Topographical Modification of Melanotropin Peptide Analogues with β-Methyltryptophan Isomers at Position 9 Leads to Differential Potencies and Prolonged Biological Activities

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We have introduced topographical constraints at the 9 position of a superpotent cyclic α-melanotropin analogue, Ac-Nle[4]-Asp[5]-His[6]-DPhe[7]-Arg[8]-Trp[9]-Lys[10]-NH_2, by incorporating a methyl group at the β-carbon of Trp[9]. These studies were performed on the Trp side chain pharmacophore to identify the bioactive topography of the indole moiety with melanocortin MC1 receptors. The four β-MeTrp[9] isomers, in addition to the stereochemical controls L- and DTrp[9], were used to probe differential receptor molecular recognition of the tryptophan moiety in two bioassay systems. Approximately a 460-fold difference in potency was observed between the diastereoisomeric peptides in the frog skin bioassay, with only 33- and 10-fold efficacy differences observed in binding and intracellular cAMP accumulation, respectively, on the human melanocortin receptor, hMC1R. The relative orders of potencies in the frog skin bioassay were 2*R*,3*S* > 2*S*,3*S* = 2*R*,3*R* ≫ 2*S*,3*R* and for the hMC1R were 2*S*,3*S* > 2*R*,3*R* > 2*R*,3*S* ≫ 2*S*,3*R*. Of particular interest is the ability of these topographically constrained ligands to differentially affect prolonged biological activity. The 2*R*,3*R* diastereoisomeric peptide possessed superprolonged activity, whereas the 2*S*,3*S* peptide lacked any residual activity in the frog skin bioassay. However, on the melanocortin receptor, the 2*S*,3*S* diastereoisomeric peptide maintained slow dissociation rates ($t_{1/2}$ = 7 h), while the other diastereoisomeric peptides possessed dissociation $t_{1/2}$ rates of ca. 2 h. These data strongly implicate ligand-receptor interactions and kinetics as contributing to the observed prolonged biological activities and clearly illustrate topographical recognition differences between these two peripheral MC1 receptors involved in skin pigmentation. This study also demonstrates that topographical modifications of pharmacophore side chain residues, in addition to identifying preferential side chain orientation, can be a useful strategy for the design of peptides to increase the duration of biological activity, relative to the native ligand.

Introduction

α-Melanocyte-stimulating hormone (α-melanotropin, α-MSH, Ac-Ser[1]-Tyr-Ser-Met-Glu[5]-His-Phe-Arg-Trp-Gly[10]-Lys-Pro-Val-NH_2) belongs to the family of melanocortin peptides derived by posttranslational processing of the proopiomelanocortin (POMC) gene.[1] Several biological activities have been attributed to this peptide hormone. Both peripheral and central nervous system activities related to this peptide hormone include cognitive affects related to learning, memory, and attention,[1,2] analgesic effects,[3] modulation of body temperature,[4] role in fetal development and parturition,[5] and immunomodulatory effects.[6] The most recognized biological activity of α-MSH is its role in regulating skin pigmentation: follicular melanogenesis and tanning.[7] Clinical applications of melanotropic peptides and related analogues include the possible prevention of skin cancer and the detection and eradication of melanoma.[8] Several distinct melanocortin receptor types have been cloned and found to have different tissue expression patterns. The peripheral melanocortin receptor, designated MC1R, has been isolated from human melanoma[9,10] and is the G-protein-coupled receptor believed to be involved in regulating skin pigmentation.

These multiple physiological effects have made the α-melanotropin tridecapeptide the target of extensive structure-activity studies[11-13] and led to the design and synthesis of the superpotent cyclic heptapeptide MTII, Ac-Nle[4]-Asp[5]-His[6]-DPhe[7]-Arg[8]-Trp[9]-Lys[10]-NH_2 (the superscript numbers refer to the location of the residues relative to the native α-MSH peptide).[40,41] This peptide has become the lead analogue for extensive studies to optimize and identify the bioactive conformations of α-MSH.[16-19] Several models have been proposed for the bioactive conformations of α-MSH, but these generally have been limited to the backbone φ,ψ torsion angles. The side chain topography also is important in identifying bioactive conformations and a preferred topology for receptor recognition. Rotation about the C^{α}-C^{β} torsion angle, χ_1, of a side chain group is an important conformational parameter. Incorporation of a methyl group at either the *pro-R* or *pro-S* position of the β-carbon of aromatic amino acid residues should decrease the freedom of rotation about this angle and limit the

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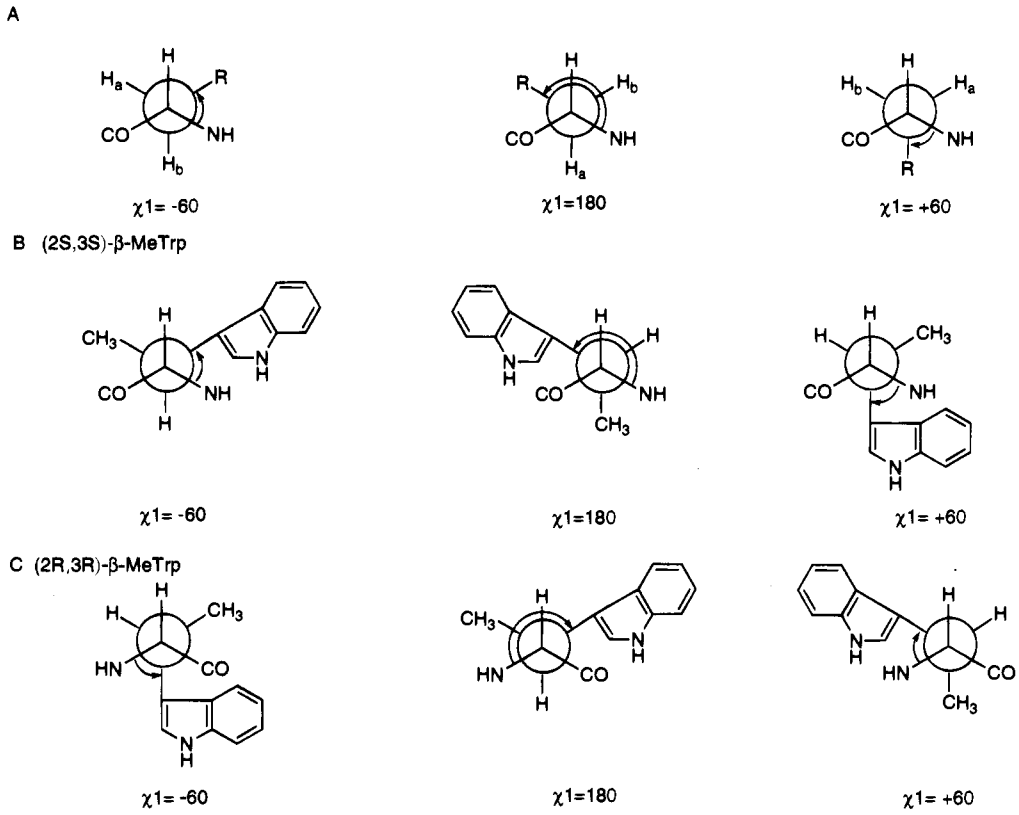

Figure 1. (A) Side chain rotamer populations for α-amino acids about the χ_1 torsion angle, (B) Newman projections of the side chain conformations of the L amino acid (2*S*,3*S*)-β-methyltryptophan, and (C) Newman projections of the side chain conformations of the D amino acid (2*R*,3*R*)-β-methyltryptophan.

available topographies to either a gauche −, trans, or gauche + conformation (Figure 1), depending on stereochemical considerations. This particular topographical constraint, in addition to other more global constraints, such as side chain-to-side chain cyclization, can be used to probe the molecular recognition processes for preferential ligand binding. Examples of this stereochemical approach for conformational and topographical studies have been successfully utilized in somatostatin,[20] enkephalin,[21,22] opioid,[23,24] and other bioactive peptides.

In this study, we have examined the effect of incorporating four isomers of β-MeTrp[25] into the 9 position of the superpotent agonist α-melanotropin template, Ac-Nle[4]-Asp[5]-His[6]-DPhe[7]-Arg[8]-β-MeTrp[9]-Lys[10]-NH_2[14,15] (Figure 2). These peptide derivatives were studied in the classical frog skin bioassay[26] and in the cloned human peripheral melanocortin receptor, hMC1R,[9,10] for binding, dissociation rates, and intracellular cAMP generation.

Results

The syntheses of the cyclic lactam diastereoisomeric analogues of Ac-Nle-Asp-His-DPhe-Arg-Xaa-Lys-NH_2 discussed herein were accomplished by solid-phase synthetic methods.[15] These analogues were purified by reversed-phase high-pressure liquid chromatography (RP-HPLC). The properties and purity of these peptides were assessed by fast atom bombardment mass spectrometry (FAB-MS), RP-HPLC, thin-layer chromatography (TLC) in three solvent systems, optical rotation, and amino acid analysis (see the Experimental Section).

Biological Evaluation. The peptide analogues, Ac-Nle-Asp-His-DPhe-Arg-Xaa-Lys-NH_2, which contained one of the four possible isomers of β-MeTrp, as well as LTrp, or DTrp at position 9, were biologically evaluated in the frog skin bioassay.[26] The relative potencies and effective concentration at 50% maximal response (EC_{50}) values (Figure 3) are summarized in Table 1. Peptide analogues MTII and **VI** were used to examine the stereochemical effects of the α-carbon at position 9 on biological activity, as well as to serve as controls for the topographically restricted analogues. Binding and intracellular cAMP accumulation were examined utilizing the cloned hMC1R[9,10] (transfected into L-cells, a mouse fibroblast-like cell line)[27] believed to be involved in skin pigmentation. The hMC1R binding concentration that inhibits 50% specific binding (IC_{50}), the EC_{50} values for the production of intracellular cAMP, and the relative potency values are also summarized in Table 1.

Examination of Table 1 (frog skin bioassay) showed ca. a 460-fold difference between the β-MeTrp[9] diastereoisomeric peptides. Analogues **III** and **VI**, which contain the (2*R*,3*S*)-β-MeTrp[9] and DTrp[9] residues, respectively, were equivalent in potency, within experimental error, to α-MSH and MTII. Analogues **II** (which contains (2*S*,3*S*)-β-MeTrp[9]), and **V** (which contains (2*R*,3*R*)-β-MeTrp[9]) were ca. 3−4-fold less potent than α-MSH and MTII. Analogue **VI** (DTrp[9]) was 3-fold more potent than the 2*R*,3*R* analogue **V** and approximately equipotent to the 2*R*,3*S* analogue **III**. Comparison of the LTrp (2*S*) compounds reveals that MTII is 4.4-fold more efficacious than the 2*S*,3*S* analogue **II** and 286-fold more potent than the 2*S*,3*R* peptide **IV**. Interestingly, but not surprisingly, analogue **IV**, containing the

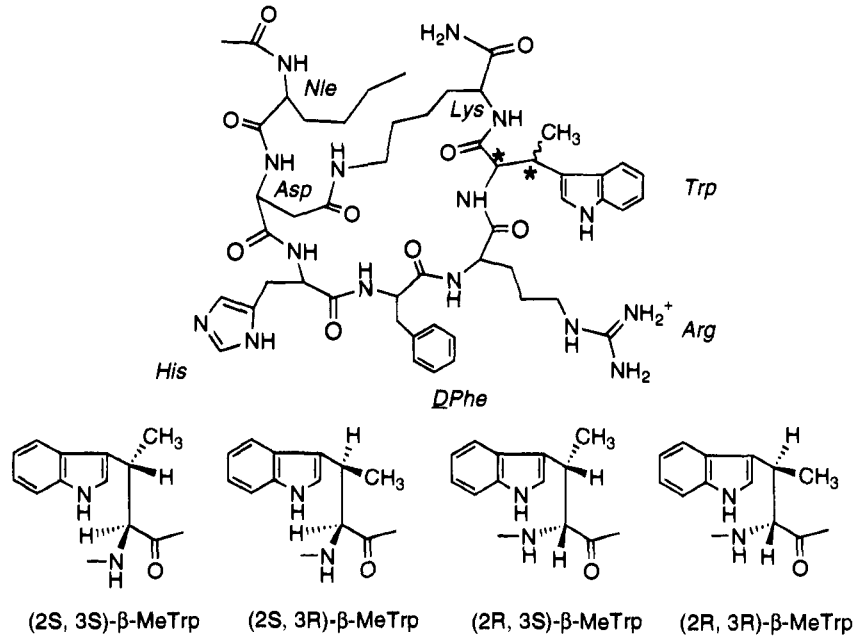

Figure 2. Illustration of the monocyclic α-melanotropin peptide template Ac-Nle^4-Asp^5-His^6-DPhe^7-Arg^8-β-MeTrp^9-Lys^{10}-NH$_2$, where the four isomers of β-MeTrp were incorporated at position 9. An asterisk indicates the presence of the two chiral centers present in the β-MeTrp^9 amino acid.

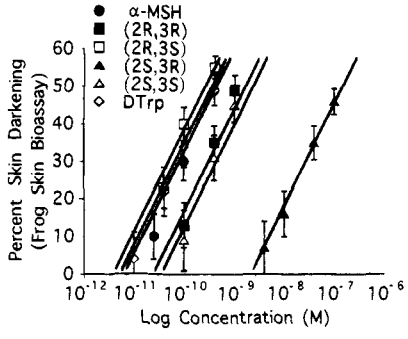

Figure 3. Dose–response plot of the diastereoisomeric peptides, Ac-Nle^4-Asp^5-His^6-DPhe^7-Arg^8-Xaa^9-Lys^{10}-NH$_2$ (with the isomer denoted in parentheses), and α-MSH as determined in the frog skin bioassay. The standard error is illustrated at each concentration (n = 5).

(2*S*,3*R*)-β-MeTrp^9 residue, resulted in a 286-fold decrease in potency compared to α-MSH and both the LTrp and DTrp analogues, which all lack the methyl group at the β-carbon of Trp^9. From this data there appears to be three groups of topographical families (Figure 3) which can be summarized as follows: (1) analogues **III** ((2*R*,3*S*)-β-MeTrp^9) and **VI** (DTrp^9), α-MSH, and MTII, (2) analogues **II** ((2*S*,3*S*)-β-MeTrp^9) and **V** ((2*R*,3*R*)-β-MeTrp^9), and (3) analogue **IV** ((2*S*,3*R*)-β-MeTrp^9).

In addition to the above physiological bioassay, evaluation of these compounds was performed utilizing the cloned hMC1R[9,10] which is believed to be involved in skin pigmentation. Binding and intracellular cAMP accumulation were examined, and the relative potencies, IC_{50} and EC_{50} values, respectively, are reported in Table 1. MTII was selected over α-MSH to represent the standard potency value of 1.0 since this was the lead compound of these studies and was the control used while performing these bioassays. In the physiological assays, α-MSH was the control for comparative potency determinations. MTII was reported to possess 10-fold greater potency than α-MSH in these assays,[27] so these results can be compared with α-MSH if so desired.

Table 1 shows the relative potencies for the peptides, which are consistent for both the binding and cAMP assays, as expected, with the exception of analogues **II** ((2*S*,3*S*)-β-MeTrp^9) and **VI** (DTrp^9). Interestingly, these latter peptides show 2- and 4-fold decreases, respectively, in intracellular cAMP accumulation potency versus binding potencies. Analogues **V** ((2*R*,3*R*)-β-MeTrp^9) and **VI** (DTrp^9) are ca. 3-fold less potent than MTII, while analogues **III** ((2*R*,3*S*)-β-MeTrp^9) and **IV** ((2*S*,3*R*)-β-MeTrp^9) are 7–25-fold less potent than MTII. The two topographical families evident from these studies are summarized as (1) analogues **II** ((2*S*,3*S*)-β-MeTrp^9), **V** ((2*R*,3*R*)-β-MeTrp^9), **VI** (DTrp^9), and MTII and (2) analogues **III** ((2*R*,3*S*)-β-MeTrp^9) and **IV** ((2*S*,3*R*)-β-MeTrp^9).

In addition to affecting biological potencies, alteration of the residues at position 9 has led to an exceptional finding regarding the biological phenomenon of prolongation, or residual activity. Prolonged activity of some melanotropic peptides has been observed in our laboratories and in those of others for over 2 decades. Figure 4 illustrates the dramatic effect that β-MeTrp^9 containing peptides exhibit on this biological activity with the chirality of both the α-carbon and β-carbon sites playing a substantial role. By stereochemically inverting the LTrp to the DTrp amino acid, a change in residual activity is observed, with the DTrp-containing analogue possessing a decreased prolonged activity (Figure 5) as compared with MTII which maintains residual activity. Incorporation of the β-Me group into the Trp residue (either L or D isomer) shows a consistent trend, with the 3*R* chirality at the β-carbon maintaining this prolonged activity more effectively than the 3*S* isomer (Figure 4). It is important to emphasize the fact that chirality at the α-carbon can effect this biological event, and the systematic decrease in prolonged activity when going from the 3*R* to 3*S* chirality at the Trp β-carbon also plays a crucial role affecting duration of action.

To further examine the prolongation phenomena, dissociation binding experiments were designed to see

Table 1. Comparative Biological Activities of Ac-Nle[4]-Asp[5]-His[6]-DPhe[7]-Arg[8]-Xaa[9]-Lys[10]-NH_2 α-MSH Analogues on the MC1 Receptors

		frog skin MC1		hMC1R binding		hMC1R cAMP	
peptide	structure	EC_{50} value (nM)	relative[a] potency	IC_{50}[b] value (nM)	relative[c] potency	EC_{50}[d] value (nM)	relative[e] potency
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.10	1.0	NA		NA	
MTII	Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2	0.10	1.0	0.50 ± 0.08	1.00	0.15 ± 0.05	1.00
II	Ac-Nle-Asp-His-DPhe-Arg-β-MeTrp-Lys-NH_2 (2*S*,3*S*)	0.44	0.225	0.50 ± 0.15	1.00	0.30 ± 0.10	0.50
III	Ac-Nle-Asp-His-DPhe-Arg-β-MeTrp-Lys-NH_2 (2*R*,3*S*)	0.06	1.6	3.00 ± 0.25	0.17	1.00 ± 0.70	0.15
IV	Ac-Nle-Asp-His-DPhe-Arg-β-MeTrp-Lys-NH_2 (2*S*,3*R*)	28.6	0.0035	15.0 ± 6.0	0.03	3.00 ± 1.70	0.05
V	Ac-Nle-Asp-His-DPhe-Arg-β-MeTrp-Lys-NH_2 (2*R*,3*R*)	0.30	0.30	2.00 ± 0.45	0.25	0.40 ± 0.20	0.38
VI	Ac-Nle-Asp-His-DPhe-Arg-DTrp-Lys-NH_2	0.10	1.00	0.40 ± 0.17	1.25	0.50 ± 0.07	0.30

[a] All peptide activities were tested at a range of concentrations (10^{-6}–10^{-12} M) and compared to the half-maximal effective dose of α-MSH in the frog skin (10^{-10} M) bioassay. [b] IC_{50} = Concentration of peptide at 50% specific binding (N = 4–6). The peptides were tested at a range of concentrations (10^{-6}–10^{-11} M). [c] Relative potencies were calculated based on the EC_{50} or IC_{50} values relative to analogue MTII. [d] IC_{50} = Concentration of peptide at 50% maximal cAMP generation (n = 4). The peptides were tested at a range of concentrations (10^{-6}–10^{-11} M). NA signifies that this analogue was not run simultaneously with these analogues.

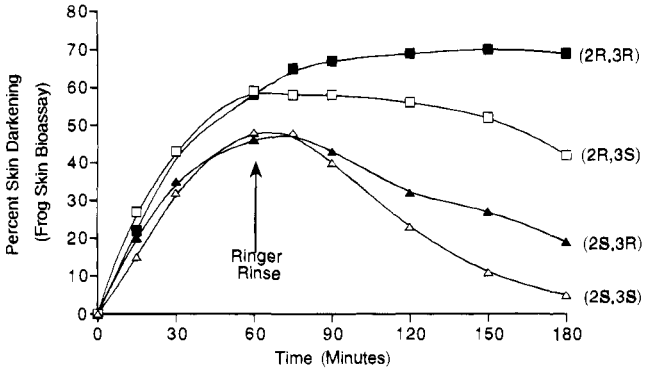

Figure 4. Differential prolonged biological activity observed for the diastereoisomeric peptides, based on the template Ac-Nle[4]-Asp[5]-His[6]-DPhe[7]-Arg[8]-β-MeTrp[9]-Lys[10]-NH_2, in the frog skin bioassay. The assay skins are incubated with the peptide for 60 min, at which time the skins are rinsed and placed in a Ringer solution lacking peptide. Subsequently, the assay skins (n = 5) are monitored for percentage skin darkening for up to 3 h. The 2*R*,3*R* analogue **V** possesses superprolonged biological activity, whereas the 2*S*,3*S* analogue **II** lacks any prolonged activity.

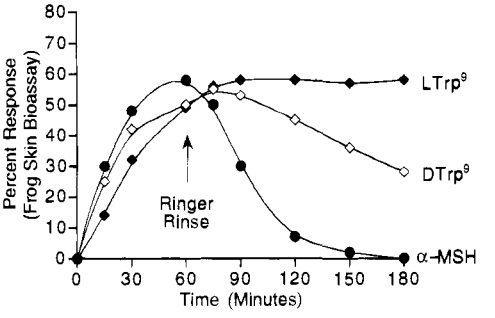

Figure 5. Illustration of the effect chirality of the α-carbon of the Trp[9] residue plays on prolonged biological activity. The LTrp-containing peptide MTII possesses superprolonged biological activity, whereas the DTrp analogue **VI** maintains prolonged biological activity and α-MSH lacks any prolonged biological activity. The assay skins (n = 5) are incubated with the peptide for 60 min, at which time the skins are rinsed and placed in a Ringer solution lacking peptide. Subsequently, the assay skins are monitored for percentage skin darkening for up to 3 h.

if ligand nonreversible binding could explain the residual activity observed. Figure 6 demonstrates the percentage of β-MeTrp[9] ligand bound at (a) 1 h and (b) 4 h incubation times of [^{125}I][Tyr[2],Nle[4],DPhe[7]]α-MSH after removal of the ligand from the assay medium. At the 1 h incubation period, all the peptides remain between 75% and 95% bound to the cells. Interestingly, at the 4 h incubation period, analogue **II** ((2*S*,3*S*)-β-MeTrp[9]) remained ca. 70% bound to the cells, with MTII also significantly bound to the cells. Analogues **V** (27%), **III** (16%), **IV** (12%), and **VI** (<5%) possess residual binding below 30%.

The observed dissociation rates for MTII and analogues **II**–**VI** on hMC1R were determined according to the first-order equation[28]

$$\ln [LR]/[LR]_e = -k_{-1}t \quad (1)$$

where [LR] is the concentration of the ligand–receptor complex at time t and $[LR]_e$ is the concentration found at the 4 h time point. A linear plot of the peptide bound (percentage of specific binding) versus time is obtained, and the apparent dissociation rate, k_{-1}, was determined from the $t_{1/2}$ value, using eq 2.

$$k_{-1} = (\ln 1/2)/t_{1/2} \quad (2)$$

Observed values obtained from this study (Figure 7) are summarized in Table 2. Because of very slow dissociation rates in some cases, the $t_{1/2}$ value was determined by extrapolation using the slopes determined from Figure 7. All the peptides examined had dissociation rates ca. 2 times faster than MTII, with the exception of analogue **II** (which dissociated about 25% slower than MTII). Unsurprisingly, the human receptor, hMC1R, prefers the topology of analogue **II** in regards to conformational properties and residual activity, whereas the frog MC1 receptor favors the topographical presentation of analogue **V** for prolonged biological activity.

Discussion

Rational design of superpotent and selective peptide ligands for structure–activity studies and to probe

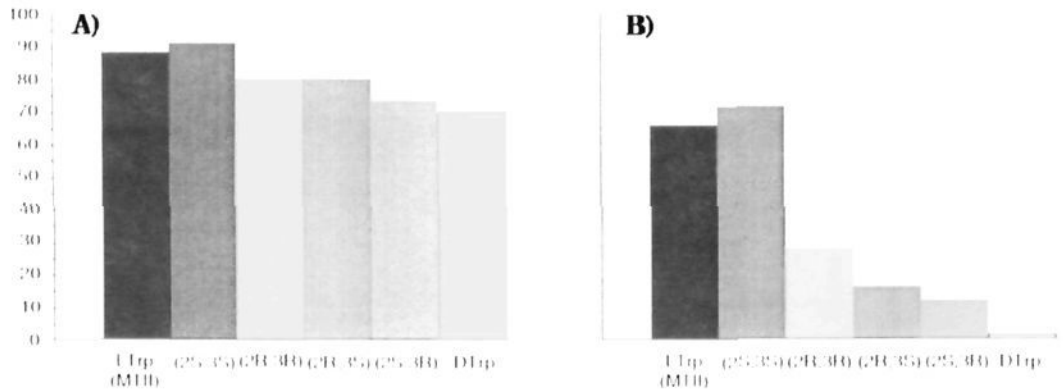

Figure 6. Dissociation studies of Ac-Nle^4-Asp^5-His^6-DPhe^7-Arg^8-Xaa^9-Lys^{10}-NH_2 (isomer denoted on the axis) from the cloned hMC1R: (A) the percent peptide bound after the peptides have been removed from the cells for 1 h and (B) the percent peptide bound after the peptides have been removed from the cells for 4 h. At the 1 h time point, all the peptides are >70% bound to the cells, whereas at the 4 h time point, only MTII and the 2*S*,3*S*-containing peptide remain >65% bound.

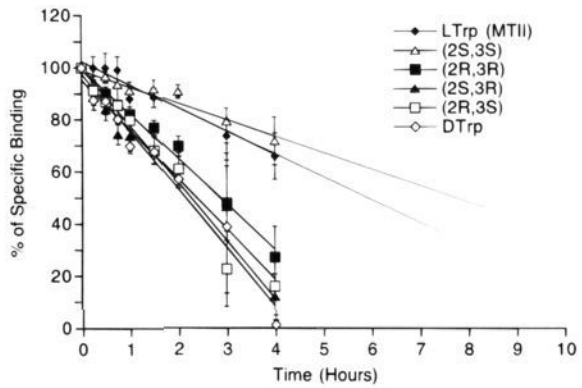

Figure 7. Dissociation rates of the diastereoisomeric peptides, Ac-Nle^4-Asp^5-His^6-DPhe^7-Arg^8-Xaa^9-Lys^{10}-NH_2 (with the isomer denoted in parentheses), from the cloned human melanocortin receptor, hMC1R. The peptides are preincubated for 1 h at 10^{-6} M to saturate the receptor sites. The peptides are removed from the cells which are washed with binding buffer for a minimal of three times followed by addition of the radioligand [^{125}I][Tyr^2,Nle^4,DPhe^7]α-MSH. The counts of radioligand associated with the cells are determined at various times (n = 4), and the respective percentage of ligand remaining bound to the receptor is determined.

Table 2. Observed Dissociation Characteristics of Melanotropin Peptides

Ac-Nle^4-Asp^5-His^6-DPhe^7-Arg^8-Xaa^9-Lys^{10}-NH_2 from the hMC1 Receptor over a Time Period of 4 h

peptide	residue at position 9	extrapolated[a] $t_{1/2}$ value	apparent[b] k_{-1} (h^{-1})	relative[c] dissociation rate
I	LTrp (MTII)	5.80	0.12	1.00
II	2*S*,3*S*	7.85	0.09	0.75
III	2*R*,3*S*	2.25	0.31	2.58
IV	2*S*,3*R*	2.30	0.30	2.50
V	2*R*,3*R*	2.50	0.28	2.33
VI	DTrp	2.10	0.33	2.75

[a] Determined by extrapolation of the slope until 50% of the peptide was bound relative to the specific binding, Figure 7. [b] Dissociation rate calculated from the first-order equation (ln 1/2)/$t_{1/2}$ = k_{-1}, using the $t_{1/2}$ value listed. [c] The relative rates were determined by setting MTII equal to 1 and dividing the apparent k_{-1} by the MTII value.

receptor topographical preference is a current goal of protein and peptide research.[29,30] To effectively design compounds possessing desired biological activities, knowledge must be acquired regarding ligand bioactive conformations and topology. Extensive structure–function activity studies in combination with biophysical methods such as nuclear magnetic resonance (NMR), circular dichroism (CD), infrared (IR), and Raman spectroscopies have become invaluable tools in determining proposed bioactive conformations. The problem remains that most of these peptide ligands are linear and have a large number of available conformations. Identification of the biologically relevant conformations has required a tremendous effort and much speculation. To overcome this problem, the use of conformational constraints such as side chain-to-side chain, side chain-to-backbone, and backbone-to-backbone cyclization has become common practice. This approach has been successful in creating ligands possessing enhanced biological potencies while decreasing the number of allowed backbone conformations. These modifications still allow for a large degree of freedom and flexibility of the amino acid side chains and their presentation to the receptor. The torsional angle about the C^α–C^β bond, χ_1 (Figure 1), is an important conformational parameter for amino acid side chain groups. This torsion angle can allow for a variety of preferred side chain rotamer populations, depending on the chirality at both α- and β-carbon atoms. To limit the flexibility and available torsion angles in this region, addition of a methyl group at either the *pro-R* or *pro-S* position should decrease the number of available side chain rotomer populations.[30]

Heat–alkali treatment of α-MSH results in prolonged biological activity, as determined on amphibian melanophores.[31–34] These studies led to the synthesis and biological characterization of [Nle^4,DPhe^7]α-MSH (NDP-MSH). This melanotropic peptide possessed superpotent and prolonged biological activity and greatly increased stability to enzymatic degradation.[35] This and related [Nle^4,DPhe^7]α-MSH-substituted melanotropins were found to stimulate prolonged activity of tyrosinase, the rate-limiting enzyme in melanin biosynthesis, in Cloudman S91 murine melanoma cells[36] and maintained prolonged activity on vertebrate chromatophores.[37] These and further studies suggested that this prolongation effect was sustained by the stereochemical inversion of LPhe to DPhe at position 7,[11,38] but not all analogues containing DPhe^7 exhibited prolonged activity.[39]

The importance of the chirality and aromatic side chain functionality of the Phe[7] initiated a study of χ_1 side chain conformational constraints. These replacements include L- or D-phenylglycine (Pgl), L- or D-1,2,3,4-tetrahydrosioquinolinecarboxylic acid (Tic),[40] and β-methylphenylalanine.[41] Interestingly, the DPgl substitution reproduced the prolonged activity of the lead compound but only in one bioassay system examined. NMR studies of these derivatives implied that ring stacking interactions between the phenylalanine and tryptophan side chains may be relevant to the prolongation phenomena.[40]

Recent studies of melanotropin analogues indicate that the tryptophan indole side chain may be an important pharmacophore for α-MSH-related peptides (unpublished results).[42] Substitutions at the Trp[9] position of α-MSH analogues has not been extensively examined. A brief discussion of structure–function studies relating to substitutions at the Trp[9] position has been reported,[43] with the substitutions of pentamethylphenylalanine (Pmp), Phe, Trp(NPS), and Leu all possessing decreased potencies compared to α-MSH in the bioassays examined.[44] Inversion of chirality of LTrp[9] to DTrp[9], however, either maintained potency or enhanced it, depending on the bioassay examined.[45] The importance of the indole ring at the 9 position appears, from these limited studies, to be biologically important. This is exemplified by the Pmp[9] substitution, which was made on the basis of maintaining similar donor properties to the native Trp residue but possessed a 50% decrease in potency.[44] This emphasized that the chemical properties of the indole ring may be crucial for α-MSH ligands to possess the necessary stereochemical characteristics for biological function.

To further probe the three-dimensional topographical presentation of the indole ring to the receptor, we limited χ_1 side chain flexibility by introducing a methyl group on the β-carbon of the Trp residue.[25] We choose the semirigid, superpotent, monocyclic peptide Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2[15] as our conformationally constrained template to examine the biological effects of introducing a β-methyl group and restricting the rotomer populations of the indole ring (Figure 1). Ranking the four β-MeTrp[9] isomers for relative potency (Table 1) in the frog skin bioassay we found 2*R*,3*S* > 2*S*,3*S* = 2*R*,3*R* ≫ 2*S*,3*R* and, in hMC1R binding and cAMP bioassays, 2*S*,3*S* > 2*R*,3*R* > 2*R*,3*S* ≫ 2*S*,3*R*.

In addition to effecting biological potencies, incorporation of these β-MeTrp residues has lead to a extraordinary finding regarding the biological activity of prolongation, or residual activity. Although extensive structure–function studies of α-MSH peptides have been undertaken (for reviews, see refs 11, 41, and 42–44), a rational approach to the design of this biological property has not been identified since residual activity could not be correlated with either primary sequence or biological potencies. Thus, the successful design of analogues maintaining this prolonged activity could not be achieved.

The analogues containing β-MeTrp[9] isomers, however, demonstrate a remarkable systematic alteration in the prolongation effect (Figure 4). All of these analogues maintain the same elemental composition, primary sequence, and, presumably, the same secondary structure. The only notable difference between these analogues is the orientation of the side chains in topographical space and the side chain rotomer populations (Figure 1). Analogue **V** possesses superprolonged biological activity, whereas analogue **II** possesses no residual biological activity whatsoever, with analogues **III** and **IV** demonstrating intermediate residual activity (Figure 4). These remarkable findings show that compounds which are structurally similar may be used as tools to identify the biological mechanism(s) responsible for the prolongation of biological activity.

One obvious explanation to account for prolonged biological activity is the enzymatic stability of the peptide to proteases. This may be a possibility for other prolonged acting peptides, such as [Nle[4],DPhe[7]]α-MSH,[35,46] but for the analogues presented in this study, this can be dismissed as the central hypothesis. These compounds contain a monocyclic lactam bridge between two side chains, a Nle at position 4, a DPhe at position 7, and a β-MeTrp at position 9, all of which are modifications which do not naturally occur in eukaryotic systems, hence leading to stability due to the lack of protease molecular recognition. The lead compound Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2 (MTII) was, in fact, found to be resistant to inactivation by the proteolytic enzymes α-chymotrypsin, trypsin, and pepsin.[47] In addition, *in vivo* human studies of the lead compound have demonstrated that this compound is excreted in the urine, intact, as monitored by RP-HPLC (unpublished results).

Another possible explanation for the prolongation effect is that the peptide interacts to a greater extent with the lipid environment. This hypothesis is based on the membrane lipid–peptide interactions discussed by Sargent and Schwyzer.[48] A study incorporating different fatty acid conjugates onto the template FA-[Asp[5],DPhe[7],Lys[10]]α-MSH(5–10)-NH_2 (FA = hexanoic (Hxl), decanoic (Dcl), myristic (Mrl), or palmitic (Pml)) demonstrated a "creeping" potency increase over time and prolonged activity, whereas the unconjugated peptide was devoid of prolonged activity.[49,50] Modifications of the lead peptide, Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2, by incorporation of amino acid residues possessing a positive charge into the lactam side chain cycle were reported to increase compartmentalization of these derivatives into the membrane phase but nonetheless lacked prolonged activity.[16] This later result is unexpected due to the membrane compartmentalization and because previous studies of a separate series of melanotropin derivatives possessing the same dibasic modifications in the lactam side chain monocycle demonstrated prolonged activity.[51] The former studies were based on the theory of increasing the peptide interactions with the lipid bilayer by increasing the ligands positive charge to interact favorably with the bilayer's negatively charged head groups. A separate study of the interaction of the melanotropin peptides α-MSH, [Nle[4],DPhe[7]]α-MSH, and the lead peptide for this study, Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2, MTII, with model lipids suggested that MTII interacted differently with the lipid environment than the other melanotropins examined.[55] Peptide–lipid interactions, as shown in these studies, can account for prolonged activity. In our study, however, the peptides consist of a template

Table 3. Physicochemical Properties of the Ac-Nle[4]-Asp[5]-His[6]-DPhe[7]-Arg-[8]Xaa[9]-Lys[10]-NH_2 Melanotropin Analogues Modified at Position 9

peptide	optical rotation $[\alpha]^{24}_{598}$ (deg) (10% aqueous HOAc)	R_f, TLC solvents[a] A	B	C	HPLC k' [b]	FAB-MS (M + 1) obsd	calcd
II	−37.50 (c = 0.37)	0.63	0.70	0.042	5.3	1039	1039
III	+1.83 (c = 0.55)	0.66	0.69	0.054	4.4	1039	1039
IV	−57.69 (c = 0.35)	0.64	0.68	0.027	5.3	1039	1039
V	−7.26 (c = 0.41)	0.66	0.75	0.042	4.9	1039	1039
VI	3.75 (c = 0.80)	0.81	0.73	0.260	2.6	1025	1025

[a] R_f values on thin-layer chromatograms of silica gel were observed in the following solvent systems: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4), (B) ammonium hydroxide/water/2-propanol (1:1:3:), and (C) upper phase of 1-butanol/acetic acid/water (4:1:5). [b] HPLC k' = (peptide retention time−solvent retention time)/solvent retention time in a solvent system of 10% acetonitrile in 0.1% trifluoroacetic acid and a gradient to 90% acetonitrile over 40 min. An analytical Vydac C_{18} column was used with a flow rate of 1.5 mL/min.

differing only in the topographical presentation of the tryptophan side chain. These peptides demonstrate large differences in their prolonged activity (Figure 4), suggesting that peptide−lipid interactions are not the only biomolecular interactions accounting for the prolongation phenomena. Theoretically, all of the peptides in this study should be able to interact with the same lipid environment to the same extent and for the same duration of time.

Due to the nature of the classical peripheral pigmentation bioassays used to monitor the potency of α-MSH analogues, which measured only the end point of melanosome aggregation, only speculation as to the possible mechanism has been available previously. Presently, however, with the advances in molecular cell biology techniques, the peripheral melanocortin receptor, designated MC1R, has been cloned from both the mouse and human and has been successfully transfected into stable cell lines.[9,10,27] The relative potencies of both binding and cAMP generation in the cloned receptor system offer no clear explanation for this residual activity, other than the unfavorable topography of the (2*S*,3*R*)-β-MeTrp side chain. The experiments examining the percentage of ligand that remained bound to the cells suggest the residual activity of these peptides may involve a mechanism that is receptor mediated and/or, perhaps, a postreceptor event associated with signal transduction.

By using the topographical constraints of the β-methyl-substituted amino acid β-methyltryptophan, we have restricted the topology of the side chain that is being presented to the receptor. Although rotation about the χ_1 and χ_2 torsion angles is affected and introduces a higher energy barrier rotation about χ_1 than the unmethylated *pro-R* or *-S* hydrogen on the β-carbon, all rotamer populations are accessible within the energy given by kT. We suggest that by restricting the indole ring presentation to the receptor, one of the rotamer populations is specifically favored by the receptor and hence has an increased binding affinity. The other rotamer populations will require increased energy to position the indole ring with the desired topology and hence may demonstrate different ligand rates of binding. Once binding has occurred, and if other topographical requirements necessary for signal transduction occur, the side chain rotamer populations may need to be furthered modified in accordance with the receptor requirements for these mechanisms.

The information provided here should be valuable for the identification of the superprolonged bioactive conformations of α-MSH analogues. We have not only limited φ,ψ space by the use of a monocyclic template, but we also have restricted χ space of the tryptophan side chain. This study demonstrates how conformational and topographical constraints can not only be used to probe receptor−peptide recognition features but can also be used as tools to design analogues with a long duration of biological activity and to study biological mechanisms associated with peptide hormone G-protein-coupled receptors.

Experimental Section

Materials. TLC was done on Merck silica gel 60 F_{254} plates using the following solvent systems: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4), (B) ammonium hydroxide/water/2-propanol (1:1:3:), and (C) upper phase of 1-butanol/acetic acid/water (4:1:5). The peptides were detected on the TLC plates using iodine vapor. Final peptide purification was achieved using a semipreparative RP-HPLC C_{18}-bonded silica gel column (Vydac 218TP1010, 1.0 × 25 cm). The peptides were eluted with a linear acetonitrile gradient (10−50%) over 40 min at a flow rate of 5.0 mL/min, with a constant concentration of TFA (0.1%, v/v). The linear gradient was generated with a Perkin-Elmer 410 LC Bio pump system. The separations were monitored at 280 nm and integrated with a Perkin-Elmer LC-235 diode array detector. Amino acid analyses were performed at the University of Arizona Biotechnology Core Facility. The system used was an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl) and precolumn phenylthiocarbamoyl amino acid (PTC-AA) analysis. No corrections were made for amino acid decomposition. Optical rotation values were measured on an Autopol III instrument at 589 nm in 10% acetic acid. The pMBHA resin (0.37 mmol of NH_2/g) was purchased from Peptides International Inc. (Louisville, KY). N^{α}-*tert*-Butyloxycarbonyl (Boc)-protected amino acids and amino acid derivatives were purchased from Bachem (Torrance, CA), with the exception of the β-methyltryptophan amino acids which were converted to their N^{α}-*tert*-butyloxycarbonyl derivative with di-*tert*-butyl bicarbonate (Bachem California, Torrance, CA) following literature procedures. All purchased amino acids were of the L configuration except for phenylalanine which was of the D configuration. The synthesis of each of the four isomers of indole-protected β-methyltryptophan has been previously reported.[25] BOC was used for N^{α} protection, and the reactive side chains of the amino acids were protected as follows: Lys, with fluorenylmethyloxycarbonyl (Fmoc); Asp, with fluorenylmethyl ester (OFm); His, with benzyloxymethyl (Bom); Arg, with tosyl (Tos); and β-MeTrp with 2-mesitylenesulfonyl (MeS). All reagents and solvents were ACS grade or better and used without further purification. The purity of the finished peptides was checked by TLC in three solvents, and analytical RP-HPLC at 280 and 220 nm in all cases was greater than 95% pure as determined by these methods. The structures of the pure peptides were confirmed by fast atom bombardment (FAB) mass spectrometry and amino acid analysis (Table 3).

Peptide Synthesis. The peptides were synthesized using a manual synthesizer adapted from previously published methods.[15] MTII, Ac-Nle-Asp-His-DPhe-Arg-Trp-Lys-NH_2, was

obtained from a previous synthesis[15] and was not resynthesized in this study.

General Procedure for the Synthesis and Cleavage of Ac-Nle-Asp-His-DPhe-Arg-Xaa-Lys-NH_2 Peptides. Approximately 0.5 mmol of *p*-methylbenzhydrylamine (pMBHA) resin (0.37 mmol of NH_2/g of resin or 0.24 mmol of NH_2/g of resin) was neutralized with 10% diisopropylethylamine (DIEA) in dichloromethane (DCM) (2 × 50 mL) followed by a DCM wash (4 × 50 mL). The amino acid N^{α}-Boc-Lys (N^{ϵ}-Fmoc) (1.2 excess) was coupled to the resin for 2 h in *N*-methylpyrrolidinone (NMP) using (benzotriazolyloxy)tris(dimethylamino)-phosphonium (BOP) (1.4-fold excess,) and DIEA (1.6-fold excess). The N^{α}-Boc protecting group was removed by washing the resin for 2 min in 50% trifluoroacetic acid (TFA), 2% anisole in DCM (1 × 50 mL) followed by a 20 min 50% TFA, 2% anisole in DCM wash (1 × 50 mL). The resin was then washed with DCM (3 × 50 mL), neutralized with 10% DIEA in DCM (2 × 50 mL), and washed with DCM (4 × 50 mL). N^{α}-Boc-Trp (N^{in}-MeS) was coupled using identical coupling conditions as described above. The sequential coupling of the amino acids N^{α}-Boc-Arg(N^{g}-Tos), N^{α}-Boc-DPhe, and N^{α}-Boc-His (N^{τ}-Bom), using DIC (6.5 mmol) and HOBT (6.5 mmol) as coupling reagents for 2 h and N^{α}-Boc removal conditions described above, added to the growing peptide chain.

Then N^{α}-Boc-Asp (β-OFm) (1.2 excess) was coupled to the peptide-resin for 2 h in NMP using BOP (1.4-fold excess) and DIEA (1.6-fold excess) and N^{α} deprotected as described above. At this stage, the N^{ϵ}-Fmoc and β-OFm protecting groups of lysine and aspartic acid, respectively, were removed by the addition of 20% piperidine/NMP (1 × 50 mL) and mixed for 20 min. The resin was washed with DCM (7 × 50 mL) followed by cyclization of the free acid side chain of Asp to the free amine side chain of Lys by the addition of BOP (5-fold excess) and DIEA (6-fold excess) in NMP for 2 h. This process was repeated until a negative Kaiser test resulted.[53]

Upon complete formation of the lactam cycle, N^{α}-Boc-Nle was coupled to the growing peptide chain using DIC/HOBT coupling conditions. After removal of the N^{α}-Boc protecting group, N-terminal acetylation was carried out by the addition of 2 mL of acetic anhydride and 1 mL of pyridine, for 20 min. The resin was washed with DCM (6 × 50 mL) and dried *in vacuo* to yield ca. 2.0 g of peptide-resin. Approximately one-half of the peptide-resin was added to an equivalent amount of both *m*-cresol and thioanisole followed by the addition of ca. 10 mL of anhydrous HF.[54] The mixture was stirred at 0 °C for 60 min followed by the removal of the scavengers and HF under high vacuum. To insure complete removal of the scavengers and nonpeptide material, anhydrous ethyl ether (5 × 30 mL) was added to each vessel and the mixture filtered using a course glass frit. The crude peptide was dissolved in glacial acetic acid (4 × 50 mL) and lyophilized to give 100–200 mg of crude peptide. A portion of the crude peptide was purified by preparative RP-HPLC and yielded 50–60% of pure peptide. The analytical properties for each peptide are given in Table 3.

Ac-Nle-Asp-His-DPhe-Arg-(2S,3S)β-MeTrp-Lys-NH_2 (II). The title peptide was synthesized using 0.37 mmol of NH_2/g of pMBHA resin on a 0.54 mmol scale to yield 1.6 g of peptide-resin. The sample was split, and 0.8 g was subjected to HF cleavage to yield 130 mg of crude peptide. A 20 mg sample of the crude peptide was purified by preparative RP-HPLC to give 9.2 mg (white powder) of the title peptide. Amino acid analysis of **II**: Nle (1.09), Asp (1.00), His (0.95), Phe (1.00), Arg (1.05), Lys (0.97). The other analytical properties of this peptide product are give in Table 3.

Ac-Nle-Asp-His-DPhe-Arg-(2R,3S)β-MeTrp-Lys-NH_2 (III). The title peptide was synthesized using 0.24 mmol of NH_2/g of pMBHA resin on a 0.50 mmol scale to yield 2.4 g of peptide-resin. The sample was split, and 1.27 g underwent HF cleavage to yield 190 mg of crude peptide. A 20 mg sample of the crude peptide was purified by preparative RP-HPLC to give 10.3 mg (white powder) of the title peptide. Amino acid analysis of **III**: Nle (1.14), Asp (0.91), His (0.98), Phe (1.00), Arg (0.90), Lys (0.91). The other analytical properties of this peptide product are give in Table 3.

Ac-Nle-Asp-His-DPhe-Arg-(2S,3R)β-MeTrp-Lys-NH_2 (IV). The title peptide was synthesized using 0.24 mmol of NH_2/g of pMBHA resin on a 0.40 mmol scale to yield 1.9 g of peptide-resin. The sample was split, and 0.98 g underwent HF cleavage to yield 200 mg of crude peptide. A 20 mg sample of the crude peptide was purified by preparative RP-HPLC to give 9.3 mg of white powder of the title peptide. Amino acid analysis of **IV**: Nle (1.10), Asp (0.91), His (0.90), Phe (1.00), Arg (0.94), Lys (0.98). The other analytical properties of this peptide product are give in Table 3.

Ac-Nle-Asp-His-DPhe-Arg-(2R,3R)β-MeTrp-Lys-NH_2 (V). The title peptide was synthesized using 0.24 mmol of NH_2/g of pMBHA resin on a 0.51 mmol scale to yield 2.32 g of peptide-resin. The sample was split, and 1.19 g underwent HF cleavage to yield 100 mg of crude peptide. A 65 mg sample of the crude peptide was purified by preparative RP-HPLC to give 17 mg of white powder of the title peptide. Amino acid analysis of **V**: Nle (1.13), Asp (0.97), His (1.04), Phe (1.00), Arg (1.07), Lys (0.91). The other analytical properties of this peptide product are give in Table 3.

Ac-Nle-Asp-His-DPhe-Arg-DTrp-Lys-NH_2 (VI). The title peptide was synthesized using 0.40 mmol NH_2/g of pMBHA resin on a 0.51 mmol scale to yield 1.77 g of peptide-resin. A 1.05 g sample of the peptide-resin was taken and underwent HF cleavage to give 50 mg of crude peptide. A 10.6 mg portion of the crude peptide was purified by preparative RP-HPLC and yielded 6.0 mg of pure title peptide. Amino acid analysis of **VI**: Nle (1.05), Asp (0.98), His (1.02), Phe (1.00), Arg (0.99), Lys (0.91). Other analytical properties of this peptide product are give in Table 3.

Bioassay. Bioassays using frog (*Rana pipiens*) skins were performed by previously published procedures.[26,55] In these bioassays skins become dark in response to a melanotropin. This darkening response can be conveniently monitored by photoreflectance methods. The potency of each peptide was determined from dose–response curves comparing the melanotropic activity of the analogues with that of the native hormone α-MSH. Prolonged residual biological activities of the peptides were monitored for up to 3 h, after removal of all peptide from the incubation medium by several washes.[55] Dose–response curves are shown in Figure 3, Table 1 lists the relative potencies and EC_{50} values, and Figures 4 and 5 show prolonged activities of the peptides prepared in this study.

Binding Assays. The coding region of hMC1R, cloned from a human genomic EMBL3 phage library (Clontech, Palo Alto, CA), was placed into the eukaryotic expression vector CMVneo and stably transfected into L-cells. Transfected cells were grown to confluence in 12-well (2.4 × 1.7 cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) containing 4.5 g/100 mL glucose, 10% fetal calf serum, 100 units/mL penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL geneticin. For the assays, this medium was removed and cells were washed twice with a freshly prepared binding buffer consisting of 97% minimum essential medium with Earle's salt (MEM; GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. A 400 μL dilution of the peptide being tested was added to the well, with the concentrations ranging between 10^{-11} and 10^{-6} M. Next, a 100 μL solution of [^{125}I]-[Tyr^2,Nle^4,DPhe^7]α-MSH (100 000 cpm/well) was added to each well, and the cells were incubated at 37 °C for 40 min. Dissociation studies used a ligand concentration of 10^{-6} M to ensure complete saturation of the receptor binding sites. The ligand remained in the assay medium until [^{125}I]-[Tyr^2,Nle^4,DPhe^7]α-MSH was added for the designated incubation time (minimum time of 60 min and maximum time of 3 h 45 min). The medium was subsequently removed, and each well was washed twice with binding buffer. The cells were lysed by the addition of 0.5 mL of 0.1 M NaOH and 0.5 mL of 1% Triton X-100. The mixture was left to react for 5 min, and the contents of each well transferred to labeled 16 × 150

mm glass tubes and quantified in a γ-counter. [^{125}I]-[Tyr^2,Nle^4,DPhe^7]α-MSH was prepared and purified by methods described previously.[56]

cAMP Assays. A commercially available cAMP assay kit (TRK 432; Amersham Corp.) was employed. L-Cells transfected with the human MC1 receptors were grown to confluence in 12-well (2.4 × 1.7 cm) tissue culture plates. The cells were maintained in DMEM (GIBCO) containing 4.5 g/100 mL glucose, 10% fetal calf serum, 100 units/mL penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL geneticin. For the assays, the medium was removed and cells were washed twice with Earle's balanced salt solution containing 10 mM HEPES (pH 7.4), 1 mM glutamine, 26.5 mM sodium bicarbonate, and 100 mg/mL bovine serum albumin. An aliquot (0.5 mL) of Earle's balanced salt solution (EBSS; GIBCO) was placed into each well along with 5 μL of 2×10^{-2} M isobutylmethylxanthine. Varying concentrations of melanotropins were added and the cells incubated for 30 min at 37 °C. Ice-cold 100% ethanol (1.0 mL/well) was added to stop the reaction. The incubation medium and scraped cells were transferred to 16 × 150 mm glass tubes and then placed on ice for 30 min. The precipitate was then centrifuged for 10 min at 1900g, and the supernatant was dried under a nitrogen stream and resuspended in 50 mM Tris and 2 mM EDTA (pH 7.5). The cAMP content was measured by competitive binding assay using the assay protocol detailed in the Amersham TRK 432 cAMP kit.

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