Articles

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

Alexander V. Mayorov,[†] Minying Cai, Erin S. Palmer, Matthew M. Dedek, James P. Cain, April R. Van Scoy, Bahar Tan, Josef Vagner, Dev Trivedi, and Victor J. Hruby*

Department of Chemistry, University of Arizona, Tucson, Arizona 85721

Received April 18, 2007

A variety of dicarboxylic acid linkers introduced between the α-amino group of Pro^6 and the ϵ-amino group of Lys^{10} of the cyclic lactam α-melanocyte-stimulating hormone (α-MSH)-derived Pro^6-D-Phe^7/D-Nal(2′)^7-Arg^8-Trp^9-Lys^{10}-NH_2 pentapeptide template lead to nanomolar range and selective hMC3R agonists and antagonists. Replacement of the Pro^6 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]-NH_2, EC_{50} = 27 nM, 70% max cAMP) and an hMC3R antagonist (analogue **13**, c[CO-2,3-pyrazine-CO-D-Nal(2′)-Arg-Trp-Lys]-NH_2, IC_{50} = 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-$(CH_2)_2$-CO-Nle-D-Nal(2′)-Arg-Trp-Lys]-NH_2, IC_{50} = 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 transmembrane 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] 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 function[68] 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

* To whom correspondence should be addressed. Phone: (520) 621-6332. Fax: (520) 621-8407 . E-mail: hruby@u.arizona.edu.

[†] Current address: Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037.

[a] 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: All, allyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; CH_3CN, 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-pentamethyldihydrobenzofuran-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-NH_2; NDP-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly Lys-Pro-Val-NH_2.

10.1021/jm070461w CCC: $40.75
Published on Web 12/19/2007

Table 1. Sequences and the Physicochemical Properties of the Cyclic α-MSH Analogues

No.	Sequence	*m/z* (M + 1) calcd	*m/z* (M + 1) obsd (ESI)	HPLC retention time, min[a]	TLC R_f[b] 1	TLC R_f[b] 2	TLC R_f[b] 3
4	c[CO-*o*-C_6H_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-*o*-C_6H_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-$(CH_2)_3$-CO-Pro-D-Phe-Arg-Trp-Lys]-NH_2	828.4521	828.4544	16.97	0.61	0.35	0.72
7	c[CO-$(CH_2)_3$-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-*cis*-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-*cis*-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

[a] HPLC column: Vydac 218TP104, 250 × 4.6 mm, 10 μm, 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. [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); TLC system 3: ethyl acetate/acetic acid/water/pyridine (5:1:3:5).

1 H-Tyr-Val-Met-Gly-His-Phe-Arg-*D*-Trp-Asp-Arg-Phe-Gly-OH (*D*-Trp^8-γ-MSH) hMC3R agonist

2 cyclo(6β→10ε)-(succinyl^6-*D*-Nal(2')^7-Arg^8-Trp^9-Lys^{10})-NH_2 (MBP10) hMC4R antagonist

3 cyclo(5β→10ε)-(succinyl^5-His^6-*D*-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.

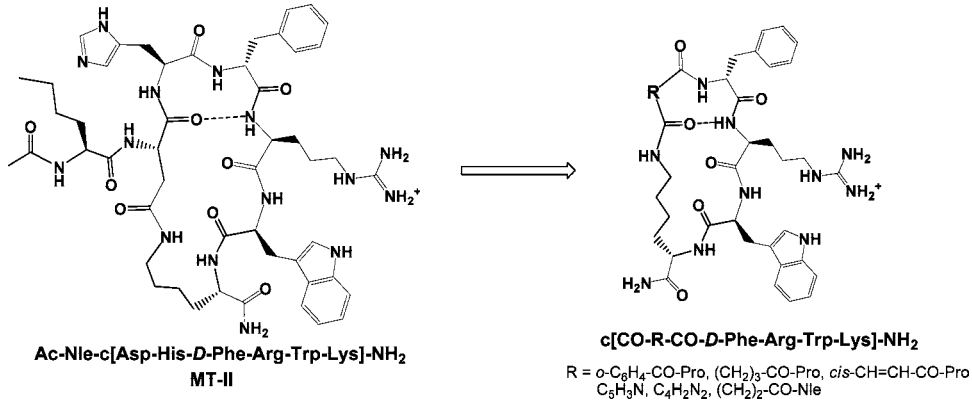

Figure 2. Design of the α-MSH-derived cyclic lactam scaffold.

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-NH_2 (Ac-NDP-γ-MSH-NH_2)[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 NH_2 (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-Nle^4-c[Asp^5, D-Nal(2′)^7, Lys^{10}]α-MSH(4–10)-NH_2 (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-NH_2) and hMC5R (Ac-c[Cys-Glu-His-D-Phe-Arg-Trp-D-Cys]-Pro-Pro-Lys-Asp-NH_2) 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-Phe^7/D-Nal(2′)^7 and the ϵ-amino group of Lys^{10} of the cyclic lactam α-MSH-derived D-Phe^7/D-Nal(2′)^7-Arg^8-Trp^9-Lys^{10}-NH_2 tetrapeptide template, which have been designed and synthesized by solid-phase methods to further pursue SAR trends leading to hMC3R selectivity.

Peptide Design. Ac-Nle^4-c[Asp^5, D-Phe^7, Lys^{10}]α-MSH(4–10)-NH_2 (MT-II), a superpotent but nonselective human melanocortin 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 Pro^6 substitution did not lead to a significant discrimination between the hMC3R and the hMC4R.[72] Furthermore, Kavarana et al. has reported that

Table 2. Binding Affinities and cAMP Activities of Cyclic α-MSH Analogues at hMCRs[a]

		hMC1R			hMC3R			hMC4R			hMC5R		
No.	sequence	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
MT-II	Ac-Nle-c[Asp-His-D-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
4	c[CO-o-C_6H_4-CO-Pro-D-Phe-Arg-Trp-Lys]-NH_2	>10000	NA	0	>1000	95 ± 11	100	777 ± 200	NA	0	>1000	NA	0
5	c[CO-o-C_6H_4-CO-Pro-D-Nal(2′)-Arg-Trp-Lys]-NH_2	410 ± 80	>5000	80	32 ± 4	NA	0	430 ± 100	NA	0	210 ± 30	NA	0
6	c[CO-$(CH_2)_3$-CO-Pro-D-Phe-Arg-Trp-Lys]-NH_2	>10000	NA	0	385 ± 50	29 ± 3	100	>1000	>2000	12	870 ± 100	570 ± 60	40
7	c[CO-$(CH_2)_3$-CO-Pro-D-Nal(2′)-Arg-Trp-Lys]-NH_2	>10000	NA	0	11 ± 2 pA_2 = 9.8	NA	0	330 ± 40	NA	0	27 ± 3	23 ± 7	40
8	c[CO-*cis*-CH=CH-CO-Pro-D-Phe-Arg-Trp-Lys]-NH_2	>10000	NA	0	>1000	100 ± 20	80	160 ± 20	NA	0	790 ± 100	NA	0
9	c[CO-cis-CH=CH-CO-Pro-D-Nal(2′)-Arg-Trp-Lys]-NH_2	>10000	NA	0	20 ± 3	NA	0	520 ± 60	NA	0	120 ± 20	NA	0
10	c[CO-2,6-pyridine-CO-D-Phe-Arg-Trp-Lys]-NH_2	>10000	>1000	15	>10000	NA	0	>10000	NA	0	>10000	NA	0
11	c[CO-2,6-pyridine-CO-D-Nal(2′)-Arg-Trp-Lys]-NH_2	>10000	NA	0	>1000	NA	0	>1000	NA	0	>10000	NA	0
12	c[CO-2,3-pyrazine-CO-D-Phe-Arg-Trp-Lys]-NH_2	412 ± 50	>10000	60	1100 ± 120	27 ± 3	70	>10000	NA	0	>10000	NA	0
13	c[CO-2,3-pyrazine-CO-D-Nal(2′)-Arg-Trp-Lys]-NH_2	707 ± 101	>3000	55	23 ± 5	NA	0	>1000	NA	0	230 ± 40	NA	0
14	c[CO-$(CH_2)_2$-CO-Nle-D-Phe-Arg-Trp-Lys]-NH_2	>10000	NA	0	84 ± 10	NA	0	930 ± 133	NA	0	520 ± 70	>1000	40
15	c[CO-$(CH_2)_2$-CO-Nle-D-Nal(2′)-Arg-Trp-Lys]-NH_2	2000 ± 200	>1000	20	12 ± 3 pA_2 = 8.3	NA	0	300 ± 100	NA	0	17 ± 2 pA_2 = 8.7	NA	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 effect = % of cAMP produced at 10 μM ligand concentration, in relation to MT-II. NA = 0% cAMP accumulation observed at 10 μM. The peptides were tested at a range of concentration from 10^{-10} to 10^{-5} M.

such a substitution in a cyclo(5β→10ϵ)-[succinyl[5]-His[6]-D-Phe[7]-Arg[8]-Trp[9]-Lys[10]]-NH_2 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 Pro[6]-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 (EC_{50} = 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 (IC_{50} = 32 nM). Interestingly, these results are in contrast with the findings previously reported by Kavarana et al.,[28] where structurally similar His[6] 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 Pro[6] 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 (EC_{50} = 29 nM) and antagonist (IC_{50} = 11 nM, pA_2 = 9.8), respectively. Comparison of the three-dimensional structures of analogue **6** and MT-II[73] 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 **7**, on the other hand, seems entirely in parallel with that of structurally similar α-MSH analogue cyclo(5γ→10ϵ)-(glutaryl[5]-His[6]-D-Nal(2′)[7]-Arg[8]-Trp[9]-Lys[10])-NH_2 (MK-9), which has been reported to be a highly potent hMC3R antagonist (K_i = 5.9 nM, pA_2 = 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 (IC_{50} = 3.3 and 1.8 nM,[70] pA_2 = 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 (EC_{50} = 100 nM, 80% cAMP stimulation), and a modest binding affinity at the hMC4R (IC_{50} = 160 nM) and the hMC5R (IC_{50} = 790 nM). The D-Nal(2′)[7]-substituted analogue **9** displayed a reversal of the hMC3R agonist activity to antagonist activity (IC_{50} = 20 nM), whereas the binding affinities at the hMC4R and hMC5R did not show significant change (IC_{50} = 520 and 120 nM, respectively). Overall, the Pro[6] cyclic α-MSH analogues exhibited higher receptor selectivity, favoring the hMC3R, than the corresponding His[6] peptides.[28]

Bednarek et al. have previously reported that deletion of His[6] 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

Table 3. Backbone Torsion Angles (°) for the Global Minima of Selected Cyclic α-MSH Analogues Based on MCMM/LMCS-OPLS2005 Calculations Compared with the NMR Structures of MT-II and SHU9119[73]

	Xaa[6]		D-Phe/D-Nal(2′)[7]		Arg[8]		Trp[9]		Lys[10]	
No.	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

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 Nle[4] 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 His[6]→Nle[6] 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 Nle[4], 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 Nle[6] and the side chains of Arg[8], which may result in partial steric hindrance of the binding space of Arg[8], analogous to the structural features of the cyclic Nle[4]-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]-NH_2 (IC_{50} = 50 nM) and a hMC3R/hMC5R antagonist Ac-Nle-c[Asp-Aba-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (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-Phe[7] analogues, whereas Figure 8 compares the binding affinities of the D-Nal(2′)[7] analogues, thus highlighting the trend toward hMC3R selectivity. The Pro[6] 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, Nle[6] 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

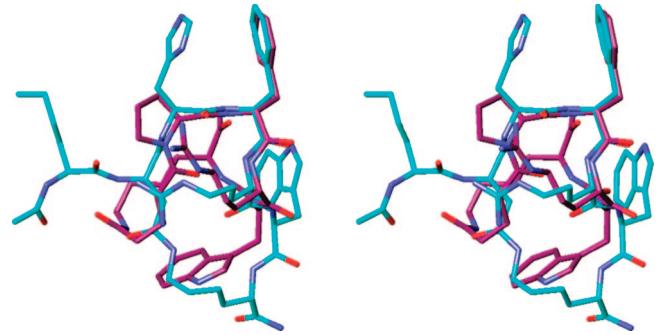

Figure 3. Stereoview of the superimposed global minimum of analogue **6** (purple), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation with the NMR-derived structure of nonselective super agonist MT-II (blue; rmsd = 1.86 Å, nonhydrogen backbone atoms of the Xaa-D-Phe-Arg-Trp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

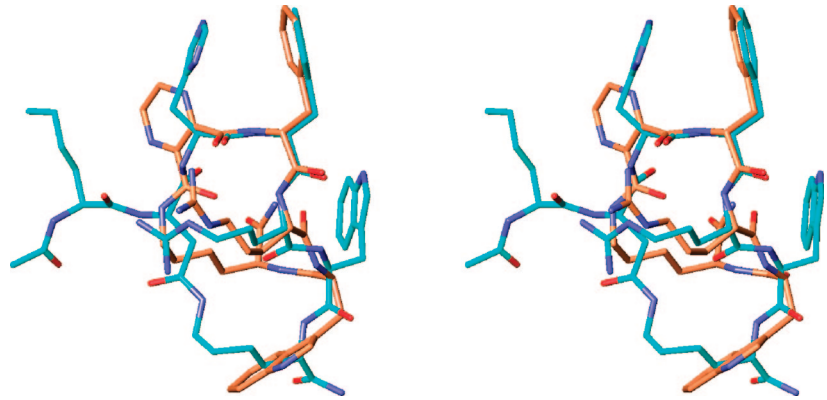

Figure 4. Stereoview of the superimposed global minimum of analogue **12** (gold), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation with the NMR-derived structure of nonselective super agonist MT-II (blue; rmsd = 1.35 Å, nonhydrogen backbone atoms of the Xaa-D-Phe-Arg-Trp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

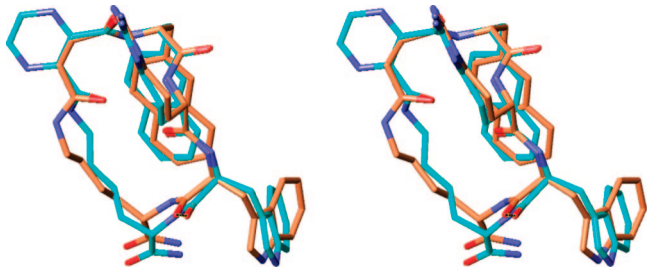

Figure 5. Stereoview of the superimposed global minimum of analogue **13** (blue) with the global minimum of selective hMC4R antagonist **2** (gold), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulations (rmsd = 0.43 Å, nonhydrogen backbone atoms of the Xaa-D-Phe-Arg-Trp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

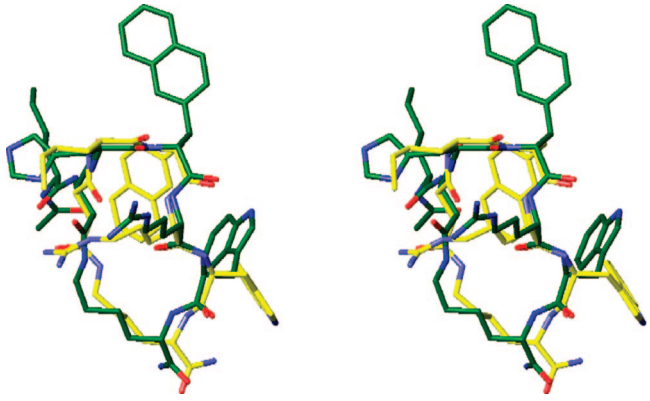

Figure 6. Stereoview of the superimposed global minimum of analogue **15** (yellow), obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation with the NMR-derived structure of hMC3R/hMC4R antagonist SHU9119 (green; rmsd = 0.39 Å, nonhydrogen backbone atoms of the Xaa-D-Nal(2′)-Arg-Trp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

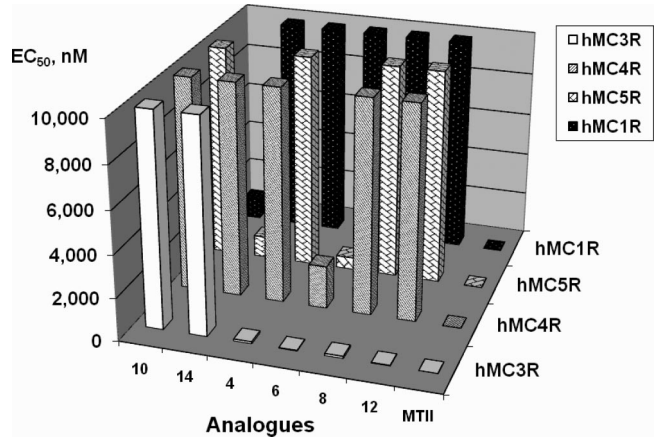

Figure 7. Graphical summary of the agonist activities, expressed in EC_{50} values (*Z* axis), of the D-Phe[7] analogues (*X* axis) at the four human melanocortin receptor subtypes (*Y* axis).

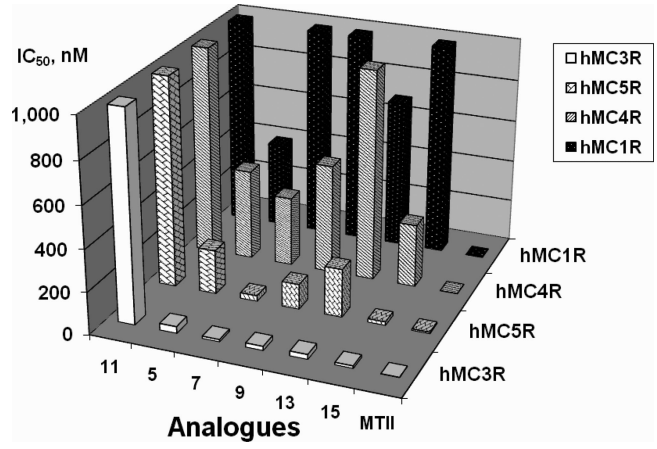

Figure 8. Graphical summary of the binding affinities, expressed in IC_{50} values (*Z* axis), of the D-Nal(2′)[7] analogues (*X* axis) at the four human melanocortin receptor subtypes (*Y* axis).

hMC3R/hMC5R antagonists (analogues **13** and **15**). Molecular modeling experiments suggested that in the Pro[6] 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 Nle[6] substitution that produced a potent hMC3R/hMC5R antagonist (analogue **15**, $pA_2 = 8.3$ (hMC3R) and 8.7 (hMC5R)). These newly developed melanotropin peptides will facilitate the elucidation of the physiologi-

cal 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(N^{in}-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 C_{18} 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 F_{254} 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 Lys^{11} and the linker (if applicable) was removed with 0.1 equiv $Pd(PPh_3)_4$/20 equiv $PhSiH_3$ 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% thioanisol, 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 μM 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 C_{18}-bonded silica column (Vydac 218TP152022, 250 × 22 mm, 15–20 μm, 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 ^{1}H 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 [^{125}I]-[Nle^4,D-Phe^7]-α-MSH (Perkin-Elmer Life Science, 20000 cpm/well, 0.14 nM) diluted in a 125 μL of freshly prepared binding buffer containing 100% MEM, 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. The assay medium was subsequently removed and the cells were washed once with basic medium and then lysed by the addition of 100 μL of 0.1 M NaOH and 100 μL of 1% Triton X-100. The lysed cells were transferred to 12 × 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 receptors[57] 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 μL of MEM buffer (GIBCO). An aliquot (100 μL) of the Earle's balanced salt solution with 0.5 mM isobutylmethylxanthine (IBMX) was placed in each well along for 1 min at 37 °C. Next, aliquots (25 μL) of melanotropin peptides of varying concentration were added, and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the assay buffer and adding 60 μL 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 × *g*. A 50 μL aliquot of the supernatant was transferred to another 96-well plate and placed with 50 μL of [^{3}H] cAMP and 100 μL of protein kinase A (PKA) buffer in an ice bath for 2–3 h. The PKA buffer consisted of Tris/EDTA buffer with 60 μg/mL PKA and 0.1% bovine serum albumin by weight. The incubation mixture was filtered through 1.0 μm glass fiber filters in MultiScreen™-FB 96-well plates (Millipore, Billerica, MA). The total [^{3}H] 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 μM concentration of each ligand was compared to the amount of cAMP produced at 10 μM 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 μM. The pA_2 values were obtained using the Schild analysis method.[82]

Data Analysis. IC_{50} and EC_{50} values represent the mean of two 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 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 field[83] and the Polak-Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient rmsd less that 0.05 kJ/Å 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 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)[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.

Acknowledgment. This research was supported by grants from the U.S. Public Health Service, National Institutes of Health, DK-17420 and DA-06284. The opinions expressed are those of the authors and not necessarily those of the USPHS.

Supporting Information Available: ^{1}H 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.

References

(1) Hadley, M. E., Ed. *The Melanotropic Peptides*; CRC Press: Boca Raton, FL, 1988; Vols. *I*−*III*.
(2) Cone, R. D. The Melanocortin System. *Ann. N.Y. Acad. Sci.* **2003**, *994*, 1–387.
(3) Cone, R. D., Ed. *The Melanocortin Receptors*. Humana Press: Totowa, NJ, 2000.
(4) Vaudry, H.; Eberle, A. N. The Melanotropic Peptides. *Ann. N.Y. Acad. Sci.* **1993**, *680*, 1–687.
(5) Gispen, W. H.; Isaacson, R. L. ACTH-Induced Excessive Grooming in the Rat. *Pharmacol. Ther.* **1981**, *12*, 209–246.
(6) Gantz, I.; Fong, T. M. The Melanocortin System. *Am. J. Physiol. Endocrinol. Metab.* **2003**, *284*, E468–E474.
(7) Chhajlani, B. Distribution of cDNA for Melanocortin Receptor Subtypes in Human Tissues. *Biochem. Mol. Biol. Int.* **1996**, *38*, 73–80.
(8) Wessells, H.; Gralnek, D.; Dorr, R.; Hruby, V. J.; Hadley, M. E.; Levine, N. Effect of an α-Melanocyte Stimulating Hormone Analogue on Penile Erection and Sexual Desire in Men with Organic Erectile Dysfunction. *Urology* **2000**, *56*, 641–646.
(9) Wessells, H.; Fuciarelli, K.; Hansen, J.; Hadley, M. E.; Hruby, V. J.; Dorr, R.; Levine, N. Synthetic Melanotropic Peptide Initiates Erections in Men with Psychogenic Erectile Dysfunction: Double-Blind Placebo Controlled Crossover Study. *J. Urol.* **1998**, *160*, 389–393.
(10) Bertolini, A.; Vergoni, W.; Gessa, G. L.; Ferrari, W. Induction of Sexual Excitement by Action of Adrenocorticotropic Hormone in Brain. *Nature* **1969**, *221*, 667–669.
(11) Bertolini, A.; Vergoni, W.; Gessa, G. L.; Ferrari, W. Erection and Ejaculation: A Central Effect of ACTH-Like Peptides in Mammals. In *Sexual Behavior: Pharmacology and Biochemistry*. Sandler, M., Gessa, G. L., Eds.; Raven Press: New York, 1975; pp 247–257.
(12) Van der Ploeg, L. H. T.; Martin, W. J.; Howard, A. D.; Nargund, R. P.; Austin, C. P.; Guan, X.; Drisko, J.; Cashen, D.; Sebhat, I.; Patchett, A. A.; Figueroa, D. J.; DiLella, A. G.; Connolly, B. M.; Weinberg, D. H.; Tan, C. P.; Palyha, O. C.; Pong, S-S; MacNeil, T.; Rosenblum, C.; Vongs, A.; Tang, R.; Yu, H.; Sailer, A. W.; Fong, T. M.; Huang, C.; Tota, M. R.; Chang, R. S.; Stearns, R.; Tamvakopoulos, C.; Christ, G.; Drazen, D. L.; Spar, B. D.; Nelson, R. J.; MacIntyre, D. E. A Role for the Melanocortin 4 Receptor in Sexual Function. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11381–11386.
(13) Li, S. J.; Varga, K.; Archer, P.; Hruby, V. J.; Sharma, S. D.; Kesterson, R. A.; Cone, R. D.; Kunos, G. Melanocortin Antagonists Define Two Distinct Pathways of Cardiovascular Control by α- and γ-Melanocyte Stimulating Hormones. *J. Neurosci.* **1996**, *16*, 5182–5188.
(14) Ni, X.-P.; Butler, A. A.; Cone, R. D.; Humphreys, M. H. Central Receptors Mediating the Cardiovascular Actions of Melanocyte Stimulating Hormones. *J. Hypertens.* **2006**, *24*, 2239–2246.
(15) Fan, W.; Boston, B. A.; Kesterson, R. A.; Hruby, V. J.; Cone, R. D. Role of the Melanocortinergic Neurons in Feeding and the Agouti Obesity Syndrome. *Nature* **1997**, *385*, 165–168.
(16) Vergoni, A. V.; Poggioloi, R.; Bertolini, A. Corticotropin Inhibits Food Intake in Rats. *Neuropeptides* **1986**, *7*, 153–158.
(17) Vergoni, A. V.; Poggioloi, R.; Marrama, D.; Bertolini, A. Inhibition of Feeding by ACTH-(1–24): Behavioral and Pharmacological Aspects. *Eur. J. Pharmacol.* **1990**, *179*, 347–355.
(18) Ramos, E. J. B.; Meguid, M. M.; Campos, A. C. L.; Coelho, J. C. U. Neuropeptide Y, α-Melanocyte-Stimulating Hormone, and Monoamines in Food Intake Regulation. *Nutrition* **2005**, *21*, 269–279.
(19) Yang, Y. K.; Harmon, C. M. Recent Developments in Our Understanding of Melanocortin System in the Regulation of Food Intake. *Obes. Rev.* **2003**, *4*, 239–248.
(20) Ellacott, K. L. J.; Cone, R. D. The Central Melanocortin System and the Integration of Short- and Long-term Regulators of Energy Homeostasis. *Recent Prog. Horm. Res.* **2004**, *59*, 395–408.
(21) Zimanyi, I. A.; Pelleymounter, M. A. The Role of Melanocortin Peptides and Receptors in Regulation of Energy Balance. *Curr. Pharm. Des.* **2003**, *9*, 627–641.
(22) Morgan, C.; Thomas, R. E.; Cone, R. D. Melanocortin-5 Receptor Deficiency Promotes Defensive Behavior in Male Mice. *Horm. Behav.* **2004**, *45*, 58–63.
(23) Morgan, C.; Thomas, R. E.; Ma, W.; Novotny, M. V.; Cone, R. D. Melanocortin-5 Receptor Deficiency Reduces a Pheromonal Signal for Aggression in Male Mice. *Chem. Senses* **2004**, *29*, 111–115.
(24) Mogil, J. S.; Wilson, S. G.; Chesler, E. J.; Rankin, A. L.; Nemmani, K. V. S.; Lariviere, W. R.; Groce, M. K.; Wallace, M. R.; Kaplan, L.; Staud, R.; Ness, T. J.; Glover, T. L.; Stankova, M.; Mayorov, A. V.; Hruby, V. J.; Grisel, J. E.; Fillingim, R. B. The Melanocortin-1 Receptor Gene Mediates Female-Specific Mechanisms of Analgesia in Mice And Humans. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 4867–4872.
(25) Vrinten, D. H.; Kalkman, C. J.; Adan, R. A. H.; Gispen, W. H. Neuropathic Pain: A Possible Role for the Melanocortin System. *Eur. J. Pharmacol.* **2001**, *429*, 61–69.
(26) Lu, D.; Willard, D.; Patel, I. R.; Kadwell, S.; Overton, L.; Kost, T.; Luther, M.; Chen, W.; Woychik, R. P.; Wilkison, W. O.; Cone, R. D. Agouti Protein Is an Antagonist of the Melanocyte-Stimulating Hormone Receptor. *Nature* **1994**, *371*, 799–802.
(27) Ollmann, M. M.; Wilson, B. D.; Yang, Y.-K.; Kerns, J. A.; Chen, Y.; Gantz, I.; Barsh, G. S. Agouti-Related Protein Is an Endogenous Antagonist of the Melanocortin-4 Receptor In Vitro and In Vivo. *Science* **1997**, *278*, 135–138.
(28) Kavarana, M. J.; Trivedi, D.; Cai, M.; Ying, J.; Hammer, M.; Cabello, C.; Grieco, P.; Han, G.; Hruby, V. J. Novel Cyclic Templates of α-MSH Give Highly Selective and Potent Antagonists/Agonists for Human Melanocortin-3/4 Receptors. *J. Med. Chem.* **2002**, *45*, 2644–2650.
(29) Grieco, P.; Lavecchia, A.; Cai, M.; Trivedi, D.; Weinberg, D.; MacNeil, T.; Van der Ploeg, L. H. T.; Hruby, V. J. Structure−Activity Studies of the Melanocortin Peptides: Discovery of Potent and Selective Affinity Antagonists for the hMC3 and hMC4 Receptors. *J. Med. Chem.* **2002**, *45*, 5287–5294.
(30) Cai, M.; Mayorov, A. V.; Ying, J.; Stankova, M.; Trivedi, D.; Cabello, C.; Hruby, V. J. Design of Novel Melanotropin Agonists and Antagonists With High Potency and Selectivity for Human Melanocortin Receptors. *Peptides* **2005**, *26*, 1481–1485.

(31) Balse-Srinivasan, P.; Grieco, P.; Cai, M.; Trivedi, D.; Hruby, V. J. Structure−Activity Relationships of γ-MSH Analogues at the Human Melanocortin MC3, MC4, and MC5 Receptors. Discovery of Highly Selective hMC3R, hMC4R, and hMC5R Analogues. *J. Med. Chem.* **2003**, *46*, 4965–4973.

(32) Nijenhuis, W. A. J.; Kruijtzer, J. A. W.; Wanders, N.; Vrinten, D. H.; Garner, K. M.; Schaaper, W. M. M.; Meloen, R. H.; Gispen, W. H.; Liskamp, R. M.; Adana, R. A. H. Discovery and In Vivo Evaluation of New Melanocortin-4 Receptor-Selective Peptides. *Peptides* **2003**, *24*, 271–280.

(33) Bednarek, M. A.; MacNeil, T.; Tang, R.; Kalyani, R. N.; Van der Ploeg, L. H. T.; Weinberg, D. H. Potent and Selective Peptide Agonists of α-Melanotropin Action at Human Melanocortin Receptor 4: Their Synthesis and Biological Evaluation In Vitro. *Biochem. Biophys. Res. Commun.* **2001**, *286*, 641–645.

(34) Bednarek, M. A.; MacNeil, T.; Kalyani, R. N.; Tang, R.; Van der Ploeg, L. H. T.; Weinberg, D. H. Selective, High Affinity Peptide Antagonists of α-Melanotropin Action at Human Melanocortin Receptor 4: Their Synthesis and Biological Evaluation In Vitro. *J. Med. Chem.* **2001**, *44*, 3665–3672.

(35) Yang, Y.-K.; Fong, T. M.; Dickinson, C. J.; Mao, C.; Li, J.-Y.; Tota, M. R.; Mosley, R.; Van der Ploeg, L. H. T.; Gantz, I. Molecular Determinants of Ligand Binding to the Human Melanocortin-4 Receptor. *Biochemistry* **2000**, *39*, 14900–14911.

(36) Ying, J.; Gu, X.; Cai, M.; Dedek, M.; Vagner, J.; Trivedi, D.; Hruby, V. J. Design, Synthesis, and Biological Evaluation of New Cyclic Melanotropin Peptide Analogues Selective for the Human Melanocortin-4 Receptor. *J. Med. Chem.* **2006**, *49*, 6888–6896.

(37) Schiöth, H. B.; Mutulis, F.; Muceniece, R.; Prusis, P.; Wikberg, J. E. S. Discovery of Novel Melanocortin-4 Receptor Selective MSH Analogues. *Br. J. Pharmacol.* **1998**, *124*, 75–82.

(38) Mayer, J. P.; Hsiung, H. M.; Flora, D. B.; Edwards, P.; Smith, D. P.; Zhang, X.-Y.; Gadski, R. A.; Heiman, M. L.; Hertel, J. L.; Emmerson, P. J.; Husain, S.; O'Brien, T. P.; Kahl, S. D.; Smiley, D. L.; Zhang, L.; DiMarchi, R. D.; Yan, L. Z. Discovery of a β-MSH-Derived MC-4R Selective Agonist. *J. Med. Chem.* **2005**, *48*, 3095–3098.

(39) Odagami, T.; Tsuda, Y.; Kogami, Y.; Koujia, H.; Okada, Y. Design of Cyclic Peptides With Agonist Activity at Melanocortin Receptor-4. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3723–3726.

(40) Arasasingham, P. N.; Fotsch, C.; Ouyang, X.; Norman, M. H.; Kelly, M. G.; Stark, K. L.; Karbon, B.; Hale, C.; Baumgartner, J. W.; Zambrano, M.; Cheetham, J.; Tamayo, N. A. Structure−Activity Relationship of (1-Aryl-2-piperazinylethyl)piperazines: Antagonists for the AGRP/Melanocortin Receptor Binding. *J. Med. Chem.* **2003**, *46*, 9–11.

(41) Richardson, T. I.; Ornstein, P. L.; Briner, K.; Fisher, M. J.; Backer, R. T.; Biggers, C. K.; Clay, M. P.; Emmerson, P. J.; Hertel, L. W.; Hsiung, H. M.; Husain, S.; Kahl, S. D.; Lee, J. A.; Lindstrom, T. D.; Martinelli, M. J.; Mayer, J. P.; Mullaney, J. T.; O'Brien, T. P.; Pawlak, J. M.; Revell, K. D.; Shah, J.; Zgombick, J. M.; Herr, R. J.; Melekhov, A.; Sampson, P. B.; King, C.-H. R. Synthesis and Structure−Activity Relationships of Novel Arylpiperazines as Potent and Selective Agonists of the Melanocortin Subtype-4 Receptor. *J. Med. Chem.* **2004**, *47*, 744–755.

(42) Sebhat, I. K.; Martin, W. J.; Ye, Z.; Barakat, K.; Mosley, R. T.; Johnston, D. B. R.; Bakshi, R.; Palucki, B.; Weinberg, D. H.; MacNeil, T.; Kalyani, R. N.; Tang, R.; Stearns, R. A.; Miller, R. R.; Tamvakopoulos, C.; Strack, A. M.; McGowan, E.; Cashen, D. E.; Drisko, J. E.; Hom, G. J.; Howard, A. D.; MacIntyre, D. E.; van der Ploeg, L. H. T.; Patchett, A. A.; Nargund, R. P. Design and Pharmacology of *N*-[(3*R*)-1,2,3,4-Tetrahydroisoquinolinium-3-ylcarbonyl]-(1*R*)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1*H*-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine (1), a Potent, Selective, Melanocortin Subtype-4 Receptor Agonist. *J. Med. Chem.* **2002**, *45*, 4589–4593.

(43) Ruel, R.; Herpin, T. F.; Iben, L.; Luo, G.; Martel, A.; Mason, H.; Mattson, G.; Poirier, B.; Ruediger, E. H.; Shi, D.; Thibault, C.; Yu, G.; Zimanyi, I. A.; Poindexter, G. S.; Macor, J. E. β-Alanine Dipeptides as MC4R Agonists. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4341–4344.

(44) Vos, T. J.; Caracoti, A.; Che, J. L.; Dai, M.; Farrer, C. A.; Forsyth, N. E.; Drabic, S. V.; Horlick, R. A.; Lamppu, D.; Yowe, D. L.; Balani, S.; Li, P.; Zeng, H.; Joseph, I. B. J. K.; Rodriguez, L. E.; Maguire, M. P.; Patane, M. A.; Claiborne, C. F. Identification of 2-{2-[2-(5-Bromo-2-methoxyphenyl)-ethyl]-3-fluorophenyl}-4,5-dihydro-1*H*-imidazole (ML00253764), a Small Molecule Melanocortin 4 Receptor Antagonist that Effectively Reduces Tumor-Induced Weight Loss in a Mouse Model. *J. Med. Chem.* **2004**, *47*, 1602–1604.

(45) Hogan, K.; Peluso, S.; Gould, S.; Parsons, I.; Ryan, D.; Wu, L.; Visiers, I. Mapping the Binding Site of Melanocortin 4 Receptor Agonists: A Hydrophobic Pocket Formed by I3.28(125), I3.32(129), and I7.42(291) is Critical for Receptor Activation. *J. Med. Chem.* **2006**, *49*, 911–922.

(46) Chen, C.; Pontillo, J.; Fleck, B. A.; Gao, Y.; Wen, J.; Tran, J. A.; Tucci, F. C.; Marinkovic, D.; Foster, A. C.; Saunders, J. 4-{(2*R*)-[3-Aminopropionylamido]-3-(2,4-dichlorophenyl)propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]phenyl}piperazine as a Potent and Selective Melanocortin-4 Receptor Antagonists - Design, Synthesis, and Characterization. *J. Med. Chem.* **2004**, *47*, 6821–6830.

(47) Tian, X.; Field, T. B.; Switzer, A. G.; Mazur, A. W.; Ebetino, F. H.; Wos, J. A.; Berberich, S. M.; Jayasinghe, L. R.; Obringer, C. M.; Dowty, M. E.; Pinney, B. B.; Farmer, J. A.; Crossdoersen, D.; Sheldon, R. J. Design, Synthesis, and Evaluation of Proline and Pyrrolidine Based Melanocortin Receptor Agonists. A Conformationally Restricted Dipeptide Mimic Approach. *J. Med. Chem.* **2006**, *49*, 4745–4761.

(48) Sun, H.; Greeley, D. N.; Chu, X.-J.; Cheung, A.; Danho, W.; Swistok, J.; Wang, Y.; Zhao, C.; Chen, L.; Fry, D. C. A Predictive Pharmacophore Model of Human Melanocortin-4 Receptor As Derived From the Solution Structures of Cyclic Peptides. *Bioorg. Med. Chem.* **2004**, *12*, 2671–2677.

(49) Yamano, Y.; Kamon, R.; Yoshimizu, T.; Toda, Y.; Oshida, Y.; Chaki, S.; Yoshioka, M.; Morishima, I. The Role of the DRY Motif of Human MC4R for Receptor Activation. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1369–1371.

(50) Tucci, F. C.; Tran, J. A.; Jiang, W.; Pontillo, J.; Marinkovic, D.; Whit, N. S.; Arellano, M.; Fleck, B. A.; Wen, J.; Saunders, J.; Foster, A. C.; Chen, C. Synthesis of Piperazinephen-1-ylethylamines as Potent and Selective Antagonists of the Human Melanocortin-4 Receptor. *Lett. Drug Des. Discovery* **2006**, *3*, 311–315.

(51) Jiang, W.; Tucci, F. C.; Chen, C. W.; Arellano, M.; Tran, J. A.; White, N. S.; Marinkovic, D.; Pontillo, J.; Fleck, B. A.; Wen, J.; Saunders, J.; Madan, A.; Foster, A. C.; Chen, C. Arylpropionylpiperazines as Antagonists of the Human Melanocortin-4 Receptor. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4674–4678.

(52) Grieco, P.; Balse, P. M.; Weinberg, D.; MacNeil, T.; Hruby, V. J. D-Amino Acid Scan of γ-Melanocyte-Stimulating Hormone: Importance of Trp^8 on Human MC3 Receptor Selectivity. *J. Med. Chem.* **2000**, *43*, 4998–5002.

(53) Cai, M.; Mayorov, A. V.; Cabello, C.; Stankova, M.; Trivedi, D.; Hruby, V. J. Novel 3D Pharmacophore of α-MSH/γ-MSH Hybrids Leads to Selective Human MC1R and MC3R Analogues. *J. Med. Chem.* **2005**, *48*, 1839–1848.

(54) Mayorov, A. V.; Cai, M.; Chandler, K. B.; Petrov, R. R.; Van Scoy, A. R.; Yu, Z.; Tanaka, D. K.; Trivedi, D.; Hruby, V. J. Development of Cyclic γ-MSH Analogues with Selective hMC3R Agonist and hMC3R/hMC5R Antagonist Activities. *J. Med. Chem.* **2006**, *49*, 1946–1952.

(55) Balse-Srinivasan, P.; Grieco, P.; Cai, M.; Trivedi, D.; Hruby, V. J. Structure−Activity Relationships of Novel Cyclic α-MSH/β-MSH Hybrid Analogues that Lead to Potent and Selective Ligands for the Human MC3R and Human MC5R. *J. Med. Chem.* **2003**, *46*, 3728–3733.

(56) Chen, C.; Yu, J.; Fleck, B. A.; Hoare, S. R. J.; Saunders, J.; Foster, A. C. Phenylguanidines as Selective Nonpeptide Melanocortin-5 Receptor Antagonists. *J. Med. Chem.* **2004**, *47*, 4083–4088.

(57) Cai, M.; Cai, C.; Mayorov, A. V.; Xiong, C.; Cabello, C. M.; Soloshonok, V. A.; Swift, J. R.; Trivedi, D.; Hruby, V. J. Biological and Conformational Study of Substituted Prolines in MT-II Template: Steric Effects Leading to Human MC5 Receptor Selectivity. *J. Pept. Res.* **2004**, *63*, 116–131.

(58) Mayorov, A. V.; Han, S.-Y.; Cai, M.; Hammer, M. R.; Trivedi, D.; Hruby, V. J. Effects of Macrocycle Size and Rigidity on Melanocortin Receptor-1 and -5 Selectivity in Cyclic Lactam α-MSH Analogues. *Chem. Biol. Drug Des.* **2006**, *67*, 329–335.

(59) Cain, J. P.; Mayorov, A. V.; Cai, M.; Wang, H.; Tan, B.; Chandler, K. B.; Lee, Y. S.; Petrov, R. R.; Trivedi, D.; Hruby, V. J. Design, Synthesis, and Biological Evaluation of a New Class of Small Molecule Peptide Mimetics Targeting the Melanocortin Receptors. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5462–5467.

(60) Bednarek, M. A.; MacNeil, T.; Tang, R.; Fong, T. M.; Cabello, M. A.; Maroto, M.; Teran, A. Potent and Selective Agonists of α-Melanotropin (αMSH) Action at Human Melanocortin Receptor 5; Linear Analogs of α-Melanotropin. *Peptides* **2007**, *28*, 1020–1028.

(61) Bednarek, M. A.; MacNeil, T.; Tang, R.; Fong, T. M.; Cabello, M. A.; Maroto, M.; Teran, A. Potent and Selective Peptide Agonists of α-Melanocyte Stimulating Hormone (αMSH) Action at Human Melanocortin Receptor 5; their Synthesis and Biological Evaluation In Vitro. *Chem. Biol. Drug Des.* **2007**, *69*, 350–355.

(62) Bednarek, M. A.; MacNeil, T.; Tang, R.; Fong, T. M.; Cabello, M. A.; Maroto, M.; Teran, A. Potent and Selective Agonists of Human

Melanocortin Receptor 5: Cyclic Analogues of α-Melanocyte-Stimulating Hormone. *J. Med. Chem.* **2007**, *50*, 2520–2526.
(63) Butler, A. A.; Kesterson, R. A.; Khong, K.; Cullen, M. J.; Pellymounter, M. A.; Dekoning, J.; Baetscher, M.; Cone, R. D. A Unique Metabolic Syndrome Causes Obesity In The Melanocortin-3 Receptor-Deficient Mouse. *Endocrinology* **2000**, *141*, 3518–3521.
(64) Chen, A. S.; Marsh, D. J.; Trumbauer, M. E.; Frazier, E. G.; Guan, X.-M.; Yu, H.; Rosenblum, C. I.; Vongs, A.; Feng, Y.; Cao, L. H.; Metzger, J. M.; Strack, A. M.; Camacho, R. E.; Mellin, T. N.; Nunes, C. N.; Min, W.; Fisher, J.; Gopal-Truter, S.; MacIntyre, D. E.; Chen, H. Y.; Van der Ploeg, L. H. I. Inactivation of the Mouse Melanocortin-3 Receptor Results in Increased Fat Mass and Reduced Lean Body Mass. *Nat. Genet.* **2000**, *26*, 97–102.
(65) Marks, D. L.; Hruby, V. J.; Brookhart, G.; Cone, R. D. The Regulation of Food Intake by Selective Stimulation of the Type 3 Melanocortin Receptor (MC3R). *Peptides* **2006**, *27*, 259–264.
(66) Getting, S. J.; Perretti, M. MC3-R as a Novel Target for Anti-inflammatory Therapy. *Drug News Perspect.* **2000**, *13*, 19–27.
(67) King, S. H.; Mayorov, A. V.; Balse-Srinivasan, P.; V. J.; Vanderah, T.; Wessells, H. Melanocortin Receptors, Melanotropic Peptides, and Penile Erection. *Curr. Top. Med. Chem.* **2007**, *7*, 1098–1106.
(68) Chen, W. B.; Kelly, M. A.; Opitz Araya, X.; Thomas, R. E.; Low, M. J.; Cone, R. D. Exocrine Gland Dysfunction in MC5-R Deficient Mice: Evidence for Coordinated Regulation of Exocrine Gland Function by Melanocortin Peptides. *Cell* **1997**, *91*, 789–798.
(69) Hruby, V. J.; Cai, M.; Cain, J. P.; Mayorov, A. V.; Dedek, M.; Trivedi, D. Design, Synthesis and Biological Evaluation of Ligands Selective for the Melanocortin-3-Receptor. *Curr. Top. Med. Chem.* **2007**, *7*, 1107–1119.
(70) Hruby, V. J.; Lu, D.; Sharma, S. D.; Castrucci, A.; de, L.; Kesterson, R. A.; Al-Obeidi, F. A.; Hadley, M. E.; Cone, R. D. Cyclic Lactam Alpha-Melanotropin Analogues of Ac-Nle^4-cyclo[Asp^5, D-Phe^7, Lys^{10}] Alpha-Melanocyte-Stimulating Hormone-(4–10)-NH_2 with Bulky Aromatic Amino Acids at Position 7 Show High Antagonist Potency and Selectivity at Specific Melanocortin Receptors. *J. Med. Chem.* **1995**, *38*, 3454–3461.
(71) Al-Obeidi, F.; Hadley, M. E.; Pettitt, B. M.; Hruby, V. J. Design of a New Class of Superpotent Cyclic α-Melanotropins Based on Quenched Dynamic Simulations. *J. Am. Chem. Soc.* **1989**, *111*, 3413–3416.
(72) Grieco, P.; Balse-Srinivasan, P.; Han, G.; Weinberg, D.; MacNeil, T.; Van der Ploeg, L. H. T.; Hruby, V. J. Extensive Structure−Activity Studies of Lactam Derivatives of MT-II and SHU-9119: Their Activity and Selectivity at Human Melanocortin Receptors 3, 4, and 5. *J. Pept. Res.* **2003**, *62*, 199–206.
(73) Ying, J.; Kövér, K. E.; Gu, X.; Han, G.; Trivedi, D. B.; Kavarana, M. J.; Hruby, V. J. Solution Structures of Cyclic Melanocortin Agonists and Antagonists by NMR. Biopolymers. *Pept. Sci.* **2003**, *71*, 696–716.
(74) Ballet, S.; Mayorov, A. V.; Cai, M.; Tymecka, D.; Chandler, K. B.; Palmer, E. S.; Van Rompaey, K.; Misicka, A.; Tourwé, D.; Hruby, V. J. Novel Selective Human Melanocortin-3 Receptor Ligands: Use of the 4-Amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (Aba) Scaffold. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 2492–2498.
(75) Krchnak, V.; Vagner, J.; Safar, P.; Lebl, M. Amino-Acids and Peptides. Part CCVI. Noninvasive Continuous Monitoring Of Solid-Phase Peptide-Synthesis by Acid-Base Indicator. *Collect. Czech. Chem. Commun.* **1988**, *53*, 2542–2548.
(76) Krchnak, V.; Vagner, J. Color-Monitored Solid-Phase Multiple Peptide Synthesis under Low-Pressure Continuous-Flow Conditions. *Pept. Res.* **1990**, *3*, 182–193.
(77) Thieriet, N.; Alsina, J.; Giralt, E.; Guibé, F.; Albericio, F. Use of Alloc-Amino Acids in Solid-Phase Peptide Synthesis. Tandem Deprotection-Coupling Reactions Using Neutral Conditions. *Tetrahedron Lett.* **1997**, *38*, 7275–7278.
(78) Sabatino, G.; Mulinacci, B.; Alcaro, M. C.; Chelli, M.; Rovero, P.; Papini, A. M. Assessment of New 6-Cl-HOBt Based Coupling Reagents for Peptide Synthesis. Part 1: Coupling Efficiency Study. *Lett. Pept. Sci.* **2003**, *9* (2–3), 119–123.
(79) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal. Biochem.* **1970**, *34*, 595–598.
(80) Haskell-Luevano, C.; Miwa, H.; Dickinson, C.; Hruby, V. J.; Yamada, T.; Gantz, I. Binding and cAMP Studies of Melanotropin Peptides with the Cloned Human Peripheral Melanocortin Receptor, hMC1R. *Biochem. Biophys. Res. Commun.* **1994**, *204*, 1137–1142.
(81) Gantz, I.; Miwa, H.; Konda, Y.; Shimoto, Y.; Tashiro, T.; Watson, S. J.; Delvalle, J.; Yamada, T. Molecular Cloning of a Novel Melanocortin Receptor. *J. Biol. Chem.* **1993**, *268*, 15174–15179.
(82) Schild, H. O. pA, a New Scale for the Measurement of Drug Antagonism. *Br. J. Pharmacol.* **1947**, *2*, 189–206.
(83) Kaminski, G. A.; Friesner, R. A.; Tirado-Rives, J.; Jorgensen, W. L. Evaluation and Reparametrization of the OPLS-AA Force Field for Proteins via Comparison with Accurate Quantum Chemical Calculations on Peptides. *J. Phys. Chem. B* **2001**, *105*, 6474–6487.
(84) Still, W. C.; Tempczyk, A.; Hawlely, R. C.; Hendrickson, T. A General Treatment of Solvation for Molecular Mechanics. *J. Am. Chem. Soc.* **1990**, *112*, 6127–6129.
(85) Kolossváry, I.; Guida, W. C. Low-Mode Conformational Search Elucidated. Application to C39H80 and Flexible Docking of 9-Deazaguanine Inhibitors to PNP. *J. Comput. Chem.* **1999**, *20*, 1671.

JM070461W