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Cyclic melanotropins

Part VII*: Modified ring structures – synthesis and biological activity

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The highly potent cyclic analogue of α-MSH, Ac-[$\overline{Cys^4, Cys^{10}}$]-α-$MSH_{4-13}$-$NH_2$, was structurally modified in position 4. Four analogues were prepared and their biological activities in the *in vitro* frog and lizard skin bioassays were determined. It was shown that removing the terminal acetylamino group to give [$\overline{Mpa^4, Cys^{10}}$]-α-$MSH_{4-13}$-$NH_2$ resulted in little change in the biological activity, but a change in the stereochemistry of cysteine in position 4 to give Ac-[$\overline{D\text{-}Cys^4, Cys^{10}}$]-α-$MSH_{4-13}$-$NH_2$ led to a small decrease of activity in both bioassays. Decreasing the size of the intramolecular ring by removing one methylene group to give [$\overline{Maa^4, Cys^{10}}$]-α-$MSH_{4-13}$-$NH_2$, resulted in an analogue with lower activities in both assays (about 3 times in the lizard and 500 times in the frog), and increasing the size of the ring by one methylene group to give Ac-[$\overline{Hcy^4, Cys^{10}}$]-α-$MSH_{4-13}$-$NH_2$ led to much lower activities in the lizard system and similar effects were seen upon decreasing the ring size in the frog skin assay.

Key words: cyclic peptides; α-melanotropin stimulating hormone (α-MSH); ring modification

Several physiological roles have been attributed to α-melanotropin (α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) (1, 2). The hormone is primarily responsible for skin pigmentation, but considerable evidence has been obtained for several neurophysiological functions as well (3,4). From studies of active site analogues of α-MSH in several laboratories (for reviews, see 5,6) the 4–10 sequence probably contains the essential structural features for its observed biological activity. A reverse turn involving the His^6-Phe^7-Arg^8-Trp^9 sequence has been proposed to be of biological importance at the frog skin receptor (7). This reverse turn structure was stabilized by preparing a cyclic analogue which involves

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Abbreviations used in this manuscript follow those recommended by the IUPAC method of nomenclature. Other abbreviations include the following: Boc, t-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DMF, *N,N'*-dimethylformamide; For, formyl; Hcy, homocysteine; HOBt, *N*-hydroxybenzotriazole; Maa, mercaptoacetic acid; MeBzl, methylbenzyl; Mpa, mercaptopropionic acid; α-MSH, α-melanotropic stimulating hormone; RP-HPLC, reversed phase-high performance liquid chromatography; Tos, tosyl; Z, benzyloxycarbonyl.

a pseudoisosteric replacement of methionine in position 4 and glycine in position 10 by cystine. This substitution led to a new class of cyclic α-MSH analogues with high biological potency (7–9). We recently investigated the roles of the *N*-terminal tripeptide Ser-Tyr-Ser (8) and of the *C*-terminal tripeptide (Lys-Pro-Val) (9) in the "superpotency" of the cyclic analogues. In this study we have examined the effect of the size of the intramolecular ring on the biological activity by modifying position 4 in cyclic melanotropins. Analogues of Ac-[Cys^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ were prepared because our previous studies had shown that the *N*-terminal tripeptide -Ser-Tyr-Ser- had little effect on the potency or efficacy of the cyclic analogues in the frog or lizard skin assay systems (8).

RESULTS AND DISCUSSION

The structures of the four cyclic analogues we have prepared as well as that of α-MSH are presented in Fig. 1. The new analogues were synthesized by solid phase methodology similar to that previously utilized (7,8). The analogues were purified by ion exchange chromatography on a carboxymethylcellulose column followed by gel filtration on Bio-gel P-4 and reverse phase-high performance liquid chromatography (RP-HPLC, when required) on a Vydac C-18 or a Waters RCM reversed phase column. The homogeneity of each analogue was determined by amino acid analysis, thin-layer chromatography (in four solvent systems), electrophoresis and RP-HPLC (various columns and mobile phases) (Table 3).

The biological activities were determined *in vitro* on the frog (*Rana pipiens*) and the lizard (*Anolis carolinensis*) bioassay (Table 1), using methods previously reported (10,11). The results of these assays are shown in Figs. 2

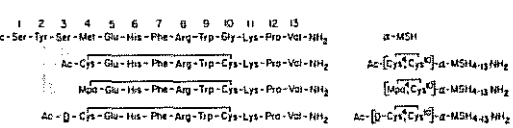

FIGURE 1

Primary structures of melanotropin analogues studied.

TABLE 1

Relative in vitro *potencies of α-MSH analogues in the frog* (Rana pipiens) *and lizard* (Anolis carolinensis) *skin bioassays*

Compound	Relative potency to α-MSH[a]	
	Frog skin	Lizard skin
α-MSH	1.00	1.00
Ac-[Cys^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (I)	30.0	2.0
[Mpa^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (II)	30.0	1.0
Ac-[D-Cys^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (III)	3.0	0.70
[Maa^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (IV)	0.06	0.70
Ac-[Hcy^4, Cys^{10}]-α-MSH$_{4-13}$NH$_2$ (V)	0.06	0.06

[a]Values derived from parallel dose response curves.

and 3. The potencies were determined over the linear portion of the dose-response curve, and are compared to those recently obtained for Ac-[Cys^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (I) under the same conditions. The potencies shown for the Ac-[Cys^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (I) are

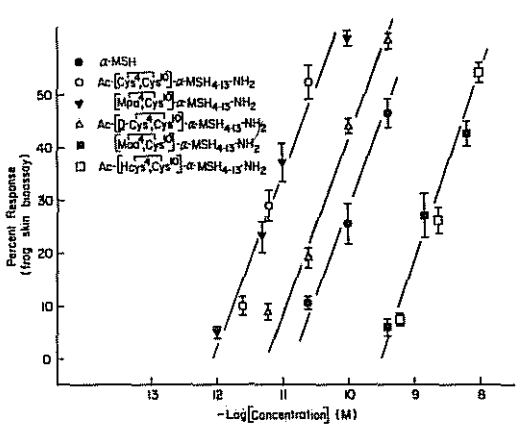

FIGURE 2

Relative *in vitro* response curves of a number of cyclic melanotropins determined in the frog (*Rana pipiens*) skin bioassay. Each values represents the mean, ± S.E., response (darkening) of the skins (N = 6 or more for each value in all experiments for the melanotropins at the concentrations indicated).

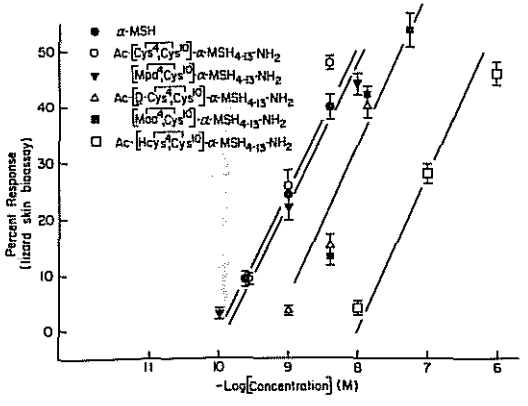

FIGURE 3
As in Fig. 2, lizard (*Anolis carolinensis*) skin bioassay.

more consistent than the previously reported minimum effective responses (7,8), which were subject to wide variability. This problem is discussed elsewhere (9).

The first modification examined was replacement of the *N*-terminal acetyl-amino functional group by a hydrogen. This was accomplished in Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ by incorporation of β-mercaptopropionic acid (Mpa) into position 4 (see Fig. 1). The cyclic analogue obtained, [Mpa^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (II) was equipotent to the cyclic 4–13 analogue (I) in the frog skin bioassay. This cyclic compound was nearly equipotent to α-MSH in the lizard skin assay (Fig. 3), but was about 30 times more active than α-MSH in the frog skin bioassay (Fig. 2). These results indicated that the acetylamino group in position 4 was not necessary for the high biological activity observed in cyclic 4–13 analogues.

In order to further study position 4, we tested the importance of chirality by replacing L-cysteine with D-cysteine in this position. It was shown (Figs. 2,3) that Ac-[D-Cys^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (III) was about 10 times less potent than the corresponding all L-amino acid-containing cyclic stereoisomer in the frog skin bioassay and about 3 times less active in the lizard skin bioassay.

Next we determined the optimal size for the intramolecular ring formed by the cysteine substitution. Intially we decreased the ring by one methylene group via substitution of mercaptoacetic acid (Maa) for cysteine in position 4. The resulting molecule, [Maa^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (IV) (22-membered ring) was less active in both bioassays than the 23-membered ring parent compound. Again we observed that *relative potency differences* exist between the analogues at the frog and the lizard skin melanocyte receptors. In the case of the frog skin assay system the loss of activity for this analogue was much more pronounced (Table 1). A further 10-fold decrease in potency in the lizard skin system was observed (Fig. 3) when the intramolecular ring was enlarged by one methylene group by substituting homocysteine in position 4 for cysteine. This compound, Ac-[Hcy^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (V), showed a greater loss of activity in the frog skin than in the lizard skin assays. These results show the greater sensitivity of the frog skin receptors to a change in structure of the cyclic α-MSH agonist molecule.

We also wish to report that Ac-[Cys^4,Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ does not exhibit any significant prolongation of skin darkening as previously reported (7,8), nor do any of the other cyclic 4–13 analogues reported here. Our recent studies with RP-HPLC suggest (12) that the previously reported prolongation of activity was probably due to a slight contamination with the D-Phe-7-containing 4–13 diastereoisomer. We have previously shown that D-Phe-7-containing analogues of α-MSH often display prolonged activity (13–15; Cody *et al.*, manuscript in preparation). The purification procedures used in previous preparations of cyclic melanotropins did not include the use of RP-HPLC conditions by which all presently synthesized L and D-Phe-7 diastereoisomers are separable (12). We are further investigating the use of RP-HPLC for the purification of synthetic melanotropins.

In summary, these studies provide new insights into the biological activities of cyclic α-MSH analogues at the frog skin and lizard skin receptor systems. They clearly show that the size of the intramolecular ring is critical to high potency in these cyclic analogues. The larger ring size (24-membered) analogue leads to a large loss of activity at the frog skin (about 500-fold) and lizard skin (about 300-fold) receptor systems, relative to the cyclic 23-membered ring, analogue I. A smaller ring

size (22-membered ring) as in analogue IV appears to be more compatible with strong receptor interaction, but still leads to reduced activity at the frog skin (about 500-fold) and lizard skin (about 3-fold) systems. A change in the chirality of the half-Cys-4 residue in the cyclic compound caused a moderate loss in activity. Interestingly, removal of the acetylamino group from the *N*-terminal amino acid had no effect on the potency of the α-MSH analogues. This is rather unexpected because while the effect of replacing the acetylamino group by a hydrogen in α-melanotropins has not previously been examined in the frog or lizard skin assay systems, removal of only the acetyl group generally leads to a large loss in potency (5,6).

EXPERIMENTAL PROCEDURES

General methods

Capillary melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. Thin-layer chromatography (t.l.c.) was performed on Silufol plates (Kavalier, Czechoslovakia) using 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); D) 2-propanol/25% aqueous NH_3/H_2O (3:1:1). Detection was by iodine vapors and ninhydrin. Proton n.m.r. spectra were recorded on a Perkin–Elmer EM-360L spectrophotometer. Electrophoresis was performed on Whatman 1 chromatography paper using an instrument by Gelman with 0.1 M pyridinium acetate (pH 5.3) as the solvent with a potential drop of 20 V/cm. Detection utilized ninhydrin and the chlorination method (16). Optical rotations were obtained on a Perkin–Elmer 241 MC polarimeter at the mercury green line (546 nm). 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. Amino acid analysis was obtained with a Beckman 120C amino acid analyzer following hydrolysis for 22 h at 110° with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (17). No corrections were made for destruction of amino acids during hydrolysis. Sulfur-containing amino acids were determined as cysteine-sulfonic acid and homocysteine-sulfonic acid after oxidation with performic acid and hydrolysis (18). High pressure liquid chromatography (HPLC) was performed on a Spectra-Physics SP-8700 instrument equipped with a SP-8400 detector, using a Vydac C18 column (25 x 0.4 cm) for analytical analysis and a Waters RCM column (15 x 0.8 cm) for preparative work. We used 0.1–0.5% trifluoroacetic acid as the buffer and acetonitrile as the organic modifier.

N^{α}-Boc-protected amino acids and amino acid derivatives were purchased from Vega Biochemicals (Tucson, AZ), or Bachem (Torrance, CA) or prepared using published procedures. Before use, all amino acid derivatives were subjected to a ninhydrin test (19) to establish their purity. Solvents used for chromatography were purified by previously reported methods (20). The *p*-methylbenzhydrylamine resin (0.22 mmol/g, 1% divinylbenzene cross-linked polystyrene) was prepared by previously reported methods (21).

Solid-phase peptide synthesis of melanotropins

The cyclic α-MSH analogues reported here were synthesized by solid-phase methods similar to those used previously for the synthesis of other cyclic α-MSH analogues (7,8). N^{α}-Boc-protected amino acid derivatives were successively coupled to a substituted *p*-methylbenzhydrylamine resin with 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 Boc protecting groups was performed by treatment with 45% trifluoroacetic acid containing 2% anisole in dichloromethane. Side-chain functional groups were protected as follows: glutamic acid, γ-benzyl ester; lysine, N^{ξ}-2,4-dichlorobenzyloxycarbonyl; tryptophan, N^{in}-formyl; histidine, N^{im}-tosyl; arginine, N^{g}-tosyl; and cysteine, S-4-methylbenzyl.

A cycle for incorporating each amino acid residue into the growing peptide chain consisted of the following: 1) washing with CH_2Cl_2 (4 x 25 ml, 1 min/wash); 2) cleaving the Boc group by adding 25 ml 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 x 25 ml, 1 min/wash); 4) neutralizing with 10% diisopropylethylamine in CH_2Cl_2 (2 x 25 ml, 2 min/wash); 5) washing with CH_2Cl_2 (3 x 25 ml, 1 min/wash); 6) adding the Boc-protected amino acid derivative in 20 ml CH_2Cl_2 followed by HOBt (dissolved in a minimal amount of dry DMF), followed by DCC and shaking for $3\frac{1}{2}$–12 h; 7) washing with CH_2Cl_2 (3 x 25 ml, 1 min/wash); 8) washing with EtOH (3 x 25 ml, 1 min/wash); 9) washing with CH_2Cl_2 (4 x 25 ml, 1 min/wash). Between steps 6 and 7, several milligrams of the resin was removed and used in a ninhydrin test to determine the progress of the coupling. After coupling all of the amino acid residues to the resin the finished protected peptides were cleaved from the resin, and all protecting groups were removed with anhydrous liquid HF (0° for 60 min) containing 10% anisole and 5% 1,2-dithioethane (22). Cyclization was carried out in a dilute solution with potassium ferricyanide (7).

[Mpa^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (II)

Starting with 3.0 g *p*-methylbenzylhydrylamine resin (0.66 mM amine total) the protected peptide resin bearing the 5–13 sequence was prepared by the stepwise coupling of the following N^{α}-Boc amino acids derivatives (in the presence of DCC and HOBt): Val, Pro, Lys(2,4Cl$_2$-Z), Cys(4-MeBzl), Trp(For), Arg(Tos), Phe, His(Tos), Glu(Bzl). The resin was divided and S-(3,4-dimethylbenzyl)-3-mercaptopropionic acid (0.45 g) was coupled to 1.4 g of the resin. The peptide was cleaved from the resin, removing all the protecting groups by treatment with a solution of anhydrous liquid HF (10 ml), 10% anisole and 5% 1,2-dithioethane (60 min, 0°). After evaporating the HF, anisole and 1,2-dithioethane *in vacuo*, the resin was washed under a stream of N_2 with EtOAc (3 x 30 ml), and subsequently extracted with 30% HOAc (3 x 60 ml), 10% HOAc (3 x 60 ml) and distilled water (3 x 60 ml). The combined aqueous extracts were lyophilized to give a white powder. The lyophilizate was dissolved in distilled water (1200 ml) and the pH of the solution was adjusted to 8.9 with aqueous ammonia. To the stirred solution was added 0.01 N $K_3Fe(CN)_6$ until a yellow color persisted (50 ml). After stirring for 1 h the pH of the solution was lowered to 5.0 with glacial HOAc and 10 ml packed Bio-Rad anion exchange resin AGX-4(Cl$^-$form) was added. After stirring for 30 min the suspension was filtered and the solution lyophilized. Half of the lyophilizate (160 mg) was dissolved in 0.01 M NH_4OAc, pH 4.5, and eluted on a carboxymethylcellulose cation exchange column (2 x 40 cm) with a discontinuous gradient (250 ml of each) of 0.01 N NH_4OAc (pH 4.5) and 0.10 N NH_4OAc (pH 6.8). The major peak (detected at 280 nm) was lyophilized to give 31.4 mg of a white powder which was further purified by gel filtration on a Bio Gel P-4 column (100 x 1.0 cm) in 1 N HOAc and by HPLC on a Waters RCM column with 0.1% trifluoroacetic acid and acetonitrile as the mobile phase. Physical properties of the peptide are presented in Tables 2 and 3. Mass spectrum: MH^+ 1288.1.

Ac-[D-Cys^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$

To the above described nonapeptide resin (0.7 g; 0.11 mM), N^{α}-*tert*-butyloxycarbonyl-S-(4-methylbenzyl)-D-cysteine (0.35 g) was coupled in the presence of DCC (0.21 g) and HOBt (0.14 g). Following removal of the Boc protecting group with trifluoroacetic acid the peptide was acetylated with a 6-fold excess of *N*-acetylimidazole (0.11 g). Cleavage from the resin, deprotection, cyclization and purification was performed as in the case of peptide II. The physical properties of this peptide are found in Tables 2 and 3.

[Maa^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (IV)

To the above described nonapeptide resin (0.7 g; 0.11 mM), S-(4-methylbenzyl)mercaptoacetic acid (0.35 g) was coupled in the presence of DCC (0.21 g) and HOBt (0.14 g). Cleavage from the resin, deprotection, cyclization and purification was performed as in the case of peptide II. The physical properties of this peptide are found in Tables 2 and 3.

Ac-[Hcy^4, Cys^{10}]-α-MSH$_{4-13}$-NH$_2$ (V)

To the above described nonapeptide resin (0.7 g; 0.11 mM), N^{α}-*tert*-butyloxycarbonyl-S-(3,4-dimethylbenzyl)homocysteine (0.35 g) was coupled in the presence of DCC (0.21 g) and

TABLE 2

Amino acid analysis of prepared analogues

Compound	Glu	His	Phe	Arg	Trp	Cys	Lys	Pro	Val
II	1.02	0.94	0.92	0.92	0.99	0.91[a]	0.98	1.08	1.09
III	1.08	1.05	0.91	0.94	0.93	1.81[a]	1.01	1.04	0.98
IV	0.98	1.01	0.97	0.97	1.04	0.99[a]	1.00	1.03	1.01
V	1.02	0.97	0.99	0.94	0.92	1.95[b]	1.10	1.11	1.00

[a]Determined as cysteine sulfonic acid after oxidation of aliquot sample.
[b]Sum of cysteine sulfonic and homocysteine sulfonic acid.

TABLE 3

Physical properties of prepared analogues

Compound	Yield (%)	T.l.c. R_F[a]				E^{His}[b] 5.3	HPLC k'[c]		$[\alpha]_{546}^{25}$	(c)[f]
		A	B	C	D		A[d]	B[e]		
II	20.4	0.05	0.57	0.56	0.59	0.51	3.19	8.58	−88°	(0.2)
III	15.6	0.05	0.63	0.55	0.59	0.47	2.64	6.03	−69.4°	(0.07)
IV	18.6	0.05	0.63	0.55	0.59	0.52	3.93	6.65	−124°	(0.2)
V	8.8	0.05	0.63	0.55	0.59	0.49	3.53	18.1	−107.7°	(0.25)

[a]Solvent systems A–D are defined in Experimental Procedures.
[b]Electrophoretic mobility relative to His at pH 5.3 under conditions defined in Experimental Procedures.
[c]k' = retention of analogue/(retention of analogue-retention of solvent).
[d]Altech C-18 (25 × 0.46 cm) column; 30% acetonitrile in 0.5% trifluoroacetic acid.
[e]Vydac C-18 (25 × 0.46 cm) column; 15% acetonitrile in 0.25 M triethylammonium phosphate buffer, pH 2.2;
[f]in 10% HOAc.

HOBt (0.14 g). Following removal of the Boc protecting group with trifluoroacetic acid the peptide was acetylated with a 6-fold excess of *N*-acetylimidazole (0.11 g). Cleavage from the resin, deprotection, cyclization and purification was performed the same as in the case of peptide II. The physical properties of this peptide are found in Tables 2 and 3.

S-(4-Methylbenzyl)mercaptoacetic acid

A solution of mercaptoacetic acid (2.3 ml; 80% in water) in water (6 ml) and ethanol (8 ml) containing triethylamine (5.6 ml) and 4-methylbenzylchloride (3.2 g) was stirred for 3 h at room temperature. The reaction mixture was diluted with water (50 ml) and extracted with ethyl ether (2 × 50 ml). The aqueous layer was acidified with concentrated HCl to pH 4 and the oily product was extracted with ethyl ether (3 × 50 ml). The combined organic phases were dried with Na_2SO_4, concentrated, and distilled, bulb-to-bulb *in vacuo* (external temperature 120°), to yield an oily product (2.38 g, 61%). ^{1}H-n.m.r. (δ, $CDCl_3$): 6.9 (q, 4H, aromatic H); 3.5 (s, 2H, CH_2-S); 2.8 (s, 2H, S-CH_2-CO); 2.02 (s, 3H, CH_3); t.l.c., R_f 0.88 (A), 0.81 (B), 0.61 (C), 0.78 (D). Synthesis of this compound was previously reported (23).

S-(3,4-Dimethylbenzyl)homocysteine

Homocysteine (1.34 g), prepared according to the literature (24), was dissolved in liquid ammonia (100 ml). Metallic sodium was added until the solution turned blue and the color remained for 5 min. Ammonium chloride was added until the blue color just disappeared and then 3,4-dimethylbenzylbromide (2.2 g) was added in petroleum ether (10 ml). After stirring the solution for 60 min, the liquid NH_3 was evaporated under a stream of nitrogen and subsequently dried *in vacuo*. The resulting solid was dissolved in H_2O (10 ml), washed with

ether (2 x 5 ml) and acidified with 1 N HCl (0°) to pH 3. Crystals were obtained after standing overnight at 5°. These were filtered, washed with H_2O, EtOH and dried *in vacuo* to yield 1.88 g (74%); m.p. 235–237° (dec.); t.l.c., R_f 0.32 (A), 0.74 (B), 0.68 (C), 0.70 (D). For elemental analysis, the sample recrystallized from EtOH/H_2O without a change in the melting point.

Anal. calc. for $C_{13}H_{19}NO_2S$ (253.3): C 61.63, H 7.56, N 5.53%. Found: C 61.23, H 7.39, N 5.38%. $[\alpha]_D^{22°}$ + 27.4° (*c* 0.25; 6 N HCl).

S-(3,4-Dimethylbenzyl)-3-mercaptopropionic acid

3-Mercaptopropionic acid (2 ml) was dissolved in liquid ammonia (150 ml) and subsequently reduced by the addition of metallic sodium, until the blue color was maintained for 5 min. Excess sodium was removed with ammonium chloride, followed by the addition of 3,4-dimethylbenzylbromide (4.8 g). After stirring for 1 h, the ammonia was removed under a stream of nitrogen and the resulting solid was dried *in vacuo*. The solid was then dissolved in H_2O (30 ml), washed with ether (2 x 10 ml), acidified with 1 N HCl to pH 3, and stored at 5°, overnight. The crystals that formed were recrystallized from H_2O to yield 4.33 g (84%) of product melting at 50–51°. ^{1}H-n.m.r. (δ, $CDCl_3$): 7.03 (s, 3H, aromatic H); 3.68 (s, 2H, Ar-C<u>H</u>); 2.60 (s, 4H, S-CH_2-CH_2-CO); 2.27 (s, 6H, 2CH_3); R_f 0.88 (A), 0.83 (B), 0.68 (C), 0.81 (D).

Anal. calc. for $C_{12}H_{16}O_2S$ (224.3): C 64.25, H 7.19%. Found: C 64.40, H 7.25%.

N^{α}-*t-Butyloxycarbonyl-S-(3,4-dimethylbenzyl) homocysteine dicyclohexylammonium salt*

S-(3,4-Dimethylbenzyl)homocysteine (1.00 g) was dissolved in a mixture of 10% Na_2CO_3 (15 ml) and dioxane (15 ml). The solution was cooled to 0° and bis-t-Boc-carbonate (1.1 g) was added. After stirring for 1 h at room temperature the solution was diluted with H_2O (50 ml) and extracted with ethyl acetate. The aqueous layer was acidified with 10% citric acid to pH 3 and extracted with ethyl acetate. The organic layer was washed with brine, dried with Na_2SO_4 and concentrated. Part of the oily product (t.l.c., R_f 0.21 (A), 0.67 (B), 0.70 (C), 0.92 (D)) (0.35 g of 1.30 g) was used immediately in solid phase synthesis and the rest was converted to the dicyclohexylammonium salt (DCHA) for subsequent analysis. The DCHA salt was recrystallized from ethanol and water (m.p. 105–107°); yield 93%.

Anal. calc. for $C_{30}H_{50}N_2O_4S$(534.8): C 67.38, H 9.42, N 5.24%. Found: C 67.45, H 9.41, N 5.20%. $[\alpha]_D^{22°}$ + 20.1 (*c* 1.1, EtOH).

Frog and lizard skin bioassay

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 (10, 25, 26). All the solutions were prepared via serial dilutions from a stock solution (10^{-4} M) by utilizing different pipettes (2). 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.

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