β-Methylation of the Phe[7] and Trp[9] Melanotropin Side Chain Pharmacophores Affects Ligand−Receptor Interactions and Prolonged Biological Activity

Carrie Haskell-Luevano,[†] Kate Toth,[†] Lakmal Boteju,[†] Constatin Job,[‡] Ana Maria de L. Castrucci,[§] Mac E. Hadley,[||] and Victor J. Hruby*,[†]

Departments of Chemistry and Anatomy, University of Arizona, Tucson, Arizona 85721, Arizona Research Laboratory, University of Arizona, Tucson, Arizona 85724, and São Paulo, Departamento de Fisiologia, Instituto de Biociencias, Universidade de Sao Paulo, Brazil

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Topographically modified melanotropin side chain pharmacophore residues Phe[7] and Trp[9] in a cyclic peptide template (Ac-Nle[4]-c[Asp-His-Xaa[7]-Arg-Yaa[9]-Lys]-NH_2) and Phe[7] in a linear peptide template (Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-Xaa[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2) result in differences in potency and prolonged biological activity in the frog and lizard skin bioassays. These topographic modifications included the four isomers of β-methylphenylalanine (β-MePhe)[7] and β-methyltryptophan (β-MeTrp)[9] and the two isomers of 1,2,3,4-tetrahydro-β-carboline (Tca).[9] Modifications in the cyclic template resulted in up to a 1000-fold difference in potency for the β-MePhe[7] stereoisomeric peptides; up to a 476-fold difference in potency resulted for the β-MeTrp[9] peptides, and about a 50-fold difference between the Tca[9]-containing peptides. Up to a 40-fold difference in potency resulted for the β-MePhe[7] stereoisomeric peptides using the linear template in these assays. The relative potency ranking for modifications in the cyclic template of β-MePhe[7] were 2*R*,3*S* > 2*S*,3*S* = 2*S*,3*R* > 2*R*,3*R* in the frog assay and 2*S*,3*R* > 2*R*,3*S* > 2*S*,3*S* > 2*R*,3*R* in the lizard assay. The relative potencies for modifications in the cyclic template of β-MeTrp[9] were 2*R*,3*S* > 2*R*,3*R* > 2*S*,3*S* ≫ 2*S*,3*R* in the frog assay and 2*S*,3*S* = 2*R*,3*R* > 2*R*,3*S* > 2*S*,3*R* in the lizard assay. The relative potencies for modifications in the cyclic template of Tca[9] were DTca > LTca in both assays. Significant differences in prolonged (residual) activities were also observed for these modified peptides and were dependent upon stereochemistry of the β-methyl amino acid, peptide template, and bioassay system. Furthermore, comparisons of β-MeTrp[9] stereoisomeric peptides on the frog, lizard, and human MC1 receptors suggest that structure−activity relationships on both the classical frog and lizard skin bioassays do not necessarily predict corresponding SAR profiles for the human melanocortin receptors, indicating a remarkable species specificity of the MC1 receptor requirements.

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 pro-opiomelanocortin (POMC) gene.[1] Several biological activities have been attributed to these peptide hormones. Both peripheral actions and central nervous system activities related to α-MSH and its analogues have been reviewed.[2−5] The most recognized biological activity of α-MSH has been its role in regulating skin pigmentation and tanning.[6,7] These multiple physiological effects have made the α-melanotropin tridecapeptide the target of extensive structure−activity studies[2−5] and led to the design and synthesis of the superpotent tridecapeptide Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-DPhe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (NDP-MSH, MTI)[8,9] and the cyclic heptapeptide Ac-Nle[4]-c[Asp[5]-His[6]-DPhe[7]-Arg[8]-Trp[9]-Lys[10]]-NH_2 (MTII).[10,11]

Structure−activity studies of truncated peptides derived from NDP-MSH identified a tripeptide possessing micromolar activity *in vitro*.[12] Further stereochemical and substitution modifications of the tripeptide led to the identification of the melanotropin side chain pharmacophores for the frog melanocortin receptor.[13] This pharmacophore model indicated the Phe[7] and Trp[9] amino acids as being necessary to generate a minimal physiological response, whereas the Arg[8] residue could be substituted, albeit with some decreased potency. Modifications of the DPhe[7] residue in the MTII template by *p*-iodo (pI) DPhe[7] and 2′-naphthylalanine DNal(2′)[7] resulted in antagonists with pA_2 values >10 in the frog skin bioassay.[14] Topographical variations of Trp[9] in the MTII template have demonstrated the importance of this residue for prolonged biological activity.[15] Conformational studies of the side chain χ_1 angles for the core DPhe-Arg-β-MeTrp sequence by ^{1}H NMR led to the hypothesis that not only was the topography of the Trp indole moiety important but that it was a combination of the Phe[7] and Trp[9] side chain rotomer populations that may be responsible for prolonged (residual) biological activity.[16,17]

The rationale for incorporating a methyl group on the β-carbon of an aromatic amino acid (Figure 1) to identify the proposed three-dimensional orientation of a particular amino acid with its corresponding receptor[18−20] has been applied to a diverse number of peptides including the opioids,[21−24] dynorphin,[25,26] glucagon,[27] oxytocin,[28] CCK-8,[29] and somatostatin.[30] Homonuclear[30] and heteronuclear[29] NMR studies of β-MePhe stereoisomers incorporated into somatostatin and CCK-8

* To whom reprint requests should be addressed at the Department of Chemistry, University of Arizona.
[†] Department of Chemistry, University of Arizona.
[‡] Arizona Research Laboratory.
[§] Universidade de Sao Paulo.
[||] Department of Anatomy, University of Arizona.
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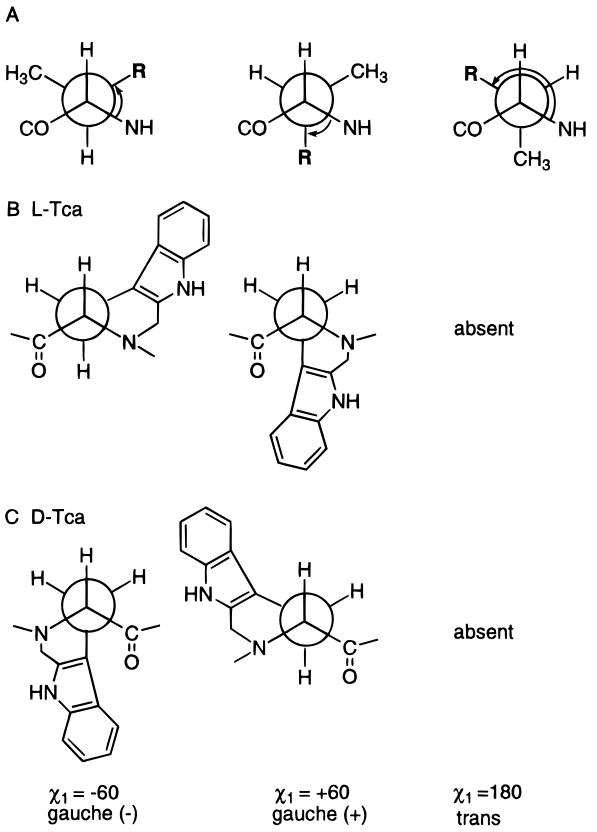


Figure 1. Newman projection of the three amino acid side chain rotomer populations (looking down the α-β carbon-carbon bond). (A) Illustrates a generic β-methylated amino acid with the side chain functional group designated by R. (B and C) Newman projections of the L- and D-isomers of the 1,2,3,4-tetrahydro-β-carboline (Tca) amino acids, respectively.

peptides, respectively, identified the *gauche (−)* (45% and 56%) side chain rotomer being the most populated for (2*S*,3*S*)-β-MePhe and *gauche (+)* (60%, CCK-8) for (2*R*,3*R*)-β-MePhe. The (2*S*,3*R*)-β-MePhe rotomer population appears to be undifferentiated between the *gauche (−)* (47%, CCK-8) and *trans* (40% and 41%) while only possessing a small percentage of the *gauche (+)* rotomer. The preferred rotomer population of (2*R*,3*S*)-β-MePhe also appears to be *gauche (+)* (64%, CCK-8) (Figure 2). Similarly, homonuclear ^{1}H NMR studies of β-MeTrp[8] and β-MeTrp[11] incorporated into somatostatin[30] reported the *gauche (−)* (44% and 45%) rotomer to be preferentially occupied by the (2*S*,3*S*)-β-MeTrp, *trans* (57−68%) for (2*R*,3*S*)-β-MeTrp, *gauche (+)* (65−72%) for the (2*R*,3*R*)-β-MeTrp, and possibly *trans* (38−57%) for the (2*S*,3*R*)-β-MeTrp isomer. 1,2,3,4-Tetrahydro-β-carboline (Tca), which is similar to 1,2,3,4-tetrahydrosioquinoline-3-carboxylic acid (Tic),[31] can only possess either *gauche (+)* or *gauche (−)* side chain rotomer populations since the indole moiety is cyclized to the peptide backbone N^{α} (Figure 1). These biophysical studies demonstrate that β-methyl aromatic amino acids incorporated into peptides provide valuable tools in identifying biologically and topographically important side chain structural information in relation to biological activity.

In this study, we examined the effect of topographical constraints on the Phe[7] and Trp[9] melanocortin pharmacophores on potency and prolonged biological activity. These constraints consisted of the four stereoisomers of β-MePhe[7] and β-MeTrp[9], the two isomers of L/D-Tca in the MTII peptide template, and the four isomers of β-MePhe[7] in the NDP-MSH template. These peptide derivatives were studied in the classical *Rana pipiens* (frog)[32] and *Anolis carolinensis* (lizard)[33,34] skin bioassays.

Results

The syntheses of the melanotropin analogues discussed herein were accomplished by solid-phase synthetic methods.[11,15,35] The optically pure stereoisomers of the unusual amino acids were synthesized as previously reported.[22,36] These peptide 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, amino acid analysis, and optical rotation (see Supporting Information).

Biological Evaluation. The biological results for peptide analogues containing β-MePhe[7] and β-MeTrp[9] modifications in the cyclic template Ac-Nle-c[Asp-His-Xaa[7]-Arg-Yaa[9]-Lys]-NH_2 are summarized in Table 1. Table 2 summarizes the biological activities of β-MePhe[7] peptide modifications of the linear NDP-MSH template Ac-Ser-Tyr-Ser-Nle-Glu-His-Xaa[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2. These peptides were biologically evaluated in both the frog and lizard skin bioassays for potency (EC_{50}) and prolonged (residual) activity. A 3-fold change in potency is within experimental error of the frog and lizard skin bioassays. These deviations are due primarily to seasonal animal variation and take into account experimental variation.

Frog Skin Bioassay. Evaluation of modifications in the cyclic peptide template, Table 1, showed that, for the frog skin bioassay, analogue **1** containing the LL stereochemical combination for Phe[7] and Trp[9], respectively, was 100-fold less potent than α-MSH, whereas analogues **2** (DPhe[7] and LTrp[9]) and **3** (DPhe[7] and DTrp[9]) were equipotent to α-MSH. The four isomers of β-MePhe[7] (**4**−**7**) resulted in 20−167-fold decreased potency as compared to α-MSH. Analogues **8** (2*S*,3*S*-β-MeTrp[9]), **9** (2*S*,3*R*-β-MeTrp[9]), and **11** (2*R*,3*R*-β-MeTrp[9]) resulted in 3−286-fold decreased potency while analogue **10** was approximately equipotent to α-MSH. Analogue **12** (LTca[9]) resulted in a 33-fold decrease in potency, and **13** (DTca[9]) resulted in a 10-fold decrease in potency, as compared to α-MSH. The 2*S*-β-MePhe[7] analogues **4** and **5** were equipotent compared with each other and were approximately equipotent (within experimental error) to analogue **1** (nonmethylated 2*S*). The 2*R* analogues **6** and **7** possessed an 8-fold difference in potencies compared to each other and 20−167-fold decreased potencies, respectively, compared to analogue **2** (nonmethylated 2*R*). Analogues **8** and **9** (2*S*-β-MeTrp[9]) resulted in a 65-fold difference in potencies compared with each other and 4−286-fold decreased potency, respectively, compared to analogue **2** (nonmethylated 2*S*). Analogues **10** and **11** (2*R*-β-MeTrp[9]) resulted in only a 6-fold difference in potencies compared to each other and were nearly equipotent to analogue **3** (nonmethylated 2*R*). Comparison of just the stereochemistry at the β-methyl position, analogues **4** and **5** (3*S*-β-MePhe[7]) showed a 3-fold difference in potencies as did analogues **5** and **7** (3*R*-β-MePhe[7]). Analogues **8** and **10**

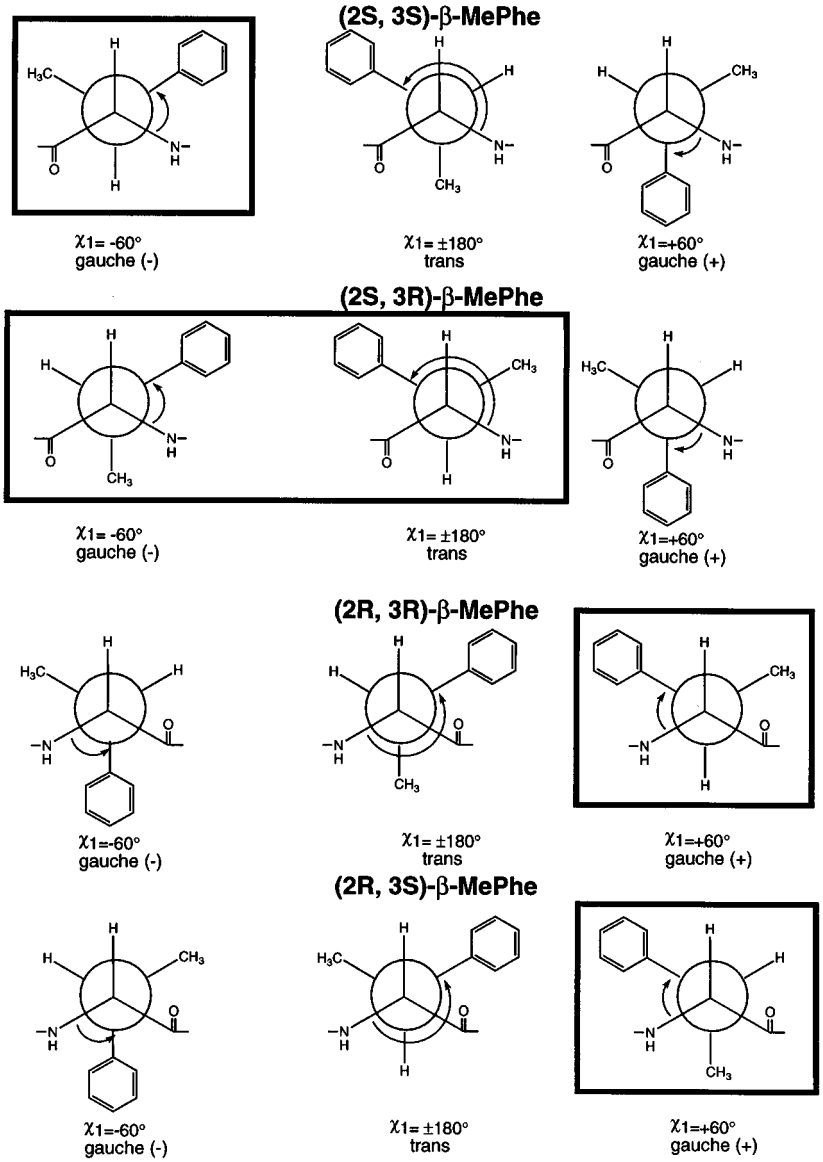


Figure 2. Illustration of the three side chain rotomer populations for each stereoisomer of β-MePhe. The populations that are enclosed in the boxes represent the populated configurations identified by homo- and heteronuclear NMR techniques of these amino acids in peptides.[29,30]

(3*S*-β-MeTrp[9]) possessed a 7-fold difference in potencies while the 3*R*-β-MeTrp[9] analogues (**9** and **11**) possessed an 86-fold difference in potencies. These latter comparisons clearly demonstrate the sensitivity of the frog skin melanocortin receptor for both the stereochemistry at the α- and β-carbons and the ultimate topographical presentation of the Trp[9] residue.

Table 2 summarizes the effect of incorporating the four isomers of β-MePhe[7] into the linear NDP-MSH template. Analogue **14** (2*S*,3*S*-β-MePhe[7]) resulted in 2-fold decreased potency compared to α-MSH and 40-fold decreased potency compared to NDP-MSH. Analogues **15** (2*S*,3*R*-β-MePhe[7]) and **17** (2*R*,3*R*-β-MePhe[7]) resulted in 5-fold increased and 2-fold decreased potencies compared to α-MSH and 4- and 40-fold decreased potencies compared with NDP-MSH, respectively. Analogue **16** (2*R*,3*S*-β-MePhe[7]) resulted in 22-fold increased potency compared to α-MSH and was equipotent to NDP-MSH in the frog skin assay. Analogues **14** and **15** (2*S*-β-MePhe[7]) showed a 10-fold difference in potencies while the 2*R*-β-MePhe[7] analogues (**16** and **17**) resulted in a 40-fold difference in potencies. Comparison of analogues **14** with **16** (3*S*-β-MePhe[7]) and **15** with **17** (3*R*-β-MePhe[7]) demonstrated 40-fold and 10-fold differences in potencies, respectively.

We have previously demonstrated that the stereochemistry at position 9 affects prolonged activity on the frog skin. After the peptides have been removed by several changes of the assay medium, analogue **2** possessed superprolonged activity, whereas analogue **3** possessed some prolonged activity (Figure 3). Analogues **2** and **3** were equipotent to each other (Table 1), suggesting that efficacy is not a factor for the prolonged activity differences observed between analogues **2** and **3**.[15] Figure 4A shows the prolonged activity of the four isomers of β-MePhe[7] (analogues **4**−**7**) in the frog skin assay. Interestingly, all stereoisomeric peptides possessed superprolonged activity except for the 2*S*,3*R*-β-MePhe[7] analogue (**5**). Figure 4B shows the differential degrees of prolonged activity for the stereoisomeric peptides containing the β-MeTrp[9] isomers in the frog skin assay.[15] The Tca[9]-containing peptides (analogues **7** and **8**) both maintained some prolonged biological activity (data not shown). Figure 4C shows the varying

Table 1. Comparative Biological Activities of Topographically Modified Peptides in the Cyclic Template

	peptide	frog skin[a] EC$_{50}$ (nM)[b]	frog skin[a] relative potency	lizard skin[a] EC$_{50}$ (nM)[b]	lizard skin[a] relative potency
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.10	1.0	1.0	1.0
1	Ac-Nle-c[Asp-His-Phe-Arg-Trp-Lys]-NH_2	10.00	0.010	ND	
2, (MTII)	Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2	0.10	1.0	0.20	5.0
3	Ac-Nle-c[Asp-His-DPhe-Arg-DTrp-Lys]-NH_2	0.10	1.0	ND	
4	Ac-Nle-c[Asp-His-(2*S*,3*S*)-β-MePhe-Arg-Trp-Lys]-NH_2	6.25	0.016	3.44	0.29
5	Ac-Nle-c[Asp-His-(2*S*,3*R*)-β-MePhe-Arg-Trp-Lys]-NH_2	6.25	0.016	0.2	5.0
6	Ac-Nle-c[Asp-His-(2*R*,3*S*)-β-MePhe-Arg-Trp-Lys]-NH_2	2.00	0.050	0.30	3.3
7	Ac-Nle-c[Asp-His-(2*R*,3*R*)-β-MePhe-Arg-Trp-Lys]-NH_2	16.67	0.006	20.0	0.05
8	Ac-Nle-c[Asp-His-DPhe-Arg-(2*S*,3*S*)-β-MeTrp-Lys]-NH_2	0.44	0.23	1.0	1.0
9	Ac-Nle-c[Asp-His-DPhe-Arg-(2*S*,3*R*)-β-MeTrp-Lys]-NH_2	28.60	0.004	6.67	0.15
10	Ac-Nle-c[Asp-His-DPhe-Arg-(2*R*,3*S*)-β-MeTrp-Lys]-NH_2	0.06	1.60	1.43	0.70
11	Ac-Nle-c[Asp-His-DPhe-Arg-(2*R*,3*R*)-β-MeTrp-Lys]-NH_2	0.33	0.30	1.0	1.0
12	Ac-Nle-c[Asp-His-DPhe-Arg-Tca-Lys]-NH_2	3.33	0.03	20.0	0.05
13	Ac-Nle-c[Asp-His-DPhe-Arg-DTca-Lys]-NH_2	1.0	0.10	11.1	0.09

[a] All peptide activities were tested at a range concentration (10^{-6}–10^{-12} M) and compared to the half-maximal effective dose of α-MSH. This assay possesses an intrinsic 3-fold experimental error. [b] EC$_{50}$ = Concentration of peptide required for 50% maximal stimulation (N = 5). These values were obtained based on the relative potency value as compared to α-MSH. The EC$_{50}$ value of α-MSH was determined (1.0×10^{-10} M in the frog skin assay and 1.0×10^{-9} M in the lizard skin assay), by the average of this peptide over several years, with the relative EC$_{50}$ values of each peptide calculated from this value for normalization.

Table 2. Comparative Biological Activities of Topographically Modified Phe^7 Peptides in the Linear Template

	peptide	frog skin[a] EC$_{50}$ (nM)[a]	frog skin[a] relative potency	lizard skin[a] EC$_{50}$ (nM)[b]	lizard skin[a] relative potency
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.10	1.00	1.0	1.0
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.005	22.00	0.50	2.0
14	Ac-Ser-Tyr-Ser-Nle-Glu-His-(2*S*,3*S*)-β-MePhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.20	0.50	3.33	0.3
15	Ac-Ser-Tyr-Ser-Nle-Glu-His-(2*S*,3*R*)-β-MePhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.02	5.00	1.0	1.0
16	Ac-Ser-Tyr-Ser-Nle-Glu-His-(2*R*,3*S*)-β-MePhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.005	22.00	0.50	2.0
17	Ac-Ser-Tyr-Ser-Nle-Glu-His-(2*R*,3*R*)-β-MePhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.20	0.50	1.0	1.0

[a] All peptide activities were tested at a range concentration (10^{-6}–10^{-12} M) and compared to the half-maximal effective dose of α-MSH. This assay possesses an intrinsic 3-fold experimental error. [b] EC$_{50}$ = Concentration of peptide required for 50% maximal stimulation (N = 5). These values were obtained based on the relative potency value as compared to α-MSH. The EC$_{50}$ value of α-MSH was determined (1.0×10^{-10} M in the frog skin assay and 1.0×10^{-9} M in the lizard skin assay), by the average of this peptide over several years, with the relative EC$_{50}$ values of each peptide calculated from this value for normalization.

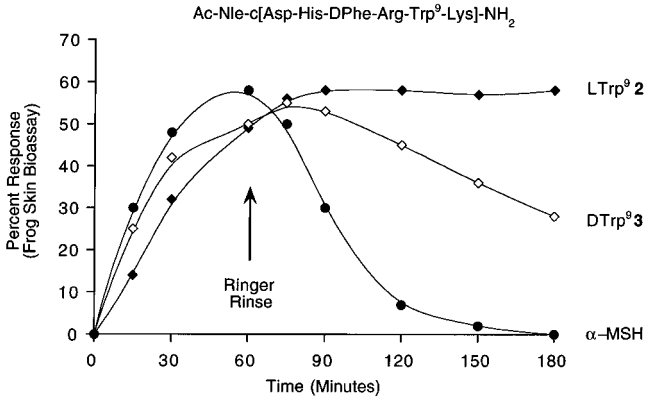


Figure 3. Prolongation activity of the diastereoisomeric peptides, Ac-Nle^4-c[Asp^5-His^6-DPhe^7-Arg^8-L/DTrp^9-Lys^{10}]-NH_2, and α-MSH as determined in the frog skin bioassay. The LTrp^9-containing peptide possesses superprolonged activity, while the DTrp^9-containing peptide possesses some residual activity, and α-MSH lacks any prolonged activity. 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.

degrees of prolonged biological activity of peptides (**14**–**17**) in this bioassay.

Lizard Skin Bioassay. In the lizard skin bioassay, a different biological activity profile was observed (Table 1) for these cyclic peptide derivatives. Analogue **2** exhibited a 5-fold increase in potency compared to α-MSH (Table 1). Analogue **4** (2*S*,3*S*-β-MePhe^7) resulted in 3-fold decreased potency, whereas analogue **5** (2*S*,3*R*-β-MePhe^7) resulted in 5-fold increased potency compared to α-MSH. Analogue **6** (2*R*,3*S*-β-MePhe^7) resulted in 3-fold increased potency while analogue **7** (2*R*,3*R*-β-MePhe^7) resulted in 20-fold decreased potency compared to α-MSH. Peptides **8** (2*S*,3*S*-β-MeTrp^9), **10** (2*R*,3*S* β-MeTrp^9), and **11** (2*R*,3*R* β-MeTrp^9) were equipotent to α-MSH, whereas the 2*S*,3*R*-β-MeTrp^9-containing peptide **9** exhibited a 6-fold decrease in potency. The L- and DTca peptides **12** and **13**, were 20–11-fold less potent, respectively, compared to α-MSH. Comparison of analogues **4** with **5** (2*S*-β-MePhe^7) and **6** with **7** (2*R*-β-MePhe^7) revealed 17- and 67-fold differences in potencies, respectively. Comparison of analogues **4** with **6** (3*S*-β-MePhe^7) and **5** with **7** (3*R* β-MePhe^7) showed 11- and 100-fold differences in potencies, respectively. Comparison of peptides **8** with **9** (2*S*-β-MeTrp^9) and **10** with **11** (2*R*-β-MeTrp^9) demonstrated 7- and 1.4-fold differences in potencies, respectively. Comparison of analogues **8** with **10** (3*S*-β-MeTrp^9) and **9** with **11** (3*R*-β-MeTrp^9) revealed 1.4- and 7-fold differences in potencies, respectively. These latter comparisons clearly demonstrate the sensitivity of the lizard skin melanocortin receptor for both the stereochemistry at the α- and β-carbons and the ultimate topographical presentation of the Phe^7 residue, which is in striking contrast to the frog skin receptor.

The effect of incorporating the four isomers of β-MePhe^7 into the linear NDP-MSH template on the lizard skin melanocortin receptor are summarized in Table 2.

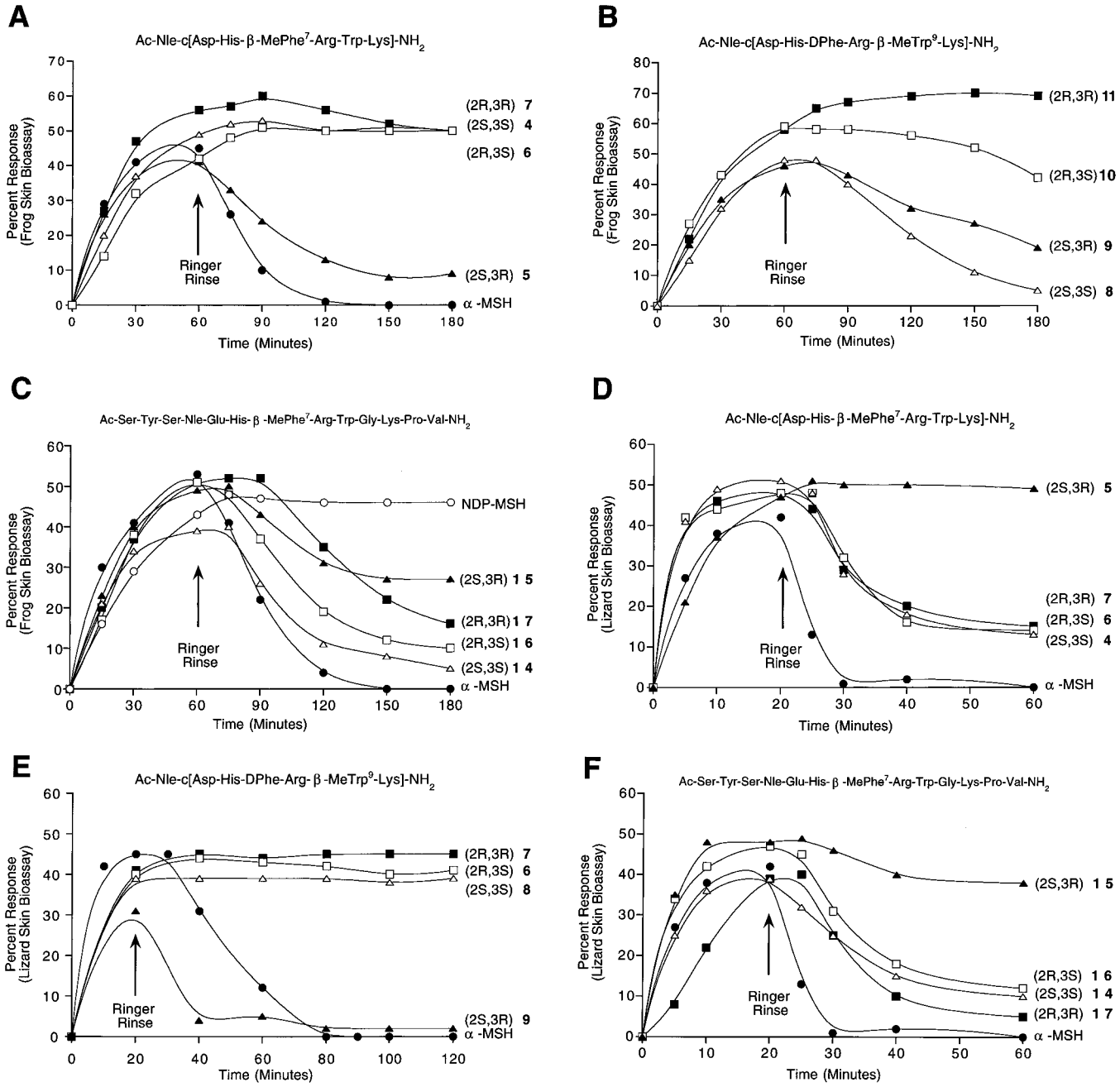


Figure 4. Differential prolonged biological activity of peptides possessing β-methylated aromatic amino acids in the frog and lizard skin bioassays. The assay skins are incubated with the peptide for the indicated period of time (designated by the arrow). The skins are then rinsed several times and placed in a Ringer solution lacking peptide. Subsequently, the assay skins ($n = 5$) are monitored for percentage skin darkening (prolonged activity) for up to 3 h.

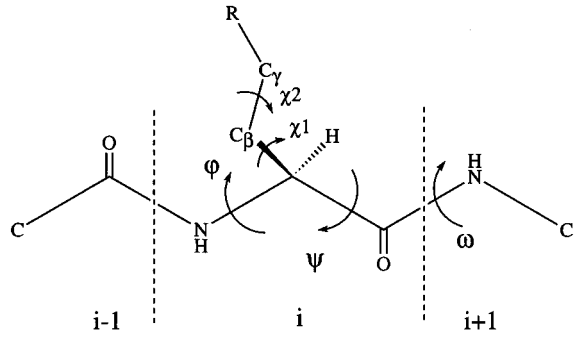
Analogue **14** (2*S*,3*S*-β-MePhe^7) resulted in 3-fold decreased potency compared to α-MSH and 7-fold decreased potency compared to NDP-MSH. Analogues **15** (2*S*,3*R*-β-MePhe^7) and **17** (2*R*,3*R*-β-MePhe^7), respectively, were equipotent to α-MSH and NDP-MSH (within experiment error). Analogue **16** (2*R*,3*S*-β-MePhe^7) resulted in a 2-fold increased potency compared to α-MSH and was equipotent to NDP-MSH. Comparison of analogues **14** with **15** (2*S*-β-MePhe^7) and **16** with **17** (2*R*-β-MePhe^7) showed equipotency with respect to each other (within experimental error). Comparison of analogues **14** with **16** (3*S*-β-MePhe^7) demonstrated 7-fold difference in potencies while analogues **15** and **17** (3*R*-β-MePhe^7) were equipotent.

Interestingly, the β-MePhe^7-containing peptides all lacked prolonged activity except analogue **5**, 2*S*,3*R*-β-MePhe^7, whereas all the β-MeTrp^9 stereoisomeric peptides possessed prolonged biological activity except for analogue **9** (2*S*,3*R*-β-MeTrp^9) (Figure 4D,E). Both analogues **12** and **13** (L- and DTca^9) possessed prolonged activity. Analogues **14**−**17** all lacked prolonged activity in the lizard skin assay, except for the 2*S*,3*R*-β-MePhe^7 which possessed prolonged activity (Figure 4F).

Discussion

The rationale for using side chain constraints such as β-methyl substitution (Figure 1) in a peptide template is to isolate the biologically relevant chi 1 (χ_1) rotomer population(s) by sterically hindering rotation about the χ side chain torsion angles (Figure 5). Theoretically, use of these types of modifications should produce stereoisomeric peptides which possess differential bio-

A) Peptide Dihedral Angles



B) Chi 1 (χ_1) Torsion Angle of Amino Acid Side Chains

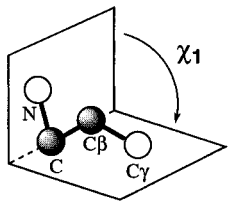


Figure 5. Definition of (A) peptide dihedral angles and (B) the atoms that define the χ_1 side chain torsion angle.

logical activities including receptor selectivity, potency, and other biological effects such as prolongation. Identification of the side chain rotomer that is present in the superpotent or bioselective ligand can provide a valuable tool to aid in the development of three-dimensional pharmacophore models of peptides.[37] One of the possible caveats in designing peptides with this type of modification is that the addition of a methyl group may change the way the peptide side chain interacts with the receptor, as illustrated in Figure 6. The four β-MePhe^7 isomers evaluated in the frog skin bioassay (Table 1) show that while both the 2*S* (L configuration)-containing analogues **4** and **5** retain near equipotency to the lead peptide **1**, the 2*R* (D configuration) analogues **6** and **7** were 20−166-fold less potent compared with analogue **3**. These results are not decisive in isolating a favorable side chain rotomer population nor for proposing a specific topographical presentation of the phenyl moiety with the frog skin receptor, as the β-methyl may be affecting ligand−receptor interactions (Figure 6C). However, in the lizard skin bioassay, peptides possessing enhanced potency (2*S*,3*R*-β-MePhe^7, **5**), equipotency (2*R*,3*S*-β-MePhe^7, **6**), and decreased potency (2*S*,3*S*-β-MePhe^7, **4**, and 2*R*,3*R*-β-MePhe^7, **7**) were observed. These data reflect the theoretical predictions, suggesting that the β-methyl may not be significantly adversely affecting ligand−receptor interactions (Figure 6A,B). The peptides containing β-MeTrp^9 also comply with the theoretically expected results in the frog skin bioassay, with the 2*R*,3*S*-β-MeTrp^9 isomer (**10**) possessing enhanced potency compared with DTrp^9 (**3**). The 2*S*,3*S*-β-MeTrp^9 (**8**) and 2*R*,3*R*-β-MeTrp^9 (**11**) analogues possess slightly decreased potencies, while the 2*S*,3*R*-β-MeTrp^9 peptide (**9**) resulted in 286-fold decreased potency. However, in the lizard skin bioassay, these β-MeTrp^9-modified peptides all resulted in 5−33-fold decreased potency compared with analogue **2**. Thus, in the lizard skin assay, the divergence from theoretically expected results suggests that addition of the methyl moiety to the β-carbon of Trp^9 not only has restricted rotation about the side chain χ_1 torsion angle but may also play a role in modifying the ligand side chain interactions with the receptor. Since in the frog or lizard skin assay, either the β-MePhe^7 or the β-MeTrp^9 stereoisomeric peptides diverge from the theoretically expected observations stated previously, identification of the proposed synergism of the side chain topography of the melanotropin pharmacophores Phe^7 and Trp^9 becomes difficult to correlate using the MTII cyclic peptide template.

Prolonged biological activity of some melanotropin peptides has been observed in the classical frog and lizard skin bioassays in our laboratories and those of others. Residual activity for these assays has been defined as (a) not prolonged if the assay skin darkening is less than 50% of the maximal response 60 min after the melanotropin has been removed from the incubation medium, (b) prolonged if the assay skin darkening remains greater than 50% of the maximal response 60 min after the melanotropin has been removed from the incubation medium, or (c) superprolonged if the assay skin darkening remains greater than 90% of the maximal response for longer than 60 min after the melanotropin has been removed from the incubation medium.[38] The observation that the MTII stereoisomeric peptides containing β-MeTrp^9 resulted in varying degrees of prolonged activity in the frog skin (Figure 4B),[15] initiated ^{1}H NMR conformational analysis of these peptides (unpublished data).[16,17] These studies showed that, for the 2*R*-β-MeTrp^9-containing peptides **8** and **9** the *gauche (+)* χ_1 rotomer was favored in both compounds, and yet they produced different degrees of prolonged activity (Figure 4B). Further examination of the other melanotropin side chain pharmacophore DPhe^7 revealed that, for the peptide-containing 2*R*,3*R*-β-MeTrp^9 (analogue **11**), the χ_1 angle of the DPhe^7-containing residue preferred the *trans* χ_1 rotomer, whereas for the 2*R*,3*S*-β-MeTrp^9-containing peptide (**10**), the *gauche (+)* rotomer for DPhe^7 was observed to be preferentially populated. These observations support our hypothesis regarding the importance of the topography of the Phe^7 side chain pharmacophore and its interactions with the melanocortin receptors for prolonged activity. Evaluation of the stereoisomer peptides containing β-MePhe^7 in the cyclic template (Figure 4A) using the frog skin assay showed that all of the analogues possessed prolonged activity, except for analogue **5** (2*S*,3*R*-β-MePhe^7). Interestingly even the 2*S*,3*S*-β-MePhe^7 (an L-amino acid) peptide **4** possessed prolonged activity. The initial hypothesis developed in our laboratories was that the DPhe^7 was important for prolonged activity,[3,39] although this hypothesis has been further modified since some DPhe^7-containing peptides did not possess prolonged activity.[15,38,40]

The importance of the topography of the Trp^9 indole moiety for prolonged activity in the frog skin assay was supported by simply inverting the chirality from L to D, as illustrated in Figure 3. When 1,2,3,4-tetrahydro-β-carboline (Tca) was incorporated at position 9 (analogues **12** and **13**), prolonged activity was observed for both the L- and D-containing analogues (data not shown). Interestingly, the use of this unusual amino acid eliminates the *trans* side chain χ_1 rotomer population by conformational constraint (Figure 1). These data agree with the ^{1}H NMR studies that identified 2*S*,3*R*-β-MeTrp^9 (**9**) as the only peptide of the series to favor

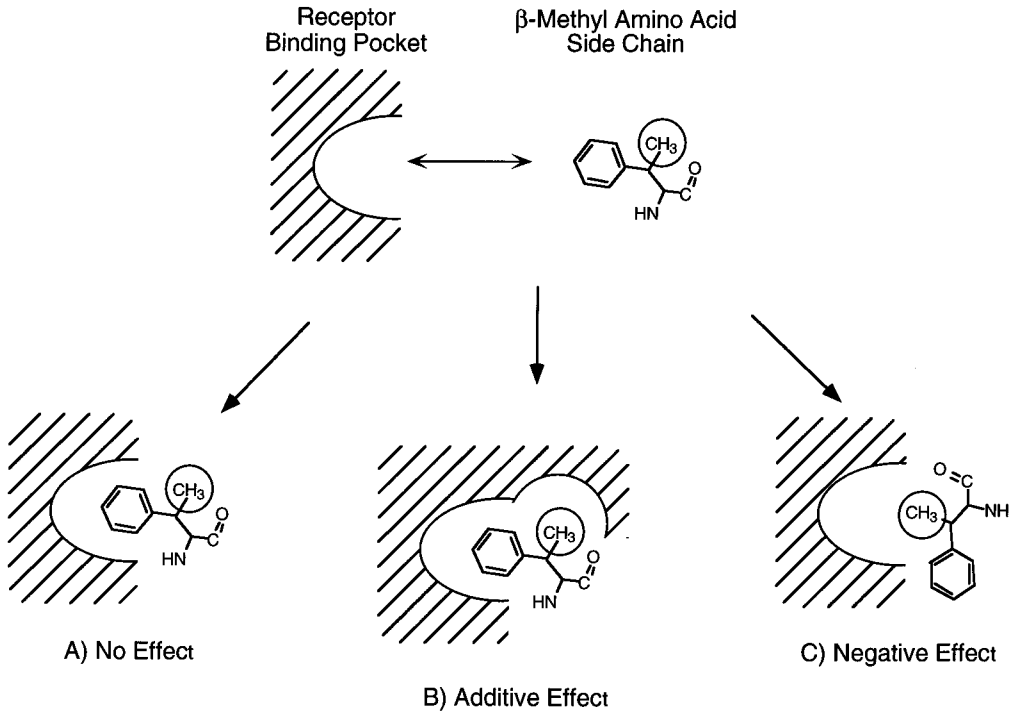


Figure 6. Illustration of the variety of possibilities of how β-methylation (circled) of aromatic ligand side chains can (A) not effect ligand−receptor interactions, (B) result in enhancing ligand−receptor interactions, or (C) sterically interfere with ligand−receptor interactions.

the *trans* χ_1 rotomer and did not possess prolonged activity in the frog skin (Figure 4B). Studies of a conformationally constrained bicyclic melanotropin peptide Ac-c[Cys^4-c(Asp^5-His^6-DPhe^7-Arg^8-Trp^9-Cys^{10}]-Lys^{11})-NH_2 (unpublished results)[17,38] resulted in prolonged activity in the frog skin identical to the DTrp^9-containing analogue **3**, as seen in Figure 3. Both the bicyclic peptide, which possesses a LTrp^9, and analogue **3** (DTrp^9) favored the *gauche (+)* χ_1 rotomer population, albeit with different α-carbon configurations at position 9.[17] Taken together, the above results suggest that for melanotropin peptides to possess prolonged activity in the frog skin assay, the side chain χ_1 rotomer population of the indole moiety at position 9 should not populate the *trans* configuration.

Different trends in prolonged activity were observed in the lizard skin bioassay. The cyclic peptides containing β-MePhe^7 (analogues **4**−**7**) all lacked prolonged activity except for the 2*S*,3*R*-β-MePhe^7 compound **5** (Figure 4D). Interestingly, this analogue has an L amino acid in position 7, and this same analogue was the only one identified not to possess prolonged activity in the frog skin assay (Figure 4A). Analogues **8**−**13** all were found to possess prolonged activity (Figure 4E and data not shown) with the exception of the 2*S*,3*R*-β-MeTrp^9 peptide **9**. This latter peptide also did not possess prolonged activity in the frog skin assay (Figure 4B). One unique aspect of the lizard skin assay is that a peptide possessing prolonged activity (2*S*,3*S*-β-MeTrp^9, analogue **8**) can be made to lose residual activity by changing the peptide concentration (Figure 7). Conversely, an analogue lacking prolonged activity (2*S*,3*R*-β-MeTrp^9, analogue **9**) at 10^{-7} M possesses some residual activity at 10^{-6} M (data not shown).[17] These data suggest that kinetics of ligand−receptor association and dissociation rates affects prolonged activity. This observation was also made when examining analogues **8**−**11** on the human melanocortin receptor, hMC1R.[15]

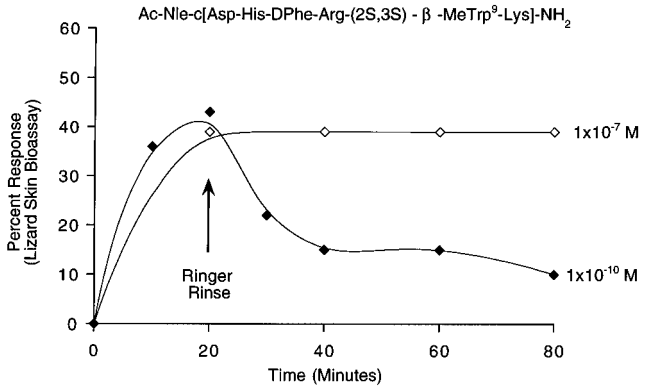


Figure 7. Demonstration of concentration dependence on prolonged activity in the lizard skin assay. The same peptide containing 2*S*,3*S*-β-MeTrp^9 (analogue **8**) possesses superprolonged activity at 10^{-7} M; however, at 10^{-10} M decreased prolonged activity is observed. These data support the hypothesis that prolonged activity may be related to ligand−receptor kinetics at the lizard skin melanocortin receptor.

In the lizard skin bioassay, the four stereoisomers of β-MePhe^7 incorporated into the more flexible NDP-MSH linear peptide template (Table 2, analogues **14**−**17**) resulted in the same trends of prolonged activity as those observed for the corresponding β-MePhe^7 isomers in the cyclic peptide template (Figure 4D,F). The 2*S*,3*R*-β-MePhe^7-containing peptides (**15** and **5**, respectively) maintained prolonged activity whereas the other isomers were not prolonged. However, in the frog skin assay (Figure 4A,C) a very different trend in prolonged activity was observed between the cyclic and linear peptide templates. The 2*S*,3*R*-β-MePhe^7 isomer in the linear template (analogue **15**) possessed some prolonged activity, as defined above, while the other stereoisomeric peptides lacked prolonged activity. In the cyclic template, however, the 2*S*,3*R*-β-MePhe^7 isomer (analogue **5**) lacked any prolonged activity, whereas the other

Table 3. Comparative Biological Activity of the Ac-Nle-c[Asp-His-DPhe-Arg-β-MeTrp-Lys]-NH_2 Peptides on the Melanocortin Receptor Indicated

melanocortin receptor	relative potency	relative prolonged activity
frog skin	(2*R*,3*S*) > (2*S*,3*S*) = (2*R*,3*R*) ≫ (2*S*,3*R*)	(2*R*,3*R*) > (2*R*,3*S*) > (2*S*,3*R*) > (2*S*,3*S*)
lizard skin	(2*R*,3*S*) = (2*S*,3*S*) = (2*R*,3*R*) ≫ (2*S*,3*R*)	(2*R*,3*R*) = (2*R*,3*S*) = (2*S*,3*S*) ≫ (2*S*,3*R*)
human MC1R	(2*S*,3*S*) > (2*R*,3*R*) > (2*R*,3*S*) ≫ (2*S*,3*R*)	(2*S*,3*S*) ≫ (2*R*,3*R*) > (2*R*,3*S*) > (2*S*,3*R*)

peptides clearly possessed prolonged activity. These data emphasize the importance of the peptide template, in addition to the local topographical constraints such as β-methylation for receptor selectivity and biological activity.

Previously reported structure−activity relationships comparing the lizard assay and human melanocyte have suggested that the lizard skin bioassay may correlate with the human peripheral melanocyte receptor, hMC1R.[3,41−43] However, when comparing the four stereoisomers of β-MeTrp in the template Ac-Nle-c[Asp-His-DPhe-Arg-β-MeTrp-Lys]-NH_2 on the frog, lizard, and human (hMC1R) skin[15] melanocortin receptors for potency and "prolonged" biological activity, differences were observed (Table 3). For example, relative potencies are approximately equivalent for the 2*R*,3*S*-, 2*S*,3*S*-, and 2*R*,3*R*-β-MeTrp[9] stereoisomers on the lizard skin receptor while a 6-fold difference in activity is observed for these isomers on the human MC1R. Interestingly, however, is the observation that the 2*S*,3*R*-β-MeTrp[9] stereoisomer is the least potent on the frog, lizard, and human receptors (Table 3). When evaluating prolonged biological activity on the human MC1 receptor, dissociation kinetics of the various ligands was evaluated for up to 4 h after the ligands had been removed from the assay medium.[15] Thus a direct comparison with the lizard skin assay is not applicable. But when comparing which analogues dissociate slower (hMC1R) or possess the greatest prolonged activities (lizard MCR), the 2*S*,3*S*-β-MeTrp isomer stands out as possessing dramatically different characteristics. This stereoisomeric peptide has the slowest dissociation kinetics ("prolonged") on the human receptor ($t_{1/2}$ = 7.85 h)[15] and exhibits prolonged activity on the lizard skin assay (Figure 4E). Furthermore, two other stereoisomeric peptides (**10** and **11**) possessed $t_{1/2}$ dissociation values on the order of 2 h (considered "not prolonged") on the hMC1R, while possessing prolonged activity on the lizard skin. These differences in activities clearly indicate that these topographical modifications are able to differentiate ligand−receptor interaction of the lizard and hMC1R melanocortin receptors.

Conclusions

These studies illustrate that incorporation of topographically constrained aromatic amino acids into peptide templates can result in large differences in potencies and biological activity of peptides. The three-dimensional orientation of the Phe[7] and Trp[9] amino acids can affect both the potencies and prolonged biological activities of melanotropin peptides. Furthermore, the peptide template chosen to incorporate topographically modified amino acids can be important as illustrated by the β-MePhe[7] analogues which possessed different trends in biological activity when using the cyclic MTII template versus the linear NDP-MSH template. Additionally, these data provide evidence that some structural differences exist between the lizard and human MC1 receptors and further suggest that previous SAR using the lizard skin assay may not predict all the SAR of the human melanocortin 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:); (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 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 phenylthiocabamoyl-amino acid (PTC-AA) analysis. No corrections were made for amino acid decomposition. FAB-MS analyses were performed at the University of Arizona Core Facility. The instrument was custom made in Breman, Germany and consists of a LIQUID SIMS4 Sectors AMD mass spectrometer. The experimental conditions consisted of a glycerol matrix scan of 200−2000 Da in the positive ion mode. Optical rotation values were measured on an Autopol III 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 and β-methylphenylalanine 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.[36] The diastereoisomeric peptides containing β-methylphenylalanine were synthesized as previously reported.[22] *tert*-Butyloxycarbonyl (Boc) was used for N^{α}-protection, and the reactive side chains of the amino acids were protected as follows: Lys, with fluorenylmethyloxycarbonyl (Fmoc) or 2,4-dichlorobenzyloxycarbonyl (2,4-Cl_2-Z); Asp, with fluorenylmethyl ester (OFm); Glu, with benzyl (OBzl); His, with benzyloxymethyl (Bom); Arg, with tosyl (Tos); β-MeTrp, with 2-mesitylenesulfonyl (MeS); Trp, with formyl (For); Ser, with *O*-benzyl (*O*-Bzl); and Tyr, with 2,6-dichlorobenzyl (Cl_2-Bzl). All reagents and solvents were ACS grade or better and were 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 were greater than 95% pure as determined by these methods. The structures of the pure peptides were confirmed by fast atom bombardment (FAB) mass spectrometry (Supporting Information).

General Procedure for the Synthesis and Cleavage of Melanotropin Ac-Nle-c[Asp-His-Xaa-Arg-Yaa-Lys]-NH_2 Peptides. The peptides were synthesized using a manual synthesizer adapted from previously published methods.[11,15] Approximately 0.5 mmol of *p*-methylbenzhydrylamine (pM-

BHA) 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 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) or N^{α}-Boc-Trp (For) 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-D-Phe, and N^{α}-Boc-His (N^{τ}-Bom) were performed using DIC (6.5 mmol) and HOBT (6.5 mmol) as coupling reagents for 2 h, and N^{α}-Boc removal conditions described above, and 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) for 20 min. The resin was washed with DCM (7 × 50 mL), followed by cyclization of the free acid side chain group of Asp to the free amine side chain group 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.[44] 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 approximately 2.0 g of peptide-resin. Approximately half of the peptide-resin was added to an equivalent amount of 10% *m*-cresol (or 8% *m*-cresol and 8% thioanisole for β-MeTrp[9]),[35] followed by the addition of approximately 10 mL of anhydrous HF.[35] The mixture was stirred at 0 °C for 60 min followed by the removal of the scavengers and HF under high vacuum. To ensure complete removal of the scavengers and non-peptide 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. The analytical properties for each peptide are given in the Supporting Information.

General Procedure for the Synthesis and Cleavage of Ac-Ser-Tyr-Ser-Nle-Glu-His-Xaa-Arg-Trp-Gly-Lys-Pro-Val-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-Val was coupled to the resin in DCM using DIC (6.5 mmol) and HOBT (6.5 mmol) as coupling reagents for 2 h. The N^{α}-Boc protecting group was removed by washing the resin for 2 min in 50% 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). The sequential addition of the amino acids, N^{α}-Boc-Pro, N^{α}-Boc-Lys (2,4-Cl_2-Z), N^{α}-Boc-Gly, N^{α}-Boc-Trp (N^{i}-For), N^{α}-Boc-Arg (N^{γ}-Tos), N^{α}-Boc-D-Phe or N^{α}-Boc-β-MePhe, N^{α}-Boc-His (N^{τ}-Tos), N^{α}-Boc-Glu (OBzl), N^{α}-Boc-Ser (*O*-Bzl), N^{α}-Boc-Tyr (2-ClZ), and N^{α}-Boc-Ser (*O*-Bzl), were performed using a 4-fold excess (6.5 mmol) and utilizing the same coupling and N^{α}-Boc removal conditions described above. The N-terminal acetylation was carried out by the addition of 2 mL of acetic anhydride and 1 mL pyridine, for 20 min. The resin was washed with DCM (6 × 50 mL) and dried *in vacuo*. The dry resin was divided equally into two HF reaction vessels, and 10% *m*-cresol and 10 mL of anhydrous HF were added to each vessel. The mixture was stirred at 0 °C for 50 min. To remove the scavengers, anhydrous ethyl ether (5 × 30 mL) was added to each vessel and the contents were 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. The analytical properties for each peptide are given in the Supporting Information.

Frog and Lizard Skin Bioassays. The frog (*Rana pipiens*)[32] and lizard (*Anolis carolinensis*)[33,34] skin bioassays were utilized to determine the relative potencies of the synthetic melanotropins.[45] The assays measure the amount of light reflected from the surface of the skins *in vitro*. In response to melanotropic peptides, the melanosomes within integumental melanocytes migrate from a perinuclear position into the dendritic processes of the pigment cells. This centrifugal organellar dispersion results in a change in color (darkening) of the skins which is measured by a Photovolt reflectometer and is expressed as the percent response compared to the initial (time zero) reflectance value. Subsequent removal of a melanotropin such as α-MSH usually results in a rapid perinuclear (centripetal) reaggregation of melanosomes within melanocytes leading to a lightening of the skins back to their original (base) value. 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 rinses in Ringer's solution (111.2 mM NaCl, 1.0 mM KCl, 1.1 mM $CaCl_2$, and 2.4 mM $NaHCO_3$).

Parallel dose−response lines are generated for α-MSH and the peptide being tested. From these parallel lines (not curves which can be fitted by nonlinear regression analysis), a relative potency of the peptide being tested is determined. The EC_{50} value is then back calculated based upon the average frog skin EC_{50} value of 0.10 nM and lizard skin value of 1.0 nM. These EC_{50} values have been the observed average over 10 years of experiments in our laboratory using a multitude of animals and takes into account seasonal and experimental variations. Additionally, these experiments were not corrected for peptide content.

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Supporting Information Available: Analytical properties of these compounds including optical rotation, TLC, *k*′ HPLC values, FAB-MS values, and amino acid analysis values (2 pages). Ordering information is given on any current masthead page.

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