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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

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It has been shown by extensive studies that α-MSH bioactivity is critically dependent on the core or central tetrapeptide sequence, His-Phe-Arg-Trp, however with poor selectivity for the human MC3R-MC5R. The structure−activity relationships study here is aimed at identifying lead structures or templates of this core sequence by the use of different conformational constraints that might impart changes in its topography and thus promote differences in potency and selectivity at these receptors. Our peptide library consists of a novel series of cyclic α-MSH analogues that have disulfide bridges between Cys or Cys-like residues at positions 4 and 10, giving rise to 23-membered rings fused at the C-terminal end with the C-terminal fragment of *β*-MSH (Pro-Pro-Lys-Asp). While such constraints of the peptide backbone with disulfide bridges of different chirality affect potency and selectivity at these receptors, further changes in the hydrophobicity at position 7 with either a D-Phe or D-Nal(2′) and replacement of a His with a Pro in position 6 cause additional effects. Thus, the most interesting lead compounds that emerged from this study are (1) compound **5**, Ac-*c*[Cys-Glu-His-D-Phe-Arg-Trp-D-Cys]-Pro-Pro-Lys-Asp-NH_2 (IC_{50} = 10 nM), which is the first potent and highly selective antagonist ligand for the hMC5R (560-fold vs the MC3R and 1000-fold vs the MC4R); (2) compound **7**, Ac-*c*[Cys-Glu-Pro-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2 (IC_{50} = 31 nM), which is a highly selective antagonist analogue for the MC3R (560-fold vs the hMC4R and about 3000-fold vs the hMC5R; and (3) compound **9**, Ac-*c*[Pen-Glu-His-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2 (IC_{50} = 3 nM), which is more potent than **7** at the MC3R but not as selective.

Introduction

α-Melanocyte-stimulating hormone (α-melanotropin, α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) and *β*-MSH (Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp-OH) belong to the family of melanocortin peptides derived by the posttranslational processing of the pro-opiomelanocortin (POMC) gene. The melanocortins, which include the melanotropins (α-, *β*-, and *γ*-MSH, Table 1) and adrenocorticotropin (ACTH), are widely distributed in both the central and peripheral autonomic nervous systems. Physiological effects of the melanocortins have been extensively studied for many years in vertebrates and mammals, including humans. These peptides have been implicated in numerous biological functions including regulation of skin pigmentation, regulation of steroid production, stimulation of nerve regeneration, modulation of immune responses, learning processes, and cell growth.[1−4] The molecular clones of five different melanocortin receptors (MC1−MC5) have provided tools for studying the molecular mechanisms involved in these physiological effects.[5−9] The receptors are G-protein-coupled receptors, and their activation leads to an elevation in cAMP. The endogenous hormones are not known to be receptor subtype selective, except that ACTH is selective for the MC2 receptor. The MC2 subtype is distinguished from the other MC subtypes in that it does not bind to the MSH peptides. α-MSH binds at the other four receptors, i.e., MC1, MC3, MC4, and MC5. The MC3 subtype has a slight preference for *γ*-MSH and the MC5 for α-MSH. The MC4 receptor has a high affinity for α-MSH and *β*-MSH but a very low affinity for *γ*-MSH. The physiological role of these receptors is not fully understood, but it has been speculated that the various physiological effects of the melanocortins are mediated through these receptors. The MC1 receptor is mainly found in skin and melanoma cells and plays a major role in skin pigmentation. The MC2 receptor, expressed in the adrenal cortex, is involved in steroidogenesis and response to stress. The MC4 receptor is found mainly in the central nervous system and is related to the feeding behavior and weight homeostasis by MSH peptides. The MC3 and MC5 receptors are found both in the brain and periphery, but their functions are not as clearly defined, though the MC3 receptor is thought to be involved in the regulation of fat stores through a metabolic pathway,[10] and the MC5 receptor in peripheral glandular secretion.[11]

As shown earlier, all the melanotropins are characterized by the core or central tetrapeptide sequence, His-Phe-Arg-Trp, flanked by N- and C-terminal residues. This core sequence is the minimal fragment essential

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Table 1. Structures of the Mammalian Melanotropin Peptides

peptide	structure
α-MSH	Ac-Ser-**Tyr**-Ser-**Met**-Glu-**His-Phe-Arg-Trp**-Gly-Lys-Pro-Val-NH_2
β-MSH	H-Asp-Glu-Gly-Pro-**Tyr**-Lys-**Met**-Glu-**His-Phe-Arg-Trp**-Gly-Ser-Pro-Pro-Lys-Asp-NH_2
γ-MSH	H-**Tyr**-Val-**Met**-Gly-**His-Phe-Arg-Trp**-Asp-Arg-Phe-Gly-OH

Table 2. Binding and Intracellular cAMP Accumulation of the Melanotropin Analogues at Human Melanocortin Receptors

		hMC3R			hMC4R			hMC5R		
peptide code	structure	IC_{50} (nM)[a]	EC_{50} (nM)[b]	% act. at 10 μM	IC_{50} (nM)[a]	EC_{50} (nM)[b]	% act. at 10 μM	IC_{50} (nM)[a]	EC_{50} (nM)[b]	% act. at 10 μM
1	Ac-*c*[Cys-Glu-His-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	130 ± 29	>10 000	-	3.4 ± 1.9	6.1 ± 1.5	17	180 ± 23	190 ± 49	100
2	Ac-*c*[D-Cys-Glu-His-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	200 ± 50	>10 000	-	3700	>10 000	-	26 000	880	100
3	Ac-*c*[Cys-Glu-His-D-Nal(2′)-Arg-Trp-D-Cys]-Pro-Pro-Lys-Asp-NH_2	2900	>10 000	-	40 000	>10 000	-	15 000	>10 000	-
4	Ac-*c*[D-Cys-Glu-His-D-Phe-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	620 ± 100	>10 000	-	37000	>10 000	-	73. ± 20	>10 000	-
5	Ac-*c*[Cys-Glu-His-D-Phe-Arg-Trp-D-Cys]-Pro-Pro-Lys-Asp-NH_2	5600	>10 000	-	>10 000	>10 000	-	10. ± 2	>10 000	-
6	Ac-*c*[Cys-Glu-Pro-D-Phe-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	>1000	401	-	3300	>10 000	-	200 ± 30	380	46
7	Ac-*c*[Cys-Glu-Pro-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	31. ± 10	>10 000	-	17 000	>10 000	-	97 000	>10 000	-
8	Ac-*c*[Pen-Glu-His-D-Phe-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	430 ± 100	2.8 ± 0.5	100	1100	6.3 ± 1.2	91	27 000	>10 000	-
9	Ac-*c*[Pen-Glu-His-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	3.0 ± 0.5	>10 000	-	750 ± 100	>10 000	-	94 ± 11	>10 000	-
10	Ac-*c*[Asp-Glu-His-D-Phe-Arg-Trp-Lys]-Pro-Pro-Lys-Asp-NH_2	>1000	>10 000	-	>10 000	>10 000	-	>10 000	>10 000	-
11 α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	30 ± 3.9	6.7 ± 1	100	5 ± 1	2.1 ± 0.6	100	18 ± 2	8.1 ± 1.5	100
12 NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	3.3 ± 0.3	0.8 ± 0.1	100	0.40 ± 0.02	0.20 ± 0.04	100	2.2 ± 0.5	1.0 ± 0.3	100
13 HS014	Ac-*c*[Cys-Glu-His-D-Nal(2′)-Arg-Trp-Gly-Cys]-Pro-Pro-Lys-Asp-NH_2	54[c]	-	-	3.1[c]	-	-	694[c]	partial agonist	-

[a] IC_{50} = Concentration of peptide at 50% specific binding ($N = 4-6$). The peptides were tested in a range of concentrations (10^{-10} to 10^{-4} M). [b] EC_{50} = Concentration of peptide at 50% maximal cAMP generation ($n = 4$). The peptides were tested in a range of concentrations (10^{-10} to 10^{-4} M). [c] These are K_i values (ref 32) instead of IC_{50} values.

for biological activity[12−14] and is believed to form the basic pharmacophore for the MC1, MC3, MC4, and MC5 receptors. Further, the "bioactive conformation" of α-MSH was first shown to possibly exist as a reverse-turn-type conformation at this His-Phe-Arg-Trp sequence by the design of the macrocyclic analogue *c*[Cys^4,Cys^{10}]-α-MSH, a superagonist in the frog skin bioassay.[15,16] In our aim to probe the topographical requirements of this pharmacophore at each of the different receptor sites that contribute to potency and selectivity, we have developed several templates for the core sequence His-Phe-Arg-Trp.

We have synthesized a new cyclic template which is a hybrid of α-MSH and *β*-MSH, starting with the N-terminal residue Glu^5 and the core sequence -His^6-Phe^7-Arg^8-Trp^9-, and then cyclizing these five residues with a disulfide bridge, combining it with the *C*-terminal sequence of *β*-MSH, i.e., -Pro-Pro-Lys-Asp-, and ending with a C-terminal carboxyamide. Variations in this library included replacements for His and Phe, different disulfide bridges such as Cys-Cys, Cys-D-Cys, Cys-Pen, etc. A 23-membered ring formed by a lactam bridge between side chains of Asp and Lys at positions 4 and 9 was also included in this focused minilibrary (Table 2).

Results

Peptide Synthesis. The peptides (Table 2) were synthesized by N^{α}-Fmoc chemistry on Rink amide resin, the standard supports for batch Fmoc SPPS of peptide amides.[17] Furthermore, couplings were carried out with standard in situ-activating reagents used in routine Fmoc SPPS, such as the uronium salts (HBTU), in the presence of a tertiary base (DIPEA), to generate HOBt esters. Cys and Trp are extremely susceptible to alkylation by cations produced during the cleavage process. The other concern is the Rink amide linker. Trialkylsilanes, such as TES, have been shown to be effective, nonodorous substitutes for ethanedithiol, particularly for peptides containing Arg(Pmc) and Trp(Boc).[18−20] They also are very efficient at quenching those highly stabilized cations that are not irreversibly scavenged by thiols, e.g., Trt and the Rink linker.[18] In our syntheses, cleavage of the peptide from the resin with a TES-based TFA cocktail was adopted since the Trp residue had a Boc-side chain-protecting group.

Lactam cyclization was performed on the resin by first removing the allyl and Aloc protecting groups using a procedure described by Thieriet and others.[21−23] The smooth removal of these groups in neutral conditions with catalytic amounts of $Pd(PPh_3)_4$ in the presence of $PhSiH_3$ as a scavenger for the allyl system and careful elimination of all oxygen[23] permits orthogonality with the Fmoc and Boc protecting groups in the sequence. The free side chains of Asp and Lys were then cyclized using the traditional coupling reagents HBTU and HOBt in the presence of a tertiary amine.

The HPLC profile of the crude linear peptide obtained following cleavage from the resin showed a single peak by FAB-MS corresponding to the desired peptide. Oxi-

dation of cysteinyl peptides to form cyclic disulfide-bonded peptides is generally carried out using dilute solutions of peptide in aqueous or aqueous/organic media at weakly basic pH values. Various oxidants have been used of which the most useful are oxygen (air), $K_3Fe(CN)_6$[24,25] iodine,[26] DMSO,[27,28] and mixtures of oxidized and reduced glutathione.[29] The classical oxidizing agent $K_3Fe(CN)_6$ was used in our procedure with the principle that a low concentration of the linear (reduced) peptide is necessary for the oxidation.[30,31] Thus, oxidation was performed by slow dropping of a solution of the peptide into a reaction vessel containing an excess (1.2 equiv) of the oxidizing agent in aqueous solution. The concentration of the reduced peptide was regulated by controlling the speed of addition of the peptide with the help of an automated syringe pump.

All the analogues in this library were purified by reversed-phase HPLC. The physicochemical properties and purity of these peptides were assessed by MALDI-TOF, RP-HPLC, and TLC in three solvent systems, and amino acid analysis (see Experimental Section).

Binding Data. The binding affinities for the hybrid analogues of α-MSH/*β*-MSH at cloned human MC3R, MC4R, and MC5R are compiled in Table 2 and are compared with that of the native α-MSH (**11**), NDP-MSH (**12**) (controlled experimental data from our laboratory for both the peptides), and a related cyclic disulfide-containing peptide (**13**) (but with an added Gly in the disulfide ring) reported by Schiöth et al.[32] It was observed that although α-MSH and NDP-α-MSH are highly potent at all three receptors, they generally lack high selectivity. Compound **13** (HS014) on the other hand was a selective antagonist for the hMC4R with a K_i of 3.1 nM. The first peptide in our series, i.e., the Cys^4-D-Nal(2′)^7-Cys^{10} analogue (analogue **1,** Table 2) was quite selective for the hMC4R (IC_{50} = 3.4 nM) over the hMC3R and hMC5R by close to 2 orders of magnitude. On the other hand, the D-Cys^4-D-Nal(2′)^7-Cys^{10} analogue (analogue **2**) was only moderately selective for hMC3R (IC_{50} = 200 nM) and much less potent at all three hMC receptors (Table 2), indicating the effect of change in conformation on receptor selectivity. Further, the Cys^4-D-Nal(2′)^7-D-Cys^{10} analogue **3** barely binds even at 1 μM at all three sites. The D-Cys^4-D-Phe^7-Cys^{10} analogue **4** (IC_{50} = 73 nM) is selective for the hMC5R, and the Cys^4-D-Phe^7-D-Cys^{10} analogue **5** (IC_{50} = 10 nM) is exceptionally potent and selective for binding to the MC5R (560-fold vs the MC3R and >1000-fold selective vs the MC4R). The Cys^4-Pro^6-D-Phe^7-Cys^{10} analogue **6** shows moderate affinity for the hMC5R (IC_{50} = 200 nM) and almost none for the hMC3R and hMC4R. The Cys^4-Pro^6-D-Nal(2′)^7-Cys^{10} analogue **7** on the other hand has high affinity and selectivity for the hMC3R (IC_{50} = 30.8 nM), compared to the hMC4R and hMC5R. The Pen^4-D-Phe^7-Cys^{10} analogue **8** shows weak affinity for the hMC3R (IC_{50} = 430 nM) and almost none at the other two sites. However, the Pen^4-D-Nal(2′)^7-Cys^{10} analogue **9** shows high affinity and selectivity for the hMC3R (IC_{50} = 2.96 nM) over the hMC4R and hMC5R by at least 2 orders of magnitude. Finally, the Asp^4-D-Phe^7-Lys^{10} analogue **10** is inactive at all three receptors.

cAMP Assays. Measurement of the intracellular cAMP accumulation (Table 2) using cloned human hMC3R, hMC4R, and hMC5R in this functional assay revealed that the Cys^4-D-Nal(2′)^7-Cys^{10} analogue (analogue **1**) shows potent and selective cAMP bioactivity at the hMC4R (EC_{50} = 6.1 nM) but with a maximum efficacy of 17% relative to α-MSH. Thus analogue **1** is a potent partial agonist at the hMC4R, a weak agonist at the hMC5R, and a weak antagonist at the hMC3R. The D-Cys^4-D-Nal(2′)^7-Cys^{10} analogue (analogue **2**), the Cys^4-D-Nal(2′)^7-D-Cys^{10} analogue **3**, the D-Cys^4-D-Phe^7-Cys^{10} analogue **4**, and the Cys^4-D-Phe^7-D-Cys^{10} analogue **5** show almost no stimulation of cAMP at all three receptors though as noted above they vary tremendously in their binding affinities at these receptors. The Cys^4-Pro^6-D-Phe^7-Cys^{10} analogue **6** shows weak cAMP activation at the hMC3R and hMC5R, while the Cys^4-Pro^6-D-Nal(2′)^7-Cys^{10} analogue **7** does not stimulate cAMP accumulation at any of the three receptors. The Pen^4-D-Phe^7-Cys^{10} analogue **8** shows potent cAMP accumulation (EC_{50} = 2.82 to 6.28 nM) at the hMC3R and hMC4R and no bioactivity at the hMC5R. The Pen^4-D-Nal(2′)^7-Cys^{10} analogue **9** and the Asp^4-D-Phe^7-Lys^{10} analogue **10** again cause no cAMP activation at any of the three hMC receptors.

Discussion

Identification of key amino acid residues in a hormone or neurotransmitter that are necessary for receptor recognition is the first step in structure−affinity and structure−biological activity relationships.[33] It has been established that three receptors, the hMC3R, hMC4R, and hMC5R, utilize the same sequence motif -His-Phe-Arg-Trp- as the principle pharmacophore for receptor interactions and signal transduction in α-MSH. However, there is poor selectivity of α-MSH for these receptors (Table 2). Our current SAR studies are aimed at identifying lead structures or templates of the core sequence by the use of different conformational constraints that might impart changes in topography of this core sequence and thus promote differences in potency and selectivity at these receptors.

We have designed a novel series of cyclic α-MSH analogues that have disulfide bridges between Cys or Cys-like residues at positions 4 and 10, giving rise to 23-membered rings as reported previously.[15] However, the novel aspect of this work is that the rings have been fused at the C-terminal end with the C-terminal fragment of *β*-MSH (Pro-Pro-Lys-Asp) to give α-MSH/*β*-MSH hybrid analogues. Furthermore, our design strategies here have included: (1) changing the hydrophobicity at position 7 with either a D-Phe or D-Nal(2′);[34] and (2) effect of replacing His with a Pro in position 6.[35]

The cyclic Cys^4-D-Nal(2′)^7-Cys^{10} analogue **1** (EC_{50} = 6.1 nM) was the most potent agonist in the series with high selectivity for the hMC4R versus the hMC3R and hMC5R. Interestingly replacing His with Pro in the D-Nal(2′) series changes the receptor selectivity for the hMC4R to the hMC3R. Thus, for example, the cyclic Cys^4-Pro^6-D-Nal(2′)^7-Cys^{10} analogue **7** (IC_{50} = 31 nM) is in an exceptionally selective antagonist for the hMC3R. Replacement of D-Nal(2′) with D-Phe, e.g., in the cyclic D-Cys^4-D-Phe^7-Cys^{10} analogue **4** (IC_{50} = 73 nM) and the cyclic Cys^4-D-Phe^7-D-Cys^{10} analogue **5** (IC_{50} = 10 nM), imparted antagonist activity at all three receptors and high selectivity for the hMC5R only, pointing out the effect of the change in hydrophobicity of residue

Table 3. Physicochemical Properties of the Melanotropin Analogues

peptide code	structure	TLC R_f[a] A	TLC R_f[a] B	TLC R_f[a] C	HPLC k'[b]	HR-MS[c] obsd	HR-MS[c] calcd
1	Ac-c[Cys-Glu-His-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	0.69	0.92	0.03	2.38	1506.65	1506.74
2	Ac-c[D-Cys-Glu-His-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	0.77	0.87	0.03	2.31	1506.31	1506.74
3	Ac-c[Cys-Glu-His-D-Nal(2′)-Arg-Trp-D-Cys]-Pro-Pro-Lys-Asp-NH_2	0.77	0.87	0.03	2.44	1506.42	1506.74
4	Ac-c[D-Cys-Glu-His-D-Phe-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	0.76	0.80	0.03	1.77	1456.73	1456.68
5	Ac-c[Cys-Glu-His-D-Phe-Arg-Trp-D-Cys]-Pro-Pro-Lys-Asp-NH_2	0.76	0.89	0.03	1.89	1456.54	1456.68
6	Ac-c[Cys-Glu-Pro-D-Phe-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	0.78	0.90	0.07	2.54	1416.56	1416.66
7	Ac-c[Cys-Glu-Pro-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	0.78	0.92	0.07	3.16	1466.51	1466.72
8	Ac-c[Pen-Glu-His-D-Phe-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	0.75	0.86	0.03	1.89	1484.62	1484.72
9	Ac-c[Pen-Glu-His-D-Nal(2′)-Arg-Trp-Cys]-Pro-Pro-Lys-Asp-NH_2	0.76	0.89	0.03	2.38	1534.66	1534.79
10	Ac-c[Asp-Glu-His-D-Phe-Arg-Trp-Lys]-Pro-Pro-Lys-Asp-NH_2	0.77	0.78	0.03	1.79	1477.60	1477.67

[a] R_f values on thin-layer chromatograms of silica gel were observed in the following solvent systems: (A) 1-butanol/pyridine/acetic acid/water (5:5:1:4); (B) ethyl acetate/pyridine/acetic acid/water (5:5:1:3); and (C) upper phase of 1-butanol/acetic acid/water (4:1:1). [b] HPLC k' = [(peptide retention time − solvent retention time)/solvent retention time] in a solvent system of 10% CH_3CN in 0.1% TFA and a gradient to 90% CH_3CN over 40 min. An analytical Vydac C_{18} column was used with a flow rate of 1 mL/min. [c] High-resolution MS were determined by MALDI-TOF spectra.

7 in the α-MSH sequence are most acutely felt at the hMC5R. Comparison of the D-Phe and D-Nal series, suggests that the D-Phe residue has a preference for hMC5R while the D-Nal(2′) prefers either hMC3R or hMC4R. This is especially noted in the cyclic Pen[4]-D-Nal(2′)[7]-Cys[10] analogue **9** (IC_{50} = 3.0 nM) which is a potent antagonist at the hMC3R with 30-fold selectivity against the hMC5R and 250-fold selectivity over the hMC4R.

To conclude, the introduction of conformational constraints on the peptide backbone through disulfide bridges of different chirality and topographical bias in the α-MSH core sequence imparts large changes in potency and selectivity at the hMC3R, hMC4R, and hMC5R. Furthermore, substitution of Cys with its optical isomer or His with Pro, or hydrophobic replacements, such as D-Phe to D-Nal(2′), also can affect potency and selectivity within the conformational changes. In this regard the most interesting lead compounds for further study are (1) compound **5**, which is the first potent and highly selective antagonist ligand for the hMC5R; (2) compound **7** (Table 2), which is a highly selective antagonist analogue for the hMC3R (560-fold vs the hMC4R and about 3000-fold vs the hMC5R; and (3) compound **9** (Table 2) which is more potent than **7** at the hMC3R but not as selective.

Experimental Section

Methods. N^{α}-Fmoc-protected amino acids and Rink-amide resin were purchased either from Advanced ChemTech (Louisville, KY) or from American Peptide Company, Inc. (Sunnyvale, CA). HBTU and HOBt were purchased from Quantum Biotechnologies (Montreal, Quebec, Canada). For the N^{α}-Fmoc-protected amino acids, the following side chain protecting groups were used: Arg(N^{g}-Pbf); Asp(β-*O*-All); Asp(β-*O*-tBu); Cys(*S*-Trt); D-Cys(*S*-Trt); (His(N^{im}-Trt); Lys(N^{ϵ}-Aloc); Lys(N^{ϵ}-Boc); Pen(*S*-Trt); Trp(*N*-Boc) and Tyr(*O*-*t*Bu). All protected amino acid derivatives were analyzed for purity by thin-layer chromatography before use. Peptide synthesis solvents, reagents, as well as CH_3CN for HPLC were reagent grade and were acquired from commercial sources and used without further purification unless otherwise noted. TLC was done on Analtech, Inc. (Newark, DE) silica gel F_{254} (250 μM) plates using the following solvent systems: (A) 1-butanol/pyridine/acetic acid/water (5:5:1:4); (B) ethyl acetate/pyridine/acetic acid/water (5:5:1:3); and (C) upper phase of 1-butanol/acetic acid/water (4:1:1). The peptides were detected on the TLC plates using iodine vapor. Amino acid analyses were performed at the University of Arizona Mass Spectrometry and Protein Sequencing Facilities. The system used was an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl) and precolumn phenylthiocarbamoyl amino acid (PTC-AA) analysis. No corrections were made for amino acid decomposition. The purity of the finished peptides was checked by TLC in three solvent systems and by analytical RP-HPLC at 214, 230, 254, and 280 nm using a Hewlett-Packard 1090 Series II Liquid Chromatograph with a built-in diode array detector. In all cases, the purity of the finished peptides was greater than 95% as determined by these methods. The structures of the pure peptides were confirmed either by fast atom bombardment (FAB) mass spectrometry or by high-resolution MALDI-TOF, both of which were performed at the University of Arizona Mass Spectrometry and Protein Sequencing Facilities. The summary of the analytical results are given in Table 3 and in the Supporting Information.

Linear Peptide Synthesis. The peptide library was synthesized by N^{α}-Fmoc chemistry on 0.15 g each of Rink amide resin (substitution 0.7 mmol/g). The synthesis was carried out on a 16-well automated multiple peptide synthesizer ACT model 396 (Advanced ChemTech, Louisville, KY) using the DOS software. Solvents used were *N*,*N*-dimethylformamide and *N*-methylpyrrolidinone. The entire synthesis was performed under an atmosphere of argon. The resin was first swollen in DCM/DMF (1:1) for 2 h. Deprotection of the Fmoc protecting group on the resin linker was accomplished with 20% piperidine in DMF over 30 min. The first amino acid was coupled using 3 equiv each of the N^{α}-Fmoc-protected amino acid, HBTU, HOBt, and 6 equiv of DIPEA in DMF for 1 h. Amino acid solutions were in concentrations of 0.25 M in NMP, HBTU in a concentration of 0.4 M in NMP, and HOBt and DIPEA in a concentration of 0.5 M each in DMF. The peptide sequences were thus assembled by alternate cycles of deprotection and coupling. After each coupling, the Kaiser test[36,37] was performed to determine the completeness of coupling while after each deprotection, the test was performed to determine removal of the Fmoc protecting group. After coupling of the N-terminal amino acid, the N-terminal Fmoc group was deblocked as before and the peptide-resin was thoroughly washed with DCM (4 × 25 mL) and dried under an atmosphere of argon to yield dried peptide-resin.

Cyclization to Lactam on Resin. After assembling the linear sequence, the orthogonal protecting groups on the aspartic and lysine side chains, namely the Asp(β-*O*-All) and the Lys(N^{ϵ}-Aloc) protecting groups, respectively, were removed by the following protocol: DCM (1 min × 3) and then DCM (1 mL/200 mg resin) and $PhSiH_3$ (24 equiv/g peptide-resin) were added, and the resin suspension was bubbled with argon for 5 min. Then a solution of $Pd(PPh_3)_4$ (0.2 equiv/g peptide-resin) in minimal amount of DCM was added, followed by bubbling with argon for 30 min. The suspension was drained, and the protocol was repeated.

Following the removal of side chain protecting groups (confirmed by Kaiser test, vide infra), the free amino and

carboxyl-containing side chains were cyclized by treating with HBTU (6 equiv), HOBt (6 equiv), and DIPEA (12 equiv) in DMF for 2 h. After thorough washing with DMF (4 × 2 min) and DCM (4 × 2 min), the Kaiser test was repeated. If the peptide-resin was still positive (blue beads) at this point, cyclization conditions were repeated until a negative Kaiser test was obtained.

Cleavage of Peptide from Resin. The peptide resin was cleaved by treating with 4 mL of a solution of Et_3SiH (7%) and water (5%) in TFA with shaking in the reaction vessels on the automated multiple peptide synthesizer at room temperature. After 3 h, the solutions, which contain the cleaved peptides, were filtered from the resin through the fritted reaction vessels into glass receptacles and the resin washed with 1 mL of the TFA cocktail. The filtrate and washings were combined and cooled to 0 °C in an ice-bath for 15 min, and anhydrous ethyl ether was added dropwise to precipitate the crude peptides. Centrifugation at 2000 rpm for 3 min followed by decantation of the supernatant ether and air-drying of the residue yielded the crude peptide as a white to pale beige-colored amorphous solid.

Oxidative Cyclization to Disulfides. A solution of $K_3Fe(CN)_6$ was prepared as follows: 1 mmol (330 mg) of $K_3Fe(CN)_6$ was dissolved in a mixture of water (100 mL) and CH_3CN (20 mL), a saturated solution of ammonium acetate (20 mL) was added to it, and the pH was adjusted to 8.5 with concentrated ammonium hydroxide. A solution of the linear peptide (0.25 mmol) in 20% aqueous CH_3CN was added to the above solution dropwise overnight with the help of a push−pull syringe.[30,31] After the overnight reaction, glacial acetic acid was added to the reaction mixture to obtain pH 4.0, followed by 20 mL of Amberlite IRA-68 anion-exchange resin (preequilibrated with 2 M HCl), and the suspension stirred for 30 min until the solution turned colorless and the resin turned yellow. The resin was suction-filtered and the filtrate rotoevaporated using 1-butanol to form a water/1-butanol azeotrope. The residual oil was lyophilized overnight to yield a pale yellow solid.

HPLC Purification. Final peptide purification was achieved using a preparative RP-HPLC Vydac C18 (218TP1520, 15−20 microns, 10 × 250 mm). The peptides were injected onto the column at a concentration of 20−30 mg/mL in 20% aqueous CH_3CN and were eluted with a CH_3CN gradient (0 to 55%) over 35 min at a flow rate of 15.0 mL/min, with a constant concentration of TFA (0.1% v/v). The gradient was generated with a Dynamax HPXL solvent delivery system (Rainin Instrument Co., Inc., Woburn, MA). The separations were monitored at 230 and 280 nm and integrated with a Dynamax dual wavelength absorbance detector model UV-D. Fractions corresponding to the major peak were collected, combined, and lyophilized to yield the final peptides as highly purified (>95%) white solids.

Kaiser Test.[36,37] After each coupling and deprotection, a small portion of the peptide-resin was heated at 100 °C for 5 min with 1 drop each of the three Kaiser test reagents: (a) 2 mL of 0.01 M KCN in 98 mL of pyridine, (b) 500 mg of ninhydrin in 10 mL of *n*-butanol, and (c) 40 g of phenol in 20 mL of *n*-butanol. The coupling is complete if the solution is yellow and the beads are colorless; an incomplete coupling is indicated by a blue solution and blue or dark beads.

Binding Assays. Competition binding experiments were performed on whole cells. The coding region of the human melanocortin receptors cloned from a human genomic EMBL3 phage library was placed into the eukaryotic transfection vector, CMVneo, and stably transfected into human embryonic kidney (HEK) cells as previously described.[38] Transfected HEK293 cell line with hMCRs cells were seeded on 24-well plates, 48 h before assay, 50 000 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, 200 mg/L bacitracin. Cells were then incubated with different concentrations of unlabeled peptide and labeled [^{125}I-Tyr^2]-[Nle^4,D-Phe^7]α-MSH (Perkin-Elmer 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 assay buffer. The cells were lysed by the addition of 500 μL of 0.1 N NaOH and 500 μL of 1% Triton X-100. The lysed cell was transferred to the 12 × 75 mm glass tubes and counted by Wallac 1470 WIZARD Gamma Counter. Data were analyzed using Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

Adenylate Cyclase Assay. HEK 293 cells transfected with human melanocortin receptors 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 24-well plates 48 h before assay (50 000 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. Varying concentrations of melanotropins (0.1 mL) were added and the cells 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). The 24-well plates were covered and placed on ice. After dislodging the cells with the help of a cell scrapper, the suspension of cells was 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. cAMP content was measured by competitive binding assay according to the assay kit instructions (TRK 432, Amersham Corp.).

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).

Appendix

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC−IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: AAA, amino acid analysis; All, allyl; Aloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; *t*Bu, *tert*-butyl; CH_3CN, acetonitrile; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; Et_3SiH, triethylsilane; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; $K_3Fe(CN)_6$, potassium ferricyanide; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; hMC3R, human melanocortin-3 receptor; NMP, *N*-methylpyrrolidinone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; $Pd(PPh_3)_4$, palladium tetrakis-(triphenylphosphine); $PhSiH_3$, phenyltrihydrosilane; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; RP-HPLC, reversed-phase high performance liquid chromatography; SPPS, solid-phase peptide synthesis; SPS, solid-phase synthesis; TFA, trifluoroacetic acid; Trt, triphenylmethyl (trityl). Amino acid symbols denote L-configuration unless indicated otherwise.

Supporting Information Available: Amino acid analysis of the melanotropin analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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