Substitution of Arginine with Proline and Proline Derivatives in Melanocyte-Stimulating Hormones Leads to Selectivity for Human Melanocortin 4 Receptor

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A new series of melanotropin analogues with His or Arg residues in the core pharmacophores of MTII, SHU9119, and Ac-NDP-γ-MSH-NH_2 replaced by Pro or *trans*-/*cis*-4-guanidinyl-Pro derivatives were designed and synthesized to introduce selectivity toward the human melanocortin 4 receptor (*h*MC4R). Analogues **1**, **2**, **3**, **6**, **7**, **8** were found to be *h*MC4R selective. Second messenger studies have demonstrated that analogues **1** and **2** are insurmountable inhibitors of MTII agonist activity at the *h*MC4R. Molecular modeling studies suggest that the *h*MC4R selectivity is due to a β-turn shift induced by the Pro ring that makes the global minimum structures of these analogues resemble the NMR solution structure of the *h*ASIP melanocortin receptor binding motif. Substitution of His in MTII also provided functional selectivity for the *h*MC3R or the *h*MC4R. These findings are important for a better understanding of the selectivity mechanism at the *h*MC3R/*h*MC4R and the development of therapeutic ligands selectively targeting the *h*MC4R.

Introduction

Melanocortin receptors along with their endogenous agonists and antagonists are involved in the modulation of a wide range of important physiological functions[1] that include skin pigmentation,[2−4] blood pressure and heart rate,[5] erectile function,[6,7] feeding behavior and energy homeostasis,[7−14] aggressive/defensive behavior,[15] and modulation of pain.[16−18] To date, five human melanocortin receptor subtypes, namely, the human melanocortin receptors 1−5 (*h*MC1−5R[a]), have been identified and cloned. All five receptor subtypes belong to rhodopsin family of seven-transmembrane G-protein-coupled receptors (GPCRs), which intracellularly mediates its effects by activating cAMP-dependent pathways.[19] The *h*MC1R is mainly expressed in the melanocytes and leukocytes and is involved in skin pigmentation, pain modulation, inflammation, and control of immune system.[18,20−22] The *h*MC2R, also known as the ACTH receptor, is expressed in the adrenal cortex and mediates glucocorticoneogenesis and other effects.[23,24] The *h*MC3R and *h*MC4R are primarily found in the central nervous system (CNS) with different patterns of distribution.[25,26] Both of them have been implicated to play roles in feeding regulation and weight control.[1,27,28] The *h*MC3R also has been suggested to play an important role in mediating some of the anti-inflammatory effects of its endogenous agonist α-MSH.[29] Erectile function, anxiety, and stress are also primarily mediated through the *h*MC4R.[6,7,30,31] The *h*MC5R is distributed in a variety of peripheral tissues and in the brain, cortex, and cerebellum. Its effects in the central nervous system have not yet been well studied, but it has been shown to be important in regulating exocrine gland function[32,33] and coordinating central and peripheral signals for aggression.[15,34] Of particular interest for therapeutic applications is the *h*MC4R,[7,35] as there is an extensive body of preclinical and clinical evidence that suggest that the *h*MC4R is intimately involved in the regulation of food intake and energy expenditure.[7,8,36] It has been shown that *h*MC4R selective agonists can be used to treat obesity, though there are other side effects to overcome.[37] On the other hand, *h*MC4R selective antagonists are promising candidates for treating anxiety and stress, anorexia, and cancer- and HIV-related weight loss.[31,38] Furthermore, it has been shown that targeting the *h*MC4R may provide an alternative approach to treat sexual dysfunctions in both male and female.[6,39] Unlike current therapies that target end organ vascular tissue, this new approach is centrally mediated and thus will have significant advantages over existing ones for the treatment of a variety of sexual dysfunctions in both sexes.[6,40] Despite the central importance of the *h*MC4R, rational design of potent selective *h*MC4R antagonists is still challenging. In this paper, we have introduced a rational structure-based design of selective *h*MC4R antagonists.

Design of Novel Ligands

The endogenous antagonists of the *h*MCRs include the agouti signaling protein (*h*ASIP or the "agouti protein"), which

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[a] Abbreviations: Aba, 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one; Ac, acetyl; Ac-NDP-γ-MSH-NH_2, Ac-Tyr-Val-Nle-Gly-His-D-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2; AGRP, agouti related protein; Alloc, allyloxycarbonyl; ASIP, agouti signaling protein; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; cAMP, cyclic adenosine monophosphate; Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; CNS, central nervous system; DCM, dichloromethane; DIC, 1,3-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DNal(2′), 3-(2-naphthyl)-D-alanine; ESI, electrospray ionization; Et_2O, diethyl ether; EtOAc, ethyl acetate; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, *N*-[(9-fluorenylmethoxycarbonyl)oxy]succinimide; GB/SA, generalized Born/surface area; GPCR, G-protein-coupled receptor; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HIV, human immunodeficiency virus; *h*MC1R, human melanocortin 1 receptor; *h*MC2R, human melanocortin 2 receptor; *h*MC3R, human melanocortin 3 receptor; *h*MC4R, human melanocortin 4 receptor; *h*MC5R, human melanocortin 5 receptor; HOAt, 1-hydroxy-7-aza-benzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; HRMS, high resolution mass spectrometry; Hyp, *trans*-4-hydroxylproline; IC_{50}, 50% inhibitory concentration; MBHA, 4-methylbenzhydrylamine; α-MSH, α melanocyte-stimulating hormone; Nle, L-norleucine; NMR, nuclear magnetic resonance spectroscopy; OPLS, optimized potential for liquid simulations; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl; POMC, proopiomelanocortin; PRCG, Polak−Ribier conjugate gradient; rmsd, root-mean-square deviation; RP-HPLC, reverse phase high-pressure liquid chromatography; SAR, structure−activity relationship; SPPS, solid phase peptide synthesis; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; TLC, thin layer chromatography; TMS, tetramethylsilane; Trt, trityl.

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*h*AGRP(110-117) central loop c[CRFFNAFC]
*h*ASIP(116-123) central loop c[CRFFRSAC]

Figure 1. Central loops of *h*AGRP and *h*ASIP and their core pharmacophore RFF.

primarily binds to the *h*MC1R and *h*MC4R and displays weaker affinity toward the *h*MC3R, and the agouti-related protein (*h*AGRP) which is selective for the *h*MC3,4R.[41] These two antagonists are expressed in different parts of the body,[42] and it has been postulated that they interact with the melanocortin receptors via the core pharmacophore RFF in their central Cys-constrained loops (Figure 1).[43−45] The high selectivity of the *h*ASIP for the *h*MC4R (46-fold, *h*MC4R/*h*MC3R) prompted us to compare its structures with potent nonselective *h*MC3R/*h*MC4R antagonist SHU9119 (Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2) by using computational modeling methods, and these studies demonstrated considerable similarity. First of all, both of these molecules' binding domains are constrained cyclic peptides with similar ring size. Second, they all have guanidinyl and aromatic moieties in their core pharmacophores and all of them have β-turn structures. However, the sequences of their β-turns are somewhat different. The NMR solution structure of the SHU9119 suggests a β-turn encompassed by the Asp-His-DNal(2′)-Arg residues,[46] whereas for the *h*ASIP, its NMR solution structure suggests a β-turn at Phe-Phe-Arg-Ser residues.[41] We envisioned that a forced turn spanning between the DNal(2′) and Arg residues in SHU9119 may provide short and stable peptide analogues that could mimic the *h*ASIP core pharmacophore, thus achieving greater selectivity for the *h*MC4R. Since SHU9119 and MTII (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2) share considerable sequence and secondary structural similarities,[46] we also decided to extend similar modifications to MTII.

One possible approach to have such a turn in the globally constrained SHU9119 and MTII is to substitute arginine[8] with structurally more constrained proline or its derivatives.[47] Presumably, the two Phe residues in the *h*ASIP central loop could be mimicked by the His[6] and DPhe[7]/DNal(2′)[7] residues in the MTII and SHU9119. In order to keep the guanidinyl groups in MTII or SHU9119 for possibly mimicking the guanidinyl group in the *h*ASIP pharmacophore sequence, proline derivatives with guanidinyl moieties on the proline ring are needed. In this regard, Tamaki et al. have developed a methodology for the synthesis of both *trans*- and *cis*-4-guanidinyl-Pro derivatives in their protected forms.[48] These novel constrained amino acids would also allow us to study the topographic importance of the guanidinyl group when it is highly constrained. Therefore, we designed MTII and SHU9119 analogues **2**, **3**, **7**, **8** in which the Arg[8] residues in their core sequences were replaced by 4-*trans*- or *cis*-guanidinyl-Pro residues (Figure 2). Additionally, to test how this modification compares with a potent nonselective linear peptide, Ac-NDP-γ-MSH-NH_2 (Ac-Tyr[1]-Val[2]-Nle[3]-Gly[4]-His[5]-DPhe[6]-Arg[7]-Trp[8]-Asp[9]-Arg[10]-Phe[11]-Gly[12]-NH_2),[49] analogues **12**, **13** were designed that contained a *trans*- or *cis*-4-guanidinyl-Pro in the key arginine[7] positions (Figure 2). For comparison, control analogues **1**, **6**, and **11** were also synthesized to investigate the importance of the guanidinyl groups. Furthermore, previous studies have indicated that the His[6] residue in the MTII and SHU9119 can be replaced with a Pro residue without causing any selectivity changes at the *h*MC3R and *h*MC4R.[50−52] However, replacement of His[6] residue in the SHU9119 with a variety of proline-like, conformationally constrained, hydrophobic amino acid residues led to the discovery of several *h*MC3R and *h*MC4R selective

1: Ac-Nle-c[Asp-His-DPhe-Pro-Trp-Lys]-NH_2
2: Ac-Nle-c[Asp-His-DPhe-trans-Xaa-Trp-Lys]-NH_2
3: Ac-Nle-c[Asp-His-DPhe-cis-Xaa-Trp-Lys]-NH_2
4: Ac-Nle-c[Asp-trans-Xaa-DPhe-Arg-Trp-Lys]-NH_2
5: Ac-Nle-c[Asp-cis-Xaa-DPhe-Arg-Trp-Lys]-NH_2
6: Ac-Nle-c[Asp-His-DNal(2')-Pro-Trp-Lys]-NH_2
7: Ac-Nle-c[Asp-His-DNal(2')-trans-Xaa-Trp-Lys]-NH_2
8: Ac-Nle-c[Asp-His-DNal(2')-cis-Xaa-Trp-Lys]-NH_2
9: Ac-Nle-c[Asp-trans-Xaa-DNal(2')-Arg-Trp-Lys]-NH_2
10: Ac-Nle-c[Asp-cis-Xaa-DNal(2')-Arg-Trp-Lys]-NH_2
11: Ac-Tyr-Val-Nle-Gly-His-DPhe-Pro-Trp-Asp-Arg-Phe-Gly-NH_2
12: Ac-Tyr-Val-Nle-Gly-His-DPhe-trans-Xaa-Trp-Asp-Arg-Phe-Gly-NH_2
13: Ac-Tyr-Val-Nle-Gly-His-DPhe-cis-Xaa-Trp-Asp-Arg-Phe-Gly-NH_2

trans-Xaa = cis-Xaa =

Figure 2. Peptide design with use of proline or *trans*/*cis*-4-guanidinyl-Pro.

antagonists.[53] Therefore, analogues **4**, **5**, **9**, **10** (Figure 2) were prepared by replacing the His[6] residues in the MTII and SHU9119 with 4-*trans*- or *cis*-guanidinyl-Pro derivatives. Such substitution will help in investigating the possible effects of introducing additional positively charged guanidinyl groups in position 6.

Peptide Synthesis

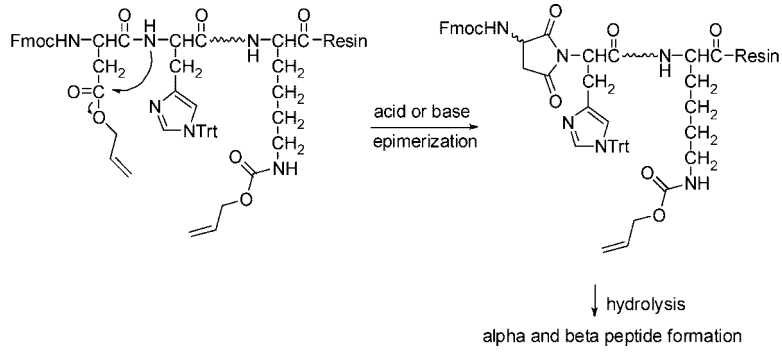
A well-known side reaction in Fmoc peptide synthesis of MTII analogues is aspartimide formation (Scheme 1).[54,55] To avoid this side reaction, the synthesis was designed in such a way that all coupling reactions were performed under neutral conditions using DIC/Cl-HOBt as coupling reagents. In addition, the side chain to side chain cyclization was performed immediately after the attachment of the Fmoc-Asp(Allyl) residue to the growing peptide chain under neutral conditions (Scheme 2). The cyclization was then followed by aspartic acid Fmoc removal and the coupling of the next residue, Nle.

Results

The protected *trans*- and *cis*-4-guanidinyl-Pro analogues were synthesized from *trans*-4-hydroxyproline following a facile method developed by Tamaki et al.[48,56] All peptides were synthesized via the Fmoc SPPS strategy outlined above with high yields and purity as determined by HPLC, TLC, NMR, and high resolution mass spectrometry (Table 1). To see whether aspartimide derivatives could be formed under basic conditions, we also synthesized Ac-Nle-Asp-His-DPhe-*trans*-4-guanidinyl-Pro-Trp-Lys-NH_2, the linear analogue of **2**, using HBTU/HOBt/DIPEA as coupling reagents. The linear peptide was cleaved from the resin after the removal of the side chain Allyl/Alloc protecting groups in the last step. Analytical HPLC and mass spectrometry identified an aspartimide derivative (35%) which has exactly the same mass as the corresponding cyclic peptide **2**. Considering the possible ring-opening of the aspartimide derivative that could lead to the formation of linear, epimerized α-peptide or β-peptide that might be difficult to separate from the desired α-peptide by HPLC,[54,57] the actual percentage of the aspartimide peptide formed could be higher. This result is consistent with the report by Flora et al. regarding the Fmoc synthesis of MTII.[55] We did not observe the aspartimide derivative during the synthesis of peptide **2** using the strategy shown in Scheme 2.

The binding affinities and biological activities of the peptides **1**−**13** were determined at the *h*MC1R, *h*MC3R, *h*MC4R, and *h*MC5R, and the results are summarized in Table 2. Considering

Scheme 1. Aspartimide Formation and the Consequence of the Side Reaction



Scheme 2. SPPS Strategy Used To Avoid Aspartimide Derivative Formation

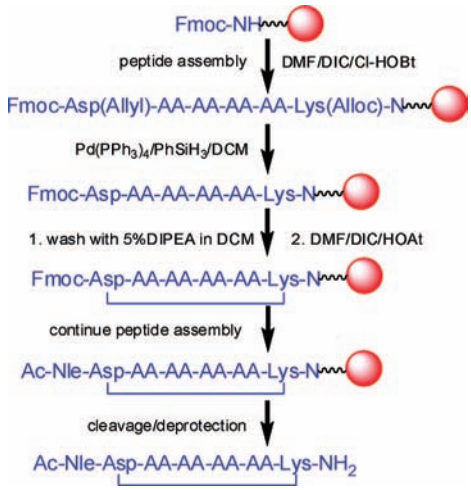


Table 1. Physicochemical Properties of the Peptides Synthesized

	HRMS (*m*/*z*)		HPLC t_R'[a] (min)		TLC R_f[b]	
compd	calcd	found	1	2	1	2
1	511.7701 (2H)	511.7725 (ESI)	7.90	11.00	0.67	0.36
2	511.7701 (2H)	511.7725 (ESI)	8.09	11.70	0.67	0.36
3	483.2538 (2H)	483.2546 (ESI)	10.26	14.81	0.74	0.4
4	521.2912 (2H)	521.2907 (ESI)	8.98	13.03	0.68	0.37
5	521.2912 (2H)	521.2889 (ESI)	8.94	12.86	0.68	0.37
6	536.7779 (2H)	536.7768 (ESI)	10.02	14.59	0.73	0.44
7	536.7779 (2H)	536.7756 (ESI)	10.00	14.50	0.73	0.44
8	508.2616 (2H)	506.2624 (ESI)	12.28	17.82	0.78	0.51
9	546.2990 (2H)	546.3014 (ESI)	10.38	15.12	0.73	0.44
10	546.2990 (2H)	546.2995 (ESI)	11.05	16.17	0.73	0.45
11	531.2693 (3H)	531.2697 (ESI)	9.70	14.31	0.71	0.33
12	531.2693 (3H)	531.2695 (ESI)	9.95	14.70	0.71	0.33
13	767.8837 (2H)	767.8868 (ESI)	11.85	17.44	0.74	0.37

[a] HPLC column, Vydac 218TP104, 250 mm × 4.6 mm, 10 μm, 300 Å. t_R' = peptide retention time minus solvent retention time. System 1: solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient 10−90% B in 40 min; flow rate 1.0 mL/min at 40 °C. System 2: solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient 10−60% B in 40 min; flow rate 1.0 mL/min at 40 °C. [b] TLC system 1: *n*-butanol/acetic acid/water/pyridine (4:1:2:1). TLC system 2: *n*-butanol/acetic acid/water (4:1:1).

very high receptor density used for the assays that may give increased apparent drug potency and/or efficacy and 10−20% errors in the experiments, we define a drug that binds to the *h*MCRs and triggers a maximal cAMP level that is 20−80% of MTII triggered maximal cAMP response as a partial agonist and when 80−100% level of MTII as a full agonist. Also, we define a drug that binds to the *h*MCRs and triggers less than 20% maximal cAMP level of MTII as an antagonist.[58] Control peptides **1**, **6** show high affinity and selectivity toward the *h*MC1,4Rs and no binding at the *h*MC3,5Rs at up to 10 μM. Peptide **1** is considered a selective antagonist for the *h*MC4R (IC_{50} = 11 nM) with very weak partial agonist activity at the *h*MC1R (EC_{50} > 1000 nM, 75% max activation). Peptide **6** is a moderate partial agonist selective for the *h*MC1R (EC_{50} = 160 nM, 20-fold, *h*MC1R/*h*MC4R). Peptide **11** is a nonselective partial agonist at the *h*MC1R (EC_{50} = 120 nM, 53% max activation) and *h*MC4R (EC_{50} = 210 nM, 46% max activation). Its binding affinity at the *h*MC4R is 25- and 8-fold lower than peptides **1** and **6**, respectively. Analogue **2** is a potent antagonist selective for the *h*MC4R (IC_{50} = 50 nM, >2000-fold, *h*MC4R/*h*MC3R). The presence of a *trans*-4-guanidinyl group also introduces weak partial agonist activity at the *h*MC5R (EC_{50} = 2500 nM, 24% max activation). Schild analyses of analogues **1** and **2** based on cAMP accumulation (Figure 3) show that both peptides **1** and **2** are not competitive inhibitors of MTII toward the *h*MC4R, which suggests that they might be allosteric binders. The binding affinity of peptide **3** is 4-fold weaker than that of the control peptide **1** at the *h*MC4R. However, the EC_{50} and maximal cAMP response at the same receptor are increased by 18-fold and 3-fold, respectively, converting peptide **3** to a potent *h*MC4R selective partial agonist (EC_{50} = 11 nM, 36% max activation, 70-fold, *h*MC4R/*h*MC1R). Compared to peptide **6**, peptide **7** displays a 5-fold increase in IC_{50}, 29-fold increase in EC_{50}, and 44% decrease in maximal cAMP response at the *h*MC4R showing moderate partial agonist activity (EC_{50} = 200 nM, 31% max activation). It also shows very weak partial agonist activity at the *h*MC5R (EC_{50} = 2400 nM, 59% max activation). Peptide **8** is an antagonist for the *h*MC4R (IC_{50} = 23 nM), with no binding at the *h*MC3,5Rs and very weak full agonist activity at the *h*MC1R (EC_{50} = 19 000 nM, 100% max activation). In the Ac-NDP-γ-MSH-NH_2 series, the additional guanidinyl groups in the 4′ positions of the proline ring in analogues **12** or **13** produce weak full agonists that are *h*MC1R selective but with a 4- to 5-fold drop in binding affinity and more than 11-fold drop in potency.

For MTII or SHU9119 analogues **4**, **5**, **9**, **10** in which the His^6 residues in the parent peptides are replaced by the *trans*- or *cis*-4-guanidinyl-Pro, the binding at the *h*MC3,4Rs remains strong and their IC_{50} values are still in the single-digit nanomolar range. However, there are large changes in ligand potency and/or efficacy at individual receptors. The *trans*-4-guanidinyl-Pro^6 analogue **4** is a potent *h*MC3R full agonist (EC_{50} = 14 nM) and *h*MC5R partial agonist (EC_{50} = 1.6 nM, 68% max activation) with high selectivity against the *h*MC4R (250-fold, *h*MC3R/*h*MC4R) and *h*MC1R (60-fold, *h*MC3R/*h*MC1R). Intriguingly, the isomeric *cis*-4-guanidinyl-Pro^6 analogue **5** displays a reversal of *h*MC3R/*h*MC4R selectivity (60-fold, *h*MC4R/*h*MC3R), showing potent *h*MC4R/*h*MC5R full agonist activity (EC_{50} = 41, 21 nM, respectively). Such functional selectivity at the *h*MC3R and *h*MC4R is not seen in peptides

Table 2. Binding Affinities and cAMP Activities of the Designed Peptides[a]

	hMC1R			hMC3R			hMC4R			hMC5R		
peptide[b]	IC_{50}, nM	EC_{50}, nM	% max	IC_{50}, nM	EC_{50}, nM	% max	IC_{50}, nM	EC_{50}, nM	% max	IC_{50}, nM	EC_{50}, nM	% max
MTII	0.20 ± 0.01	0.30 ± 0.04	100	1.3 ± 0.2	1.9 ± 0.2	100	1.1 ± 0.3	2.9 ± 0.5	100	7.5 ± 0.2	3.3 ± 0.7	100
1	>4800	>1000	75 ± 10	NB	0	0	11 ± 0.9	200 ± 40	13 ± 5	NB	0	0
2	NB	0	0	NB	0	0	50 ± 10	43 ± 8	3.9 ± 0.3	590 ± 90	2500 ± 480	24 ± 3
3	3500 ± 400	760 ± 150	91 ± 8	NB	0	0	39 ± 0.2	11 ± 2	36 ± 12	NB	0	0
4	2.7 ± 0.5	820 ± 150	77 ± 17	1.0 ± 0.2	14 ± 2.5	91 ± 7	0.60 ± 0.20	3500 ± 660	64 ± 17	2.5 ± 0.4	1.6 ± 0.2	68 ± 8
5	1.1 ± 0.2	2700 ± 200	100 ± 5	2.8 ± 0.1	2500 ± 500	68 ± 15	5.7 ± 0.5	41 ± 4	89 ± 15	1.1 ± 0.4	21 ± 3	100
SHU9119	1.0 ± 0.2	1.5 ± 0.1	100	2.3 ± 0.2	0	0	0.60 ± 0.10	0	0	0.90 ± 0.20	1.2 ± 0.1	97 ± 10
6	600 ± 120	160 ± 30	66 ± 10	NB	0	0	33 ± 3	5700 ± 1000	55 ± 7	NB	0	0
7	>3000	0	0	>10000	620 ± 120	56 ± 7	7.2 ± 1.4	200 ± 10	31 ± 1	22 ± 3	2400 ± 460	59 ± 8
8	400 ± 80	19000 ± 3700	100 ± 5	NB	0	0	23 ± 1	250 ± 30	7.3 ± 1	NB	0	0
9	2.3 ± 0.4	620 ± 110	25 ± 5	2.3 ± 0.1	130 ± 25	28 ± 2	1.5 ± 0.3	200 ± 40	17 ± 2	0.70 ± 0.20	0.30 ± 0.06	18 ± 2
10	1.2 ± 0.2	1700 ± 300	90 ± 18	2.2 ± 0.2	0	0	2.5 ± 0.5	1400 ± 280	6.2 ± 2	2.2 ± 0.4	85 ± 15	52 ± 17
Ac-NDP-γ-MSH-NH_2	0.50 ± 0.01	1.5 ± 0.1	100	2.0 ± 0.2	2.0 ± 0.2	100	1.2 ± 0.2	1.4 ± 0.1	100	2.4 ± 0.3	1.9 ± 0.2	100
11	190 ± 35	120 ± 20	53 ± 10	NB	0	0	270 ± 30	210 ± 30	46 ± 5	NB	0	0
12	830 ± 150	1400 ± 250	92 ± 14	NB	0	0	NB	0	0	NB	0	0
13	1000 ± 200	>5000	100	NB	0	0	NB	0	0	NB	0	0

[a] IC_{50} = concentration of peptide at 50% specific binding ($N = 4$). EC_{50} = effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation ($N = 4$). % max is ratio of the highest cAMP level triggered by peptides over the highest cAMP level triggered by MTII. The peptides were tested at a range of concentrations from 10^{-10} to 10^{-5} M. NB: no binding at 10^{-5} M. [b] Xaa = 4-guanidinyl-Pro.

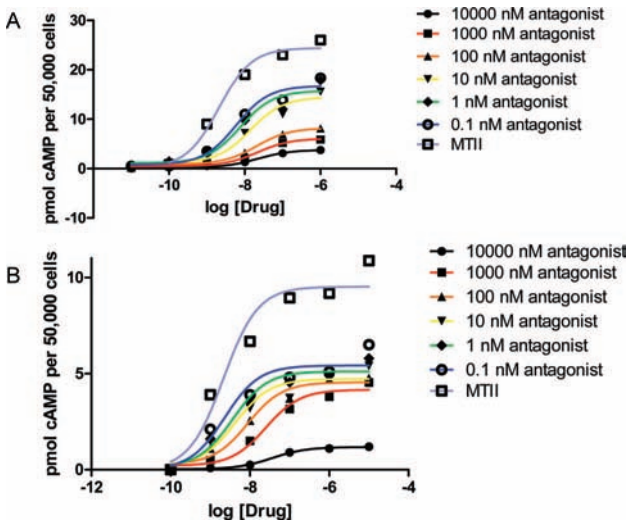


Figure 3. (A) Analysis of **1** inhibition of MTII triggered cAMP response by the antagonist peptide **1**. The panel shows the inhibition of the agonist MTII log dose response curve in the presence of increasing concentration of **1**. (B) Analysis of the **2** inhibition of MTII triggered cAMP response by the antagonist peptide **2**. The panel shows the inhibition of the agonist MTII log dose response curve in the presence of increasing concentration of **2**.

9, **10** that show no or low partial agonist activity. Evidently, the melanocortin receptor 3/4 binding pockets are extremely sensitive to slight modifications of the structure of residue 6 in MTII template.

Peptides **1** and **2** were chosen for further molecular modeling study because of their selective antagonist activity at the *h*MC4R vs *h*MC3R. The modeling experiments were performed using Kolossvary's Monte Carlo multiple minima/low frequency mode conformational search method (MCMM/LMCS)[59] with the OPLS_2005 force field[60] and the GB/SA continuum dielectric water solvent model.[61] It was revealed that the type II β-turn spanning the His^6-$DPhe^7$/DNal(2′)^7 residues, prominent in the NMR solution structures of MTII or SHU9119,[46] was no longer possible in Pro^8-analogue **1** (Figure 4). The 3D structure of analogue **2** displays a shift of the β-turn motif to Asp^5-His^6 residues (Figure 5), which is now closer to a type II′ turn ($\varphi_5 = 75°$, $\Psi_5 = -74°$, $\varphi_6 = -143°$, and $\Psi_6 = 9°$). At the same time, the 3D structure of analogue **2** was found to produce a good fit within the pharmacophore region of the NMR solution structure of the *h*ASIP central loop (Figure 5).[41] The side chains of His^6 and $DPhe^7$ residues in analogue **2** align well with the side chains of the Phe^{118} and Phe^{119} residues, respectively, in the putative Arg^{117}-Phe^{118}-Phe^{119}-Arg^{120}-Ser^{121} pharmacophore sequence of the *h*ASIP, as well as the guanidinyl groups of guanidinyl-Pro^8 of analogue **2** and Arg^{120} of the *h*ASIP. Yet, analogue **2** lacks the functionality to mimic the potentially critical Arg^{117} residue of the *h*ASIP pharmacophore. The unique conformational profiles of the peptides in this study stemmed from employment of the dihedrally constrained arginine analogues and inferred an opportunity to discover peptide analogues with enhanced melanocortin receptor selectivity.

Discussion

It has been known that it is difficult to separate ligand interactions with the *h*MC3R from those with the *h*MC4R based on rational modifications on MTII or SHU9119. In the present study, Pro residues in the positions of Arg residues in the MTII, SHU9119, and Ac-NDP-γ-MSH-NH_2 have been shown to be able to introduce high binding selectivity for the *h*MC4R vs *h*MC3R as indicated by the binding data of the three control peptides **1**, **6**, **11**. It should be pointed out that Bednarek et al. reported lower selectivity and weaker binding of peptides **1**, **6** to the *h*MC4R.[62] These discrepancies may be caused by different assay systems used. Nonetheless, it was shown in both systems that peptide **1** was an antagonist at the *h*MC4R. The altered selectivity/activity of those novel peptides is presumably caused by constrained proline-induced peptide backbone conformational changes as suggested in Figure 4. These changes probably altered the topography of the core pharmacophore elements in a way that favors interaction with the *h*MC1R and/or *h*MC4R. The new 3D positions of the core pharmacophore at least partially resemble that of the *h*ASIP as supported by the molecular modeling results and receptor selectivity displayed by the three control peptides. The much larger affinity drop seen in peptide **11** is likely due to its flexible linear structure, which is easily altered by the presence of a proline residue.

The replacement of Arg with Ala in MTII/SHU9119 has been previously reported to be able to cause at least a 100-fold affinity drop at the *h*MC3R, *h*MC4R, and *h*MC5R due to loss of direct interactions between the guanidinyl group and the melanocortin receptors.[63−66] However, there is only 1.5- to 5-fold difference in affinity between peptides **2**, **3**, **7**, **8** and their respective control peptides at the *h*MC4R. In addition, control peptide **1** shows

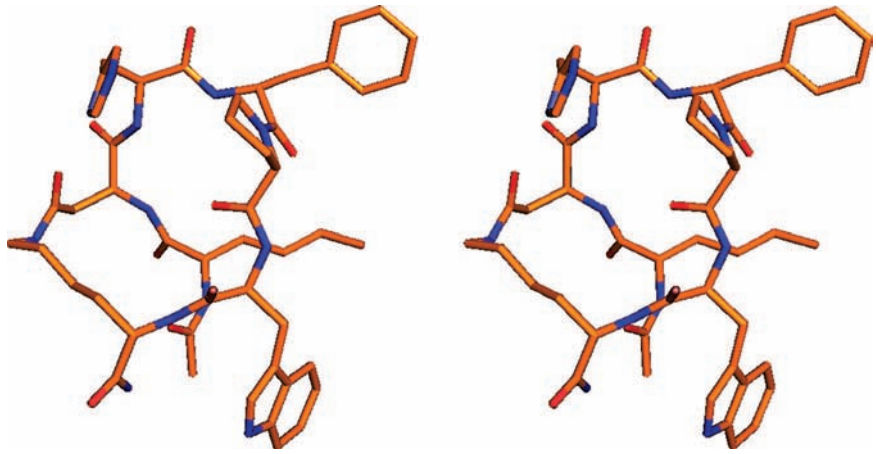


Figure 4. Stereoview of the global minimum of analogue **1** obtained by MCMM/LMCS (Monte Carlo multiple minima-low frequency mode)−OPLS 2005 simulation.

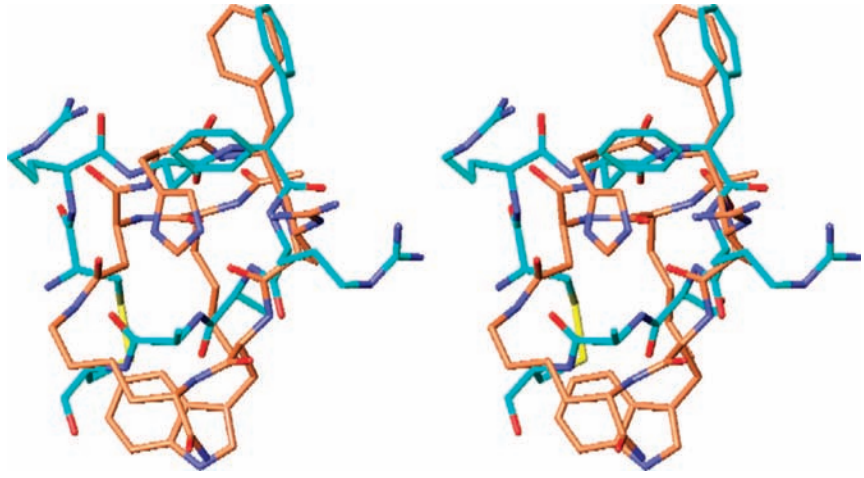


Figure 5. Stereoview of the global minimum structure of analogue **2** (orange) obtained by MCMM/LMCS (Monte Carlo multiple minima-low frequency mode)−OPLS 2005 simulation, superimposed on the NMR solution structure of the *h*ASIP central loop (blue).

stronger binding (4- to 5-fold) than its analogues **2**, **3** that have additional guanidinyl groups in position 8. These data suggest that the additional guanidinyl groups in peptides **2**, **3**, **7**, **8** do not contribute to binding to the *h*MC4R. And their presence is not required for excluding binding to the *h*MC3R as indicated by the lack of control peptides binding to the *h*MC3R at up to 10 μM concentration. The molecular modeling study (Figure 5) shows that the guanidinyl group in peptide **2** is quite far away from the crucial guanidinyl group in Arg^{117} of the *h*ASIP core pharmacophore. Therefore, the additional guanidinyl groups in novel analogues **2**, **3**, **7**, **8** are probably not mimicking Arg^{117} of the *h*ASIP.[41] The poor mimicry of the Arg^{117} guanidinyl group is at least in part due to the sequence difference. To better mimic the core pharmacophore of *h*ASIP central loop, future analogues based on the templates of analogues **1** and **6** will incorporate an Arg residue before the His^6 residue.

Although the additional guanidinyl groups in positions 8 of peptides **2**, **3**, **7**, **8** do not appear to bind to the *h*MC4R, they do seem to affect ligand potency (up to 29-fold) and efficacy (up to 3-fold) at the *h*MC4R and ligand selectivity over other receptor subtypes depending on the stereochemistry of the guanidinyl groups. Figure 5 shows that the guanidinyl group is in proximity with the imidazole group of His^6, which mimics Phe^{118} of the *h*ASIP. Therefore, it is possible that the additional guanidinyl groups can change the 3D positions of the critical His^6 side chain, which may affect ligand functional activity and selectivity.

In addition to position 8 of these novel MTII/SHU9119 analogues, position 7 also displays SAR that is different from MTII/SHU9119 SAR. In the MTII/SHU9119 binding sites on the *h*MC3R and *h*MC4R, the proper positioning of different types of side chains in position 7 determines ligand functional activity. A phenyl group usually provides agonist activity as in MTII, while a 2′-naphthyl group often displays antagonist activity as in SHU9119. Such observations are not closely followed by the MTII analogues **1**, **2**, **3** and SHU9119 analogues **6**, **7**, **8**. The different SARs in positions 7, 8 further support the hypothesis based on Schild analysis of peptides **1** and **2** that analogues **1**, **2** are noncompetitive inhibitor of hMC4R and might be allosteric binders for the *h*MC4R.

From the cAMP experiment-based Schild analyses, it is clear that the maximal stimulation of cAMP level has decreased, and the EC_{50} values are right shifted along with increasing the concentration of antagonists (Figure 3). Similar observations were reported by other laboratories for ASIP and AGRP at the *h*MC4R.[42,67] Allosteric binding sites are also suggested for other novel *h*MC4R selective antagonists designed on the basis of MTII.[68,69]

Another approach to achieve high *h*MC4R/*h*MC3R selectivity is via His^6 substitution in the SHU9119 template. Previously investigations have shown that the side chain of His^6 in MTII/SHU9119 was not important for binding/activation of *h*MCRs[51,63,66] and that replacement of His^6 in MTII and SHU9119 with proline does not change ligand biological profiles at the *h*MC3R and

*h*MC4R.[53,66] However, Grieco et al. showed that other His[6] substitutions in the SHU9119 template dramatically changed the selectivity between the *h*MC3R and *h*MC4R, suggesting slightly different binding pockets for interactions with residues in position 6.[53] Furthermore, Cai et al.[70] suggested that the *h*MC3R and *h*MC4R binding pocket could not accommodate a bulky *trans*-3-substituent on the proline ring in position 6 of MTII, whereas the *h*MC5R binding pocket can tolerate such bulky substituents. While retaining strong binding of peptides **4**, **5**, **9**, **10** at the *h*MC5R supports such hypothesis, the lack of binding selectivity of these analogues at the *h*MC3R and *h*MC4R suggests that the additional positively charged guanidinyl groups are not involved in binding to the proposed binding pockets. Grieco et al. also showed that His[6] substitutions were less likely to affect other key residue 3D conformations, and a small *trans*-4-hydroxyl group in position 6 of Ac-Nle-c[Asp-Hyp-DNal(2′)-Arg-Trp-Lys]-NH_2 did not affect binding affinity/selectivity at the *h*MC3R and *h*MC4R. Therefore, this lack of influence on binding to the *h*MC3,4Rs may be due to the relatively small size of the guanidinyl groups.[53] However, the opposite functional selectivity between peptides **4** (250-fold, *h*MC3R/*h*MC4R) and **5** (60-fold, *h*MC4R/*h*MC3R) at the *h*MC3/4R does suggest that the guanidinyl groups can affect receptor activation by interacting with the slightly different binding pockets. It has been established that DNal(2′)[7] side chain in SHU9119 is the sole determinant of antagonist activity by interacting with Leu[133] in the *h*MC4R TM3.[71] And Leu[165] in TM3 of the *h*MC3R is critical for agonist or antagonist selectivity at this receptor.[72] Both receptor binding pockets are sensitive to positioning of Phe/Nal(2′) side chains. It is possible that the phenyl moieties in peptides **4** and **5** can induce receptor functional conformational changes at the *h*MC3/4R, but such changes are partially or completely blocked by the constrained guanidinyl groups depending on their orientations. Other MTII analogues with His[6] substitution (Ac-Nle-c-[Asp-Aba-DPhe-Arg-Trp-Lys]-NH_2 and c[CO-$(CH_2)_2$-CO-Nle-DPhe-Arg-Trp-Lys]-NH_2) also have been reported to be able to bind to the *h*MC3,4Rs without agonist activity and can be explained by this theory.[73,74] Peptides **9** and **10** do not show functional selectivity at the *h*MC3/4R probably because the bulky naphthyl moiety can prevent receptor conformational changes in the first place. The positive charges on the guanidinyl groups in these analogues may be important for high functional selectivity because [Hyp(Bzl)[6]]MTII shows lower *h*MC3R/*h*MC4R (23-fold) functional selectivity than peptide **4** (250-fold).[50] Though less likely, we cannot exclude the possibility that the presence of the 4-guanidinyl-Pro[6] can alter the 3D positioning of the DPhe[7]/DNal(2′)[7] side chain in position 7, thus changing ligand potency/efficacy at melanocortin receptors. In any case, this study confirms previous findings by us and others that a constrained side chain in position 6 can influence not only ligand binding affinity but also receptor activation at melanocortin receptors.[75] These findings are important for understanding the different binding mechanisms of *h*MC3R and *h*MC4R and for the rational design of selective ligands for melanocortin receptors.

Summary

Both cyclic and linear analogues of melanotropins were designed to have a Pro or 4-guanidinyl-Pro residues in the His or Arg positions of the melanotropin pharmacophore. The Pro residue was incorporated to investigate the effects of a backbone conformational change on biological activity in those analogues, and the guanidinyl group on the Pro ring was used to probe possible electrostatic interactions with the melanocortin receptors. These peptides were synthesized successfully via a carefully designed solid phase peptide synthesis strategy to avoid the formation of the aspartimide derivatives. Competitive binding and cAMP assays revealed a series of novel ligands selective for the *h*MC1R and/or *h*MC4R. Some of these analogues are high affinity noncompetitive antagonists for the *h*MC4R. The results are consistent with the finding from our molecular modeling studies of analogues **1** and **2**, whose global minimum structures fit well with the NMR solution structure of the endogenous *h*MC1/4R selective antagonist *h*ASIP. The β-turn shift induced by the proline ring was identified to be responsible for this selectivity. The guanidinyl group on Pro[8] is not required for *h*MC4R selectivity. In addition, the 4-guanidinyl group on Pro[6] was shown to be able to affect ligand functional selectivity for the *h*MC3R or *h*MC4R. These findings are useful for the rational design of novel selective ligands targeting melanocortin receptors, especially the *h*MC4R, which is involved in many major diseases.

Experimental Section

Materials. N^{α}-Fmoc-amino acids and Rink amide MBHA (w/Nle) resin were obtained from Chem-Impex International (Wood Dale, IL). DIC and Cl-HOBt were purchased from Bachem (King of Prussia, PA). $Pd(PPh_3)_4$ and $PhSiH_3$ were purchased from Sigma-Aldrich (Milwaukee, WI). HSW syringes (Torviq, Niles, MI) (10 mL) with frits on the bottom were used for all peptide syntheses. Analytical HPLC was performed using a Vydac C_{18} 218TP104 column (Western Analytical Products, Murrieta, CA) monitored at 230 and 254 nm. Preparative HPLC was done on XTerra Prep MSC18 column (10 μm, 19 mm × 250 mm, part no. 186002259) obtained from Waters (Milford, MA). Analytical TLC was carried out on Anatech Uniplate silica gel TLC plates (250 μm) (Newark, DE). The TLC chromatograms were visualized by UV light and by potassium permanganate solution. ^{1}H NMR spectra were recorded on Bruker DRX600 or -500 or Varian Inova600 spectrometers with DMSO-d_6 as internal standard. Mass spectra were obtained from Mass Spectrometry Facility, Department of Chemistry, University of Arizona.

Peptide Synthesis. All peptides in this study were synthesized manually by Fmoc solid phase methodology using DIC and Cl-HOBt or HOAt as coupling reagents. The following procedures were used for the synthesis of the cyclic peptides. The linear peptides were synthesized likewise. Rink amide MBHA resin (250 mg, 0.7 mmol/g) was placed into a 10 mL HSW polypropylene syringe and swollen in DCM (5 mL) for 30 min. After removal of the Fmoc protecting group (25% piperidine in DMF, 5 mL, 5 and 10 min), the resin was washed with DMF (4 × 5 mL) and DCM (4 × 5 mL). Then, Fmoc-Lys(Alloc)-OH, Fmoc-Trp(Boc)-OH, (2*S*,4*R*)-N^{α}-Fmoc-4-*N*,*N*′-di-Boc-guanidinyl-Pro-OH or (2*S*,4*S*)-N^{α}-Fmoc-4-*N*,*N*′-di-Boc-guanidinyl-Pro-OH or Fmoc-Arg(Pbf)-OH, Fmoc-DPhe-OH or Fmoc-DNal(2′)-OH, Fmoc-His(Trt)-OH or (2*S*,4*R*)-N^{α}-Fmoc-4-*N*,*N*′-di-Boc-guanidinyl-Pro-OH or (2*S*,4*S*)-N^{α}-Fmoc-4-*N*,*N*′-di-Boc-guanidinyl-Pro-OH, Fmoc-Asp(Allyl)-OH were coupled to the resin following the above order. For each coupling, 3 equiv of amino acids, Cl-HOBt, DIC were used with 10 min of preactivation in DMF. All couplings were performed for 1 h and monitored by either the Kaiser test or the chloranil test. In case of a positive test result, the coupling was repeated until a negative test result was observed. Fmoc protecting groups were removed as described above. Once the Fmoc-Asp(Allyl)-OH was coupled to the peptide chain, the side chain Alloc protecting group of Lys and Allyl protecting group of Asp were removed simultaneously with 0.25 equiv of $Pd(PPh_3)_4$ and 20 equiv of $PhSiH_3$ in 5 mL of dry DCM (30 min × 2). The cyclization was performed with 6 equiv of DIC and HOAt in DMF for 72 h and was monitored by the Kaiser test. Upon completion of the cyclization, the resin was treated with 5 mL of 5% sodium diethyldithiocarbamate trihydrate in DMF (10 min) and then washed with DMF (4 × 5 mL), DCM

(4 × 5 mL). Fmoc-Nle-OH was then coupled to the peptide chain following the procedures described above. Next the N^{α}-Fmoc was removed and the amino group was acetylated with 10 equiv of acetic anhydride and 2 equiv of DIPEA in 5 mL of DCM for 30 min. The resin was washed with DCM (4 × 5 mL) and DCM/diethyl ether (1:1, 4 × 5 mL) and dried under vacuum for 4 h. The peptides were cleaved from the resin with a mixture of 95% TFA, 2.5% water, and 2.5% TIPS for 3 h. After evaporation of the TFA under vacuum, the peptides were precipitated and washed 3 times with 5 mL of cold diethyl ether for each wash. The liquid was separated from the solid by centrifuge and was decanted. The crude peptides were dried in air and dissolved in acetonitrile and 0.1% TFA in water (2:1) before it was purified by preparative RP-HPLC and eluted with a linear gradient of 15−40% acetonitrile in aqueous 0.1% TFA solution over 35 min with a 15 mL/min flow rate. The fractions containing the desired products were collected, concentrated, and lyophilized. The purified peptides were isolated in 40−50% overall yields and were >95% pure as determined by analytical RP-HPLC. The structures of the pure peptides were confirmed by high-resolution mass spectrometry and ^{1}H NMR in DMSO-d_6.

Binding Assays. Competition binding experiments were performed on whole cells. Stably transfected HEK293 cell lines having the individual *h*MCRs[70,76,77] were seeded on 96-well plate 48 h before the assay and grown to 100 000 cells/well. For the assay, the medium was removed and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated with different concentrations of unlabeled peptides and ^{125}I-labeled [Nle^4,DPhe^7]-α-MSH (PerkinElmer Life Science, 100 000 cpm/well, 0.1386 nM) for 40 min at 37 °C. The medium was subsequently removed, and each well was washed twice with the binding buffer. The cells were lysed by the addition of 250 μL of 0.1 mM NaOH and 250 μL of 1% Triton X-100. The lysed cells were transferred to the 12 mm × 75 mm glass tubes, and the radioactivity was measured using a Wallac 1470 WIZARD γ counter. Data were analyzed using Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

Adenylate Cyclase Assays. HEK 293 cells stably transfected with individual human melanocortin receptors[70] 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 and grown to 100 000 cells/well. For the assay, the medium was removed and cells were rinsed with 1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with isobutylmethylxanthine (IBMX, 5 μL, 0.5 mM) for 1 min at 37 °C. Next, various concentrations of melanotropins (0.1 mL) were added and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the buffer and adding ice cold Tris/EDTA buffer to each well (0.15 mL). After the cells were dislodged with the help of trypsin, the cells were transferred to polypropylene microcentrifuge tubes, capped, and placed in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min (6500 rpm), and 50 μL of the supernatant was aliquoted into a clean Eppendorf tube. The total cAMP content was measured by competitive binding assay according to the assay kit instructions (TRK 432, Amersham Corp., Arlington Heights, IL). The antagonist properties of analogues **1**, **2** were evaluated by its ability to competitively displace the MTII agonist in a dose dependent manner, at up to 10 μM.

Data Analysis. IC_{50} and EC_{50} values represent the mean of duplicate experiments performed in triplicate. IC_{50} and EC_{50} estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis, with the help of Graphpad Prism 3.1 (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.[78] Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS_2005 force field[60] and the Polak−Ribier conjugate gradient (PRCG).[79] Optimizations were converged to a gradient rmsd less than 0.05 kJ/(Å mo) or continued until a limit of 50 000 iterations was reached. Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA).[61] Extended cutoff distances were defined at 8 Å for van der Waals, 20 Å for electrostatics, and 4 Å for H-bonds.

Conformational profiles of the cyclic peptides were investigated by the hybrid Monte Carlo/low frequency mode (MCMM/LMCS)[59] 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 20 000 search steps were performed, and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. The superimpositions of peptide structures were performed using the α-carbons of the core sequence His-DPhe/DNal(2′)-Xaa-Trp.

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Supporting Information Available: ^{1}H NMR spectra of peptide analogues **1**−**10** in DMSO-d_6. This material is available free of charge via the Internet at http://pubs.acs.org.

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