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Structure–Activity Relationships of Cyclic Lactam Analogues of α -Melanocyte-Stimulating Hormone (α -MSH) Targeting the Human Melanocortin-3 Receptor

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Abstract

A variety of dicarboxylic acid linkers introduced between the α -amino group of Pro⁶ and the ε -amino group of Lys¹⁰ of the cyclic lactam α -melanocyte-stimulating hormone (α -MSH)-derived Pro⁶-D-Phe⁷/D-Nal(2')⁷-Arg⁸-Trp⁹-Lys¹⁰-NH₂ pentapeptide template lead to nanomolar range and selective hMC3R agonists and antagonists. Replacement of the Pro⁶ residue and the dicarboxylic acid linker with 2,3-pyrazine-dicarboxylic acid furnished a highly selective nanomolar range hMC3R partial agonist (analogue **12**, c[CO-2,3-pyrazine-CO-D-Phe-Arg-Trp-Lys]-NH₂, EC₅₀ = 27 nM, 70% max cAMP) and an hMC3R antagonist (analogue **13**, c[CO-2,3-pyrazine-CO-D-Nal(2')-Arg-Trp-Lys]-NH₂, IC₅₀ = 23 nM). Modeling experiments suggest that 2,3-pyrazinedicarboxylic acid stabilizes a β -turn-like structure with the D-Phe/D-Nal(2') residues, which explains the high potency of the corresponding peptides. Placement of a Nle residue in position 6 produced a hMC3R/hMC5R antagonist (analogue **15**, c[CO-(CH₂)₂-CO-Nle-D-Nal(2')-Arg-Trp-Lys]-NH₂, IC₅₀ = 12 and 17 nM, respectively), similarly to the previously described cyclic γ -melanocyte-stimulating hormone (γ -MSH)-derived hMC3R/hMC5R antagonists. These newly developed melanotropins will serve as critical biochemical tools for elucidating the full spectrum of functions performed by the physiologically important melanocortin-3 receptor.

Introduction

The five known subtypes of human melanocortin receptors (hMC1–5R) are members of the superfamily of seven trans-membrane G-protein-coupled receptors (GPCRs) expressed in various tissues, including skin (hMC1R),^{1–4} the adrenal cortex (hMC2R),^{5,6} and throughout the central nervous system (hMC3R, hMC4R, hMC5R).⁷ The melanocortin system has received much attention in recent years due to its involvement in a large number of important physiological functions, such as skin pigmentation,^{1–3} control of the immune system,^{1–4}

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^aAbbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977–983. The following additional abbreviations are used: All, allyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; CH₃CN, acetonitrile; Cl-HOBt, 1-hydroxy-6-chlorobenzotriazole; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DIC, diisopropyl carbodiimide; HOBt, *N*-hydroxybenzotriazole; hMCR, human melanocortin receptor; MSH, melanocyte-stimulating hormone; Nal(2'), 2'-naphthylalanine; Pbf, 2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl; TFA, trifluoroacetic acid; SPPS, solid-phase peptide synthesis; RP-HPLC, reverse-phase high performance liquid chromatography; hMC3R, human melanocortin-3 receptor; α -MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly Lys-Pro-Val-NH₂; NDP- α -MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly Lys-Pro-Val-NH₂.

Supporting Information Available: ¹H NMR spectra of peptide analogues **4–15** in DMSO-*d*₆ and the list of chemical shifts and coupling constants. This material is available free of charge via the Internet at <http://pubs.acs.org>.

erectile function,^{8–12} blood pressure and heart rate,^{13,14} control of feeding behavior and energy homeostasis,^{15–21} modulation of aggressive/defensive behavior,^{22,23} and mediation of pain.^{24,25} The endogenous melanocortin agonists include α -, β -, and γ -melanocyte-stimulating hormones (MSH) and adrenocorticotropin (ACTH), while Agouti-signaling and Agouti-related proteins have been identified as the endogenous antagonists.^{26,27}

A considerable effort has been made toward the development of highly potent hMC4R-selective agonists and antagonists^{28–51} due to the involvement of this receptor in the regulation of feeding^{15–21} and sexual behavior.^{8–12} At the same time, comparatively little attention has been given to obtaining selective ligands for the hMC3R^{28–31,52–55} and the hMC5R,^{30,31,54–62} owing to the dearth of specific evidence on their physiological functions.²¹ However, recent reports have demonstrated that inactivation of the mouse MC3R leads to increased fat mass, reduced lean mass, and higher feed efficiency than their wild type littermates.^{63,64} Furthermore, peripheral injections of the hMC3R selective agonist **1**⁵² (Figure 1) can stimulate food intake in mice,⁶⁵ suggesting an important role of this receptor subtype in the regulation of feeding and energy partitioning. In addition, possible involvement of the hMC3R in the regulation of inflammatory responses and cardiovascular function has also been proposed.⁶⁶ Finally, the emerging evidence points to a potential role of the hMC3R in regulation of erectile function and sexual behavior, which provides further impetus for the development of highly selective hMC3R agonists and antagonists.⁶⁷ The hMC5R, on the other hand, is found in a variety of peripheral tissues and plays a role in regulating exocrine gland function⁶⁸ and coordinating central and peripheral signals for aggression.^{22,23}

Several approaches to the design of hMC3R-selective agonists and antagonists have been described in the literature.⁶⁹ Among the natural melanocyte-stimulating hormones, γ -MSH exhibits substantial hMC3R selectivity, whereas α -MSH and β -MSH show little selectivity for any specific receptor subtype.^{52,53} A D-amino acid scan of the γ -MSH sequence revealed the importance of position 8 in hMC3R selectivity and led to the discovery of a highly selective hMC3R agonist **1**.⁵² Structure–activity relationships of γ -MSH have yielded linear peptide analogues with enhanced potency and selectivity, most notably, the nonselective superagonist Ac-Tyr-Val-Nle-Gly His-D-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH₂ (Ac-NDP- γ -MSH-NH₂)⁵³ and a potent hMC3R/hMC5R antagonist and hMC4R agonist H-Tyr-Val-Nle-Gly His-D-Nal(2')-Arg-Trp-Asp-Arg-Phe-Gly NH₂ (PB-II-94).³¹ Recently, our laboratories have produced several potent and selective hMC3R agonists and hMC3R/hMC5R antagonists by placing a bulky hydrophobic Nle residue next to the melanocortin pharmacophore Xaa-Phe-Arg-Trp in a cyclic γ -MSH-derived template.⁵⁴ Some cyclic α -MSH templates have also been described, where increased selectivity in hMC3R agonists and antagonists was observed. Thus, Kavarana et al.²⁸ have found that enhancing the hydrophobic properties of the cyclic α -MSH analogues, increasing the peptide macrocycle size, resulted in improved hMC3R selectivity. Furthermore, Grieco et al.²⁹ have shown that certain dihedrally constrained amino acid substitutions at position 6 of Ac-Nle⁴-c[Asp⁵, D-Nal(2')⁷, Lys¹⁰] α -MSH(4–10)-NH₂ (SHU9119)⁷⁰ led to potent and highly hMC3R- and hMC4R-selective antagonists. Balse-Srinivasan et al. have reported a series of cyclic disulfide α -MSH/ β -MSH hybrid peptides with highly selective hMC3R (Ac-c[Pen-Glu-His-D-Nal(2')-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH₂) and hMC5R (Ac-c[Cys-Glu-His-D-Phe-Arg-Trp-D-Cys]-Pro-Pro-Lys-Asp-NH₂) antagonists.⁵⁵

Described herein are a series of novel cyclic α -MSH analogues possessing a variety of constrained linkers introduced between the α -amino group of D-Phe⁷/D-Nal(2')⁷ and the ϵ -amino group of Lys¹⁰ of the cyclic lactam α -MSH-derived D-Phe⁷/D-Nal(2')⁷-Arg⁸-Trp⁹-Lys¹⁰-NH₂ tetrapeptide template, which have been designed and synthesized by solid-phase methods to further pursue SAR trends leading to hMC3R selectivity.

Peptide Design

Ac-Nle⁴-c[Asp⁵, D-Phe⁷, Lys¹⁰]- α -MSH(4–10)-NH₂ (MT-II), a superpotent but nonselective human melano-cortin receptor agonist,⁷¹ along with the potent nonselective hMC3R/hMC4R antagonist SHU9119,⁷⁰ were chosen as templates for the design of more selective melanotropin peptides. Conformationally constrained linkers, including *o*-phthaloyl-prolyl, glutaryl-prolyl, maleyl-prolyl, 2,6-pyridinyl, 2,3-pyrazinyl, and succinyl-norleucinyl, were introduced between the α -amino group of D-phenylalanine or D-(2')naphthylalanine and the ϵ -amino group of lysine to achieve higher receptor selectivity (Figure 2). Earlier SAR studies on MT-II and SHU9119-based cyclic α -MSH analogues revealed that Pro⁶ substitution did not lead to a significant discrimination between the hMC3R and the hMC4R.⁷² Furthermore, Kavarana et al. has reported that such a substitution in a cyclo(5 β \rightarrow 10 ϵ)-[succinyl⁵-His⁶-D-Phe⁷-Arg⁸-Trp⁹-Lys¹⁰]-NH₂ α -MSH template resulted in a considerable decline in binding affinities as well as agonist activity.²⁸ This study was aimed at further optimization of the conformationally constrained cyclic Pro⁶- α -MSH template toward achieving higher receptor selectivity. In addition, the effects of steric factors on hMC3R selectivity were also investigated by placing a bulky hydrophobic Nle residue into position 6 of this cyclic α -MSH template. The sequences of the peptides discussed in this report, as well as their physicochemical properties, are summarized in Table 1.

Results and Discussion

Table 2 summarizes the binding affinities and the *in vitro* biological activities of the cyclic α -MSH analogues. Analogue **4** was found to have modest binding affinities to the hMC3R and hMC4R and exhibited full agonist activity at the hMC3R (EC₅₀ = 95 nM, 100% cAMP stimulation), while being evidently inactive at the hMC1R and hMC5R. D-Nal(2')⁷ substitution (analogue **5**) resulted in improved binding affinities at all four receptor subtypes and converted a full hMC3R agonist (analogue **4**) to a good affinity hMC3R antagonist (IC₅₀ = 32 nM). Interestingly, these results are in contrast with the findings previously reported by Kavarana et al.,²⁸ where structurally similar His⁶ α -MSH analogues exhibited little or no selectivity between the hMC3R and the hMC4R. It seems plausible that increased conformational constraint within this cyclic peptide template brought about by the Pro⁶ substitution is responsible for the enhanced hMC3R selectivity. Replacement of the phthalic acid linker with the glutaric acid linker produced analogues **6** and **7**, which followed the same trend toward a potent and hMC3R-selective agonist (EC₅₀ = 29 nM) and antagonist (IC₅₀ = 11 nM, pA₂ = 9.8), respectively. Comparison of the three-dimensional structures of analogue **6** and MT-II⁷³ revealed a good fit between their secondary structures (Table 3, Figure 3), which may explain the high agonist potency of this peptide. Pharmacology of the analogue, on the other hand, seems entirely in parallel with that of structurally similar α -MSH analogue cyclo(5 γ \rightarrow 10 ϵ)-(glutaryl⁵-His⁶-D-Nal(2')⁷-Arg⁸-Trp⁹-Lys¹⁰)-NH₂ (MK-9), which has been reported to be a highly potent hMC3R antagonist (K_i = 5.9 nM, pA₂ = 10.6), with a fair selectivity against the hMC4R and the hMC5R.²⁸ These peptides show an improved antagonist potency and selectivity toward the hMC3R compared to the parent peptide, the nonselective hMC3R/hMC4R antagonist SHU9119 (IC₅₀ = 3.3 and 1.8 nM,⁷⁰ pA₂ = 8.7 and 9.1,²⁹ respectively). Analogues **8** and **9** were obtained by employing a maleic acid linker, which provides a similar conformational constraint to the phthalic acid linker but is less sterically demanding. Analogue **8** showed no activity at the hMC1R, partial agonist activity at the hMC3R (EC₅₀ = 100 nM, 80% cAMP stimulation), and a modest binding affinity at the hMC4R (IC₅₀ = 160 nM) and the hMC5R (IC₅₀ = 790 nM). The D-Nal(2')⁷-substituted analogue **9** displayed a reversal of the hMC3R agonist activity to antagonist activity (IC₅₀ = 20 nM), whereas the binding affinities at the hMC4R and hMC5R did not show significant change (IC₅₀ = 520 and 120 nM, respectively). Overall, the Pro⁶ cyclic α -MSH analogues exhibited higher receptor selectivity, favoring the hMC3R, than the corresponding His⁶ peptides.²⁸

Bednarek et al. have previously reported that deletion of His⁶ from the sequence of the structurally related cyclic α -MSH analogues results in enhanced receptor selectivity, most notably, yielding a hMC4R selective antagonist **2**.³⁴ It was suggested in that report that the tetrapeptide core His-D-Nal(2')-Arg-Trp was required for high binding affinity toward the hMC3R and hMC5R, whereas a shorter tripeptide core D-Nal(2')-Arg-Trp was sufficient for high binding affinity toward the hMC4R. To further test this hypothesis, we have used heterocyclic dicarboxylic acid linkers to constrain the tripeptide sequence D-Phe/D-Nal(2')-Arg-Trp, and the biological activities of the resulting peptides were determined. Analogues **10** and **11**, which employed a 2,6-pyridinedicarboxylic acid linker, were found to be inactive at all the receptor subtypes (hMC1, 3–5R). Analogues **12** and **13** contained the 2,3-pyrazinedicarboxylic acid linker, which was structurally better suited to mimic a β -turn motif (Table 3, Figure 4), suggested to be important for melanotropin bioactivities.⁷³ As was revealed by biological evaluation of analogue **12**, it showed a weak partial agonist activity at the hMC1R and a potent partial agonist activity at the hMC3R ($EC_{50} = 27$ nM, 70% cAMP stimulation), while exhibiting no activity at the hMC4R and hMC5R. Furthermore, analogue **13** retained a weak partial agonist activity at the hMC1R and a marginal binding affinity to the hMC4R. On the other hand, this peptide was found to be a potent hMC3R antagonist ($IC_{50} = 23$ nM) and showed a modest hMC5R binding affinity ($IC_{50} = 230$ nM). It is evident from our results that, contrary to the earlier hypotheses,³⁴ the tripeptide sequence D-Phe/D-Nal(2')-Arg-Trp is sufficient for high binding affinity and agonist activity not only at the hMC4R but also at the hMC3R. The observed receptor selectivity in both of these cases seems to be strongly affected by the nature of the linker, including its conformational, steric, and electrostatic properties, which is evident from the remarkable similarity between the 3D conformational structures of peptides **2** and **13**, as illustrated by Figure 5. Thus, manipulation of the linker structure is proving to be a powerful tool in the development of highly selective melanotropin peptides.

Our recent report described the influence of the steric hindrance of the Nle⁴ residue in cyclic γ -MSH analogues on the hMC3R receptor selectivity.⁵⁴ To further test the importance of steric factors in the melanocortin receptor selectivity of cyclic α -MSH analogues, a norleucine residue was introduced into position 6 of the hMC4R-selective agonist **3**.²⁸ This His⁶ \rightarrow Nle⁶ substitution led to the conversion of the hMC3R/hMC4R agonist **3** ($EC_{50} = 70$ and 1.6 nM, respectively) to the hMC3R/hMC4R antagonist (analogue **14**, $IC_{50} = 84$ and 930 nM, respectively). Interestingly, while the hMC3R binding affinity was largely retained, the drastic loss of the hMC4R binding affinity, as well as agonist activity, provides additional evidence for our hypothesis that steric hindrance of the melanocortin pharmacophore has a strong effect on the receptor–ligand interaction. Furthermore, the D-Nal(2')⁷ analogue **15** showed a weak partial agonist activity at the hMC1R and antagonist activities at the hMC3–5R ($IC_{50} = 12$ nM, 300 nM and 17 nM, respectively; $pA_2 = 8.3$ (hMC3R) and 8.7 (hMC5R)). This peptide demonstrated a hMC3R/hMC5R antagonist activity trend similar to one observed in cyclic Nle⁴, D-Nal(2')⁶- γ -MSH analogues.⁵⁴ Figure 6 illustrates the three-dimensional structure of analogue **15**, featuring hydrophobic interactions between the side chains of Nle⁶ and the side chains of Arg⁸, which may result in partial steric hindrance of the binding space of Arg⁸, analogous to the structural features of the cyclic Nle⁴- γ -MSH analogues.⁵⁴ These results are remarkably consistent with the recent findings by Ballet et al.,⁷⁴ who reported that placing a conformationally constrained and sterically demanding 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (Aba) building block in position 6 of the MT-II/SHU9119 cyclic lactam template produced a good affinity and very selective hMC3R antagonist Ac-Nle-c[Asp-Aba-D-Phe-Arg-Trp-Lys]-NH₂ ($IC_{50} = 50$ nM) and a hMC3R/hMC5R antagonist Ac-Nle-c[Asp-Aba-D-Nal(2')-Arg-Trp-Lys]-NH₂ ($IC_{50} = 43$ and 87 nM, respectively). These findings provide additional support for our hypothesis of the importance of steric factors in hMC3R selectivity.

In summary, the structure–activity relationships of the cyclic lactam α -MSH analogues were established to evaluate the multiple factors that contribute to the melanocortin receptor selectivity and are illustrated by the Figures 7 and 8. In particular, Figure 7 describes the agonist activities of the D-Phe⁷ analogues, whereas Figure 8 compares the binding affinities of the D-Nal(2')⁷ analogues, thus highlighting the trend toward hMC3R selectivity. The Pro⁶ template yielded several selective hMC3R agonists and antagonists, whereas employment of heterocyclic linkers and deletion of residue 6 from the sequence resulted in substantially augmented hMC3R selectivity. Finally, Nle⁶ substitution produced a potent hMC3R/hMC5R antagonist, consistent with our earlier findings.^{54,74}

Conclusions

Our SAR studies of cyclic α -MSH analogues have identified new selective hMC3R agonists (analogues **6** and **12**) and hMC3R/hMC5R antagonists (analogues **13** and **15**). Molecular modeling experiments suggested that in the Pro⁶ series the observed hMC3R selectivity is brought about by the increased rigidity of the template. It is noteworthy that replacement of an aliphatic linker in the hMC4R selective antagonist **2** with a heterocyclic pyrazine linker led to a high affinity hMC3R antagonist with good selectivity against the hMC4R (analogue **13**). Such a drastic conversion of an hMC4R antagonist into an hMC3R antagonist underlines the importance of the conformational, steric and electrostatic properties of the linker for the melanocortin receptor selectivity. Steric factors were found to be particularly prominent in the Nle⁶ substitution that produced a potent hMC3R/hMC5R antagonist (analogue **15**, pA₂ = 8.3 (hMC3R) and 8.7 (hMC5R)). These newly developed melanotropin peptides will facilitate the elucidation of the physiological roles of the hMC3R and the hMC4R in feeding behavior, obesity, sexual dysfunction, and related disorders.

Experimental Section

Materials

N^α-Fmoc-amino acids, peptide coupling reagents, and Rink amide AM resin were obtained from Novabiochem (San Diego, CA), except N^α-Fmoc-Lys(Alloc)-OH, which was purchased from NeoMPS (San Diego, CA). The following side chain protecting groups were used: Trp (Nⁱⁿ-Boc), Arg(N^ε-Pbf), and Lys(N^ε-Alloc). ACS grade organic solvents were purchased from VWR Scientific (West Chester, PA) and other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and used as commercially available. The polypropylene reaction vessels (syringes with frits) were purchased from Torviq (Niles, MI). The purity of the peptides was checked by analytical reverse-phase HPLC using a Vydac C₁₈ 218TP104 column (Western Analytical Products, Murrieta, CA), monitored at 230 and 254 nm, and by thin-layer chromatography (TLC), which was performed using three different solvent systems. Analytical thin-layer chromatography (TLC) was carried out on 0.25 mm glass-backed silica gel 60 F₂₅₄ plates (EM Science 5715, VWR Scientific). The TLC chromatograms were visualized by UV light and by dipping in potassium permanganate solution followed by heating (hot plate).

Peptide Synthesis

All peptides in this study were synthesized manually by the N^α-Fmoc solid phase methodology,^{54,58} using bromophenol blue pH indicator to monitor the extent of coupling reactions, as described by Krchnak et al.^{75,76} Rink amide AM resin (4-(2',4'-dimethoxyphenyl)-Fmoc-aminomethyl)phenoxy resin, 0.5 g, 0.637 mmol/g) was placed into a 50 mL polypropylene syringe with the frit on the bottom and swollen in DMF (20 mL) for 1 h. The Fmoc protecting group on the Rink linker was removed by 25% piperidine in DMF (1 × 5 min and 1 × 15 min). The resin was washed with DMF (4 × 15 mL), then washed with 0.02 M HOBt solution in DMF, stained with 0.05 mM solution of Bromophenol Blue in 0.02 M HOBt/DMF solution,

and washed with 0.02 M HOBt/DMF solution (4×15 mL). The first N^{α} -Fmoc amino acid was coupled using preactivated ester (3 equiv of N^{α} -Fmoc amino acid, 3 equiv of HOBt, and 3 equiv of DIC) in DMF. The coupling mixture was transferred into the syringe with the resin and shaken for 60 min, at which point the blue color of the resin changed to yellow, indicating complete coupling. The resin was washed with DMF (3×15 mL) and thrice with DCM (3×15 mL), the unreacted amino groups were capped using acetic anhydride (2 mL) and pyridine (2 mL) in DCM (15 mL) for 30 min, and the resin was once again washed with DMF (6×15 mL). The peptide sequences were completed by consecutively coupling the appropriate amino acids and then the dicarboxylic acid linkers using the procedure described above.

Pyrazinedicarboxylic and succinic acids were converted into their corresponding monoallyl esters prior to appending to the peptides to minimize competing formation of cyclic imides, as previously described.^{28,58} The other dicarboxylic acids were used as commercially available. The orthogonal allylic protection for the side chain of Lys¹¹ and the linker (if applicable) was removed with 0.1 equiv Pd(PPh₃)₄/20 equiv PhSiH₃ in DCM (2×30 min) prior to the peptide cyclization.^{58,77} The deprotected resin-bound peptide was washed with DCM (6×5 mL) and DMF (3×5 mL). The peptide cyclizations were accomplished as described previously,⁵⁴ with 6 equiv DIC, and 6 equiv Cl-HOBt in THF (36 h),⁷⁸ and were monitored by Kaiser ninhydrin test.⁷⁹ The DIC/Cl-HOBt treatment was repeated until a negative Kaiser test was obtained.

Upon completion of cyclization the resin was treated with 5% solution of sodium diethyldithiocarbamate trihydrate in DMF (20 min) to remove any remaining traces of the Pd catalyst,⁵⁴ then washed with DMF (5×15 mL), DCM (3×15 mL), methanol (5×15 mL), and diethyl ether (5×15 mL), and dried under reduced pressure (16 h). The cyclized peptides were cleaved off the solid support with 82.5% v/v TFA, 5% water, 5% thioanisole, 2.5% 1,2-ethanedithiol, and 5% phenol (5 mL, 3 h), and the crude peptides were precipitated out by the addition of a chilled 3:1 mixture of diethyl ether and petroleum ether (50 mL) to give white precipitates. The resulting peptide suspensions were centrifuged for 10 min at 6500 rpm, and the liquid was decanted. The crude peptides were washed with diethyl ether (4×50 mL), and after the final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues were dissolved in 2 M acetic acid, and the insoluble impurities were removed, by passing the solutions through Gelman Laboratory Acrodisc 13 mm syringe filters with 0.45 μ M PTFE membranes (Pall Corporation, East Hills, NY). The clear filtrates were lyophilized, the obtained white powders (50–80 mg) were redissolved in glacial acetic acid (1 mL), and the resulting solutions were diluted with water (4 mL) to a peptide concentration of about 10–15 mg/mL and passed through a Sephadex G-15 column (520×30 mm) using 1 M aqueous acetic acid as the eluent. Fractions containing the target peptides, as determined by TLC, were combined and lyophilized. Final purification was accomplished by preparative RP-HPLC on a C₁₈-bonded silica column (Vydac 218TP152022, 250×22 mm, 15–20 μ m, 300 Å) using a Shimadzu SCL-10A HPLC system. The peptides were eluted with a linear gradient of 20–80% acetonitrile in 0.1% aqueous TFA solution over 50 min with 10 mL/min flow rate. The purified peptides were isolated in 25–30% overall yield. The structures of the pure peptides were confirmed by ¹H NMR in DMSO-*d*₆ and by high resolution electrospray ionization (ESI) mass spectrometry using an IonSpec Fourier transform mass spectrometer with a HiRes ESI source.

Biological Activity Assays. Receptor Binding Assay

Competition binding experiments were carried out using whole HEK293 cells stably expressing human MC1, MC3, MC4, and MC5 receptors. HEK293 cells transfected with hMCRs^{57,80,81} were seeded on 96-well plates 48 h before assay (50000 cells/well). For the assay, the cell culture medium was aspirated, and the cells were washed once with a freshly prepared MEM buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO) and 25 mM sodium bicarbonate. Next, the cells were incubated for 40 min at 37 °C with different concentrations of unlabeled peptide and labeled [¹²⁵I]-[Nle⁴, D-Phe⁷]- α -MSH (Perkin-Elmer Life Science, 20000 cpm/well, 0.14 nM) diluted in a 125 μ L of freshly prepared

binding buffer containing 100% MEM, 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. The assay medium was subsequently removed and the cells were washed once with basic medium and then lysed by the addition of 100 μ L of 0.1 M NaOH and 100 μ L of 1% Triton X-100. The lysed cells were transferred to 12 \times 75 mm borosilicate glass tubes, and the radioactivity was measured by a Wallac 1470 WIZARD Gamma Counter.

Adenylate Cyclase Assay

HEK 293 cells transfected with human melanocortin receptors⁵⁷ were grown to confluence in MEM medium (GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96-well plates 48 h before assay (50000 cells/well). For the assay, the cell culture medium was removed and the cells were rinsed with 100 μ L of MEM buffer (GIBCO). An aliquot (100 μ L) of the Earle's balanced salt solution with 0.5 mM isobutylmethylxanthine (IBMX) was placed in each well along for 1 min at 37 $^{\circ}$ C. Next, aliquots (25 μ L) of melanotropin peptides of varying concentration were added, and the cells were incubated for 3 min at 37 $^{\circ}$ C. The reaction was stopped by aspirating the assay buffer and adding 60 μ L of ice-cold Tris/EDTA buffer to each well, then placing the plates in a boiling water bath for 7 min. The cell lysates were then centrifuged for 10 min at 2300 \times g. A 50 μ L aliquot of the supernatant was transferred to another 96-well plate and placed with 50 μ L of [³H] cAMP and 100 μ L of protein kinase A (PKA) buffer in an ice bath for 2–3 h. The PKA buffer consisted of Tris/EDTA buffer with 60 μ g/mL PKA and 0.1% bovine serum albumin by weight. The incubation mixture was filtered through 1.0 μ m glass fiber filters in MultiScreenTM-FB 96-well plates (Millipore, Billerica, MA). The total [³H] cAMP was measured by a Wallac MicroBeta TriLux 1450 LSC and Luminescence Counter (PerkinElmer Life Science, Boston, MA) The cAMP accumulation data for each peptide analogue was determined with the help of a cAMP standard curve generated by the same method as described above. The maximal cAMP produced at 10 μ M concentration of each ligand was compared to the amount of cAMP produced at 10 μ M concentration of the standard agonist MT-II and is expressed in percent (as % max effect) in Table 2. The antagonist properties of the lead compounds were evaluated by their ability to competitively displace the MT-II agonist in a dose-dependent manner at up to 10 μ M. The pA₂ values were obtained using the Schild analysis method.⁸²

Data Analysis

IC₅₀ and EC₅₀ values represent the mean of two experiments performed in triplicate. IC₅₀ and EC₅₀ estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis, with the help of GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Computational Procedures

Molecular modeling experiments employed MacroModel version 9.1 equipped with Maestro 7.5 graphical interface (Schrödinger, LLC, New York, NY, 2005), installed on a Linux Red Hat 9.0 system, and were performed as previously described.⁵⁴ Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS 2005 force field⁸³ and the Polak-Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient rmsd less than 0.05 kJ/ \AA mol or continued until a limit of 50000 iterations was reached. Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA).⁸⁴ Extended cutoff distances were defined at 8 \AA for Van der Waals, 20 \AA for electrostatics, and 4 \AA for H-bonds.

Conformational profiles of the cyclic peptides were investigated by the hybrid Monte Carlo/Low Frequency Mode (MCOMM/LMCS)⁸⁵ procedure, as implemented in MacroModel using

the energy minimization parameters, as described above. MCMM torsional variations and Low Mode parameters were set up automatically within Maestro graphical user interface. A total of 20000 search steps were performed and the conformations with an energy difference of 50 kJ/mol from the global minimum were saved. Interatomic dihedral angles were measured for each peptide analogue using the Maestro graphical user interface and they are described in Table 3. The superimpositions of peptide structures were performed using the α -carbons of the core sequence Xaa-DPhe/D-Nal(2')-Arg-Trp.

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1	H-Tyr-Val-Met-Gly-His-Phe-Arg- <i>D</i> -Trp-Asp-Arg-Phe-Gly-OH (<i>D</i> -Trp ⁸ - γ -MSH)	hMC3R agonist
2	cyclo(6 β →10 ϵ)-(succinyl ⁶ - <i>D</i> -Nal(2') ⁷ -Arg ⁸ -Trp ⁹ -Lys ¹⁰)-NH ₂ (MBP10)	hMC4R antagonist
3	cyclo(5 β →10 ϵ)-(succinyl ⁵ -His ⁶ - <i>D</i> -Phe ⁷ -Arg ⁸ -Trp ⁹ -Lys ¹⁰)-NH ₂ (VJH-085)	hMC4R agonist

Figure 1.
Some leading hMC3R- and hMC4R-selective melanotropin peptide ligands.

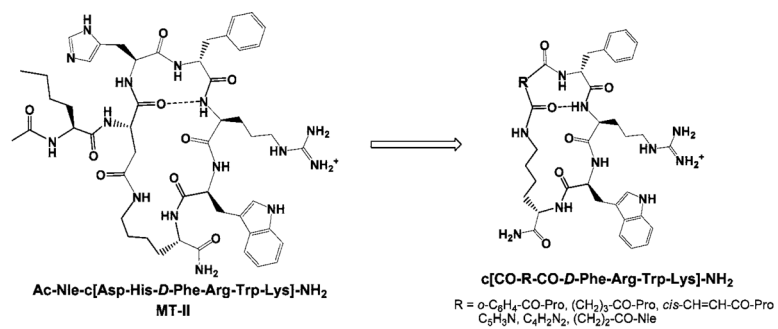


Figure 2.
Design of the α -MSH-derived cyclic lactam scaffold.

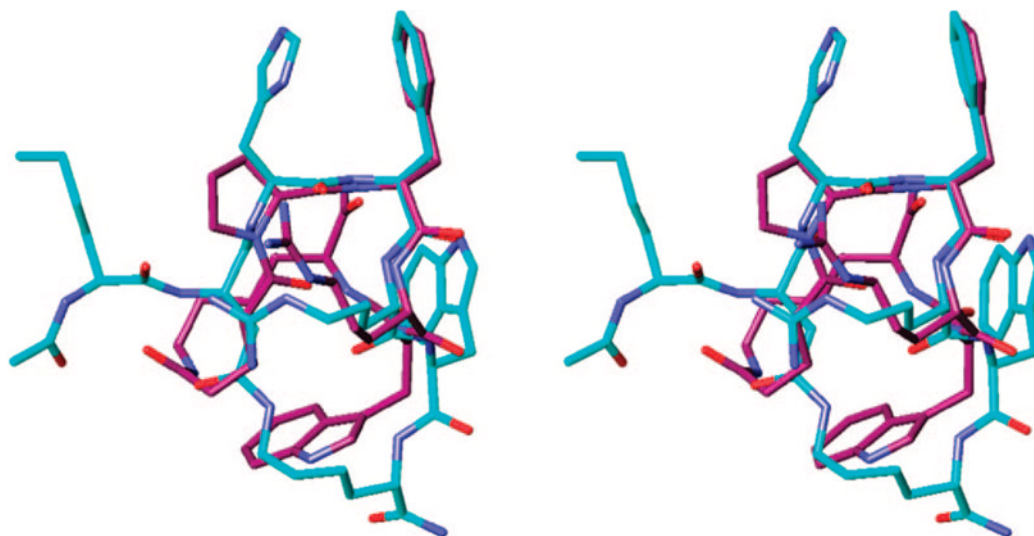


Figure 3. Stereoview of the superimposed global minimum of analogue **6** (purple), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation with the NMR-derived structure of nonselective super agonist MT-II (blue; rmsd = 1.86 Å, nonhydrogen backbone atoms of the Xaa-D-Phe-Arg-Trp pharmacophore only). Hydrogens are omitted for clarity.

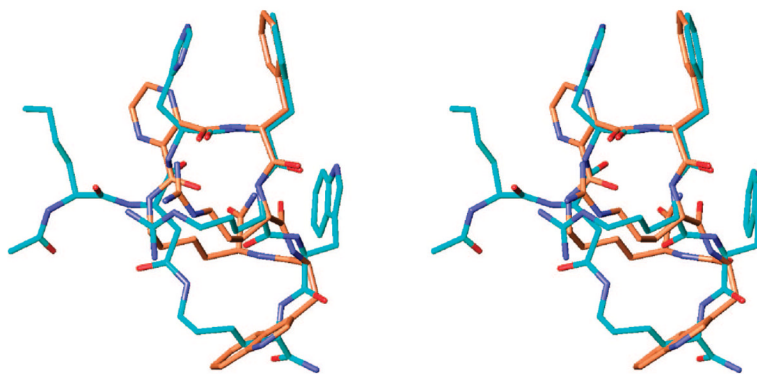


Figure 4. Stereoview of the superimposed global minimum of analogue **12** (gold), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation with the NMR-derived structure of nonselective super agonist MT-II (blue; rmsd = 1.35 Å, nonhydrogen backbone atoms of the Xaa-D-Phe-Arg-Trp pharmacophore only). Hydrogens are omitted for clarity.

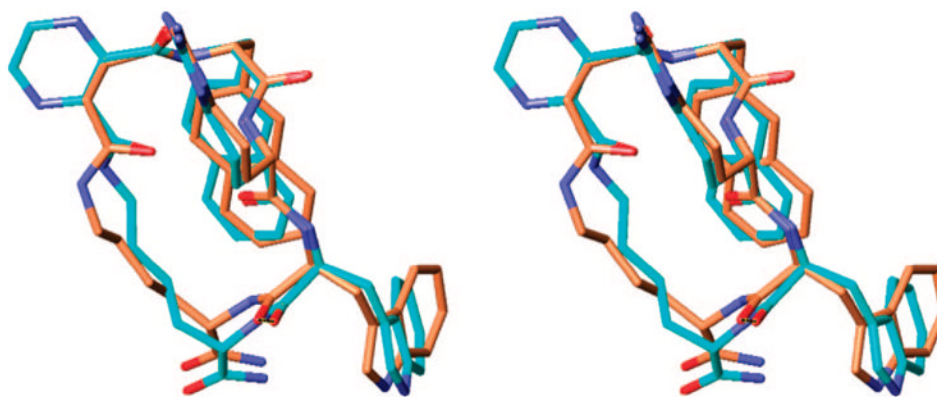


Figure 5. Stereoview of the superimposed global minimum of analogue **13** (blue) with the global minimum of selective hMC4R antagonist **2** (gold), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulations (rmsd = 0.43 Å, nonhydrogen backbone atoms of the Xaa-D-Phe-Arg-Trp pharmacophore only). Hydrogens are omitted for clarity.

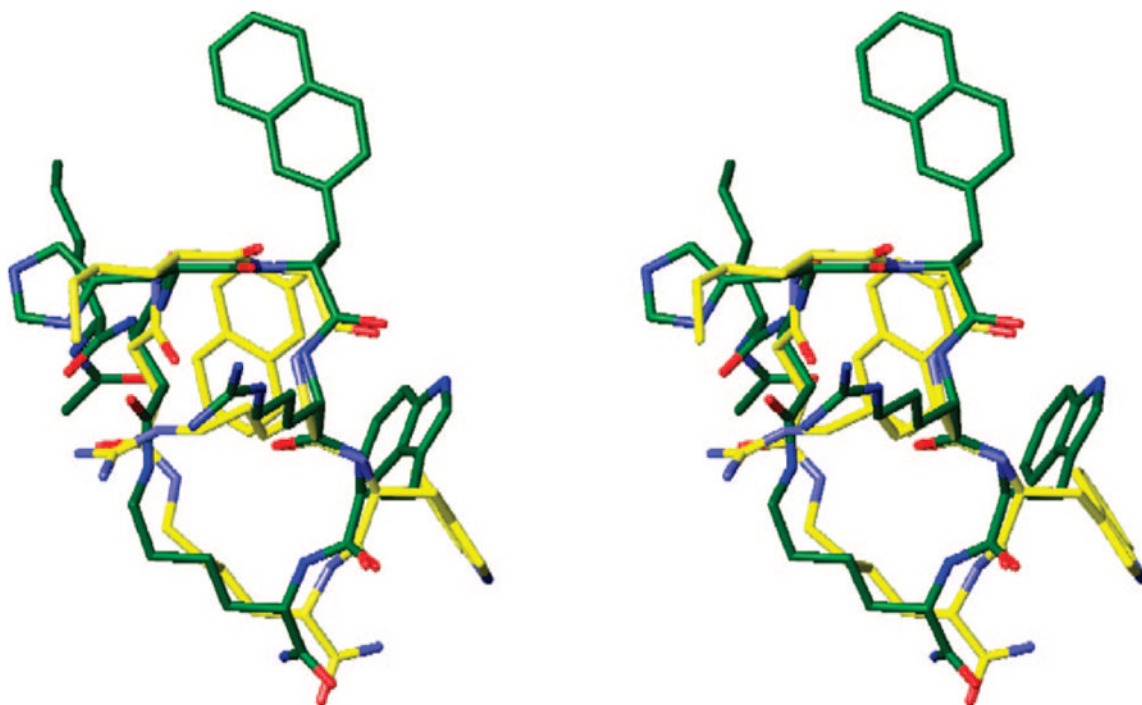


Figure 6. Stereoview of the superimposed global minimum of analogue **15** (yellow), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation with the NMR-derived structure of hMC3R/hMC4R antagonist SHU9119 (green; rmsd = 0.39 Å, nonhydrogen backbone atoms of the Xaa-D-Nal(2')-Arg-Trp pharmacophore only). Hydrogens are omitted for clarity.

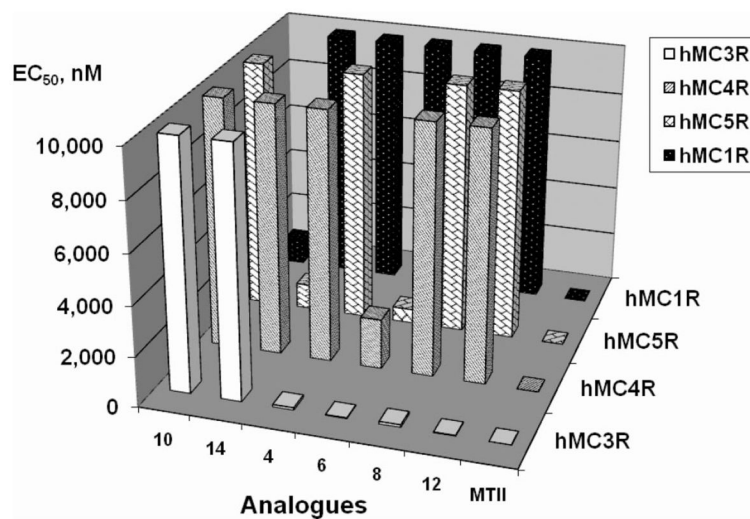


Figure 7. Graphical summary of the agonist activities, expressed in EC₅₀ values (Z axis), of the D-Phe⁷ analogues (X axis) at the four human melanocortin receptor subtypes (Y axis).

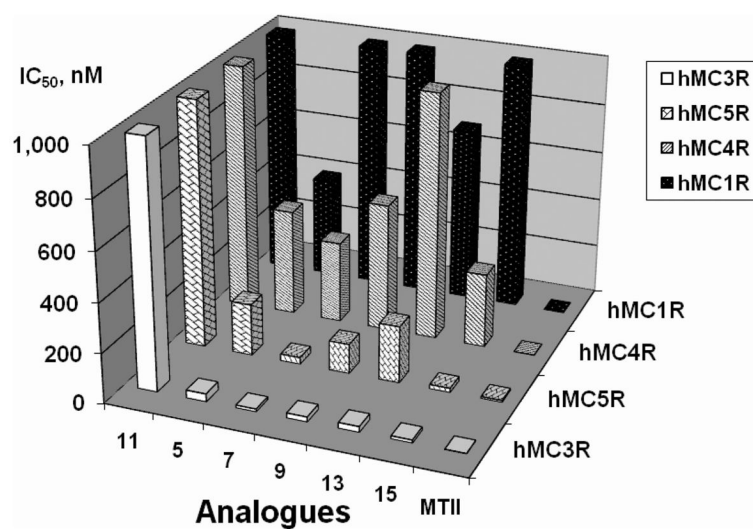


Figure 8. Graphical summary of the binding affinities, expressed in IC₅₀ values (Z axis), of the D-Nal (2')⁷ analogues (X axis) at the four human melanocortin receptor subtypes (Y axis).

Sequences and the Physicochemical Properties of the Cyclic α -MSH Analogues

Table 1

No.	Sequence	m/z (M + 1)		obsd (ESI)	HPLC retention time, min ^d	TLC R_f^b		
		calcd				1	2	3
4	c[CO- <i>o</i> -C ₆ H ₄ -CO-Pro-D-Phe-Arg-Trp-Lys]-NH ₂	862.4364		862.4345	17.27	0.68	0.45	0.75
5	c[CO- <i>o</i> -C ₆ H ₄ -CO-Pro-D-Nal(2')-Arg-Trp-Lys]-NH ₂	912.4521		912.4536	19.64	0.69	0.46	0.78
6	c[CO-(CH ₂) ₅ -CO-Pro-D-Phe-Arg-Trp-Lys]-NH ₂	828.4521		828.4544	16.97	0.61	0.35	0.72
7	c[CO-(CH ₂) ₅ -CO-Pro-D-Nal(2')-Arg-Trp-Lys]-NH ₂	878.4677		878.464	19.41	0.64	0.38	0.74
8	c[CO- <i>cis</i> -CH=CH-CO-Pro-D-Phe-Arg-Trp-Lys]-NH ₂	812.4208		812.4179	17.05	0.64	0.38	0.74
9	c[CO- <i>cis</i> -CH=CH-CO-Pro-D-Nal(2')-Arg-Trp-Lys]-NH ₂	862.4364		862.4397	18.43	0.66	0.41	0.76
10	c[CO-2,6-pyridine-CO-D-Phe-Arg-Trp-Lys]-NH ₂	766.3789		766.3821	16.45	0.65	0.52	0.77
11	c[CO-2,6-pyridine-CO-D-Nal(2')-Arg-Trp-Lys]-NH ₂	816.3946		816.393	19.10	0.67	0.49	0.78
12	c[CO-2,3-pyrazine-CO-D-Phe-Arg-Trp-Lys]-NH ₂	767.3742		767.3763	16.24	0.62	0.4	0.73
13	c[CO-2,3-pyrazine-CO-D-Nal(2')-Arg-Trp-Lys]-NH ₂	817.3898		817.3906	19.02	0.65	0.42	0.76
14	c[CO-(CH ₂) ₂ -CO-Nle-D-Phe-Arg-Trp-Lys]-NH ₂	830.4677		830.4704	18.80	0.68	0.45	0.78
15	c[CO-(CH ₂) ₂ -CO-Nle-D-Nal(2')-Arg-Trp-Lys]-NH ₂	880.4834		880.4806	21.35	0.69	0.47	0.78

^aHPLC column: Vydac 218TP104, 250 × 4.6 mm, 10 μ m, 300 Å; HPLC solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient: 10–90% B in A over 40 min, flow rate 1.0 mL/min.

^bTLC system 1: *n*-butanol/acetic acid/water/pyridine (4:1:2:1); TLC system 2: *n*-butanol/acetic acid/water (4:1:1); TLC system 3: ethyl acetate/acetic acid/water/pyridine (5:1:3:5).

Table 2
 Affinities and cAMP Activities of Cyclic α -MSH Analogues at hMCRs^a

	hMCR1R			hMCR3R			hMCR4R			hMCR5R		
	IC ₅₀ nM	EC ₅₀ nM	% max effect	IC ₅₀ nM	EC ₅₀ nM	% max effect	IC ₅₀ nM	EC ₅₀ nM	% max effect	IC ₅₀ nM	EC ₅₀ nM	% max
Phe-Arg-Trp-Lys]-NH ₂	0.2 ± 0.01	0.3 ± 0.04	100	1.25 ± 0.2	1.85 ± 0.2	100	1.07 ± 0.3	2.87 ± 0.52	100	7.47 ± 0.23	3.3 ± 0.7	100
-D-D-Phe-Arg-Trp-Lys]-NH ₂	>10000	NA	0	>10000	95 ± 11	100	777 ± 200	NA	0	>1000	NA	0
-D-D-Nal(2')-Arg-Trp-Lys]-NH ₂	410 ± 80	>5000	80	32 ± 4	NA	0	430 ± 100	NA	0	210 ± 30	NA	0
-D-D-Phe-Arg-Trp-Lys]-NH ₂	>10000	NA	0	385 ± 50	29 ± 3	100	>10000	>20000	12	870 ± 100	570 ± 60	40
-D-D-Nal(2')-Arg-Trp-Lys]-NH ₂	>10000	NA	0	11 ± 2 pA ₂	NA	0	330 ± 40	NA	0	27 ± 3	23 ± 7	40
-D-Pro-D-Phe-Arg-Trp-Lys]-NH ₂	>10000	NA	0	>10000	100 ± 20	80	160 ± 20	NA	0	790 ± 100	NA	0
-D-D-Phe-Arg-Trp-Lys]-NH ₂	>10000	NA	0	20 ± 3	NA	0	520 ± 60	NA	0	120 ± 20	NA	0
-D-D-Phe-Arg-Trp-Lys]-NH ₂	>10000	>10000	15	>10000	NA	0	>10000	NA	0	>10000	NA	0
-D-D-D-D-Nal(2')-Arg-Trp-Lys]-NH ₂	>10000	NA	0	>10000	NA	0	>10000	NA	0	>10000	NA	0
-D-D-Phe-Arg-Trp-Lys]-NH ₂	412 ± 50	>10000	60	1100 ± 120	27 ± 3	70	>10000	NA	0	>10000	NA	0
-D-D-Nal(2')-Arg-Trp-Lys]-NH ₂	707 ± 101	>3000	55	23 ± 5	NA	0	>10000	NA	0	230 ± 40	NA	0
-D-D-Phe-Arg-Trp-Lys]-NH ₂	>10000	NA	0	84 ± 10	NA	0	930 ± 133	NA	0	520 ± 70	>1000	40
-D-D-Nal(2')-Arg-Trp-Lys]-NH ₂	2000 ± 200	>10000	20	12 ± 3 pA ₂	NA	0	300 ± 100	NA	0	17 ± 2 pA ₂	NA	0
				= 9.8						= 8.7		

^a IC₅₀ = effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (N = 4). EC₅₀ = 50% maximal intracellular cAMP accumulation observed at 10 μM. The peptides were tested at a range of concentration from 10⁻¹⁰ to 10⁻¹ μM ligand concentration.

Table 3
 Backbone Torsion Angles ($^{\circ}$) for the Global Minima of Selected Cyclic α -MSH Analogues Based on MCOMM/LMCS-OPLS2005
 Calculations Compared with the NMR Structures of MT-II and SHU9119⁷³

No.	Xaa ⁶		D-Phe/D-Nal(2) ⁷		Arg ⁸		Trp ⁹		Lys ¹⁰	
	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ
6	-73	110	74	9	-89	158	-59	-17	-83	-14
7	-78	72	74	15	-76	105	-75	144	-145	22
12			86	0	-100	178	-52	-22	-77	-15
13			76	6	-96	154	-70	126	-151	23
14	-139	30	91	-22	-90	118	-83	149	-140	18
15	72	18	82	9	-83	118	-79	129	-98	-4
MT-II	-108	109	84	0	-122	90	-77	108	-101	103
SHU9119	-90	49	82	-6	-99	117	-79	111	-90	-68