. J. Simpson, M. R. Neuberger, and T. V. Liu, *J. Biol. Chem.*, **251**, 1936 (1976).
(23) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
(24) V. J. Hruby and C. M. Groginsky, *J. Chromatogr.*, **63**, 432 (1971).
(25) W. König and R. Geiger, *Chem. Ber.*, **103**, 2034 (1970).
(26) T. Fujii and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, **47**, 3146 (1974).
(27) S. Lemaire, D. Yamashiro, and C. H. Li, *J. Med. Chem.*, **19**, 373 (1976).

vacuo (4.65 g), and a portion of this material (1.00 g) was treated with 15 mL of anhydrous HF in the presence of 10% anisole for 60 min at 0 °C. After evaporation of the HF and anisole in vacuo, the dried product was washed, under a stream of N_2, with three 30-mL portions of EtOAc and extracted with three 60-mL portions of 30% HOAc. The combined extracts were lyophilized to give 290.3 mg of Ac-[half-Cys(SH)^4,N^i-For-Trp^9,half-Cys(SH)^{10}]-α-MSH_{4-13}-NH_2.

The N^i-For-Trp disulfhydryl peptide was deformylated in the same manner as above. The deformylated peptide solution was diluted with 500 mL of 10% HOAc and the pH adjusted to 8.5 with concentrated NH_4OH. Cyclization was achieved via oxidation with 0.01 N $K_3Fe(CN)_6$ (44 mL, 100% excess) for 1 h at 25 °C. The reaction was terminated by the addition of 30% HOAc to a final pH of 5.0. Excess ferro- and ferricyanide ions were removed by addition of Rexyn 203 (Cl^- form). After filtration, the solution was lyophilized to give 1.28 g of crude Ac-[$\overline{Cys^4,Cys^{10}}$]-$\alpha$-$MSH_{4-13}$-$NH_2$. A portion of the crude peptide (310 mg) was dissolved in 3 mL of 0.01 N NH_4OAc (pH 4.5) and chromatographed on carboxymethylcellulose under the same conditions as above. The major peak eluted during the 0.1 N NH_4OAc (pH 6.8) fraction and was lyophilized to give 11.03 mg of white powder. The ion exchange was repeated on the remaining crude peptide to give a total yield of 38.9 mg. This material was then chromatographed on Sephadex G-25 (2.0 × 36.5 cm) with 0.2 N HOAc and gave one symmetric peak (280 nm detection), which was collected and lyophilized to give 18.6 mg of white, fluffy powder. The purified peptide gave single uniform spots on TLC: R_f 0.13 (A), 0.42 (B), 0.52 (C), 0.54 (D). Amino acid analysis gave the following molar ratios: Glu, 1.00; His, 0.93; Phe, 0.98; Arg, 1.04; Trp, 1.02; half-Cys, 1.94; Lys, 1.00; Pro, 0.96; Val, 1.06. Paper electrophoresis (centimeters from origin): 12.1; α-MSH, 11.5. FAB mass spectrum: MH^+ calcd, 1345; found, 1345.

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 by the frog and lizard bioassays as previously described.[15,28,29] The frogs (*Rana pipiens*) used in these studies were obtained from Lemberger Co., Germantown, WI, and the lizards (*Anolis carolinensis*) were from the Snake Farm, La Place, LA.

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Registry No. II, 81854-62-8; III, 82219-24-7; IV, 83877-16-1; V, 83897-18-1; N^α-Boc-N^i-For-Trp, 47355-10-2; N^α-Boc-N^g-Tos-Arg, 13836-37-8; N^α-Boc-Phe, 13734-34-4; N^α-Boc-N^{im}-Tos-His, 35899-43-5; N^α-Boc-γ-Bzl-Glu, 13574-13-5; N^α-Boc-(*S*-3,4-Me_2Bzl)-Cys, 41117-66-2; Ac-[half-Cys(SH)^4,N^i-For-Trp^9,half-Cys(SH)^{10}]-αMSH_{4-10}-NH_2, 83877-18-3; N^α-Boc-Pro, 15761-39-4; N^α-Boc-N^ϵ-2,4-Cl_2-Z-Lys, 42294-64-4; Ac-[half-Cys(SH)^4,*N*-For-Trp^9,half-Cys(SH)^{10}]-α-MSH_{4-13}-NH_2, 83877-19-4.

(28) T. Huntington and M. E. Hadley, *Endocrinology*, **66**, 599 (1970).
(29) K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 533 (1954).

Synthesis of Partially Modified Retro-Inverso Substance P Analogues and Their Biological Activity†

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Partial retro-inverso modification of a single peptide bond was applied to pGlu-Phe-Phe-Gly-Leu-Met-NH_2 (I), a C-terminal hexapeptide analogue of the neuropeptide substance P. Two analogues with reversed peptide bonds, between the pGlu-Phe and Phe-Gly residues, were prepared, purified and characterized. The analogue gpGlu-(*RS*)-mPhe-Phe-Gly-Leu-Met-NH_2 (II) was devoid of either agonistic or antagonistic activity. The second pseudopeptide analogue, i.e., pGlu-Phe-gPhe-mGly-Leu-Met-NH_2 (III), was found to be a full agonist with 22% of the potency of I in the guinea pig ileum assay.

Substance P (SP) is an undecapeptide that is widely distributed in the central and peripheral nervous system. It was isolated and purified to homogeneity by Chang and Leeman,[1] and its sequence was established to be the following:[2]

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2

Synthetic SP has been prepared by Tregear et al.[3] and proved to be identical with the endogenous material.

SP belongs to the class of tachykinin-like peptides. Some of its pharmacological activities are vasodilation and spasmogenic activity,[4] salivation,[5] release of histamine from mast cells,[6] and depolarization of spinal motoneurons.[7] SP is rapidly degraded by various enzymes present in different brain preparations[8-10] and in plasma.[11,12] The major sites

† Abbreviations according to IUPAC-IUB Commission (1972), *Biochemistry*, 11, 1726–1732, and Specialist Periodical Reports, "Amino Acids, Peptides and Proteins", Volume 11 (The Chemical Society, London, 1980, R. C. Sheppard, Ed.), are used throughout. The following special abbreviations for the partially modified retro-inverso peptides are used: The standard three-letter notation for amino acid residues preceded by the prefix g represents the *gem*-diamino alkyl residue derived from the specified amino acid. The prefix m represents the malonic acid residue derived from the amino acid specified by the three-letter notation. Configurational designation of the retro-inverso residues follows those of the amino acids.

(1) M. M. Chang and S. E. Leeman, *J. Biol. Chem.*, **245**, 4784 (1970).
(2) M. M. Chang, S. E. Leeman, and H. D. Niall, *Nature (London) New Biol.*, **232**, 86 (1971).
(3) G. W. Tregear, H. D. Niall, J. T. Potts, Jr., S. E. Leeman, and M. Otsuka, *Brain Res.*, **73**, 59 (1974).
(4) U. S. von Euler and J. H. Gaddum, *J. Physiol.*, **72**, 74 (1931).
(5) F. Lembeck and K. Starke, *Naunyn-Schmiedeberg's Arch. Pharmakol.*, **259**, 375 (1968).
(6) A. R. Johnson and E. G. Erdos, *Proc. Soc. Exp. Biol. Med.*, **142**, 1252 (1973).
(7) S. Konishi and M. Otsuka, *Brain Res.*, **65**, 397 (1974).
(8) B. Gullbring, *Acta Physiol. Scand.*, **6**, 246 (1943).

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