# Structure−Activity Studies of the Melanocortin Peptides: Discovery of Potent and Selective Affinity Antagonists for the *h*MC3 and *h*MC4 Receptors†

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We have designed and synthesized several novel cyclic SHU9119 analogues (Ac-Nle[4]-[Asp[5]-His[6]-DNal(2′)[7]-Arg[8]-Trp[9]-Lys[10]]-$NH_2$) modified in position 6 with nonconventional amino acids. SHU9119 is a high affinity nonselective antagonist at *h*MC3R and *h*MC4R with potent agonist activity at *h*MC1R and *h*MC5R. We measured the binding affinity and agonist potency of the novel analogues at cloned *h*MC3R, *h*MC4R, and *h*MC5R receptors and identified several selective, high affinity *h*MC3R and *h*MC4R antagonists. Compound **4** containing Che substitution in position 6 is a high affinity *h*MC4R antagonist ($IC_{50}$ = 0.48 nM) with 100-fold selectivity over *h*MC3R antagonist. Analogue **7** with a Cpe substitution in position 6 is a high affinity *h*MC4R antagonist ($IC_{50}$ = 0.51 nM) with a 200-fold selectivity vs the *h*MC3R. Interestingly, analogue **9** with an Acpc residue in position 6 is a high affinity *h*MC3R antagonist ($IC_{50}$ = 2.5 nM) with 100-fold selectivity vs the *h*MC4R antagonist based on its binding affinities. This compound represents the first cyclic lactam antagonist with high selectivity for the *h*MC3R vs *h*MC4R. To understand the possible structural basis responsible for selectivity of these peptides at *h*MCR3 and *h*MCR4, we have carried out a molecular modeling study in order to examine the conformational properties of the cyclic peptides modified in position 6 with conformationally restricted amino acids.

## Introduction

The melanotropin peptides include α-, *β*-, and *γ*-melanocyte-stimulating hormone (MSH) and adrenocorticotropin (ACTH). All of these hormones are derived by posttranslational processing of the proopiomelanocortin (POMC) gene transcript, and each of them possess a central "core sequence" His-Phe-Arg-Trp, which is essential for its agonist biological activity.[1−3] They are known to have several physiological actions including thermoregulation,[4] regulation of melanocyte pigmentation,[5] adrenal gland function,[5] and obesity,[6] control of the cardiovascular system,[7] effects on memory and learning,[8] and inflammatory effects.[9] All these actions are mediated through five seven-transmembrane G-protein-coupled receptors, MC1R-MC5R.[10−13] These receptors differ in ligand binding specificity as well as in tissue distribution, and the development of selective ligands is needed to understand the physiological functions of these melanocortin receptor system. Therefore, it is essential to identify ligands that can discriminate between the MC3R and MC4R. Both receptors are present at high concentrations in the hypothalamus and in particular, MC3R is much more widely expressed, being detected in brain, adipose tissue, and skeletal muscle. Many studies have established the importance of MC4R in the control of energy homeostasis, and it has been reported that the MC4R deficient mice are severely obese and hyperphagic.[14,15]

The MC4R, due to its involvement in feeding behavior, is a current drug target for the design of selective agonist therapeutics to treat obesity and the design of selective antagonists for potential treatment of anorexia.[16−18] The melanocortin receptor family also has two endogenous antagonists, agouti and agouti-related protein (AGRP), which are the only natural antagonists of these receptors discovered to date.[19−21] Development of MCR selective antagonists are an important task to identify the molecular interactions responsible for affinity of α-MSH and AGRP binding to the MC4R, promoting the design of antiobesity drugs.[22−24]

The present study was devoted to study and design of new cyclic lactam analogues of **1** (PG-901**)**, in which proline was replaced by several conformationally constrained amino acids. The incorporation of these residues is effective in limiting orientational freedom of the side chain in position 6, giving accessibility only to well-

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† Abbreviations 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: AAA, amino acid analysis; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; *t*Bu, *tert*-butyl; $CH_3CN$, acetonitrile; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; $Et_3SiH$, triethylsilane; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; GFC, glass fiber/c; HOBt, *N*-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hyp, hydroxyproline; Pbf, 2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulfonyl; $PhSiH_3$, phenylsilane; RP-HPLC, reversed-phase high performance liquid chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; Trt, triphenylmethyl (trityl). Amino acid symbols denote L-configuration unless indicated otherwise; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; Acpc, 1-aminocyclopropane-1-carboxylic acid; Che, 1-amino-1-cyclohexane-carboxylic acid; Cpe, 1-amimo-1-cyclopentane carboxylic acid; Aic, 2-aminoindane-2-carboxylic acid; Oic, octahydroindole-2-carboxylic acid; Ioc, indoline-2-carboxylic acid; MCR, melanocortin receptor.

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**Table 1.** Biological Activity of the Cyclic α-MSH Analogues at Human Melanocortin Receptors

| | | | *h*MC3R | | | *h*MC4R | | | *h*MC5R | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| compd | code | structure | $IC_{50}$ (nM) | $EC_{50}$ (nM) | % act. | $IC_{50}$ (nM) | $EC_{50}$ (nM) | % act. | $IC_{50}$ (nM) | $EC_{50}$ (nM) | % act. |
| **1** | PG-901 | Ac-Nle-c[Asp-Pro-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.19 ± 0.048 | - | 0 | 0.087 ± 0.007 | - | | 0.031 ± 0.006 | 0.072 ± 0.022 | 95 |
| **2** | PG-911 | Ac-Nle-c[Asp-Hyp-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.21 ± 0.049 | - | 0 | 0.23 ± 0.13 | - | 0 | 0.027 ± 0.0033 | 0.031 ± 0.014 | 86 |
| **3** | PG-913 | Ac-Nle-c[Asp-Oic-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 2.5 ± 0.29 | - | 0 | 0.16 ± 0.02 | - | 0 | 0.11 ± 0.008 | 0.20 ± 0.046 | 85 |
| **4** | PG-914 | Ac-Nle-c[Asp-Che-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 41 ± 14 | - | 0 | 0.48 ± 0.046 | - | 0 | 4.6 ± 0.26 | 10 ± 1.1 | 79 |
| **5** | PG-915 | Ac-Nle-c[Asp-Aic-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 190 ± 43 | - | 0 | 1.3 ± 0.27 | - | 0 | 12 ± 1.2 | 17 ± 4.7 | 73 |
| **6** | PG-916 | Ac-Nle-c[Asp-Ioc-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 290 ± 61 | - | 0 | 3.5 ± 0.77 | - | 0 | 21 ± 5.5 | >100 | <5 |
| **7** | PG-917 | Ac-Nle-c[Asp-Cpe-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 110 ± 30 | - | 0 | 0.51 ± 0.21 | - | 0 | 16 ± 4.5 | 45 ± 9.7 | 79 |
| **8** | PG-944 | Ac-Nle-c[Asp-Tic-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 6.70 ± 0.6 | $>10^5$ | 0 | 3.70 ± 0.3 | $>10^5$ | 0 | 1.24 ± 0.13 | 2.05 ± 0.2 | 102 |
| **9** | PG-946 | Ac-Nle-c[Asp-Acpc-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 2.50 ± 0.2 | $>10^5$ | - | 270 ± 30 | $>10^5$ | 0 | 4.1 ± 0.51 | 390 ± 50 | 50 |
| | SHU9119 | Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.23 ± 0.02 | - | 0 | 0.06 ± 0.01 | - | 0 | 0.09 ± 0.02 | 0.12 ± 0.01 | 97 |
| | MBP10[a] | cyclo(6β→10(ε)−(succinyl$^6$-D-(2′)Nal$^7$-Arg$^8$-Trp$^9$-Lys$^{10}$)-$NH_2$ | 150 ± 12 | - | 0 | 0.5 ± 0.064 | - | 0 | 540 ± 200 | 530 ± 190 | - |
| | HS014[a] | Ac-c[Cys-Glu-His-D(2′)Nal-Arg-Trp-Gly-Cys]-Pro-Pro-Lys-Asp-$NH_2$ | 25 ± 4.1 | - | 0 | 1.5 ± 0.3 | - | 0 | 16 ± 3.1 | 42 ± 15 | - |

[a] Data from refs 47 and 48.

defined regions of conformational space. The aim is to confirm our hypothesis that position 6 of the cyclic core peptides has an important role for interaction and selectivity at human melanocortin receptors, as previously reported.[25] In support of this study, we report here new compounds which are selective antagonists at the *h*MC4R, as well as ligand which is a selective antagonist for the *h*MC3R.

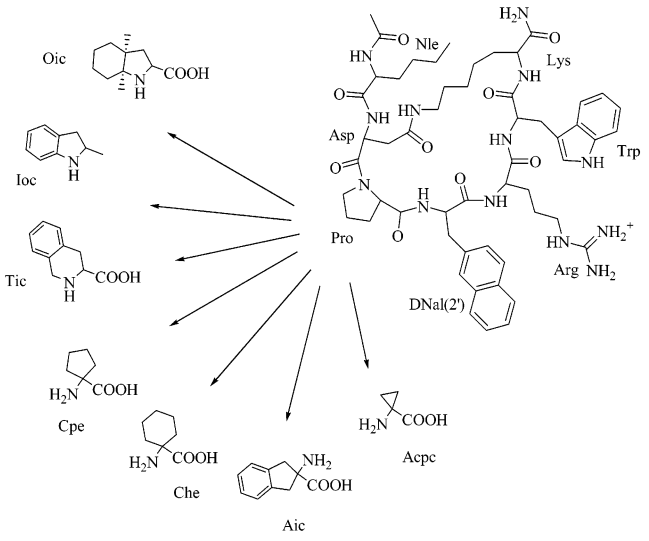
**Peptide Synthesis.** The peptides (Table 1) were synthesized by methods recently developed in our laboratory[26] using $N^{\alpha}$-Fmoc chemistry with an appropriate orthogonal protection strategy. Coupling was carried out with standard in situ activating reagents used in $N^{\alpha}$-Fmoc SPPS, such as the uronium salts (HBTU) in the presence of a tertiary base (DIPEA), to generate HOBt esters. The cyclization of the peptides was performed on the solid support after removing the allyl and Alloc protecting groups under neutral conditions with catalytic amounts of $Pd(PPh_3)_4$ in the presence of $PhSiH_3$ and argon, ensuring an orthogonal deprotection of the side chain protecting groups used during the synthesis. One concern was that Trp residues are extremely susceptible to alkylation by cations produced during the cleavage process. Trialkylsilanes, such as $Et_3$-SiH, have been shown to be effective, particularly for peptides containing Arg(Pbf) and Trp(Boc).[27,28] In our syntheses, cleavage of the peptide from the resin with a $Et_3SiH$-based TFA cocktail was adopted since the Trp residue had a Boc-side chain-protecting group.

The crude peptide obtained following cleavage from the resin showed a single major peak by analytical RP-HPLC. Purification was accomplished by preparative RP-HPLC. The physicochemical properties and purity of the final peptides were assessed by FAB-MS, RP-HPLC, TLC in three solvent systems, and amino acid analysis (see Experimental Section for details).

## Results and Discussion

We have recently designed and developed a novel ligand for the *h*MC5R **1** (PG-901), where we replaced $His^6$ with a Pro residue.[25] We demonstrated, in contrast with some other authors,[29] that position 6 could be very important for ligand-melanocortin receptor interactions. In fact, the $His^6$ residue in HFRW sequence may have different modes of interaction with *h*MCRs, as already suggested for the MC1R, MC3R and MC4R.[30,31] This report extends the structure−activity relationship (SAR) studies for melanotropin peptides and is focused on the importance of position 6 by syntheses of new cyclic lactam analogues of **1** (PG-901) where we replace $Pro^6$ with nonconventional amino acids Oic, Ioc, Tic, as well as with α,α′-dialkylglycine derivatives including Che, Cpe, Acpc, and Aic (Figure 1). All of these new peptides were tested for binding affinities and agonist potencies on cloned *h*MC3R, *h*MC4R, and *h*MC5R.

Analogue **3** (PG-913), with an Oic residue instead of $Pro^6$, resulted in lower binding affinity at all receptors compared to **1** (PG-901) with an $IC_{50}$ = 2.5 nM at the *h*MC3R, an $IC_{50}$ = 0.16 nM at the *h*MC4R, and an $IC_{50}$ = 0.11 nM at the *h*MC5R, respectively (Table 1). Interestingly, in analogue **2** (PG-911) where Hyp was substituted for Pro, we found that this analogue is 10-fold more selective for *h*MC5R when compared to the binding affinities of *h*MC3R and *h*MC4R. This result is slightly different from what was observed for analogue **1**. We believe that the hydroxyl group of Hyp in analogue **2** may cause intramolecular hydrogen bonding that results in the conformational change. Thus, analogue **2** prefers this new conformation which is selective for the *h*MC5R. Analogue **6** (PG-916), in which we substituted $Pro^6$ with Ioc, showed a 1500-fold decrease in affinity at the *h*MC3R ($IC_{50}$ = 290 nM), a 40-fold decrease at the *h*MC4R ($IC_{50}$ = 3.5 nM) and a 680-fold decrease at the *h*MC5R ($IC_{50}$ = 21 nM) when compared




Ac-Nle-c[Asp-*His*-DNal(2')-Arg-Trp-Lys]-$NH_2$ **SHU9119**

Ac-Nle-c[Asp-*Pro*-DNal(2')-Arg-Trp-Lys]-$NH_2$ **PG-901**

**Figure 1.** Structure of the potent monocyclic peptide Ac-Nle-c[Asp-Pro-DNal(2′)-Arg-Trp-Lys]-$NH_2$, referred to as PG-901, and the conformationally constrained amino acids.

to **1**. Interestingly, when Pro[6] was substituted by the unusual amino acid Tic, a widely used cyclic Phe analogue, as well as a pipecolic acid analogue, a different behavior regarding affinity and selectivity for the *h*MC3R, *h*MC4R, and *h*MC5R was observed. The analogue **8** (PG-944) had an $IC_{50} = 6.7$ nM at the *h*MC3R, an $IC_{50} = 3.73$ nM at the *h*MC4R, and an $IC_{50} = 1.21$ nM at the *h*MC5R. Furthermore, analogue **8** (PG-944) was an antagonist at the *h*MC3 and the *h*MC4 receptors and a full agonist at the *h*MC5R. These results demonstrate that specific changes in position 6 could play an important role in binding affinity as well as in selectivity for the *h*MC3R and the *h*MC4R. On the basis of these results, and also considering our previous SAR studies,[25] we decided to further evaluate the effects of structural change at this position by synthesizing analogues where Pro[6] was replaced by α,α′-dialkylglycine derivatives. These residues have been described as useful new type of conformational constraint in peptides.[32−35] Through this backbone modification, information may be obtained about the bioactive conformation of a peptide molecule, and the biological activity can be improved due to enhanced potency and resistance to enzyme degradation. In this context, the α,α′-symmetrically disubstituted Gly, like cycloaliphatic residues, are useful not only for their ability to limit the $\phi$, $\psi$ conformational space, but also for their propensity to adopt a type-II $\beta$-turn motif. Starting from this observation, we decided to synthesize analogues of **1** having the α,α′-disubstituted Gly residues 1-amino-1-cyclopentane carboxylic acid (Cpe), Che, Aic and Acpc in position 6.

Compound **7** (PG-917) containing Cpe[6] had an $IC_{50} = 110$ nM at the *h*MC3R, an $IC_{50} = 0.51$ nM at the *h*MC4R, and an $IC_{50}$ of 16 nM at the *h*MC5R, thus exhibiting 200-fold selectivity for the *h*MC4 vs *h*MC3 receptor as an antagonist. The $pA_2$ values for *h*MC3R and *h*MC4R were 6.2 and 8.0, respectively (Table 2) which further supports that analogue **7** has selective affinity for the *h*MC4R as an antagonist. Compound **4** (PG-914) with Che,[6] (a six-membered ring amino acid), resulted in an analogue with $IC_{50}$ binding affinities of 41 nM at the *h*MC3R, 0.48 nM at the *h*MC4R and 4.6 nM at the *h*MC5R, respectively. The $pA_2$ values found for **4** were 6.5 and 8.4 at *h*MC3R and *h*MC4R, respectively, a binding selectivity of almost 100-fold as an antagonist at the *h*MC4R vs *h*MC3R (Tables 1 and 2). Based on these results these compounds can be considered as new highly selective *h*MC4R peptide antagonist ligands vs the *h*MC3R. Compound **9** (PG-946) which contains an Acpc[6] residue, which has the smallest ring size, resulted in an analogue with high affinity at the *h*MC3R ($IC_{50} = 2.50$ nM), lower affinity at the *h*MC4R ($IC_{50} = 268$ nM), and modest affinity at the *h*MC5R ($IC_{50} = 4.1$ nM). This compound had no agonist activity at the human MC3R and MC4R, but was a weak partial agonist at the *h*MC5R (Table 1). Finally, analogue **5** (PG-915), having an Aic in position 6, resulted in a weak antagonist ($IC_{50} = 190$ nM) at the *h*MC3R, but a potent antagonist ($IC_{50} = 1.3$ nM) at the *h*MC4R and weak agonist ($IC_{50} = 12$ nM) at the *h*MC5R. Thus, this analogue is about 150-fold selective for the *h*MC4R when compared to the *h*MC3R.

The biological results of this series of compounds clearly indicate that a conformational restriction in position 6 can be an extremely useful to design selective ligands at the *h*MCRs. To understand the possible structural basis responsible for selectivity of these peptides at *h*MCR3 and *h*MCR4, we have carried out a molecular modeling study in order to examine the conformational properties of the cyclic peptide **1** and its analogues modified in position 6 with conformationally restricted amino acids.

**Molecular Modeling.** Molecular dynamics (MD) simulations using the TRIPOS force field[36,37] in SYBYL 6.2 software package[38] were performed on cyclic peptide **1** (PG-901**,** Figure 1) in order to study its conformational characteristics, especially if preferred turn geometries exist in the sequence region 5−9. This structural motif has been previously evaluated as the bioactive conformation of α-MSH analogues with the His[6]-Phe[7]-Arg[8]-Trp[9] core.[38−42]

Since the superpotent and *h*MCR prolonged acting cyclic peptide Ac-Nle[4]-c[Asp[5]-His[6]-DPhe[7]-Arg[8]-Trp[9]-Lys[10]]-$NH_2$ (MTII)[41−43] differs from **1** by having a His in position 6 and a DPhe residue in position 7 instead of Pro and DNal(2′), we used its two low-energy conformers obtained from previous calculations[41,44] as templates to build up the peptide cycle in **1**. As evident in Table 3, the two representative conformers for MTII differ from one another only by slight rotations of the planes of amide bonds between some residues, giving rise to a geometrical shape of the peptide backbone nearly identical to those previously found. Therefore, we selected only the first of the two MTII conformers to model **1**. The resulting structure was then minimized by molecular mechanics using a combination of steepest descent and conjugate gradient algorithms, as reported in the Experimental Section.

As illustrated in Figure 2, the 5−9 region of the peptide consists of a double turn with residues Asp[5] to Arg[8] forming a type II $\beta$-turn ($\gamma_L$-$\beta_E$)[45] and residues DNal(2′)[7] to Lys[10] a type $\beta_E$-$\gamma_L$ turn. A hydrogen bond is observed between the Arg[8] HN and Asp[5] CO groups.

**Table 2.** $pA_2$ Values for Melanocortin Peptides

| | | *h*MC3R | | *h*MC4R | |
|---|---|---|---|---|---|
| compd | code | $pA_2$ | $K_b$ (nM) | $pA_2$ | $K_b$ (nM) |
| **1** | PG-901 | 9. 0 ± 0.17 | 1.0 ± 0.39 | 9.3 ± 0.13 | 0.53 ± 0.15 |
| **2** | PG-911 | 9.4 ± 0.019 | 0.38 ± 0.02 | 9.8 ± 0.042 | 0.17 ± 0.015 |
| **3** | PG-913 | 8.2 ± 0.17 | 5.8 ± 0.2 | 8.9 ± 0.03 | 1.2 ± 0.3 |
| **4** | PG-914 | 6.5 ± 0.056 | 290 ± 40 | 8.4 ± 0.009 | 4.2 ± 0.05 |
| **5** | PG-915 | 6.4 ± 0.055 | 440 ± 55 | 7.7 ± 0.02 | 21 ± 1.0 |
| **6** | PG-916 | 6.1 ± 0.1 | 640 ± 160 | 8.6 ± 0.03 | 3.2 ± 0.3 |
| **7** | PG-917 | 6.2 ± 0.12 | 590 ± 150 | 8.0 ± 0.18 | 10 ± 4.0 |
| **8** | PG-944 | 9.2 ± 0.18 | 0.58 ± 0.05 | 8.0 ± 0.2 | 21 ± 1.3 |
| **9** | PG-946 | 7.6 ± 0.02 | 22 ± 4 | 6.5 ± 0.06 | 290 ± 40 |
| | SHU9119 | 8.7 ± 0.006 | 2.2 ± 0.05 | 9.1 ± 0.15 | 0.89 ± 0.32 |

**Table 3.** Backbone Torsion Angles (deg) of Selected Low-Energy Conformers of $Asp^5$-$Trp^9$ Fragment Common for MTII and PG-901

| | Asp | | His/Pro | | DPhe/DNal(2′) | | Arg | | Trp | |
|---|---|---|---|---|---|---|---|---|---|---|
| peptide | Φ | Ψ | Φ | Ψ | Φ | Ψ | Φ | Ψ | Φ | Ψ |
| MTII[a] | −101 | 18 | −56 | 121 | 85 | 11 | −144 | 122 | 58 | 48 |
| | −75 | 80 | −130 | 130 | 82 | −61 | −78 | 89 | −82 | −20 |
| PG-901[b] | −77 | 166 | −60 | 74 | 127 | −16 | −138 | 179 | −134 | 48 |

[a] Previously proposed lowest-energy conformers for MTII.[39,41] [b] Low-energy conformer of PG-901 obtained from free MD simulation.

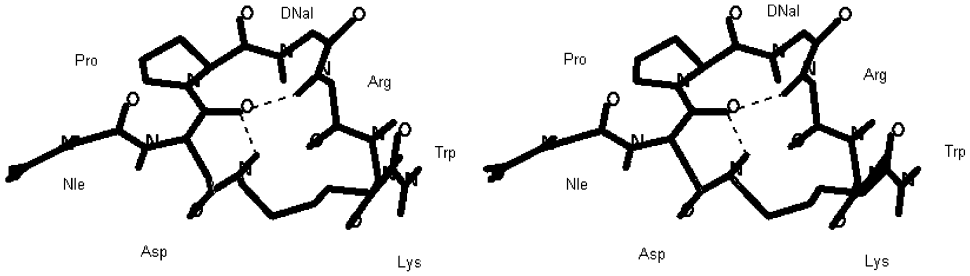



**Figure 2.** Stereoview of the cyclic peptide **1** generated by restrained energy minimization. Only backbone heavy atoms are shown. The hydrogen bond within the β-turn formed between $Asp^5$ CO and $Arg^8$ HN is indicated by a dotted line.

The first turn is further stabilized by a hydrogen bond between the $NH^{\epsilon}$ of $Lys^{10}$ and the $Asp^5$ CO side chain groups.

To obtain more insight in the conformational flexibility of the cyclic peptide, **1** was subjected to a constraint free MD simulation using simulated annealing as the sampling protocol. Seventeen structures, within 6 kcal/mol of the lowest energy conformer observed, were analyzed further as a basis for predicting the preferred conformation of peptide. All these conformers are characterized by the presence of a trans proline peptide bond, suggesting that this bond is trans in the bioactive conformation of this melanocortin antagonist. Families with similar conformational characteristics were classified into clusters based on the torsional root-mean-square deviation (rmsd) calculated for heavy backbone atoms (N, Cα, and C′). To cluster β-turns, the Φ and Ψ angles were used. When the distance from the Cα of the first residue to Cα of the fourth residue is less than 7 Å and the tetrapeptide sequence is not part of an α-helical region, it is considered to be a β-turn.[46] Only one of the 17 conformations identified fulfilled this distance criterion. This structure adopts two consecutive β-turns in sequence region 5−9: residues $Asp^5$-$Pro^6$-DNal(2′)$^7$-$Arg^8$ form a type II β-turn and residues $Pro^6$-DNal(2′)$^7$-$Arg^8$-$Trp^9$ form a type $\beta_E$-$\gamma_L$ turn. The superposition of restrained structure with that generated from free MD simulation using the heavy backbone atoms of the five residues involved in the turns shows a good fit with a rmsd value of 0.5.

To shed more light on the possible structural elements responsible for selectivity and/or potency of these compounds at *h*MCR subtypes, we have compared **3**−**7**, **8**, and **9** on the basis of the template conformation of **1** (Figure 3). All compounds were built as described in the Experimental Section and superimposed by a rmsd fit of the heavy backbone atoms.

Interestingly, the comparison of these compounds suggests that the side chain of residues in position 6 adopt a specific orientation that could be decisive for selectivity. Inspection of the model reveals the presence of two putative contiguous pockets (referred to as A and B) in the *h*MCR4 and *h*MCR3 subtypes, which are able to accommodate the different conformationally constrained residues of this peptide.

As can be observed in Figure 3, the most *h*MC4R selective analogues **4** and **7** accommodate their Che and Cpe residues into pocket A, while the Tic residue of **8** mainly occupies pocket B. This result could explain the nonselectivity of **8** for the *h*MC3R vs *h*MC4R ($IC_{50}$ = 6.70 and 3.70 nM at *h*MC3R and *h*MC4R, respectively), while **5** and **6** are selective at the *h*MC4R. The model suggests that the Aic and Ioc residues interact with pocket A, highlighting that the occupancy of this region might be important for *h*MC4R selectivity. The modeling comparison results for **3** also are consistent with the experimental data showing that it is not very selective for either the *h*MC3R and *h*MC4R. In fact, the Oic residue projects its peculiar conformation toward a region situated between pockets A and B in the two

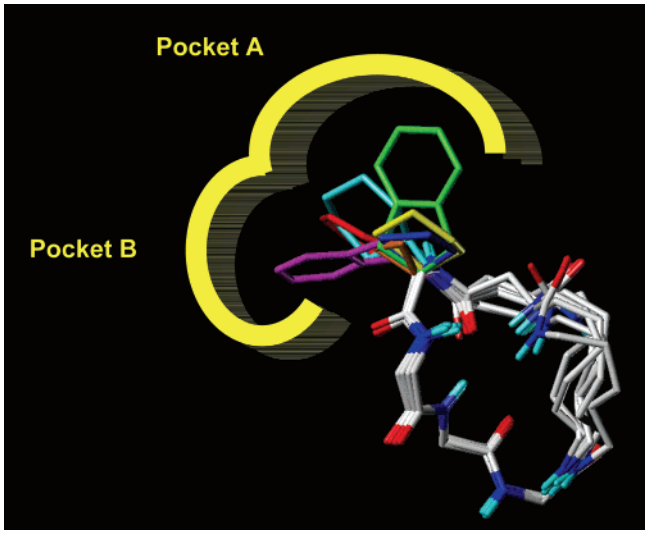



**Figure 3.** Overlay comparison of melanocortin antagonist analogues **3**−**7** (cyan, yellow, green, red and blue, respectively) **8** (magenta) and **9** (orange) on the energy-minimized conformation of **1**. The superimposition was performed by a rmsd fit of the heavy backbone atoms. For clarity, only the peptide backbone atoms are shown. Region A highlights the space occupied by position 6 side chain groups of analogues **4**-**7**, while Region B is the space occupied by position 6 side chain groups of analogues **8** and **9**.

receptor subtypes, as evidenced in Figure 3. Surprisingly, the conformationally constrained Acpc, characterized to have the smallest ring size, makes **9** a potent and reasonably selective *h*MC3R antagonist. At the moment we cannot explain this interesting result, but it is possible to speculate that the small side chain of Acpc residue accommodates itself better into the hypothetical pocket B.

## Conclusions

In conclusion, these SAR studies of synthetic melanocortin ligands at the *h*MCR3, *h*MCR4, and hMCR5 have identified new selective antagonists for the *h*MC4R, **5** and **7**, both of which are better than SHU-9119 (Table 1) and HS014[47] (Table 1) for their *h*MC4R selectivities, and similar in selectivity to the recently reported MBP10[48] (Table 1). Interestingly, we also found a potent selective antagonist for the *h*MC3R, **9**, which is about 100-fold selective for the *h*MC3R. Several modifications of position 6 by insertion of appropriate conformationally restricted amino acids demonstrate that this position represents an important "key" to identify potent and selective ligands at melanocortin receptors. The comparison of these compounds, in a molecular modeling study, revealed the presence of two putative contiguous but different pockets in the *h*MC3R and *h*MC4R subtypes, where the different conformationally constrained residues can be accommodated. This possibility could explain the different profiles of selectivity observed for this series of peptides at the two melanocortin receptor subtypes *h*MC3R and *h*MC4R.

## Experimental Section

**Materials.** Thin-layer chromatography (TLC) was done on Merck silica gel 60 $F_{254}$ plates using the following solvent system: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4); (B) ethyl acetate/pyridine/acetic acid/water (5:5:1:3); (C) upper phase of 1-butanol/acetic acid/water (4:1:1). 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 30 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 Dynamax HPLC solvent delivery system (Rainin Instrument Co., Inc., Woburn, MA). The separations were monitored at 280 and 230 nm and integrated with a Dynamax dual wavelength absorbance detector model UV-D. Fractions corresponding to the major peak were collected, pooled, and lyophilized. 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), a precolumn phenylthiocarbamyl-amino acid (PTC-AA) analysis. No corrections are 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 stors AMD mass spectrometer. The experimental conditions consisted of a glycerol matrix-scan of 200−2000 Da in the positive ion mode. The Rink Amide resin, $N^{\alpha}$-Fmoc amino acids and amino acid derivatives ($N^{\alpha}$-Fmoc-Cpe-OH, $N^{\alpha}$-Fmoc-Che-OH, $N^{\alpha}$-Fmoc-Ioc-OH, $N^{\alpha}$-Fmoc-Tic-OH) were purchased from Advanced Chemtech (Louisville, KY). $N^{\alpha}$-Fmoc-Aic-OH, $N^{\alpha}$-Fmoc-Oic-OH, and $N^{\alpha}$-Fmoc-Acpc-OH were purchased from Chem-Impex International Inc. (Wood Dale, IL). All purchased amino acids were of the L configuration. Fluorenylmethoxycarbonyl (Fmoc) was used for $N^{\alpha}$ protection, and the reactive side chains of the amino acids were protected as follows: Lys, with allyloxycarbonyl (Alloc); Asp, with allyl ester; Arg, with Pbf; Trp, with *tert*-butyloxycarbonyl (Boc). All reagents and solvents were ACS grade or better and were used without further purification. The purity of the finished peptides were checked by TLC in three different solvents and analytical RP-HPLC at 280 and 230 nm in all cases and they were greater than 95% pure as determined by these methods. The structures of the pure peptides were confirmed by fast atom bombardment (FAB) and in some cases with electon spray ionization (ESI) mass spectrometry. The analytical data for each compound is presented in Tables4 and 5.

**General Method for Peptide Synthesis and Purification.** The peptides were synthesized on 0.5 g of Rink amide resin (0.7 mmol of $NH_2$/g of resin) using $N^{\alpha}$-Fmoc chemistry and an orthogonal side chain protection strategy using manual solid-phase synthesis methods. The first amino acid, $N^{\alpha}$Fmoc-Lys($N^{\epsilon}$-Alloc)-OH, was linked on to the resin previously deprotected by a 25% piperidine solution in DMF for 30 min. The following amino acids were then added to the growing peptide chain by stepwise addition of $N^{\alpha}$-Fmoc-Trp($N^{i}$-Boc)-OH, $N^{\alpha}$-Fmoc-Arg($N^{G}$-Pbf)-OH, $N^{\alpha}$-Fmoc-*D*Nal(2′)-OH, $N^{\alpha}$-Fmoc-Che-OH, $N^{\alpha}$-Fmoc-Cpe-OH, $N^{\alpha}$-Fmoc-Aic-OH, $N^{\alpha}$-Fmoc-Oic-OH, $N^{\alpha}$-Fmoc-Ioc-OH, $N^{\alpha}$-Fmoc-Asp($\beta$-allyl)-OH, and $N^{\alpha}$-Fmoc-Nle-OH using standard solid-phase methods. Each coupling reaction was achieved using a 3-fold excess of amino acid, of HBTU/HOBt in the presence of DIEA. The $N^{\alpha}$-Fmoc protecting group was removed by treating the protected peptide resin with 25% piperidine solution in DMF (1 × 50 mL, 5 min, 1 × 50 mL, 20 min). The peptide resin was washed with DMF (3 × 50 mL), DCM (3 × 50 mL) and again with DMF. Upon complete formation of the protected linear peptide the final $N^{\alpha}$-Fmoc protecting group was removed in the usual manner with 25% piperidine and acetylated with 25% acetic anhydride in DCM for 20 min. At this step the Alloc and allyl protecting groups of lysine and aspartic acid, respectively, were removed as detailed below. All procedures were done in an argon atmosphere.

In a typical example, to a peptide resin (500 mg) washed with DCM (3 × 2 min) in the presence of argon was added a solution of $PhSiH_3$ (24 equiv) in 2 mL of DCM, and the resin was manually stirred for 2 min. Subsequently, a solution of $Pd(PPh_3)_4$ (0.25 equiv) in 6 mL of DCM was added as argon was bubbled continuously through the resin. The reaction was

**Table 4.** Physiochemical Properties of the Melanocortin Peptides

| code | peptide | solv. A | solv. B | solv. C | MW | MS | HPLC[b] |
|---|---|---|---|---|---|---|---|
| PG-901 | Ac-Nle-c[Asp-Pro-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.82 | 0.04 | 0.41 | 1034.5 | 1035.2 | 4.47 |
| PG-911 | Ac-Nle-c[Asp-Hyp-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.78 | 0.04 | 0.38 | 1050.5 | 1051.4 | 4.15 |
| PG-913 | Ac-Nle-c[Asp-Oic-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.85 | 0.08 | 0.43 | 1088.6 | 1089.9 | 5.13 |
| PG-914 | Ac-Nle-c[Asp-Che-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.82 | 0.04 | 0.41 | 1063.6 | 1062.8 | 5.47 |
| PG-915 | Ac-Nle-c[Asp-Aic-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.85 | 0.04 | 0.47 | 1096.6 | 1096.7 | 5.80 |
| PG-916 | Ac-Nle-c[Asp-Ioc-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.86 | 0.05 | 0.46 | 1083.5 | 1083.8 | 4.33 |
| PG-917 | Ac-Nle-c[Asp-Cpe-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.80 | 0.04 | 0.39 | 1048.5 | 1048.8 | 5.44 |
| PG-944 | Ac-Nle-c[Asp-Tic-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.92 | 0.10 | 0.55 | 1096.4 | 1096.6 | 5.23 |
| PG-946 | Ac-Nle-c[Asp-Acpc-DNal(2′)-Arg-Trp-Lys]-$NH_2$ | 0.77 | 0.05 | 0.37 | 1020.2 | 1020.7 | 4.32 |

[a] Solvent systems: (A) 1-butanol/HOAc/pyridine/$H_2O$ (5:5:1:4); (B) EtOAc/pyridine/AcOH/$H_2O$ (5:5:1:3); (C) 1-butanol/AcOH/$H_2O$ (4:1:1) [b] Analytical HPLC performed on a C18 column (Vydac 218TP104) using a gradient of acetonitrile in 0.1% aqueous TFA at 1 mL/min. The following gradient was used: 10−90% acetonitrile in 40 min.

**Table 5.** Amino Acid Analysis[a] of the Melanocortin Peptides

| code | Nle | Asp | Xaa | DNal | Arg | Trp | Lys |
|---|---|---|---|---|---|---|---|
| PG-901 | 0.98 | 1.02 | 0.97 | 0.98 | 0.95 | - | 0.96 |
| PG-911 | 0.96 | 0.98 | 1.03 | 0.93 | 0.97 | - | 0.98 |
| PG-913 | 0.98 | 1.02 | 0.89 | 0.96 | 0.95 | - | 0.96 |
| PG-914 | 1.00 | 1.03 | 0.84 | 0.99 | 0.95 | - | 0.96 |
| PG-915 | 0.99 | 0.98 | 0.93 | 1.00 | 1.01 | - | 0.99 |
| PG-916 | 1.03 | 0.98 | 0.83 | 1.00 | 1.02 | - | 1.03 |
| PG-917 | 0.97 | 0.95 | 0.96 | 0.98 | 0.97 | - | 1.03 |
| PG-944 | 0.95 | 1.04 | 0.96 | 1.06 | 0.96 | - | 0.97 |
| PG-946 | 1.01 | 0.92 | 0.98 | 0.91 | 0.93 | - | 0.96 |

[a] The analyses were performed using 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 a precolumn phenyl-thiocarbamyl-amino acid (PTC-AA) analysis. No correction is made for amino acid decomposition. Trp was not well-determined due to decomposition under these conditions.

mechanically stirred for 30 min under argon. Then, the peptide resin was washed with DCM (3 × 50 mL, 2 min), DMF (3 × 50 mL, 1 min), and again with DCM (4 × 50 mL, 2 min), and the process was repeated. The removal of allyl and Alloc groups under neutral conditions with catalytic amounts of $Pd(PPh_3)_4$ in the presence of $PhSiH_3$ and complete absence of oxygen[26] permitted orthogonal deprotection of the side chain protecting groups used during the synthesis. Then the peptide resin was suspended in 20 mL of *N*-methylpyrrolidone (NMP), followed by cyclization of the peptide via the free carboxylic acid side chain of Asp and the free amino group side chain of Lys by addition of HBTU (6 equiv), HOBt (6 equiv), and DIEA (12 equiv) for 2 h. This process was repeated until a negative Kaiser test resulted.[49] The acylation step was accomplished using $Ac_2O$ (20 equiv) and DIEA (1 equiv) in DCM.

The peptide was cleaved from the resin and the other side chain protecting groups removed using the following mixture: TFA/TES/$H_2O$ (9:0.5:0.5) for 3 h. The resin was removed from solution by filtration and the crude peptide was recovered by precipitation with cold anhydrous ethyl ether giving a white powder that was purified by preparative HPLC on a C18-bonded silica gel column (Vydac 218TP1010, 1.0 × 25 cm) eluted with a linear gradient of acetonitrile in aqueous 0.1% of trifluoroacetic acid (v/v). The purification was monitored at 280 nm, and the fractions corresponding to the major peak were collected, combined, and lyophilized to give final compound as a pure (>95%) white powder (yield: 45−50% as cyclic peptide). Analytical HPLC, TLC, and amino acid analysis was used to characterize the peptides (Tables 4 and 5).

**Binding Assays.** Binding assays were carried out using membranes prepared from CHO cells stably expressing human MC3, MC4, and MC5 receptors by a procedure described previously.[50] The binding assay mixture contained 0.2 nM of $^{125}$I-[Tyr$^2$][Nle,$^4$DPhe$^7$]α-MSH ($^{125}$I-NDP-α-MSH), varying concentrations of the peptide being tested, and an appropriate amount of membranes so that the total bound radioligand was less than 10% of the added radioligand. The above mixture in binding buffer (50 mM Tris, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, pH 7.2) was incubated at room temperature for 2 h, followed by filtration through GFC paper. The bound ligand was quantitated in a $\gamma$ counter. $IC_{50}$ values were calculated as previously described.[50] Assays were performed in triplicate and were repeated 2−4 times.

**cAMP Assays.** Intracellular cAMP concentration was measured by the New England Nuclear cAMP-[$^{125}$I] Flask Plate assay. CHO cells stably expressing the human MC3R, MC4R, and MC5R were resuspended in Earle's balanced salt solution, 10 mM HEPES, 5 mM $MgCl_2$, 1 mg/mL BSA, and 0.5 mM IBMX incubated with the peptide being tested for 45 min and lysed by 0.1 M HCl as described.[50] Assays were performed in triplicate and were repeated 2−4 times.

**Computational Procedures.** Molecules were modeled using standard bond lengths and angles, with the SYBYL software package[37] running on an Octane R10000 Silicon Graphics workstation. Conformational energies were calculated through the molecular mechanics TRIPOS force field[36] including the electrostatic contribution. Atom-centered partial charges were calculated according to the Gasteiger−Hückel method[51,52] with a dielectric constant of 80 (representing water). The Kollman force field[53] was not used because of the presence of the nonconventional amino acids Oic, Che, Aic, Ioc, Cpe, Tic, Acpc, and *D*Nal(2′). The starting structure of **1** (PG-901**)**, obtained as described in Molecular Modeling section, was minimized by consecutive 1000 steps of steepest descent and 1000 steps of the conjugate gradient algorithm until a final root-mean-square (RMS) gradient not larger than 0.05 kcal/mol/A$^2$ was reached. Φ and Ψ torsional constraints were applied on peptide backbone during the minimization, using a *k* force constant of 10 kcal/mol/A$^2$. A simulated annealing MD protocol was used to sample the conformational space accessible to the selected molecule (NTV ensemble). The calculation was performed as free simulation without any constraints on peptide backbone. One hundred twenty simulated annealing cycles consisting of a 5 ps high temperature interval at 900 K followed by a 5 ps annealing interval in which the temperature was decreased exponentially from 900 to 300 K were carried out during each simulation. An integration time step of 1 fs was used. Conformers was sampled at the end of each cycle and stored in a database for further minimization. The resulting 120 "low-temperature" conformers were energy-minimized as described before. Structures within 6 kcal/mol above the global minimum were compared with each other and clustered into families by an rmsd fit of the heavy backbone atoms (N, Cα, and C).

Molecular models of **3**−**7**, **8**, and **9** were derived from structure of **1** by replacing Pro$^6$ with the above-mentioned nonconventional amino acids. The resulting structures were then minimized, as described above and superimposed on **1** by a rmsd fit of the peptide backbone atoms.

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