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Cyclic Lactam Analogues of Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂[†]

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ABSTRACT: Two side-chain cyclic lactam analogues of the 4–11 fragment of α-melanocyte-stimulating hormone (α-MSH), Ac-[Nle⁴,D-Orn⁵,Glu⁸]α-MSH₄₋₁₁-NH₂ and Ac-[Nle⁴,D-Orn⁵,D-Phe⁷,Glu⁸]α-MSH₄₋₁₁-NH₂, were prepared on *p*-methylbenzhydrylamine resin by using a combination of N^α-Boc and N^α-Fmoc synthetic strategies with diphenyl phosphorazidate mediated cyclization. The melanotropin activities of these two analogues were examined and compared relative to those of α-MSH, Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂, and Ac-[Nle⁴,D-Phe⁷]α-MSH₄₋₁₁-NH₂. In the frog (*Rana pipiens*) skin bioassay, the L-Phe⁷ 17-membered ring cyclic analogue was slightly more potent than the linear Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂ and exhibited prolonged melanotropic bioactivity (≥4 h). In this same assay, the D-Phe⁷ cyclic analogue was more than 100-fold less potent than the L-Phe cyclic analogue and was 10000 times less potent than linear Ac-[Nle⁴,D-Phe⁷]α-MSH₄₋₁₁-NH₂. In the lizard skin (*Anolis carolinensis*) bioassay, the L-Phe⁷ cyclic analogue was 100-fold less potent than Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂, while the D-Phe⁷ cyclic analogue was 10000-fold less potent than both Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂ and the D-Phe⁷ linear derivative Ac-[Nle⁴,D-Phe⁷]α-MSH₄₋₁₁-NH₂. The solution conformation of these two cyclic analogues in dimethyl sulfoxide-d_6 was examined by 1D and 2D 500-MHz ¹H NMR spectroscopy. Our analysis suggests an H bond stabilized C_{10} (or C_{13}) turn for the D-Phe⁷ cyclic structure while the L-Phe⁷ analogue is more conformationally flexible. More importantly, these results suggest that melanotropic potency may be correlated with a close spatial relationship between the side chains of His⁶, Phe⁷, and Trp⁹.

We recently proposed a topographical model for the solution conformation of α-melanocyte stimulating hormone (α-MSH,[1] 1, Figure 1) based on proton NMR analysis of the aqueous solution conformation of a series of linear Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂ diastereomers (Sugg et al., 1986). The predominant backbone solution conformation observed for all analogues was a non-hydrogen-bonded β-structure ($\phi = -139°$, $\psi = +135°$ for L-amino acid residues; $\phi = +139°$, $\psi = -135°$ for D-amino acid residues). This is illustrated in Figure 2A for the 5–9 region of α-MSH. The proposed key features for melanotropic potency were a left-handed turn of the backbone going for $C_{6\alpha}$ to $C_{9\alpha}$ and a close (i.e., gauchelike) spatial relationship between the side chains of His⁶ and Phe⁷.

In order to examine the relevance of our solution model to the bioactive conformation of the melanotropins, we designed analogues with a cyclic lactam bridging positions 5 and 8 of the linear 4–11 fragment. To stabilize the left-handed turn of the backbone, D stereochemistry is required at position 5.

Figure 2B illustrates the linear 5–9 region in β-structure with a D-amino acid at position 5. Finally, to examine the spatial relationships between the His⁶ and Phe⁷ side chains, both L-Phe⁷ and D-Phe⁷ diastereoisomers must be prepared. Figure 2C illustrates the linear 5–9 region in β-structure with D-amino acids at positions 5 and 7. On the basis of these considerations (vide infra) we first chose to examine the cyclic 17-membered ring lactam analogues Ac-[Nle⁴,D-Orn⁵,Glu⁸]α-MSH₄₋₁₁-NH₂ (2, Figure 1) and Ac-[Nle⁴,D-Orn⁵,D-Phe⁷,Glu⁸]α-MSH₄₋₁₁-NH₂ (3, Figure 1).

Here we report on the synthesis and bioactivities of these new melanotropin analogues. Analysis by 500-MHz ¹H NMR has permitted a refinement of our original proposal for the
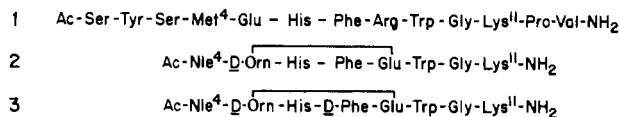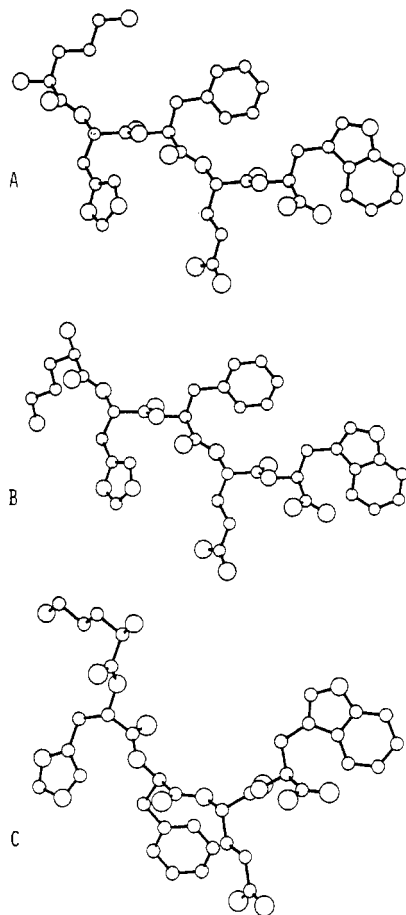
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[1] Abbreviations: Nle, norleucine; α-MSH, α-melanocyte-stimulating hormone or α-melanotropin; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DCM, dichloromethane; Fmoc, fluorenylmethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; FAB-MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance; DCZ, 2,6-dichlorobenzyloxycarbonyl; DPPA, diphenyl phosphorazidate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; PMBHA, *p*-methylbenzhydrylamine. The standard abbreviations and nomenclature for amino acids, peptides, and peptide derivatives and analogues of the IUB/IUPAC are used throughout. All amino acids except glycine are of the L configuration unless otherwise noted.

1   Ac-Ser-Tyr-Ser-Met⁴-Glu — His — Phe-Arg-Trp-Gly-Lysⁱⁱ-Pro-Val-NH₂

2   Ac-Nle⁴-D-Orn- His — Phe — Glu-Trp-Gly-Lysⁱⁱ-NH₂

3   Ac-Nle⁴-D-Orn-His-D-Phe-Glu-Trp-Gly-Lysⁱⁱ-NH₂

FIGURE 1: Primary structure of α-MSH and the analogues reported.

FIGURE 2: β-Structure model ($\phi = -139°$, $\phi = +135°$ for L-amino acid residues; $\phi = +139°$, $\phi = -135°$ for D-amino acid residues) for the 5–9 region of α-MSH: (A) all L-amino acid residues; (B) D-amino acid residue at position 5; (C) D-amino acid residues at positions 5 and 7.

bioactive conformation of the melanotropins at the melanocyte receptor.

EXPERIMENTAL PROCEDURES

General Materials and Methods. Thin-layer chromatography (TLC) was performed on 20-cm silica gel G plates (EM Reagents) using the following solvent systems: B, 1-butanol/acetic acid/pyridine/water (15:3:12:10); C, 1-butanol/acetic acid/pyridine/water (6:1.2:6:4.8); E, ethyl acetate/acetic acid/pyridine/water (60:6:20:11). Detection was by Cl_2/tolidine or ninhydrin. Analytical and preparative reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Perkin-Elmer Series 3B chromatograph, equipped with a Model LCI 100 laboratory computing integrator using hydrocarbon-bonded octadecylsilane (Vydac, 218TP, 10 μm, 25 cm × 4.6 mm and 25 cm × 10 mm) as the stationary phase. The mobile phase employed 0.1% trifluoroacetic acid (pH 2.2) with acetonitrile as the organic modifier. Peaks were monitored at 214 nm.

HPLC solvents (Burdick and Jackson) were purchased from American Scientific Products (Phoenix, AZ). N^α-*tert*-Butyloxycarbonyl(Boc)-N^ϵ-2,6-dichlorobenzyloxycarbonyl-(DCZ)-L-Lys was purchased from Bachem (Torrence, CA). N^α-Boc-Gly, N^α-Boc-L-Trp, and N^α-Boc-L-Nle were prepared

with Boc dicarbonate (Chemical Dynamics Corp., South Plainfield, NJ) according to published procedures (Stewart & Young, 1984). N^α-Fluorenylmethoxycarbonyl(Fmoc)-L-Glu(γ-O-tBu) and N^α,N^{im}-(Fmoc)₂-L-His were purchased from Bachem and Bachem-AG (Bubendorf, Switzerland), respectively. N^α-Fmoc-L-Phe, N^α-Fmoc-D-Phe, and N^α-Fmoc-D-Orn(N^δ-Boc) were prepared with Fmoc-N-hydroxysuccinimide according to published procedures (Paquet, 1982). Trifluoroacetic acid (TFA, Halocarbon Products) solutions (50%) in dichloromethane (DCM) contained 2% anisole and were bubbled with and stored under nitrogen. N,N-Diisopropylethylamine (DIEA, Chemical Dynamics Corp.) solutions (10% in DCM) were also bubbled with and stored under nitrogen. N,N-Dimethylformamide (DMF) was bubbled with nitrogen and stored over 4-Å sieves. Piperidine (Aldrich, Milwaukee, WI) solutions (50% in DMF) for deprotection of the N^α-Fmoc moiety were prepared fresh for each step. Analogues 2 and 3 were prepared by automated solid-phase synthesis (Vega Model 250) on p-methylbenzhydrylamine resin (PMBHA, 1.1 mM/gm amine substitution) (Orlowski et al., 1976). N^α-Boc amino acid couplings utilized symmetrical anhydrides (Hagenmeier & Frank, 1972) performed at 0 °C in DCM with dicyclohexylcarbodiimide (DCC, Chemical Dynamics Corp.). The precipitated dicyclohexylurea was filtered prior to coupling. Because of poor solubility in DCM, N^α-Fmoc amino acid couplings were performed in DMF/DCM mixtures (50/50) with DCC and N-hydroxybenzotriazole (HOBt) (Stewart & Young, 1984). Completion of coupling was monitored with the Kaiser test (Kaiser et al., 1970).

Fast atom bombardment mass spectra (FAB-MS) determinations were performed by the Midwest Center for Mass Spectroscopy (Lincoln, NE), a National Science Foundation Regional Instrument Facility (Grant, CHE 8211164).

NMR Methods. NMR spectra were recorded at 303 K on a Bruker AM-500 equipped with an Aspect 3000 computer. The trifluoroacetate salts of analogues 2 (1.1 mg) and 3 (3.5 mg) were dissolved in dimethyl sulfoxide-d_6 (DMSO-d_6, 0.3 mL). Two-dimensional correlation spectra (2D-COSY) were acquired with an 80-ms delay (Wynants & Van Binst, 1984). Two-dimensional nuclear Overhauser enhanced spectra (20-NOESY) were acquired with a 120-ms mixing time (Kumar et al., 1980). A $\sin^2$ window function was applied in both t_1 and t_2. Unsymmetrized and symmetrized spectra were examined, and the latter were used only when no cross-peaks were detected. The residual peak for DMSO was used as the internal reference with a chemical shift of 2.5 ppm. Temperature dependencies of the amide protons were examined in 5-deg intervals from 298 to 318 K. Accurate chemical shifts and coupling constants were obtained by 1D scalar homodecoupled difference spectra.

N^α-Fmoc-Glu(O-tBu)-Trp-Gly-Lys(N^ϵ-DCZ)-PMBHA. A portion (2.0 g) of PMBHA–resin was washed (30 mL, 2 × 2 min) with DCM, ethanol, and DCM and then shaken with N^α-Boc-L-Lys(N^ϵ-DCZ) symmetrical anhydride (1.43 g, 1.6 mmol) in DCM (30 mL) for 15 min. The amino acid derivative was removed and the resin was washed (as above). Acetylimidazole (2 g, 18 mmol) in DMF/DCM (1:1, 30 mL) was added and allowed to react for 4 h. The resin was again washed (as above) prior to removal of the N^α-Boc group with 50% TFA in DCM (25 mL, 1 × 2 min, 1 × 20 min). The resin was washed with DCM (30 mL, 3 × 2 min), neutralized with 10% DIEA in DCM (30 mL, 3 × 2 min), and rinsed once with DCM (30 mL, 1 × 2 min). N^α-Boc-Gly symmetrical anhydride (0.56 gm, 1.6 mmol) and N^α-Boc-L-Trp symmetrical anhydride (0.97 g, 1.6 mmol) were coupled by the same series

of steps. After removal of the N^α-Boc group of L-Trp, the resin was washed with DCM, neutralized with 10% DIEA in DCM, and washed (30 mL, 3 × 2 min) with ethanol and DMF to remove any traces of DIEA. N^α-Fmoc-L-Glu(γ-O-tBu) (1.02 g, 2.3 mmol), HOBt (0.26 g, 2.3 mmol), and DCC (0.4 g, 1.9 mmol) in DMF/DCM (1:1, 30 mL) were added. The coupling was judged complete in 4 h, and the resin was washed (30 mL, 3 × 2 min) with DMF, ethanol and DCM.

N^α-Fmoc-D-Orn(N^δ-Boc)-His-Phe-Glu(O-tBu)-Trp-Gly-Lys(N^ϵ-DCZ)-PMBHA (4). Half (1.92 g) of the N^α-Fmoc-tetrapeptide resin was washed with DMF, and the N^α-Fmoc group was removed with 50% piperidine in DMF (30 mL, 1 × 5 min, 1 × 20 min). The resin was washed (30 mL, 3 × 2 min) with DMF, ethanol, and DMF prior to the addition of N^α-Fmoc-L-Phe (0.62 g, 1.6 mmol), HOBt (0.18 g, 1.6 mmol), and DCC (0.27 g, 1.3 mmol) in DMF/DCM (1:1, 20 mL). N^α,N^{im}-(Fmoc)₂-L-His (0.73 g, 1.6 mmol) and N^α-Fmoc-D-Orn(N^δ-Boc) (0.73 g, 1.6 mmol) were coupled by the same sequence.

N^α-Fmoc-D-Orn-His-Phe-Glu-Gly-Lys(N^ϵ-DCZ)-PMBHA (5). The N^α-Fmoc-heptapeptide resin was reacted with 50% TFA in DCM (25 mL, 1 × 2 min, 1 × 30 min) to remove the N^α-Boc and γ-O-tBu moieties and washed with DCM (30 mL, 3 × 2 min), neutralized with 10% DIEA in DCM (25 mL, 3 × 2 min), and washed with DMF (25 mL, 3 × 2 min). Diphenyl phosphorazidate (DPPA; 0.5 g, 1.8 mmol) in DMF (30 mL) was added, and the vessel was protected from light and shaken at room temperature. The resin was washed with DMF and the DPPA replenished every 24 h. After 3 days, the resin was washed and acetylimidazole (1 g, 9 mmol) in DMF/DCM (1:1, 20 mL) was added to acetylate any remaining free amino groups.

Ac-Nle-D-Orn-His-Phe-Glu-Trp-Gly-Lys(N^ϵ-DCZ)-PMBHA (6). The N^α-Fmoc group was removed, the resin was washed, and N^α-Boc-L-Nle symmetrical anhydride (0.37 g, 0.8 mmol) in DCM (25 mL) was coupled. After N^α-Boc deprotection, neutralization, and resin wash, acetylimidazole (1 g, 9 mmol) in DMF (25 mL) was added. When the acetylation was judged complete (Kaiser et al., 1970), the resin was washed and dried.

Ac-Nle-D-Orn-His-Phe-Glu-Trp-Arg-Gly-Lys-NH₂ (2). The peptide was cleaved from resin 6, and all protecting groups were removed with anhydrous liquid HF (10 mL/g of resin, 45 min at 0 °C) with 10% anisole as scavenger. The crude peptide was extracted from the resin with 30% acetic acid and lyophilized to a powder. Ion-exchange chromatography on (carboxymethyl)cellulose utilized a linear gradient from 0.01 M acetic acid, pH 4.5, to 0.3 M NH₄OAc, pH 6.8. Preparative HPLC was utilized for final purification to give 8.1 mg of product: FAB-MS, M⁺ = 1053 (calcd, 1054); TLC (B) 0.44, (C) 0.51, (E) 0.19; HPLC k' (20% acetonitrile, 80% 0.1% TFA) 5.4.

Ac-[Nle⁴,D-Orn⁵,D-Phe⁷,Glu⁸]α-MSH₄₋₁₁-NH₂ (3). This analogue was prepared and purified in an analogous manner from the second portion of N^α-Fmoc-tetrapeptide resin 4 by using N^α-Fmoc-D-Phe, yield 17.5 mg: FAB-MS, M⁺ = 1053 (calcd, 1053); TLC (B) 0.46, (C) 0.56, (E) 0.23; HPLC k' (20% acetonitrile, 80% 0.1% TFA) 3.5.

Frog and Lizard Skin Bioassays. The migration of melanosomes within integumental melanocytes from a perinuclear position into the dendritic processes of pigment cells results in a change (darkening) in color. Reflectance changes were measured quantitatively by means of a Photovolt reflectometer and are expressed as a percentage of the original control value
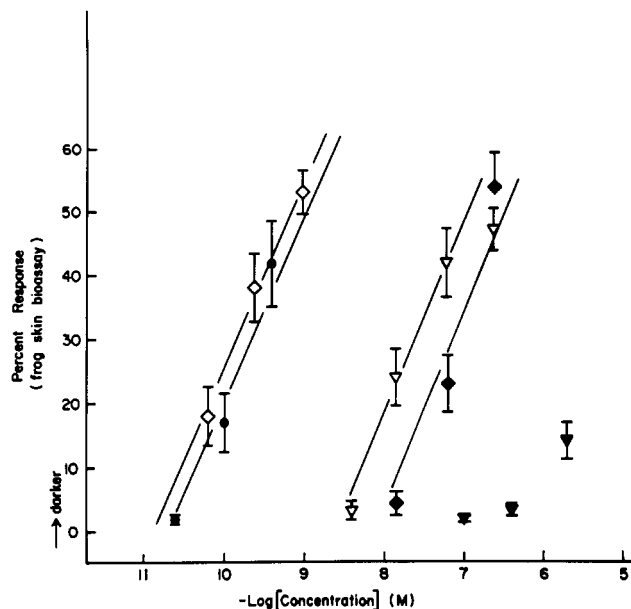

FIGURE 3: Potency of melanotropic response in the frog skin ($R.$ $pipiens$) bioassay: (●) α-MSH; (◇) Ac-[Nle⁴,D-Phe⁷]α-MSH₄₋₁₁-NH₂; (♦) Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂; (▽) Ac-[Nle⁴,D-Orn⁵,Glu⁸]α-MSH₄₋₁₁-NH₂; (▼) Ac-[Nle⁴,D-Orn⁵,D-Phe⁷,Glu⁸]α-MSH₄₋₁₁-NH₂.

(Shizume et al., 1954). Each analogue was analyzed in at least two assays, and each assay employed the skins of six frogs (*Rana pipiens*) and six lizards (*Anolis carolinensis*) for each analogue. A control (no melanotropin) and an α-MSH standard were run with each assay.

RESULTS

Chemistry. Two side-chain to side-chain cyclic lactam analogues, Ac-[Nle⁴,D-Orn⁵,Glu⁸]α-MSH₄₋₁₁-NH₂ (2) and Ac-[Nle⁴,D-Orn⁵,D-Phe⁷,Glu⁸]α-MSH₄₋₁₁-NH₂ (3), were prepared by a combination of N^α-Boc and N^α-Fmoc solid-phase synthesis on p-methylbenzhydrylamine resin, down-substituted to 0.45 mmol/g. This synthetic approach permitted differential protection of the Lys¹¹ ε-amino group and D-Orn⁵ δ-amino group. The diphenyl phosphorazidate mediated cyclization was effected on the PMBHA resin by modification of literature procedures (Lebl & Hruby, 1984; Schiller et al., 1985). The yield of the D-Phe⁷ analogue (3) was twice that of the L-Phe⁷ analogue (2). Both analogues gave a single spot in three TLC systems, gave a single peak on reversed-phase HPLC, and had the appropriate monomeric molecular weight. High-field (500-MHz) proton NMR spectra were consistent with the structure (vide supra).

Biological Assays. In the frog (*R. pipiens*) skin bioassay, the L-Phe⁷ cyclic analogue 2 was slightly more potent than linear Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂ (Figure 3), while the D-Phe⁷ cyclic analogue 3 was ≥10 000 times less potent than linear Ac-[Nle⁴,D-Phe⁷]α-MSH₄₋₁₁-NH₂ (Figure 2). In the lizard (*A. carolinensis*) skin bioassay, the L-Phe⁷ cyclic analogue 2 was 100-fold less active than linear Ac-[Nle⁴]α-MSH₄₋₁₁-NH₂ (Figure 4), and the D-Phe⁷ cyclic analogue 3 was again inactive (Figure 4). The D-Phe⁷ cyclic analogue 3 did not antagonize the response to α-MSH in either bioassay. In both assays the melanotropic response to the L-Phe⁷ cyclic analogue was quite prolonged (>4 h; data not shown).

NMR Studies. The solution conformation of the two cyclic analogues in DMSO-d_6 was examined by 500-MHz proton NMR spectroscopy. The amide (Figure 5) and α- (Figure 6) and β- (Figure 7) proton regions of the NMR spectra are
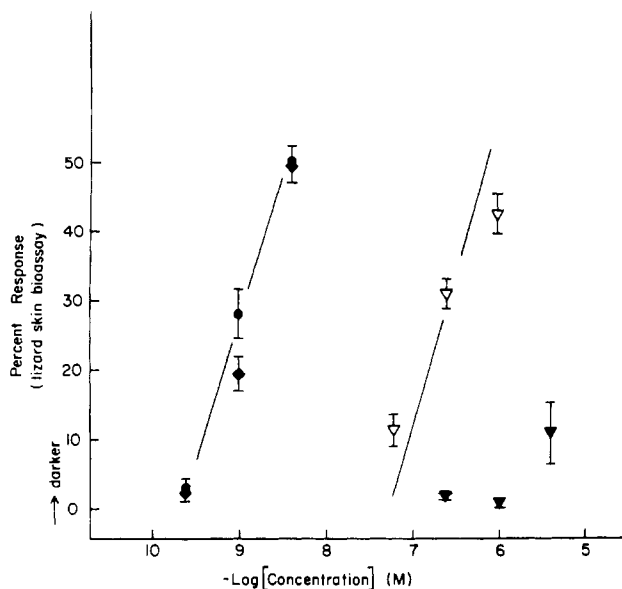
FIGURE 4: Potency of the melanotropic response in the lizard skin (*A. carolinensis*) bioassay. Legend as for Figure 3.
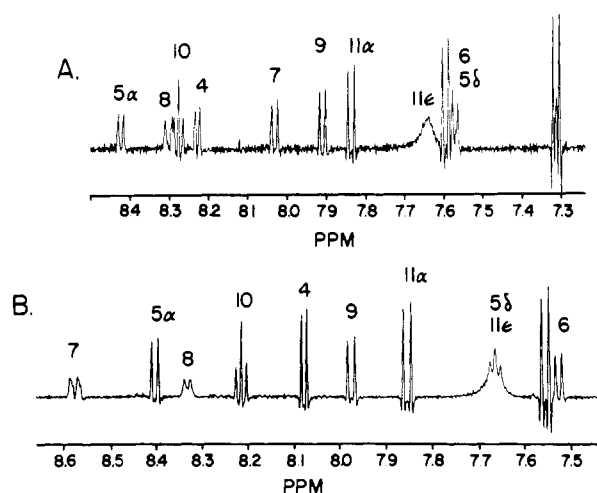

FIGURE 5: Amide region of the 500-MHz ^{1}H NMR spectra of (A) Ac-[Nle^4,D-Orn^5,Glu^8]α-MSH$_{4-11}$-NH$_2$ and (B) Ac-[Nle^4,D-Orn^5,D-Phe^7,Glu^8]α-MSH$_{4-11}$-NH$_2$.

FIGURE 6: α-Proton region of the 500-MHz ^{1}H NMR spectra of (A) Ac-[Nle^4,D-Orn^5,Glu^8]α-MSH$_{4-11}$-NH$_2$ and (B) Ac-[Nle^4,D-Orn^5,D-Phe^7,Glu^8]α-MSH$_{4-11}$-NH$_2$.

FIGURE 7: β-Proton region of the 500-MHz ^{1}H NMR spectra of (A) Ac-[Nle^4,D-Orn^5,Glu^8]α-MSH$_{4-11}$-NH$_2$ and (B) Ac-(Nle^4,D-Orn^5,D-Phe^7,Glu^8]α-MSH$_{4-11}$-NH$_2$.

illustrated for the two cyclic analogues.

Assignments for the individual proton resonances required a combination of 2D-COSY with delay (Wynants & Van Binst, 1984) (80 ms), 2D-NOESY (Kumar et al., 1980) (t_m = 120 ms), and 1D scalar homodecoupling experiments. The resonances for the protons of Glu^8 and Gly^{10} were assigned by inspection on the basis of chemical shift predictions (Wüthrich, 1976; Hruby, 1974). Long-range (J^4) coupling between the β-protons of His^6 and Trp^9 and the C2 aromatic protons were observed by 2D-COSY (not shown), permitting assignment of the proton resonances of these residues. While the resonances for the α-protons of His^6 and Trp^9 were well-separated in the L-Phe^7 analogue (Figure 6A), these resonances overlapped in the D-Phe^7 analogue (Figure 6B). Selective decoupling of the appropriate CH$_\beta$ and NH resonances permitted assignment of the high-field amide resonance (δ = 7.53 ppm, Figure 5B) to His^6 and the lower field amide resonance (δ = 7.8 ppm, Figure 5B) to Trp^9.

The diastereotopic δ-protons of D-Orn^5 were assigned by connection with the NH$^\delta$ triplet in the 2D-COSY spectra. Unfortunately, the high-field alkyl region of the NMR spectra is exceedingly complex, making assignment of the β-protons
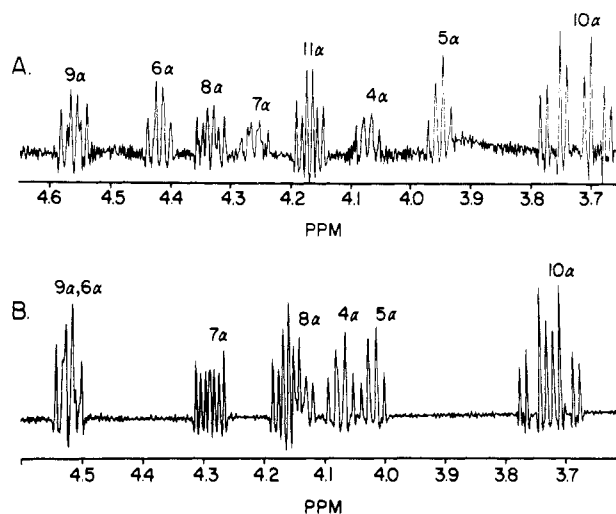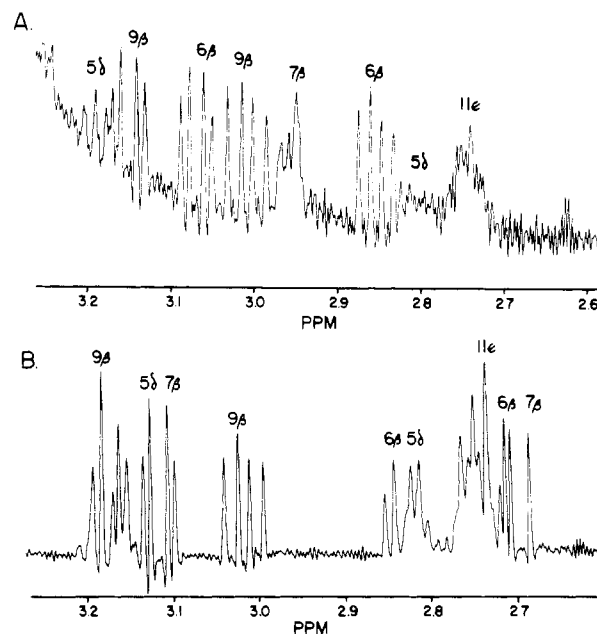
of Nle^4, D-Orn^5, and Lys^{11} difficult. Long-range coupling between the N-acetyl methyl and the Nle^4 CH$_\alpha$ (not shown) permitted assignment of the CH$_\alpha$ resonance of this residue.

Assignment of the CH$_\alpha$ and NH resonances of D-Orn^5, Phe^7, and Lys^{11} required 2D-NOESY spectra. For the L-Phe^7 cyclic analogue, CH$_\alpha^i$ to NH^{i+1} NOE's were observed between residues 4 and 5, 8 and 9, and 9 and 10 (Figure 8), permitting assignment of the D-Orn^5 NH and confirming other assignments. The ambiguity of the L-Phe^7 and Lys^{11} NH resonances was resolved by the observation of an NH^i to NH^{i+1} NOE between His^6 and L-Phe^7 (Figure 9). The remaining amide resonance (δ = 7.84 ppm, Figure 5A) was assigned to Lys^{11}.

For the D-Phe^7 analogue, CH$_\alpha^i$ to NH^{i+1} NOE's were observed between residues 4 and 5, 6 and 7, 8 and 9, 9 and 10, and 10 and 11 (Figure 10). Interresidue NH^i to NH^{i+1} NOE's were observed between D-Orn^5 and His^6 and D-Phe^7 and Glu^8 (Figure 11). Thus, all NH, CH$_\alpha$, and CH$_\beta$ resonances were unambiguously assigned for this analogue.

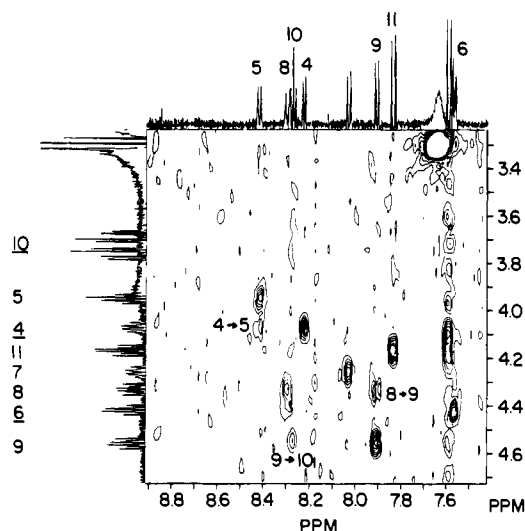FIGURE 8: Two-dimensional NOESY spectrum of Ac-[Nle^4,D-Orn^5,Glu^8]α-MSH$_{4-11}$-NH$_2$ illustrating CH$_\alpha^i$ → NH^{i+1} interresidue NOE's.
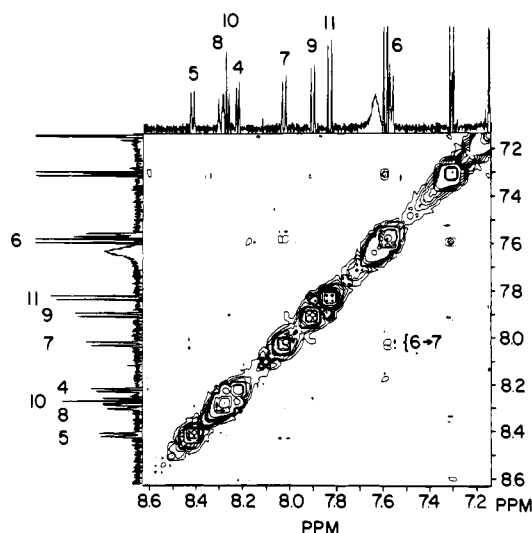
FIGURE 9: Two-dimensional NOESY spectrum of Ac-[Nle^4,D-Orn^5,Glu^8]α-MSH$_{4-11}$-NH$_2$ illustrating NH^i → NH^{i+1} interresidue NOE's.

The observed amide temperature dependencies were normal for all residues except for His^6 ($\Delta\delta/T$ = +1.2 ppb/°C) and L-Phe^7 ($\Delta\delta/T$ = -1.7 ppb/°C) of analogue 2 and His^6 ($\Delta\delta/T$ = -0.1 ppb/°C) in analogue 3. Assignments, coupling constants, and amide temperature dependencies are listed in Table I (alphatic and amide resonances) and Table II (aromatic resonances).

DISCUSSION

Design and Synthesis Strategies. The cyclic amide analogues reported here were designed to test a hypothesis recently proposed (Sugg et al., 1986) for a bioactive conformation of the melanotropins. The 5 to 8 cyclization was suggested by computer modeling studies which predicted a salt bridge between the Glu^5 and Arg^8 side chains (Nikiforovich et al., 1981, 1984). In order to promote cyclization and to stabilize the backbone to a left-handed turn, D stereochemistry is required at position 5. Finally, in order to examine the spatial relationship between the side chains of His^6 and Phe^7, both L-Phe^7 and D-Phe^7 isomers were prepared. Either D-Glu^5,Orn^8 or D-Orn^5,Glu^8 cyclic structures would fulfill the basic design
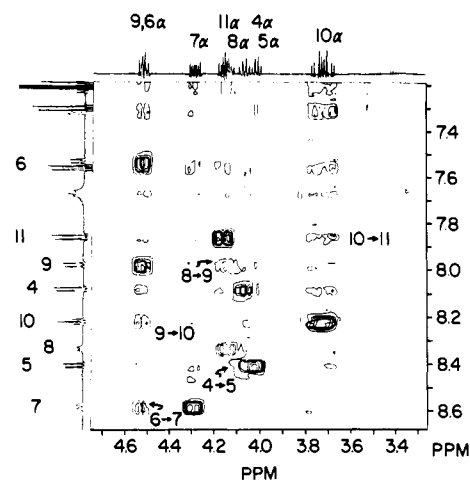
FIGURE 10: Two-dimensional NOESY spectrum of Ac-[Nle^4,D-Orn^5,D-Phe^7,Glu^8]α-MSH$_{4-11}$-NH$_2$ illustrating CH$_\alpha^i$ → NH^{i+1} interresidue NOE's.
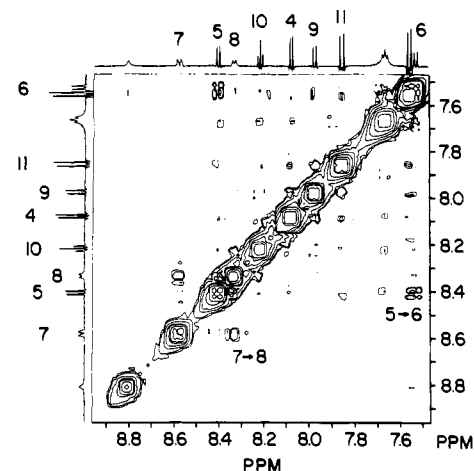
FIGURE 11: Two-dimensional NOESY spectrum of Ac-[Nle^4,D-Orn^5,Phe^7,Glu^8]α-MSH$_{4-11}$-NH$_2$ illustrating NH^i → NH^{i+1} interresidue NOE's.

requirements. Both of these analogues required differential protection for the Orn NH$^\delta$ and the Lys^{11} NH$^\epsilon$. The recently reported (Lebl & Hruby, 1984; Schiller et al., 1985) successes with resin-bound amide cyclizations encouraged us to use this synthetic approach. However, the N^α-Fmoc strategy introduced constraints on both design and synthesis. Since significant down-substitution was reported (Atherton et al., 1981) after SPPS incorporation of N^α-Fmoc-Lys(N^ϵ-Boc), we chose to prepare analogues with the D-Orn^5,Glu^8 substitution pattern. Further, we down-substituted the PMBHA resin to 0.4 mmol/g and limited cyclization time to 72 h in order to minimize dimerization (Schiller et al., 1985) and/or cleavage (Bodanszky et al., 1979) of the N^α-Fmoc group of D-Orn^5 by the free primary amine of the D-Orn^5 side chain. Despite these possible problems and side reactions, both the L-Phe^7 and D-Phe^7 cyclic analogues 2 and 3 were obtained in reasonable yields.

Melanotropic Bioactivity. Despite the elimination of the charged carboxylate and guanidino functionalities of Glu^5 and Arg^8, the L-Phe^7 analogue ^{2} retained the potency of the parent linear L-Phe^7 4-11 fragment in the frog skin bioassay. Interestingly, the cyclic L-Phe^7 analogue 2 was >100-fold more potent than the cyclic D-Phe^7 diastereoisomer 3, whereas in the case of linear analogues of α-MSH, D-Phe^7 analogues are always more potent. Furthermore, prolonged melanotropic

Table I: Resonance Assignments, Coupling Constants, and Amide Temperature Dependencies for the Aliphatic and Amide Protons of **2** and **3**

|			chemical shift (ppm)^a	
			[Nle^4,Orn^5,Glu^8]α-MSH$_{4-11}$-NH$_2$	[Nle^4,Orn^5,D-Phe^7,Glu^8]α-MSH$_{4-11}$-NH$_2$
Ac			1.79	1.77
Nle^4		NH	8.23 ($J = 5.8$; $\Delta\delta/T = -3.9$)	8.08 ($J = 6.3$; $\Delta\delta/T = -4.7$)
		CHα	4.07 ($J = 6.4$; 7.9)	4.07 ($J = 7.0$, 7.6)
		CHβ	(1.5)	(1.5)
		CHγ	(1.3)	(1.5)
		CHδ	(1.3)	(1.25)
		CHϵ	0.86 ($J = 7.1$)	0.84 ($J = 7.1$)
D-Orn^5		NH	8.42 ($J = 6.7$; $\Delta\delta/T = -5.4$)	8.40 ($J = 7.3$; $\Delta\delta/T = -4.7$)
		CHα	3.95 ($J = 5.0$, 7.6)	4.02 ($J = 6.1$, 6.3)
		CHβ	(1.6, 1.22)	(1.5, 1.6)
		CHγ	(1.27)	(1.42)
		CHδ	3.19/2.80 ($J = 13.8$)	3.17/2.82 ($J = 13.0$)
		NHδ	7.576 ($J = 5.9$, 7.2; $\Delta\delta/T = -2.7$)	7.66 ($J = 5.4$; $\Delta\delta/T = -3.8$)
His^6		NH	7.57 ($J = 7.0$; $\Delta\delta/T = +1.2$)	7.53 ($J = 6.9$; $\Delta\delta/T = -0.1$)
		CHα	4.42 ($J = 5.1$, 7.0)	4.52 ($J = 6.3$, 6.6)
		CHβ	3.07/2.85 ($J = 13.7$)	2.83/2.74 ($J = 15.3$)
Xxx^7		NH	8.03 ($J = 7.5$; $\Delta\delta/T = -1.7$)	8.58 ($J = 7.9$; $\Delta\delta/T = -4.2$)
		CHα	4.26 ($J = 7.9$, 5.0)	4.3 ($J = 4.0$, 11.0)
		CHβ	(2.95)	3.12/2.71 ($J = 14.1$)
Glu^8		NH	8.3 ($J = 8.6$; $\Delta\delta/T = -4.2$)	8.33 ($J = 6.9$; $\Delta\delta/T = -2.4$)
		CHα	4.33 ($J = 9.0$, 4.7)	4.14 ($J = 4.9$, 8.6)
		CHβ	(1.95, 1.59)	(1.85, 1.79)
		CHγ	(2.1)	2.04 ($J = 6.1$, 6.6, 15.1)
				2.17 ($J = 7.5$, 7.1, 14.6)
Trp^9		NH	7.91 ($J = 7.4$; $\Delta\delta/T = -4.6$)	7.97 ($J = 7.5$; $\Delta\delta/T = -3.9$)
		CHα	4.56 ($J = 5.0$, 8.1)	4.52 ($J = 8.0$, 6.3)
		CHβ	3.15/3.01 (14.6)	3.17/3.02 ($J = 14.5$)
Gly^{10}		NH	8.28 ($J = 5.6$; $\Delta\delta/T = -5.1$)	8.217 ($J = 5.5$; $\Delta\delta/T = -4.3$)
		CHα	3.76/3.69 ($J = 16.6$)	3.75/3.69 ($J = 16.7$)
Lys^{11}		NH	7.84 ($J = 8.1$; $\Delta\delta/T = -4.4$)	7.86 ($J = 8.2$; $\Delta\delta/T = -4.2$)
		CHα	4.17 ($J = 5.1$, 8.6)	4.17 ($J = 4.7$, 8.7)
		CHβ	(1.69, 1.5)	(1.50, 1.68)
		CHγ	1.27	(1.25)
		CHδ	1.27	(1.50)
		CHϵ	2.75	2.75 ($J = 5.6$, 13.6)
		NHϵ	7.64	7.67
CONH$_2$			7.31	7.32 ($\Delta\delta/T = -2.8$)
				7.04 ($\Delta\delta/T = -4.0$)

^{a}500 MHz; DMSO-d_6; $T = 303$ K; J in Hz; $\Delta\delta/T$ in ppb/°C.

Table II: Resonance Assignments for the Aromatic Proton Resonances of **2** and **3**

		chemical shift (ppm)^a	
		L-Phe^7	D-Phe^7
His	C2H	8.72	8.87
	C4H	6.53	6.63
Xxx^7		7.05	7.20
		7.04	7.15
Trp^9	C2H	7.15 ($J = 2.6$)	7.28 ($J = 2.5$)
	C4H	7.31 ($J = 8.1$)	7.3 ($J = 8.1$)
	C5H	7.05 ($J = 7.7$)	7.04 ($J = 7.3$)
	C6H	6.96 ($J = 7.6$)	6.95 ($J = 7.9$)
	C4H	7.59 ($J = 7.9$)	7.56 ($J = 7.9$)
	NH	10.79 ($J = 2.6$)	10.81 ($J = 2.6$)

^{a}500 MHz in DMSO-d_6; $T = 303$ K; J in Hz.

bioactivity was only observed with the L-Phe^7 cyclic analogue **2** (data not shown). Thus the conformational restraints imposed by the 5 to 8 cyclization with the L-Phe^7 isomer are fully compatible with the bioactive conformation required for the frog skin melanotropic receptor. While the potency of the L-Phe^7 cyclic analogue **2** was reduced in the lizard skin bioassay, the observed prolongation of response (data not shown) again suggest that the imposed conformation is compatible with the lizard skin melanotropic receptor, albeit to a much lesser degree than for the frog.

Solution Conformation in DMSO-d_6. The $J_{\text{NH-CH}\alpha}$ coupling constants are all within the range of 6–8 Hz (Table I), indicating that the backbone conformation of these analogues is not severely restricted by the cyclic structure. Only the
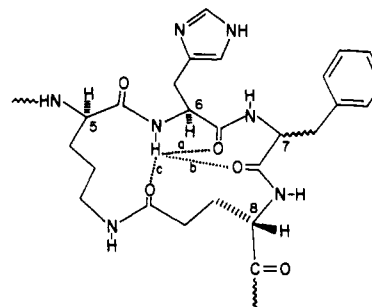
FIGURE 12: Illustration of the three possible hydrogen-bonding schemes for analogues **2** and **3**: a, C$_5$; b, C$_7$-like; c, C$_{10}$.

$J_{\text{NH-CH}\alpha}$ of D-Orn^5, Xxx^7, and L-Glu^8 vary significantly with the change in position 7 stereochemistry.

Assuming that the temperature independence observed for the His^6 NH (Table I) is due to hydrogen bonding, three hydrogen-bonding schemes can be envisioned involving the His^6 carbonyl (path a, Figure 12), Xxx^7 carbonyl (path b, Figure 12), or Glu^8 γ-carbonyl (path c, Figure 12). Paths a and b are C$_5$ and C$_7$ turns, respectively, while path c could be either a C$_{13}$ α-turn or a C$_{10}$ β-turn, depending on the direction in which the atoms are connected. The His^6 NH to L-Phe^7 NH NOE observed with **2** (Figure 9) eliminates the possibility of a His^6 NH to His^6 carbonyl hydrogen bond. However, the other two turn structures are possible and cannot be differentiated by the data obtained here. The D-Phe^7 NH to Glu^8 NH NOE observed in **3** (Figure 11) suggests that in this
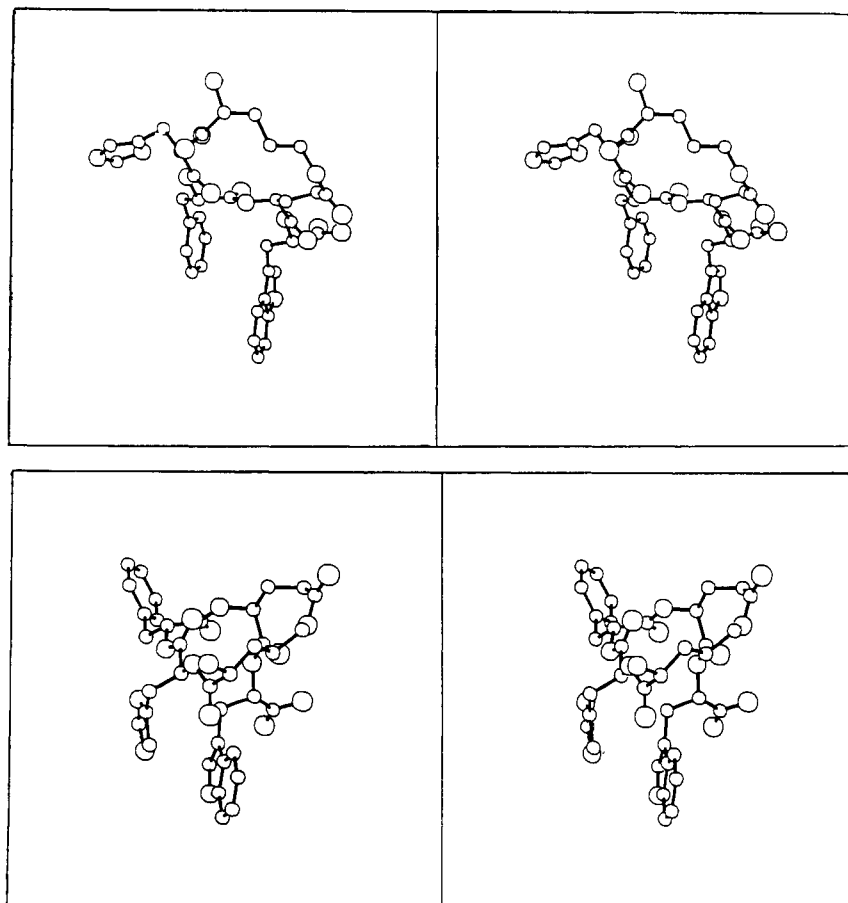
FIGURE 13: Stereo-ORTEP views of the 5–9 region of Ac-[Nle^4,D-Orn^5,Glu^8]α-MSH$_{4-11}$-NH$_2$ (top) and Ac-[Nle^4,D-Orn^5,D-Phe^7,Glu^8]α-MSH$_{4-11}$-NH$_2$ (bottom).

analogue both backbone amide bonds are oriented with the carbonyls pointed out of the ring, leaving only the Glu^8 γ-carbonyl available for H bonding. This suggests a β-type or α-type turn structure for this analogue (path c, Figure 12).

The side-chain rotamer populations calculated (DeLeeuw & Altona, 1982) from the $J_{CH\alpha-CH\beta}$ for amino acid residues 5–9 of these two cyclic analogues are listed in Table III. The Nle^4 and Lys^{11} rotamer populations are unaffected by the stereochemistry of position 7. An ambiguity in the assignment of the *pro-R* and *pro-S* β-protons prevents differentiation of the gauche(–) and trans rotamers. Nonetheless, some conformational variations in the side chains of residues 5–7 and 9 can be noted. The D-Orn^5 side chain is predominantly trans [or gauche(–)] in the L-Phe^7 diastereoisomer, while in the D-Phe^7 derivative almost equal populations of all three rotamers are observed (Table III). Both the His^6 and Trp^9 side chains lose some contribution of the gauche(+) rotamer in the D-Phe^7 diastereomer. The Xxx^7 residue is almost exclusively trans [or gauche(–)] in **3**. The Glu^8 side-chain rotamer populations are the same in both diastereomers.

Our original proposal for the bioactive conformation of the melanotropins suggested that important features included β-type structure with a left-handed turn of the backbone (His^6 CH$_\alpha$ to Trp^9 CH$_\alpha$) and a close (i.e., "gauchelike") relationship between the side chains of His^6 and Phe^7. From the NMR studies reported here, the backbone conformations of these two cyclic analogues seem to be quite similiar. However, these two analogues differ in the orientation of the L-His^6, L- or D-Phe^7, and L-Trp^9 side chains, as reflected in the shielding patterns observed for the L-His^6 and L- or D-Phe^7 residues.

In both cyclic analogues, the L-His^6 imidazole protons are significantly shielded (Table II). However, the L-His^6 α-proton

Table III: Side-Chain Rotamer Populations^a for Amino Acid Residues 5–9 of **2** and **3**

	Xxx^7 =			Xxx^7 =	
	L-Phe (**2**)	D-Phe (**3**)		L-Phe (**2**)	D-Phe (**3**)
D-Orn^5			Glu^8		
gauche(+)	0.22	0.25	gauche(+)	0.09	0.11
gauche(–)	0.25	0.37	gauche(–)	0.22	0.25
trans	0.53	0.38	trans	0.69	0.64
His^6			Trp^9		
gauche(+)	0.28	0.19	gauche(+)	0.16	0.01
gauche(–)	0.26	0.39	gauche(–)	0.25	0.41
trans	0.46	0.42	trans	0.59	0.58
Xxx^7					
gauche(+)	0.19	0.0			
gauche(–)	0.25	0.16			
trans	0.56	0.84			

^{a}DeLeeuw & Altona, 1982.

is deshielded (+0.10 ppm) and the β-protons are shielded (–0.10 to –0.20 ppm) in the D-Phe^7 cyclic analogue **3**, relative to the L-Phe^7 cyclic analogue **2**. The chemical shift difference for the L-His^6 β-proton resonances is largest in the L-Phe^7 cyclic analogue **2** ($\Delta\delta$ = 0.24 ppm). The L-Phe^7 amide proton is significantly shielded (+0.55 ppm) relative to the D-Phe^7 amide proton. The chemical shift difference for the β-proton resonances of D-Phe^7 is quite large ($\Delta\delta$ = 0.41 ppm) but is essentially zero for the β-proton resonances of L-Phe^7.

These variations in shielding patterns can be simply correlated with the side-chain topography of His^6, L- or D-Phe^7, and Trp^9. Stereo-ORTEP views of the 5–9 region of analogues **2** and **3** are illustrated in the top and bottom panels of Figure 13, respectively. In analogue **2**, the His^6 imidazole, L-Phe^7

phenyl, and Trp[9] indole moieties are closely oriented in space along the backbone of the molecule. The shielding observed for the imidazole protons can be ascribed to the L-Phe[7] phenyl ring, and the shielding observed for the phenyl protons (Table II) can be ascribed to the Trp[9] indole. In analogue **3**, the D-Phe[7] phenyl moiety is oriented away from the His[6] imidazole and Trp[9] indole moieties. With this analogue, the shielding of the imidazole protons can be ascribed to the Trp[9] indole, and the absence of shielding for the D-Phe[7] phenyl protons can be ascribed to the absence of nearby aromatic residues.

In conclusion, two novel side-chain cyclic analogues of the 4–11 fragment of α-MSH have been designed, prepared, and evaluated. Of these two cyclic derivatives, the L-Phe[7] analogue was markedly more potent than the D-Phe[7] analogue. It represents a new, unique melanotropic pharmacophore. While the two analogues appear to retain reasonable flexibility and to have similar solution backbone conformations as assessed by proton NMR, the side-chain topographies of the aromatic residues are quite different. Melanotropic activity is only observed with the analogue in which the imidazole, phenyl, and indole moieties are in close proximity in space. The higher melanotropic potency of the L-Phe analogue was correlated by NMR analysis, to a time-averaged conformation in which the imidazole and phenyl side-chain moieties are "gauche-(+)like" relative to each other.

Registry No. **2**, 116375-28-1; **3**, 116375-29-2; Ac-[Nle[4]]α-MSH$_{4-11}$-NH$_2$, 89362-12-9; Ac-[Nle[4],D-Phe[7]]α-MSH$_{4-11}$-NH$_2$, 89289-99-6; N^{α}-Boc-L-Lys(N^{ϵ}-DCZ) anhydride, 83038-58-8; N^{α}-Boc-Gly anhydride, 51499-90-2; N^{α}-Boc-L-Trp anhydride, 70889-81-5; N^{α}-Fmoc-L-Glu(γ-O-t-Bu), 71989-18-9; N^{α}-Fmoc-L-Phe, 35661-40-6; N^{α},N^{im}-(Fmoc)$_2$-L-His, 98929-98-7; N^{α}-Fmoc-D-Orn(N^{δ}-Boc), 109425-55-0; N^{α}-Boc-L-Nle anhydride, 68623-44-9; N^{α}-Fmoc-D-Phe, 86123-10-6; DPPA, 26386-88-9.

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