CONFORMATIONAL AND BIOLOGICAL ANALYSIS OF α-MSH FRAGMENT ANALOGUES WITH STERICALLY CONSTRAINED AMINO ACID RESIDUES

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Received February 15th, 1988
Accepted May 12th, 1988

Dedicated to the memory of Dr Karel Bláha.

Conformational and biological analysis of the linear 4-11 fragment analogues, Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ (*II*) and Ac-[Nle^4, D-Phe^7]-α-MSH$_{4-11}$-NH$_2$ (*III*) and related analogues have been undertaken. In solution, the peptide backbone is flexible, but in the case of D-Phe^7 analogues an interaction of the His^6, D-Phe^7 and Arg^8 amino acid side chain groups may be present based on the shielding patterns observed in the proton NMR and on comparison of NT_1 values. The importance of the position 7 to the biological and conformational properties was further examined by substitution of either L- or D-phenylglycine (Pgl) or L- and D-1,2,3,4-tetrahydroisoquinoline carboxylic acid (Tic) for phenylalanine-7. Ac-[Nle^4, Pgl^7]-α-MSH$_{4-11}$-NH$_2$ (*IV*), Ac-[Nle^4,D-Pgl^7]-α-MSH$_{4-11}$-NH$_2$ (*V*), Ac-[Nle^4, Tic^7]-α-MSH$_{4-11}$-NH$_2$ (*VI*), and Ac-[Nle^4, D-Tic^7]-α-MSH$_{4-11}$-NH$_2$ (*VII*) were prepared. These substituted analogues were examined for their biological activities and conformational properties with emphasis on the three-dimensional orientation of the aromatic ring in the position 7, and the effects of the aromatic ring on adjacent amino acids and on biological activities. The relative potencies of the analogues in the frog skin assay system were: *II* (1.00); *III* (118); *IV* (82.4); *V* (0.18); *VI* (0.18); and *VII* (0.14); and in the lizard skin bioassay they were: *II* (1.00); *III* (10.0); *IV* (0.14); *V* (0.005); *VI* (0.00025); and *VII* (0.01). On the basis of the NMR studies the L-phenylglycine substitution results in an enhanced ring stacking interaction between the phenyl ring of Pgl^7 and the indole ring of Trp^9. The 1,2,3,4-tetrahydroisoquinoline carboxylic acid (Tic) substitution leads to significant backbone restriction and an interaction of the alpha proton of His^6 with the carbonyl of Glu^5. The possible relationships of these effects to biological activity are discussed.

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α-Melanocyte stimulating hormone (α-MSH, α-melanotropin)*, ACTH, and a number of other peptide hormones are derived from pro-opiomelanocortin. α-MSH is a linear tridecapeptide (Ac-Ser[1]-Tyr[2]-Ser[3]-Met[4]-Glu[5]-His[6]-Phe[7]--Arg[8]-Trp[9]-Gly[10]-Lys[11]-Pro[12]-Val[13]-NH_2) which comprises the first thirteen amino acid residues of ACTH. However, unlike ACTH, α-MSH is acetylated at the N-terminus and contains a carboxamide at the C-terminus. α-Melanotropin is primarily known for its ability to stimulate integumental melanocytes[1,2] and more recently the hormone has been implicated in a variety of other physiological[3,4] and neurological processes. Classical structure-function studies have been directed at the elucidation of the active site sequence of α-MSH and determination of those residues that are particularly important for the observed biological activities of the hormone. Interpretation of such studies requires a priori assumptions regarding the effect of the substitution on the structure and/or conformation on the resulting analogue. In order to examine the effect of structural changes on melanotropic activity, conformational analysis of α-MSH and related fragment analogues is necessary.

Previously, we have reported on the importance of the stereochemistry of phenylalanine in the position 7 of α-MSH (refs[5-9]). Substitution of Phe[7] with D-Phe[7] into the tridecapeptide and several shorter fragment analogues led to peptides with much greater potency. In many cases these peptides displayed exceptional prolongation of the biological response[6-12] and resistance to enzymatic degradation by both serum and purified proteolytic enzymes[13,14]. It was proposed[10,11,15] that this substitution might stabilize some type of a reverse turn[16] conformation. To date, the validity of this model has been investigated primarily by conformational considerations, biological methods and model building[2,6,10] and to a less extent by biophysical or physico-chemical methods[2,9,10,17].

Presently, one of the most powerful techniques for conformational analysis of biologically active organic compounds from simple amino acids to small proteins and peptides is nuclear magnetic resonance (NMR) spectroscopy. This type of analysis has been extensively applied to the examination of the preferred solution phase conformation of peptide analogues based upon: (i) observation of individual nuclei (^1H, ^{13}C, and ^{15}N) and their spectroscopic properties; (ii) the analysis of specific spectral parameters which can be directly related to conformational properties; and (iii) advances in making assignments and

* Nomenclature and abbreviations follow published recommendations (Eur. J. Biochem. *138*, 9 (1984)). Other abbreviations: α-MSH, α-melanocyte stimulating hormone, α-melanotropin; Bzl, benzyl; Chx, cyclohexyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; HF hydrofluoric acid; For, formyl; HOBt, 1-hydroxybenzotriazole; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline carboxylic acid; Pgl, phenylglycine; 2,4-Cl_2-Z, 2,4-dichlorobenzyloxycarbonyl.

identifying pertinent spectroscopic data, especially due to the development of two-dimensional NMR techniques[18]. In this study, we have applied this methodology to the analysis of α-MSH analogues substituted with constrained amino acid residues in the critical 7 position.

We have performed detailed studies of Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ (*II*) and Ac-[Nle^4, D-Phe^7]-α-MSH$_{4-11}$-NH$_2$ (*III*) (hereafter referred to as the model peptides) and a variety of analogues by both proton and carbon-13 NMR in order to determine the possible relationships of the three-dimensional solution conformations of these α-MSH fragments to their observed biological activity. The conformational analysis by NMR has included a study of *1*) proton chemical shifts; *2*) geminal and vicinal coupling constants (corresponding rotamer populations); *3*) temperature dependence of the amide protons; *4*) carbon-13 chemical shifts; and *5*) carbon spin lattice relaxation times (T_1). The conformational analysis has been coupled to a critical examination of biological activities in both the frog (*Rana pipiens*) and the lizard (*Anolis carolinensis*) skin bioassays.

Based on the known importance of position 7 to the biological activity of α-MSH (refs[2,5,7,8,10,11]), a large variety of natural amino acids have been substituted in this position. In order to understand the relationship between the orientation of the phenyl ring in the position 7 to the biological and conformational properties of the peptide, we have selected amino acid residues for this position that can reduce the conformational freedom of the aromatic ring of Phe^7. For this purpose, phenylglycine (Pgl)[1] and 1,2,3,4-tetrahydroisoquinoline carboxylic acid (Tic) were chosen as suitable conformationally restricted replacement residues. These residues were substituted in the position 7 of Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$, an analogue equipotent to α-MSH in the lizard skin bioassay (Table I). The biological activities in two bioassays will be reported and the conformational implications of these substitutions will be examined with respect to the aromatic ring in the position 7 and the topography of the surrounding residues.

RESULTS

We have previously reported on the potencies of a series of Nle^4-substituted linear melanotropins[7,11,19]. Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ was equipotent to the parent tridecapeptide on the lizard skin bioassay (Fig. 1), but almost 1 000 times less potent than α-MSH on the frog skin bioassay (Fig. 2). Incorporation of D-Phe^7 into the sequence resulted in an analogue that was significantly more potent than its L-Phe^7 counterpart in both bioassays (Table I). The D-Phe^7 analogue was actually more potent than α-MSH on the lizard bioassay system (Fig 1). Previous studies[5,7–9] have provided evidence that stereochemistry of the

amino acid in the position 7 can have significant effects on the prolongation of melanotropic activity. In particular, the substitution of D-Phe^7 in α-MSH and various fragments (including Ac-[Nle^4,D-Phe^7]-α-MSH$_{4-11}$-NH$_2$) led to analogues with exceptionally prolonged biological responses in several in vitro and in vivo bioassays.

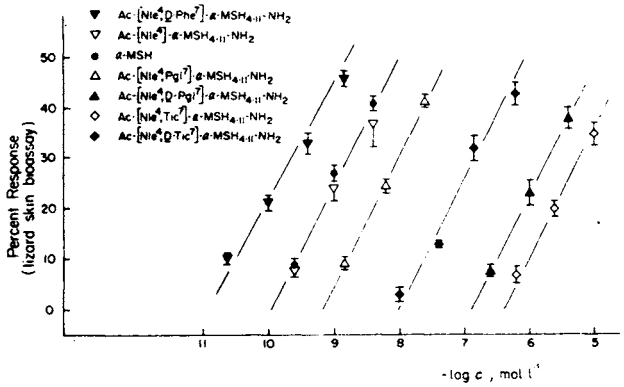

FIG. 1

Comparative in vitro dose-response curves for the linear position 7 substituted melanotropins as determined on the lizard skin (*Anolis carolinensis*) bioassay. Each value represents the mean, ± SE, response (darkening) of the skins (n = 6 or more in all experiments) to the melanotropins at the indicated concentrations

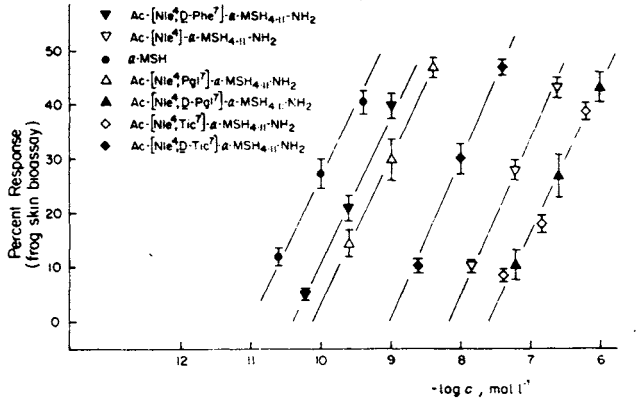

FIG. 2

Same as in Fig. 1, except these are the results of the frog (*Rana pipiens*) skin bioassay

The Pgl^7 and Tic^7 analogues also showed dramatic differences in potency depending upon stereochemistry of the amino acid in the position 7. The phenylglycine substituted analogue, Ac-[Nle^4, Pgl^7]-α-MSH_{4-11}-NH_2 (*IV*), was only slightly less potent than α-MSH in both bioassays (Table I, Figs 1 and 2), and much more active than Ac-[Nle^4]-α-MSH_{4-11}-NH_2 in the frog skin assay. The D-Pgl^7 substitution led to analogue that was less potent than α-MSH and Ac-[Nle^4, Pgl^7]-α-MSH_{4-11}-NH_2 in both bioassay systems (Table I, Figs 1 and 2).

Substitution of either enantiomer of 1,2,3,4-tetrahydroisoquinoline carboxylic acid (L-Tic and D-Tic) in the $[Nle^4]_{4-11}$ sequence led to a dramatic loss of biological potency relative to α-MSH (Table I, Figs 1 and 2). This was especially dramatic in the case of L-Tic^7, which was approximately 3 500 times less active than α-MSH in both bioassay systems. The D-Tic^7 analogue was still about 100 fold less potent than α-MSH in both bioassays, but the compound was about 400 times more potent than the L-Tic^7 diastereoisomeric analogue.

The Pgl^7 and Tic^7 substituted analogues also showed vast differences in the prolongation of their biological responses. For example, the D-phenylglycine-containing analogue exhibited this property, but primarily on the frog skin and much less so on the lizard skin bioassay (Figs 3 and 4). Neither of the L-Tic or D-Tic-containing peptides were prolonged in either bioassay (data not shown).

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

Compound	Peptide	Potency relative to α-MSH[a]		Potency relative to 4–11 model[b]	
		frog skin	lizard skin	frog skin	lizard skin
I	α-MSH	1.00	1.00	–	–
II	Ac-[Nle^4]-α-MSH_{4-11}-NH_2	0.0017	1.00	1.00	1.00
III	Ac-[Nle^4, D-Phe^7]-α-MSH_{4-11}-NH_2	0.2	10.0	1.00	1.00
IV	Ac-[Nle^4, Pgl^7]-α-MSH_{4-11}-NH_2	0.14	0.14	82.4	0.14
V	Ac-[Nle^4, D-Pgl^7]-α-MSH_{4-11}-NH_2	0.0003	0.0005	0.0015	0.00005
VI	Ac-[Nle^4, Tic^7]-α-MSH_{4-11}-NH_2	0.0003	0.00025	0.18	0.00025
VII	Ac-[Nle^4, D-Tic^7]-α-MSH_{4-11}-NH_2	0.012	0.01	0.016	0.001

[a] Values are derived from the parallel dose-response curves in Figs 1 and 2; [b] these values are obtained by comparison of position 7 substituted fragments to either Ac-[Nle^4]-α-MSH_{4-11}-NH_2 or Ac-[Nle^4, D-Phe^7]-α-MSH_{4-11}-NH_2 depending on the stereochemistry of the amino acid substituted in position 7 (i.e. on the frog skin: Potency of *IV*/Potency of *II* = 82.4 and Potency of *V*/Potency of *III* = 0.0015).

For both the model peptides the proton chemical shifts were near those expected, except for the gamma protons of arginine and the C4 protons of histidine (Tables II and III). Individual proton chemical shifts were assigned with the aid of double resonance, pH titration, literature values, analysis of shorter fragments of α-MSH, and two-dimensional COSY techniques (not

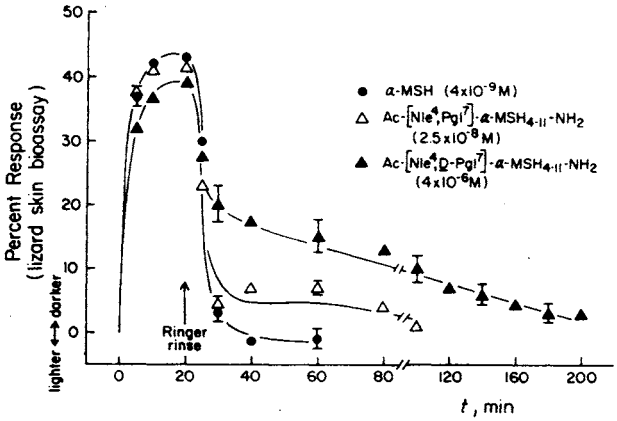

FIG. 3

In vitro demonstration of the prolonged bioactivity (skin darkening) of Ac-[Nle^4, D-Pgl^7]-α-MSH$_{4-11}$-NH$_2$ (▲) versus Ac-[Nle^4, Pgl^7]-α-MSH$_{4-11}$NH$_2$ (△) and α-MSH (●) on the lizard skin bioassay. Each value represents the mean ± SE response of the skins (n = 6) to the melanotropins at the indicated concentrations

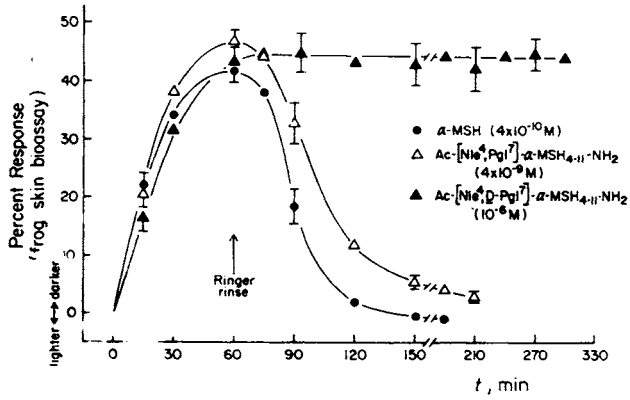

FIG. 4

Same as in Fig. 3, except these are the results of the frog (*Rana pipiens*) skin bioassay

TABLE II

The ^{1}H chemical shifts and assignments for 4–11 position 7 substituted α-MSH 4–11 analogues in D_2O (pD = 3.5)

Residue	Signal	Analogue					
		II	*III*	*IV*	*V*	*VI*	*VII*
Ac		2.04	2.02	2.08	2.05	2.07	2.08
Nle[4]	$C_\gamma H$	4.15	4.13	3.97	4.11	4.13	4.18
	$C_\beta H$	1.65	1.62	1.60	1.64	1.58	1.58
	$C_\gamma H$	1.29	1.26	1.26	1.26	1.27	1.27
	$C_\delta H$	1.26	1.24	1.23	1.23	1.24	1.23
	$C_\varepsilon H$	0.85	0.83	0.84	0.84	0.86	0.85
Glu[5]	$C_\gamma H$	4.24	4.22	4.23	4.27	4.23	4.32
	$C_\beta H$	1.88	1.87	1.90	1.90	1.87	1.91
	$C_\gamma H_A$	2.38	2.29	2.33	2.32	2.30	2.36
	$C_\gamma H_B$	2.32	2.23	2.26	2.26	2.24	2.29
His[6]	$C_\gamma H$	4.59	4.66	4.75	4.72	5.26	5.27
	$C_\beta H_A$	3.08	3.13	3.28	3.16	3.02	3.22
	$C_\beta H_B$		3.01	3.14			
XXX[7]	$C_\gamma H$	4.54	4.52	5.40	5.34	4.72	4.87
	$C_\beta H_A$	2.91	3.01	–	–	3.09	3.06
	$C_\beta H_B$	2.87	2.96	–	–	2.94	2.98
						(4.59)[a]	(4.65)[a]
Arg[9]	$C_\gamma H$	4.28	4.13	4.27	4.23	4.16	4.20
	$C_\beta H$	1.80	1.40	1.81	1.50	1.65	1.68
	$C_\gamma H$	1.40	1.01	1.60	1.09	1.08	1.23
	$C_\delta H$	2.99	2.93	2.99	2.86	2.99	3.01
Trp[9]	$C_\gamma H$	4.68	4.66	4.60	4.70	4.69	4.58
	$C_\beta H_A$	3.35	3.34	3.26	3.34	3.30	3.30
	$C_\beta H_B$	3.22	3.22	3.04	3.20	3.22	3.19
Gly[10]	$C_\gamma H_A$	3.94	3.91	3.91	3.88	3.83	3.88
	$C_\beta H_B$	3.85	3.82	3.81	3.80	3.83	3.81
Lys[11]	$C_\gamma H$	4.23	4.26	4.25	4.25	4.27	4.26
	$C_\beta H$	1.67	1.67	1.68	1.68	1.70	1.67
	$C_\gamma H$	1.40	1.40	1.41	1.40	1.41	1.40
	$C_\delta H$	1.67	1.67	1.70	1.65	1.65	1.68
	$C_\varepsilon H$	3.07	2.98	3.03	2.96	2.96	2.98

[a] Corresponds to chemical shift of protons on methylene carbon between alpha amine and *ortho* position on the phenyl ring: XXX[7] is Phe in *II*, D-Phe in *III*, Pgl in *IV*, D-Pgl in *V*, Tic in *VI* and D-Tic in *VII*.

shown) and reference to the literature[18,20,21,45]. In the D-Phe[7] containing analogue the arginine gamma protons are shifted upfield approximately 0.40 ppm from their expected chemical shifts values, and a similar large upfield shift of 0.40 ppm is seen for the β CH protons of Arg[8]. The C4 protons of histidine are shifted upfield approximately 0.10 ppm in the D-Phe[7] analogue.

The proton coupling constants in the model peptides have been studied in order to examine the rotamer populations[22] (from αCH–βCH coupling constants) and the phi (φ) dihedral angle (from the ^3J(NH–αCH) coupling constants). In general, the α–β vicinal coupling constants are in the normal range (5.0–8.0 Hz) (Table IV) which results in rotamer populations that favor primarily the *gauche*(−) and the *trans* rotamers. Likewise, the ^3J(NH–αCH) coupling constants were in the normal range (6.1–8.1 Hz) (Table V) which indicates the peptide backbone may be flexible, or fixed in a conformation with these coupling constants.

The carbon-13 chemical shifts for the model peptides were generally normal (Table VI). Assignment of individual resonances was made with the aid of heteronuclear decoupling, off-resonance decoupling, and comparison to the literature[9,23–24]. An examination of the spin lattice relaxation times (T_1) showed that the beta carbons of the 5–9 sequence had relatively short spin lattice relaxation times (~ 60–90 ms) (Table VI).

TABLE III
^{1}H Chemical shifts and assignments for aromatic protons in position 7 substituted α-MSH 4–11 analogues in D_2O (pD 3.5)

Residue	Carbon	Analogue					
		II	*III*	*IV*	*V*	*VI*	*VII*
His[6]	C2	8.57	8.54	8.53	8.44	8.48	8.51
	C4	7.15	7.08	7.22	7.10	7.20	7.24
Trp[9]	C2	7.24	7.23	7.10	7.21	7.15	7.20
	C4	7.62	7.60	7.48	7.61	7.55	7.59
	C5	7.12	7.11	7.12	7.11	7.07	7.13
	C6	7.21	7.17	7.21	7.17	7.10	7.20
	C7	7.41	7.44	7.43	7.45	7.37	7.44
XXX[7]	[a]	7.16	7.22	7.37	7.28	7.16	7.18
		7.24	7.32	7.44	7.41	7.25	7.28

[a] Corresponds to the center of respective multiplet; peptide *II*, XXX[7] = Phe; peptide *III*, XXX[7] = D-Phe; peptide *IV*, XXX[7] = Pgl; peptide *V*, XXX[7] = D-Pgl; peptide *VI*, XXX[7] = Tic; peptide *VII*, XXX[7] = D-Tic.

TABLE IV
Coupling constants and corresponding rotamer populations for α-MSH fragments in D_2O (pD 3.5)

Residue	Parameter	Analogue					
		II	*III*	*IV*	*V*	*VI*	*VII*
Nle[4]	αβ	6.2	6.2	6.1	6.2	6.4	6.6
	αβ′	7.2	8.1	8.0	7.9	8.0	7.8
	(+60)	0.05	0.01	0.04	0.04	0.05	0.00
	(180)	0.39	0.39	0.39	0.39	0.41	0.43
	(−60)	0.56	0.60	0.57	0.57	0.59	0.57
Glu[5]	αβ	5.2	5.4	6.0	5.8	5.6	5.3
	αβ′	8.8	9.3	8.9	8.5	9.5	9.0
	(+60)	0.05	0.00	0.00	0.01	0.00	0.01
	(180)	0.28	0.31	0.37	0.35	0.33	0.29
	(−60)	0.67	0.69	0.63	0.64	0.67	0.70
His[6]	αβ	5.5	5.8	5.5	6.6	6.2	7.2
	αβ′	8.7	8.3	8.9	7.9	7.3	7.2
	ββ′	−10.8	−9.4	−12.3	−9.5	−9.4	–
	(+60)	0.02	0.04	0.00	0.00	0.11	0.00
	(180)	0.67	0.62	0.68	0.57	0.57	0.50
	(−60)	0.31	0.34	0.32	0.43	0.32	0.50
XXX[7a]	αβ	7.2	8.0	–	–	5.3	5.2
	αβ′	7.2	8.0	–	–	6.3	6.4
	ββ′	–	–	–	–	−11.0	−12.5
	(+60)	0.00	0.00	–	–	–	–
	(180)	0.50	0.60	–	–	–	–
	(−60)	0.50	0.40	–	–	–	–
Arg[8]	αβ	6.1	5.6	4.9	5.4	6.3	5.4
	αβ′	8.5	8.8	8.6	9.2	9.0	9.0
	(+60)	0.00	0.00	0.11	0.00	0.01	0.00
	(180)	0.38	0.33	0.25	0.31	0.41	0.30
	(−60)	0.62	0.67	0.64	0.69	0.58	0.70
Trp[9]	αβ	6.0	6.4	6.1	6.3	6.5	6.4
	αβ′	8.1	8.5	8.6	8.3	7.7	8.1
	ββ′	−10.8	−10.6	−12.5	−10.5	−10.8	−11.9
	(+60)	0.04	0.00	0.00	0.00	0.02	0.00
	(180)	0.37	0.42	0.38	0.40	0.42	0.41
	(−60)	0.59	0.58	0.62	0.60	0.56	0.59
Gly[10]	αα′	−16.7	−16.9	−16.9	−16.8	−16.9	−16.8

TABLE IV
(*Continued*)

Residue	Parameter	Analogue					
		II	*III*	*IV*	*V*	*VI*	*VII*
Lys[11]	αβ	5.6	5.2	5.6	6.0	4.9	5.0
	αβ′	7.5	9.1	7.5	9.2	9.3	9.3
	(+60)	0.16	0.01	0.16	0.00	0.02	0.01
	(180)	0.32	0.28	0.32	0.37	0.24	0.26
	(−60)	0.52	0.71	0.52	0.63	0.74	0.73

[a] Rotamer populations were calculated by the method of Leeuw and Altona[22]. XXX[7] is as in Table II.

In general, the proton NMR parameters of the L- or D-Pgl[7] and L- or D-Tic[7] analogues gave similar chemical shifts as the same residues in the model peptides. However, when either D-Pgl or D-Tic were substituted in the 7 position, a shielding of the gamma protons of arginine was observed (Table II). The C4 protons of histidine are also shielded as a result of the D-Pgl[7] substitution. Unlike in the model peptides, an upfield shift of the C2 and C4 protons of tryptophan was observed when L-phenylglycine was substituted in the position 7. The Tic[7] substitution (regardless of stereochemistry), resulted in a deshielding of the alpha proton of histidine (Table II) and of the C4 proton of histidine (Table III). An analysis of the other proton NMR parameters (amide protons and alpha–beta coupling constants) did not show any unusual trends for either the amino acid substituted in the position 7 or for most of the adjacent residues. However, the vicinal coupling constants obtained for the Tic[7]-containing analogues indicate that this residue exists mainly in a pseudo chair form (*gauche*(+) rotamer – Fig. 5).

DISCUSSION

It was previously reported[2,5,9,11] that the substitution of D-phenylalanine in position 7 of α-MSH can enhance the biological potency of α-MSH and its analogues 10 – 100 fold, and we have found similar results with the α-MSH$_{4-11}$ analogues reported here (Table I, Figs 1 and 2). Although the L-Tic[7] analogue has very low potency in both bioassays (Table I), a large increase in potency relative to the L-Tic[7] occurs when D-Tic is placed in position 7 (*VII*), (Table I).

On the other hand, since the L-phenylglycine-7 analogue *IV* is potent in both bioassay systems, it was very interesting to note that when D-phenylglycine (D-Pgl) was placed in position 7, a very large decrease in potency was seen in both bioassay systems. Since both Tic and Pgl would be expected to put

TABLE V
Coupling constants ^3J(NH αCH), chemical shifts and temperature coefficients ($\times 10^3$ $\Delta\delta/\Delta T$) for NH protons of γ-MSH$_{4-11}$ fragments in 10% D_2O 90% H_2O (pH 3.5)

Residue	Parameter	Analogue					
		II	*III*	*IV*	*V*	*VI*	*VII*
Nle[4]	δNH	8.22	8.17	8.16	8.18	8.15	7.96
	J(NH-αCH)	6.4	7.2	5.6	6.7	6.5	7.1
	$\Delta\delta/\Delta T$	6.8	6.2	6.4	5.4	5.4	8.0
Glu[5]	δNH	8.13	8.26	8.37	8.34	8.27	8.44
	J(NH-αCH)	7.1	7.0	6.3	6.9	7.2	6.6
	$\Delta\delta/\Delta T$	7.0	7.6	5.7	5.0	7.0	6.0
His[6]	δNH	8.38	8.36	8.45	8.53	8.55	8.63
	J(NH-αCH)	6.8	7.7	7.0	6.6	8.4	6.9
	$\Delta\delta/\Delta T$	6.0	5.0	4.6	4.6	7.2	5.3
XXX[7]	δNH	8.19	8.39	8.47	8.69	–	–
	J(NH-αCH)	8.1	6.1	5.6	6.3	–	–
	$\Delta\delta/\Delta T$	7.0	7.6	4.6	5.3	–	–
Arg[8]	δNH	8.38	8.17	8.47	8.27	8.12	8.24
	J(NH-αCH)	6.8	7.2	5.6	7.6	6.1	6.5
	$\Delta\delta/\Delta T$	6.0	6.2	6.7	4.5	6.2	7.4
Trp[9]	δNH	8.00	8.11	7.70	8.16	7.84	7.89
	J(NH-αCH)	6.7	8.1	6.3	6.7	6.9	7.0
	$\Delta\delta/\Delta T$	6.8	6.0	6.3	4.8	5.0	6.6
Gly[10]	δNH	8.23	8.19	8.17	8.26	8.18	8.19
	J(NH-αCH)	6.8	7.6	6.6	6.2	5.7	6.5
	$\Delta\delta/\Delta T$	5.4	5.6	6.1	4.2	7.0	5.0
Lys[11]	δNH	8.16	8.08	8.10	8.11	8.09	8.15
	J(NH-αCH)	7.2	7.5	7.4	7.2	6.5	7.3
	$\Delta\delta/\Delta T$	6.6	6.0	5.4	4.8	6.6	7.6

[a] XXX[7] is as in Table II.

TABLE VI

^{13}C Chemical shifts and T_1 values (ms) for Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ and Ac-[Nle^4, D-Phe^7]-α-MSH$_{4-11}$-NH$_2$ in D_2O (pD 3.5)

Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ (*III*)					Ac-[Nle^4, D-Phe^7]-α-MSH$_{4-11}$-NH$_2$ (*III*)				
residue	carbon	shift	T_1	NT_1	residue	carbon	shift	T_1	NT_1
Nle^4	C_α	54.0	188	188	Nle^4	C_α	54.0	180	180
	C_β	27.9	202	404		C_β	27.1	202	404
	C_γ	31.2	173	346		C_γ	31.5	180	360
	C_δ	22.4	506	1 012		C_δ	22.6	405	810
	C_ε	13.9	722	2 166		C_ε	14.2	971	2 913
Glu^5	C_α	53.9	188	188	Glu^5	C_α	54.1	180	180
	C_β	26.8	65	130		C_β	27.2	58	116
	C_γ	31.3	159	318		C_γ	32.2	144	288
His^6	C_α	53.2	159	159	His^6	C_α	53.2	144	144
	C_β	28.8	79	158		C_β	28.4	61	122
	C_γ	129.2	–	–		C_γ	129.1	–	–
	$C_{\delta 2}$	117.9	332	332		$C_{\delta 2}$	118.0	221	221
	$C_{\varepsilon 1}$	134.3	289	289		$C_{\varepsilon 1}$	133.4	188	188
Phe^7	C_α	55.2	181	181	Phe^7	C_α	55.4	152	152
	C_β	37.6	87	174		C_β	37.8	65	130
	C_γ	136.8	–	–		C_γ	136.9	–	–
	C_δ	129.9	260	260		C_δ	129.7	176	176
	C_ε	129.5	260	260		C_ε	130.0	188	188
	C_ζ	128.0	173	173		C_ζ	128.2	196	196
Arg^8	C_α	54.1	188	188	Arg^8	C_α	54.2	159	159
	C_β	26.8	65	130		C_β	27.2	58	116
	C_γ	24.8	108	216		C_γ	24.8	101	202
	C_δ	41.3	137	274		C_δ	41.3	170	340
	C_ζ	157.5	–	–		C_ζ	157.5	–	–
Trp^9	C_α	55.8	159	159	Trp^9	C_α	56.5	141	141
	C_β	27.7	58	116		C_β	27.8	58	116
	C_γ	109.7	–	–		C_γ	110.0	–	–
	$C_{\delta 1}$	127.7	–	–		$C_{\delta 1}$	127.8	–	–
	$C_{\delta 2}$	125.2	260	260		$C_{\delta 2}$	125.3	196	196
	$C_{\varepsilon 2}$	137.0	–	–		$C_{\varepsilon 2}$	137.0	–	–
	$C_{\varepsilon 3}$	119.0	188	188		$C_{\varepsilon 3}$	119.2	191	191
	$C_{\zeta 2}$	112.7	188	188		$C_{\zeta 2}$	112.7	208	208
	$C_{\zeta 3}$	120.1	238	238		$C_{\zeta 3}$	120.2	221	221
	$C_{\eta 2}$	122.8	216	216		$C_{\eta 2}$	122.9	208	208
Gly^{10}	C_α	43.3	108	216	Gly^{10}	C_α	43.4	100	200

TABLE VI
(*Continued*)

Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ (*II*)					Ac-[Nle^4, D-Phe^7]-α-MSH$_{4-11}$-NH$_2$ (*III*)				
residue	carbon	shift	T_1	NT_1	residue	carbon	shift	T_1	NT_1
Lys^{11}	C_α	55.2	181	181	Lys^{11}	C_α	55.1	173	173
	C_β	31.2	159	318		C_β	31.3	185	370
	C_γ	22.8	260	520		C_γ	23.0	303	606
	C_δ	27.0	405	810		C_δ	28.2	321	642
	C_ε	40.1	592	1 184		C_ε	40.1	584	1 168

important conformational constraints on the position 7, we decided to investigate the conformational consequences of these substitutions using NMR spectroscopy in order to get some insight into the conformational effects which might help explain the biological activities. For this purpose, it is necessary to completely and correctly assign the proton NMR spectra (Tables II and V). This was accomplished by a variety of techniques (see Experimental), but the most powerful for this study were double resonance techniques and COSY two-dimensional NMR spectroscopy. In general, double resonance was utilized to assign vicinal proton connectivities and to tentatively relate them to specific protons that were consistent with the data and reports in the literature. The assignments were confirmed via a COSY experiment in which the long-range coupling constants were enhanced[25]. Specifically, this allowed a ready assignment of the NMR signals corresponding to His^6, Phe^7 and Trp^9. In certain cases where further ambiguities existed, pH titrations, and preparation and evaluation of specifically deuterated analogues were employed (data not shown).

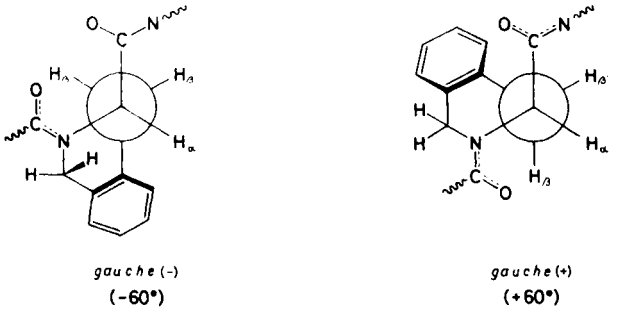

FIG. 5
Newman projections of the rotamers (*gauche*(+) or *gauche*(−)) for tetrahydroisoquinoline carboxylic acid (Tic) in a peptide

In order to examine whether a reverse turn conformation or more extended β-like conformation is preferred for the D-Phe[7]-containing α-MSH fragment analogues, a careful examination of the backbone conformation of the model peptides was made. The ^3J(NH–αCH) proton coupling constants of individual amino acids can be related to the phi (φ) dihedral angle of the αCH–NH fragment by the method of Bystrov et al.[26]. Most of the values for the model peptides (Table II) are in the range of 5.5 – 7.5 Hz, suggesting that the backbone of the model peptides may possess considerable conformational flexibility in aqueous solution, though a more fixed conformation cannot be ruled out. However, the peptide bond NH protons have large temperature coefficients (5.0 to 7.6 × 10^{-3} ppm/deg) (Table V). If an amide proton is involved in an intramolecular hydrogen bond, or is solvent shielded, smaller temperature coefficients (less than 3 × 10^{-3} ppm/deg) are generally observed[22]. Thus this indicates that the model peptides *II* and *III* do not possess a stable intramolecular hydrogen bond or solvent-shielded amide protons.

From the above data, it does not appear that *II* and *III* possess a preferred backbone conformation. However, this does not rule out the existence of a preferred secondary structure as a result of preferred interactions of the individual amino acid side chains as found previously for diastereoisomeric α-MSH_{4-11} analogues[9]. This possibility was examined by an analysis of possible anisotropic effect on the chemical shifts of the side chain protons, of the alpha–beta coupling constants (and corresponding rotamer populations), and on carbon-13 spin–lattice relaxation times (T_1).

In the model peptides, substitution of D-Phe into position 7 resulted in an upfield shift of the beta and gamma protons of Arg[8] and the C4 protons of His[5] (Tables II and III). This shift can be attributed to the proximity of shielding nucleus, such as an aromatic ring[27–30]. Based upon the previous analysis of the NMR spectra of Ac-[Nle[4], D-Phe[7]]-α-MSH_{4-11}-NH_2 (ref.[9]) and other closely related peptides (Ac-[Nle[4], Ala[7]]-α-MSH_{4-11}-NH_2, Ac-[Nle[4], D-Ala[7]]-α--MSH_{4-11}-NH_2, Ac-[Nle[4], Leu[7]]-α-MSH_{4-11}-NH_2 and Ac-[Nle[4], D-Phe[7], Leu[9]]-α-MSH_{4-11}-NH_2, unpublished results), the observed shielding is due to the phenyl ring of phenylalanine. In neither of the alanine-containing peptides were the gamma protons of arginine or the C4 protons of histidine shifted. The NMR results with the Leu[9] substitution analogues provide further support that the phenylalanine-7 aromatic ring is responsible for the observed shieldings, since the same shielding patterns are observed in these peptides as in *III*, indicating that the tryptophan aromatic ring is not responsible for the observed chemical shifts[9].

The shielding interactions observed when a D-Phe is substituted in the position 7 of 4–11 α-MSH analogues can be accounted for if the peptide backbone assumes a preferred topography with a β-like structure (Fig. 6) (i.e.

$\varphi = -139°$ and $\psi = +135°$ for L-amino acids; $\varphi = +139°$ and $\psi = -135°$ for D-amino acids[9]. A simple view of this conformation would situate the side chains of the $n - 1$ (His^6) and $n + 1$ (Arg^8) amino acids on the same side of the backbone in the case of L-amino acids and possibly allow an interaction between these moieties. However, if n corresponds to a D-amino acid, the side chains of the n, $n - 1$ and $n + 1$ amino acids would now be in closer proximity. In α-MSH, a D-amino acid in the position 7 would place the side chains of His^6, D-Phe^7 and Arg^8 closer to each other and account for the observed shielding of the C4 protons of histidine and the gamma protons or arginine, by the aromatic nucleus of phenylalanine.

From the vicinal coupling constants of individual amino acids in the model peptides *II* and *III*, the corresponding rotamer populations can be calculated (Table IV). The rotamer populations were calculated based upon the parameters of Leeuw and Altona[22]. These parameters were chosen over Pachler's[31] since the former approach considers the electronegativity of the individual side chain substituents in peptides. In these linear peptides, the *gauche*(+) (+60°) rotamer was essentially unpopulated (Table IV).

The carbon-13 NMR spectra of α-MSH has never been investigated, although the literature does contain reports on studies of various fragments of ACTH[23,24]. In one of these studies[24] it was suggested that certain of the carbon-13 resonances (specifically for Phe^7, Arg^8 and Trp^9) were "doubled" in the ACTH structure. Also, the extremely short spin–lattice relaxation times in the D-Phe^7-containing ACTH pentapeptide H-His-D-Phe-Arg-Trp-Gly-OH were suggested to indicate significant restriction of the side chain of Arg^8. Our

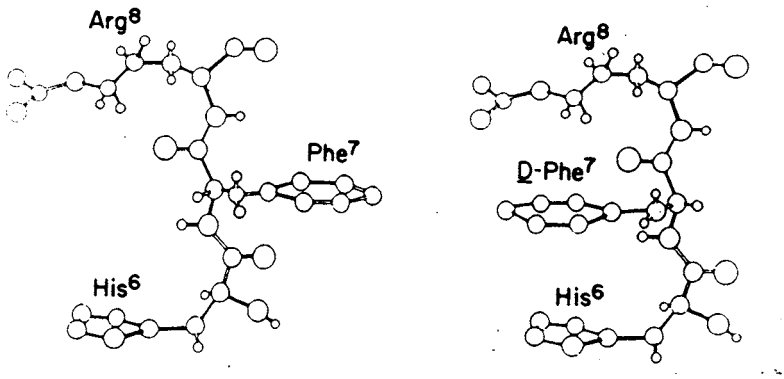

FIG. 6

A representation of a β-like conformation for the tripeptide sequence of His^6-Phe^7-Arg^8 with either an L- (left) or a D- (right) phenylalanine in position 7. The figure may be viewed in stereo to see the differences between the side chain groups at position 7

analysis of the model α-MSH peptides *II* and *III* did not provide any evidence for "doubling" of the C-13 resonances. Even though *II* and *III* are not ACTH analogues and thus are N-acetyl- and C-terminal carboxamide-containing peptides, we would expect that similar trends should be observed. Closer comparison of the literature spectra[23] to our spectra allows us to suggest that the "doubling" of certain C-13 resonances may have been due to an impurity.

Carbon-13 spin–lattice relaxation times (T_1) can provide an approximation of the mobility of individual carbon atoms. Unfortunately, it is not easy to compare our NT_1 values to those in the literature, since the two studies were done under somewhat different conditions. Therefore, the NT_1 values in Table VI can only be compared to each other. The NT_1 values for the beta carbons of the 5 – 9 sequence (Glu-His-Phe-Arg-Trp) in both Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ and Ac-[Nle^4, D-Phe^7]-α--MSH$_{4-11}$-NH$_2$ are somewhat lower than NT_1 values for the α carbons suggesting reduced side chain mobility. In comparing the NT_1 values of *II* and *III* (Table VI) it is particularly noteworthy that the NT_1 values for the β carbons of the His^5 and Phe^7 side chain groups (and to a lesser extent the Glu^5 and Arg^8) have shorter values in the D-Phe^7 analogue *III*, which provides further evidence supporting the interaction of these side chain groups in *III*.

Though the above studies and those previously reported[9] suggest the importance of the topographical relationship of the aromatic ring of position 7 with other side chain groups for high potency, further insight into the importance of this position by the use of conformationally constrained or constraining amino acid residues[17,32] in this position and their effect on biological and conformational properties. In this study, we have focused on the importance of the orientation of the aromatic ring in the position 7 in linear 4–11 melanotropins to these properties. In particular, analogues with either enantiomer of phenylglycine (Pgl) or tetrahydroisoquinoline carboxylic acid (Tic) substituted in the position 7 have been examined.

Substituting phenylglycine for phenylalanine has the effect of shortening the amino acid side chain and reducing the conformational freedom of the aromatic ring due to steric effects. Interestingly, the L-Pgl-containing analogue Ac-[Nle^4, Pgl^7]-α-MSH$_{4-11}$-NH$_2$ (*IV*) was approximately 300 – 400 fold more potent than the D-Pgl^7 containing fragment analogue *V* on both assays (Table I), and in fact was much more potent then the L-Phe^7 analogue, Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$.

As with the model peptides, both Pgl^7-containing analogues *IV* and *V* appear to have quite similar conformations as *II* and *III*, respectively, based upon the alpha proton chemical shifts (Tables II and III), the amide proton chemical shifts and the amide proton coupling constants (Table V). Likewise, these peptides do not appear to possess a conformation stabilized by an intramolecular hydrogen bond, since all the amide protons have normal temperature dependencies (Table V).

The shielding patterns for Ac-[Nle^4, D-Pgl^7]-α-MSH$_{4-11}$-NH$_2$ (*V*) are very similar to those observed for Ac-[Nle^4, D-Phe^7]-α-MSH$_{4-11}$-NH$_2$ (*III*). In particular, both the gamma protons of arginine (Table II) and the C4 protons of histidine are shifted upfield an amount consistent with the model peptide, but unlike the model peptide the C2 protons of histidine are also shielded to a small extent (Table III). Furthermore, the aromatic protons of phenylglycine are shielded (0.12 – 0.22 ppm). * This suggests that the aromatic rings of histidine and phenylglycine are oriented such that there is a greater overlap of the shielding portions of their aromatic rings in *V* than in *III*. Further support for this interaction is available from an analysis of the rotamer populations of the amino acids in positions 6 – 9 of these analogues (Table IV). For histidine, the *trans* rotamer population has been enhanced at the expense of the $-60°$ rotamer as a result of the phenylglycine substitution (compare *V* and *III* in Table IV). Apparently, the particular relationship of the phenyl and imidazole rings found in the D-Pgl^7, analogue *V*, may lead to a three-dimensional solution conformation that is not commensurate with high biological potency, but which is commensurate with prolonged activity.

The shielding patterns in the proton NMR of Ac-[Nle^4, Pgl^7]-α-MSH$_{4-11}$-NH$_2$ (*IV*) are quite differrent than those observed in the model peptide *II*. In particular, the C2 and C4 protons of the tryptophan indole ring shifted upfield (0.12 ppm), which is also accompanied by a slight upfield shift of some phenylglycine's aromatic protons (Table III).* However, based upon model studies of this analogue, the phenyl ring of phenylglycine alone cannot be responsible for shielding both the C2 and C4 protons of tryptophan. It appears that the phenylglycine aromatic ring is responsible for shielding the C4 indole protons, and this particular orientation of the aromatic rings results in shielding of the C2 proton by the carbonyl of arginine. In this case, the L-Pgl^7 substitution results in an orientation of the phenyl ring such that it is in close proximity to the indole ring of tryptophan, suggesting that this type of an interaction may be important for high biological activity.

Restriction of the flexibility of the aromatic ring of phenylalanine by the substitution of both enantiomers of Tic in the position 7 had dramatic effects on the biological potency of the resulting analogue. Both Ac-[Nle^4, Tic^7]-α-MSH$_{4-11}$-NH$_2$ and Ac-[Nle^4, D-Tic^7]-α-MSH$_{4-11}$-NH$_2$ are weak agonists in both bioassays, but the D-Tic-containing peptide was approximately 40 times more potent than the L-Tic analogue. Interestingly, neither of these analogues exhibited prolongation. Once again the differences in the biological potencies of these analogues cannot be attributed to the conformational preferences of the

* The "normal" chemical shifts for Pgl protons were determined by the synthesis and NMR analysis of Ac-Ala-Pgl-Ala-Lys-NH$_2$ (δ = 7.44 for aromatic protons) (see Experimental).

peptide backbone and side chain moieties based upon an examination of the alpha, aromatic and amide protons (Tables II – V).

A careful scrutiny of the shielding patterns reveals that in both cases the alpha protons of the histidine-5 residue are shifted downfield (deshielded) ~ 0.50 ppm for both analogues *VI* and *VII*. Otherwise no major chemical shift differences were observed. Examination of models shows that this large chemical shift change is probably a result of the proximity of the amide carbonyl of glutamic acid. Also, in both cases the gamma protons of arginine are slightly shielded by the aromatic ring of Tic, but to a smaller extent than in either the D-Phe[7] or the D-Pgl[7]-containing 4–11 peptides.

In Tic, the methylene bridge between the *ortho* position of the aromatic ring and the alpha amino group forms a highly constrained six-membered ring due to steric effects resulting from its pattern of substitution and the sp^2 hybridization of two carbons in the ring. This allows the conformational properties of the amino acids and its effects on adjacent amino acids and the peptide backbone to be more stringently defined. Examination of models shows that the Tic[7] substitution provides significant restriction of the peptide backbone (as evidenced by the shielding of histidine's alpha proton). Furthermore, the *trans* rotamer about the χ_1 torsional angle is no longer possible, and Tic must distribute itself between the *gauche*(+) (chair form) and the *gauche*(−) (probably boat form) rotamers. The magnitude of the ^{1}H alpha–beta coupling constants (5.2–6.3 Hz) suggests[33] that the *gauche*(+) rotamer is preferred. This is further supported by the minimization of allylic strain in the *gauche*(+) rotamer[34] (Fig. 5). Normally, this rotamer is not preferred for amino acids in α-MSH, and the predominance of this rotamer may explain the low biological activity of these analogues. The Tic[7] substitutions also effect the rotamer populations of the adjacent amino acids (histidine and tryptophan) (Table IV) providing further evidence for a conformational modification of this peptide.

The very weak biological activities for both *VI* and *VII* and the highly constrained nature of the Tic amino acid residue suggests that the constraints imposed by this amino acid in position 7 of α-MSH are not compatible with a strong interaction with the melanotropin receptor. However, it would appear that full transduction of the biological message is still possible since both analogues *VI* and *VII* are full agonist with no antagonistic activities.

EXPERIMENTAL

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) butanol–AcOH–pyridine–H_2O (15:3:10:12); B) butanol–pyridine–AcOH–H_2O (5:5:1:4); C) 2-propanol–NH_4OH–H_2O (3:1:1). Detection was by iodine vapors and ninhydrin. Single spots were obtained in all cases. Amino acid analyses were obtained with a Beckman 120C amino acid analyzer following hydrolysis for 48 h at 110°C with 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole and subsequent neutralization with 3.5 M-NaOH. No corrections were made for destruction of amino acids during hydrolysis.

N^{α}-Boc-protected amino acids and amino acid derivatives were purchased from Vega Biochemicals (Tucson, AZ), Peninsula Laboratories (San Carlos, CA), Bachem (Torrance, CA), or were prepared using published procedures. Before use, all amino acid derivatives were tested for purity by TLC in solvent systems A,B and C, melting point, and the ninhydrin test[35]. Solvents used for gel filtration, TLC, ion exchange chromatography, and high performance liquid chromatography (HPLC) were purified by redistillation before use. The *p*-methylbenzhydrylamine resin (*p*-MBHA) (1% divinylbenzene cross-linked polystyrene) was prepared by previously reported methods[11].

Solid Phase Synthesis of Melanotropins

The α-MSH fragment analogues were prepared by solid-phase methods similar to those reported previously[7,11]. Each N^{α}-Boc-protected amino acid derivative was successively coupled to the *p*-methylbenzhydrylamine resin using a 3-fold excess of the protected amino acid, a 3-fold excess of N-hydroxybenzotriazole (HOBt) and 2.4-fold excess of dicyclohexylcarbodiimide (DCC). Cleavage of the N^{α}-Boc-protecting group was performed by treatment with 45% trifluoroacetic acid containing 2% anisole in dichloromethane. Side chain functionalities were protected as follows: arginine, N^{g}-tosyl; glutamic acid, γ-benzyl ester; lysine, N^{ε}-2,4-dichlorobenzyloxycarbonyl; tryptophan, N^{in}-formyl; and histidine, N^{im}-tosyl. It has been reported that HOBt removes the tosyl protecting group from histidine under the conditions used for synthesis[36]. Therefore HOBt was not used once histidine had been incorporated into the peptide. The procedure utilized for incorporation of each individual amino acid residue into the growing peptide chain is outlined in Table VII.

Ac-[Nle^4]-α-MSH_{4-11}-NH_2 (*II*)

The title peptide was prepared as previously reported[7]. The purification scheme was modified to include preparative HPLC as the final step to remove all trace impurities. A portion (30 mg) of the partially purified peptide was dissolved in 2.5 ml of 10% AcOH and chromatographed on a Vydac C18 column (25.0 × 0.46) with a mobile phase of 83% aqueous trifluoroacetic acid (0.1%) and 17% acetonitrile to give 25.6 mg of the title compound. Analytical data are found in Table VIII.

Ac-[Nle^4, D-Phe^7]-α-MSH_{4-11}-NH_2 (*III*)

The title peptide was prepared as previously reported[7]. The purification scheme was modified to include preparative HPLC as the final step to remove all trace impurities. A portion (100 mg) of the peptide was further purified by RP-HPLC on a Vydac C18 column (25.0 × 0.46) with a mobile phase of 85% aqueous trifluoroacetic acid (0.1%) and 15% acetonitrile to give 30.3 mg of the title peptide. Analytical data are found in Table VIII.

Ac-[Nle^4, Pgl^7]-α-MSH_{4-11}-NH_2 (*IV*)

Starting with 1.07 g of N^{α}-Boc-Lys(2,4-Cl_2-Z)-*p*MBHA resin (0.67 mmol of N^{α}-Boc-Lys(2,4-Cl_2-Z)),

the protected title peptide was prepared by stepwise coupling of the following amino acids (in order of addition): Boc-Trp(N^{in}-For), Boc-Arg(N^{g}-Tos), Boc-Pgl, Boc-His(N^{im}-Tos), Boc-Glu(γ-OBzl), Boc-Nle. Coupling of the last amino acid and removal of the Boc group was followed by acetylation with a 6-fold excess of N-acetylimidazole in 25 ml CH_2Cl_2. The resulting peptide resin, Ac-Nle-Glu(OBzl)-His(Tos)-Pgl-Arg(Tos)-Trp(For)-Gly-Lys(2,4-Cl_2-Z)-*p*MBHA was dried in vacuo. The protected peptide was cleaved from the resin and all protecting groups were removed by treatment with anhydrous HF (13.0 ml), anisole (1.4 ml) and 1,2-ethanedithiol (0.7 ml)[37] (60 min at 0°C). After evaporation of the HF and anisole, the resin was washed with ethyl acetate (3 × 30 ml) and extracted successively with 30% aqueous AcOH (3 × 30 ml), 10% aqueous AcOH (3 × 30 ml) and H_2O (3 × 30 ml). The aqueous fractions were combined and lyophilized to give the crude title peptide (365 mg). The residue was then dissolved in 1.5 ml of 0.01 M-NH_4OAc (pH 4.5) and chromatographed on a carboxymethylcellulose column (CMC) (20 × 2.0 cm) with a discontinuous gradient (250 ml each) of 0.01 M-NH_4OAc (pH 4.5), 0.10 M-NH_4OAc (pH 6.8) and 0.20 M-NH_4OAc (pH 6.8). The major peak (280 nm detection) eluted with the 0.10 M-NH_4OAc buffer and was lyophilized to give 88 mg of a white powder. A portion of this powder (80 mg) was subsequently dissolved in 2.0 ml of 10% AcOH and chromatographed on a Vydac C18 column (25.0 × 0.46) with a mobile phase of 85% aqueous trifluoroacetic acid (0.1%) and 15% acetonitrile to give 36.1 mg of the title peptide. Analytical data are found in Table VIII.

Ac-[Nle^4, D-Pgl^7]-α-MSH_{4-11}-NH_2 (*IV*)

Starting with 1.50 g Boc-Lys(2,4-Cl_2-Z)-*p*-MBHA resin (0.66 mmol of Boc-Lys(2,4-Cl_2-Z) total, the title peptide was prepared by stepwise coupling of the appropriate Boc protected amino acids. Acetylation of the N-terminus of the protected peptide was performed as above. The resulting protected peptide-*p*-MBHA resin, was dried in vacuo. Cleavage, deprotection and purification was performed as in peptide *IV* to give 36.1 mg of the title peptide. A mobile phase of 87% aqueous

TABLE VII
Solid-phase peptide synthetic protocol

Reagent[a]	Repetition	Time, min
1) CH_2Cl_2	4	1
2) 45% TFA, 2% anisole/CH_2Cl_2	1	2
	1	20
3) CH_2Cl_2	3	1
4) 10% DIEA/CH_2Cl_2	3	2
5) CH_2Cl_2	4	1
6) N^{α}-Boc-Amino acid, DCC, HOBt/CH_2Cl_2	1	60–120
7) CH_2Cl_2	3	1
8) EtOH	3	1
9) CH_2Cl_2	4	1

[a] After steps 5 and 9, a few milligrams of the resin were removed and a ninhydrin test[35] was used to monitor the coupling. In most cases only one coupling was required for each amino acid except when the ninhydrin test after step 9 was positive. In this case either the amino acid was recoupled or the reactive site was acetylated with N-acetylimidazole.

trifluoroacetic acid (0.1%) and 13% acetonitrile was used for preparative HPLC. Analytical data are found in Table VIII.

Ac-[Nle^4, Tic^7]-α-MSH$_{4-11}$-NH$_2$ (*VI*)

Starting with 0.75 g Boc-Lys(2,4-Cl_2-Z)-*p*-MBHA resin (0.66 mmol of Boc-Lys(2,4-Cl_2-Z) total), the title peptide was prepared by stepwise coupling of the appropriate Boc protected amino acids. Acetylation of the N-terminus of the protected peptide was performed with a 10-fold excess of N-acetylimidazole in 25 ml of CH_2Cl_2. Cleavage, deprotection and purification was performed as in peptide *IV* to give 23.5 mg of the title peptide. A mobile phase of 85% aqueous trifluoroacetic acid (0.1%) and 15% acetonitrile was used for preparative HPLC. Analytical data are found in Table VIII.

Ac-[Nle^4, D-Tic^7]-α-MSH$_{4-11}$-NH$_2$ (*VII*)

Starting with 1.80 g Boc-Lys(2,4-Cl_2-Z)-*p*-MBHA resin (0.72 mmol of Boc-Lys(2,4-Cl_2-Z) total), the title peptide was prepared by stepwise coupling of the appropriate Boc protected amino acids. Acetylation was performed as above. Cleavage, deprotection and purification was performed as in peptide *IV* to give 33.5 mg of the title peptide. A mobile phase of 86% aqueous trifluoroacetic acid

TABLE VIII
Analytical data for linear melanotropins

Compound	Amino acid analysis				TLC			HPLC[a]		
	Nle^4 Arg^8	Glu^5 Trp^9	His^6 Gly^{10}	XXX^7 Lys^{11}	A	B	C	A	B	C
II	0.99 0.98	1.01 0.98	1.02 1.02	0.99 1.04	0.11	0.35	0.35	15.18	5.07	25.24
III	1.00 1.07	0.94 0.97	1.00 0.98	0.99 1.04	0.11	0.38	0.32	7.91	3.40	12.41
IV	0.93 1.02	1.04 0.96	0.99 1.04	0.99 1.05	0.13	0.38	0.30	5.82	2.93	9.24
V	0.95 0.98	1.06 0.99	1.01 1.04	0.95 1.01	0.13	0.40	0.26	2.54	2.07	4.00
VI	0.98 1.00	1.02 0.95	0.99 0.99	1.00 1.04	0.13	0.40	0.23	5.64	3.00	10.18
VII	0.96 1.06	0.98 0.95	0.94 1.04	1.02 1.05	0.19	0.44	0.31	3.91	2.80	7.35

[a] All compounds were chromatographed on a C-18 Vydac column (25 × 0.46 cm) 16 μm reverse phase column with the following conditions: A) 86% triethylammonium phosphate (pH 2.2)/14% CH_3CN, 1.5 ml/min; B) 50% heptafluorobutyric acid (0.1%) 50% CH_3CN, 2.0 ml/min; C) 68% trifluoroacetic acid (0.1%) 32% MeOH, 2.0 ml/min.

(0.1%) and 14% acetonitrile was used for preparative RP-HPLC. Analytical data are found in Table VIII.

Ac-Ala-Pgl-Ala-Lys-NH_2 (*VIII*)

Starting with 0.75 g Boc-Lys(2,4-Cl_2-Z)-*p*-MBHA resin (0.75 mmol of Boc-Lys(2,4-Cl_2-Z) total), the title peptide was prepared by stepwise coupling of the folowing amino acids (in order of addition): Boc-Ala, Boc-Pgl, and Boc-Ala. The peptide chain was acetylated with a 6-fold excess of N-acetylimidazole in 25 ml of CH_2Cl_2. The protected peptide was cleaved as described above for peptide *IV* and purified by preparative RP-HPLC on a Vydac C18 column (25.0 × 0.46 cm) with a mobile phase of 98% aqueous trifluoroacetic acid (0.1%) and 2% acetonitrile to give 65.2 mg of the title peptide. Amino acid analysis: Ala 2.02; Pgl 0.97; Lys 1.00. Thin layer chromatography (R_f): 0.35 (A); 0.48 (B); 0.00 (C). HPLC on Vydac C18 column (25.0 × 0.46 cm) using 97% aqueous trifluoroacetic acid (0.1%) and 3% acetonitrile.

L-1,2,3,4-Tetrahydroisoquinoline Carboxylic Acid (*IX*)

The title amino acid was prepared by the method of Pictet and Spengler[38] starting from L-phenylalanine to give *IX* (6.2 g, 64%); m.p. 308 – 311 C; $[\alpha]_{589}$ – 131.7 (*c* 0.65, 30% HOAc). (The low value for the optical rotation indicates that partial racemization may have occurred[39,40], or that some other impurity was present. However, the optically pure amino acid apparently was obtained on fractional crystallization of the N^{α}-Boc derivative).

Boc-L-1,2,3,4-Tetrahydroisoquinoline Carboxylic Acid (*X*)

A solution of 2.78 g L-Tic in 40 ml dioxane and 20 ml water was cooled to 0°C. With stirring, 2.2 g of di-tert-butyl dicarbonate was slowly added. The pH was then adjusted and maintained at 10.7 with 3.5 M-NaOH until the pH was constant. The solution was concentrated to remove the dioxane, overlaid with ethyl acetate (50 ml) and the pH was adjusted to 2.2 with 10% HCl at 0°C. The organic layer was separated, washed with ice cold 4% HCl (2 × 25 ml), water (2 × 25 ml), dried with $MgSO_4$, filtered and concentrated. The white foam was recrystallized from ethyl acetate–hexane to yield white crystals (2.10 g, 55%); m.p. 122 – 123.5°C; $[\alpha]_{589}$ +17.6° (*c* 1.0, CH_3OH); ^1H NMR: 1.38, 1.47, 2s, 9H ($(CH_3)_3C$); 3.12 m, 2H (β-CH_2); 4.43, 4.65 dq, 2H (N-CH_2); 4.99, 4.70 br s, 1H (α-CH); 7.13 m, 4H (aromatic-H). For $C_{15}H_{19}O_4N$ (277) calculated: 64.97% C, 6.91% H, 5.05% N; found: 64.81% C, 6.91% H, 4.93% N.

D-1,2,3,4-Tetrahydroisoquinoline Carboxylic Acid (*XI*)

The synthesis of this compound was performed exactly as in compound *IX* except that the starting amino acid was D-phenylalanine; yield, 53.8%; m.p. 306 – 309°C; $[\alpha]_{589}$ +126.9° (*c* 0.67, 30% AcOH).

N^{α}-Boc-D-1,2,3,4-Tetrahydroisoquinoline Carboxylic Acid (*XII*)

The synthesis of this compound was performed exactly as in compound *X* except that the starting amino acid was D-Tic; yield, 61.1%; m.p. 123 – 125°C; $[\alpha]_{589}$ – 18.9° (*c* 1.0, CH_3OH). ^1H NMR, see Boc-L-Tic. For $C_{15}H_{19}O_4N$ (277) calculated: 64.97% C, 6.91% H, 5.05% N; found: 64.68% C, 6.78% H, 4.91% N.

Frog and Lizzard Skin Bioassays

The biological activities of α-MSH and the linear analogues were determined by their ability to stimulate melanosome dispersion in vitro in the frog and lizard bioassay systems as previously described[41-44]. All the solutions were prepared via serial dilutions from a stock solution (10^{-4} mol l). 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.

Nuclear Magnetic Resonance Data

The proton NMR of the peptides were recorded on a WM-Bruker 250 MHz Fourier transform NMR spectrometer. For all spectra, except for those of the amide protons, approximately 6 mg of purified peptide was dissolved in 250 – 300 μl of D_2O. The pH of the resulting solution was adjusted to 3.5 with perdeuterated acetic acid. The chemical shifts are referenced to sodium-3-trimethylsilyl-tetradeuteriopropionate (TSP, $\delta = 0.00$), (Tables II and III).

In order to observe the amide resonances, 8 – 12 mg of the purified peptide was dissolved in a solution of 80% H_2O: 20% D_2O (250 – 300 μl) and the pH was adjusted to 3.5 with perdeuterated acetic acid. The peptide amide resonances were assigned (Table V) based upon decoupling of the previously assigned alpha protons and the J(NH–αCH) proton coupling constants were measured directly from the spectra (Table V). The temperature dependence ($\Delta\delta\ \Delta T$) of the amides were determined at five temperatures (295, 300, 305, 310 and 315 K), in which at least 30 min was allowed for equilibration of the sample before acquisition of the spectra.

Measurement of carbon-13 NMR spin relaxation times (T_1) was performed on the model peptides, Ac-[Nle^4]-α-MSH$_{4-11}$-NH$_2$ and Ac-[Nle^4, D-Phe^7]-α-MSH$_{4-11}$-NH$_2$. The peptides (approximately 40 mg) were dissolved in 1.5 ml D_2O and the pH was adjusted to 3.5 with perdeuterated acetic acid. Assignment of carbon-13 chemical shifts (Table VI) (referenced to dioxane at 67.4 ppm) was based on: off-resonance decoupling, heteronuclear decoupling and comparisons with the literature[24]. The T_1 values were determined by the inversion recovery method with a $(-180° - \tau - 90° -)$ pulse sequence, where $\tau_{(\text{infinity})} = 2.0$ s; τ was subsequently varied from 2.0 s to 0.04 s. The T_1 and NT_1 values were calculated and are only reported for the protonated carbons (Table VI).

This research was supported by grants from the U.S. Public Health (AM-17420) *and the National Science Foundation. A.M.C. was supported in part by a fellowship from the Counselho Nacional de Desenvolvimento Technologico e Cientifico of Brazil. Grant 200 430/82. We thank Dr Michal Lebl for his helpful suggestions.*

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