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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

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It has been shown by extensive studies that melanotropin bioactivities are critically dependent on the core or central tetrapeptide sequence, His-Phe-Arg-Trp, and in α-MSH it has been demonstrated further that a reverse-turn type conformation exists at this pharmacophore. To probe the receptor active conformation of the pharmacophore His-Phe-Arg-Trp in γ-MSH, two different series of γ-MSH analogues have been designed and synthesized and their biological activities determined at hMC3R, hMC4R, and hMC5R. The 1st series consists of a cyclic scan using different disulfides or lactam bridges. It was found that cyclization of the native γ-MSH around the highly conserved sequence can lead to shifts in affinity and selectivity for hMC4R instead of the hMC3R as seen in the native peptide. Furthermore, a 23-membered ring is desirable for potency (e.g., analogues **6** and **10**) whereas a 26-membered ring (analogue **1**, H-Tyr-Val-c[Cys-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2 with Gly^4) is more important for selectivity. The 2nd series is made of D-2-naphthylalanine (D-Nal(2′)) scan of the γ-MSH sequence at position 6 and 8 and the replacement of His^5 with Pro (analogue **13**). Analogue **12**, H-Tyr-Val-Nle-Gly-His-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2, is a potent and selective antagonist at the hMC4R, and analogue **15**, H-Tyr-Val-Nle-Gly-Aib-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2, is a highly selective and potent agonist of the hMC5R. A most promising analogue is **13,** H-Tyr-Val-Nle-Gly-Pro-D-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH_2, which is a very potent agonist of the hMC4R, and this analogue can be further evaluated for feeding behavior and the regulation of fat stores.

Introduction

γ-Melanocyte-stimulating hormone (γ-melanotropin, γ-MSH, Table 1, Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH) belongs to the family of melanotropin peptides, that also includes α-MSH, β-MSH, and adrenocorticotropin (ACTH), and is derived by the posttranslational cleavage of the precursor pro-opiomelanocortin (POMC). Physiological effects of the melanotropins have been extensively studied for many years in vertebrates and mammals including humans.[1−3] 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, feeding behavior, erectile function, learning processes, and cell growth.[1,4,5] At present, five subtypes of human receptors that bind melanotropins have been characterized, hMC1R−hMC5R.[6−10] The receptors are G-protein-coupled receptors and for the most part their activation leads to an elevation in cAMP. Each of the receptors possesses unique affinities for the melanotropins. The MC1 receptor, binding mainly to α-MSH, is found in skin and melanoma cells and plays a role in skin pigmentation. The MC2 receptor, found in the adrenal cortex, binds only to ACTH and is involved in steroidogenesis. The MC3 and MC5 receptors are found both in the brain and periphery, but their functions are not very clear, though they appear to be involved in energy homeostasis and glandular secretions, respectively.[11] Both α- and γ-MSH show about equal affinity for the MC3R, while the MC5R binds mainly to α-MSH. The MC4 receptor, found mainly in the central nervous system, has a higher affinity for α-MSH and β-MSH than for γ-MSH.

While most of the research on the melanotropins has concentrated on α-MSH, the most potent of the three melanotropins, little is known of γ-MSH in terms of its structure−activity relationships (SAR) to the various melanocortin receptors. So our initial SAR study of γ-MSH involved an L-alanine scan[12] and a D-amino acid scan,[13] to understand the importance of each of the residues in its sequence and their topographical requirements for biological activity, as determined from activities at the cloned human MCRs.[12,13] The next step would be to further probe the conformation or topographical distribution of the core pharmacophoric residues in the sequence. As shown before, 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 for biological activity[14,15] and is believed to form the pharmacophore.[14−17] Furthermore, 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

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Table 1. Structures of the Mammalian α-MSH and γ-MSH Peptides

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

Table 2. Structures of the γ-Melanotropin Analogues

peptide code	structure
Series 1	
1	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2
2	H-Tyr-Val-*c*[D-Cys-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2
3	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-D-Cys]-Arg-Phe-Gly-NH_2
4	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-Pen]-Arg-Phe-Gly-NH_2
5	H-Tyr-Val-*c*[Pen-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2
6	H-Tyr-Val-Nle-*c*[Cys-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2
7	H-Tyr-Val-Nle-*c*[D-Cys-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2
8	H-Tyr-Val-Nle-*c*[Cys-His-Phe-Arg-Trp-D-Cys]-Arg-Phe-Gly-NH_2
9	H-Tyr-Val-Nle-*c*[Pen-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2
10	H-Tyr-Val-Nle-*c*[Asp-His-Phe-Arg-Trp-Lys]-Arg-Phe-Gly-NH_2
Series 2	
11	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
12	H-Tyr-Val-Nle-Gly-His-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2
13	H-Tyr-Val-Nle-Gly-Pro-D-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
14	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2
15	H-Tyr-Val-Nle-Gly-Aib-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2
16	H-Tyr-Val-Nle-Gly-MePhe-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2

macrocyclic analogue *c*[Cys^4,Cys^{10}]-α-MSH, a superagonist in the frog skin bioassay[18] based on the results from the linear analogue Ac-[Nle^4 D-Phe^7]-α-MSH.[19] Thus, to probe the receptor-active conformation of this pharmacophore in γ-MSH, we have carried out a cyclic scan (Series **1**, Table 2). We formed 20- or 23-membered cyclic disulfides of the core sequence by introducing cysteine or its analogues at positions 3 and 9 or 4 and 9 of γ-MSH, respectively. A 23-membered ring formed by a lactam bridge between side chains of Asp and Lys at positions 4 and 9 also was included in this library.

Our second series (Series **2**, Table 2) was a D-2′-naphthylalanine (D-Nal(2′)) scan of the γ-MSH sequence at residues 6 and 8. Phe^6 and Trp^8 were earlier shown in our D-amino acid scan to be critical for γ-MSH activity. Furthermore, replacement of Phe^6 with D-Nal[20] in α-MSH, has been shown to convert an MCR agonist to an MCR antagonist.[20,21] Also, replacement of His^5 with Pro or its analogues,[22] and other constrained amino acids,[23] has been shown to improve the affinity and selectivity of α-MSH at MCRs, and we have examined the Pro substitution in our cyclic scan. In both the series, where required, Met^3 has been replaced by its bioisostere norleucine (Nle). Previous studies have shown that [Nle^4]-α-MSH is more potent than α-MSH on both amphibian melanophores and on stimulating melanoma adenylate cyclase,[24] and unlike Met, is also resistant to oxidation.[25,26] Both peptide series **1** and **2** were then evaluated for their binding affinities and their effect on intracellular cAMP generation in cloned human MC3, MC4, and MC5 receptors.

Results

Peptide Synthesis. Both peptide series were synthesized by Fmoc chemistry on a Rink amide resin, which is a standard support for batch Fmoc SPPS of peptide amides.[27] Further coupling was carried out with standard in situ activating reagents used in routine N^{α}-Fmoc SPPS, such as the uronium salts (HBTU), in the presence of a tertiary base (DIPEA), to generate HOBt esters. The use of the correct scavenger is extremely critical during the cleavage of a peptide from the bound resin. 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,[28] particularly for peptides containing Arg(Pmc) and Trp(Boc).[29,30] They also are very efficient at quenching those highly stabilized cations that are not irreversibly scavenged by thiols, e.g., Trt[28] and the Rink linker. 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 All and Aloc protecting groups using a procedure described by Thieriet and others.[31−33] 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 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. Oxidation 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. 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.[34] 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 the two libraries were purified by reversed-phase HPLC. The physicochemical properties and purity of these peptides were assessed by MALDI-TOF MS, RP-HPLC, TLC in three solvent systems, and amino acid analysis (see Experimental Section).

Binding Data. Table 3 summarizes the binding affinities of the cyclic analogues (our laboratory) with that of the native γ-MSH and its Nle^3-C-terminal amidated analogue (Merck Laboratory) at the cloned human MC3R, MC4R, and MC5R. In our earlier collaboration with Merck, we have found that our biological activity data is in parallel with the one reported by them. The native dodecapeptide γ-MSH with a free amino terminal and a C-terminal carboxylic acid shows weak binding at all three receptors (i.e., hMC3R, hMC4R, and hMC5R) although it is about 1 order of magnitude more selective at the hMC3 and hMC4 receptors than at the hMC5R (K_i = about 700 nM to 2.1 μM). With the Nle-analogue, there is an increase in binding affinity at all three receptors by about 1 order of magnitude, K_i = 43 nM, 62 nM, and 193 nM, respectively, for the hMC3, hMC4, and hMC5 receptors. Next, comparing the cyclic disulfides (Series **1**) with γ-MSH data, the Cys^3-Cys^9 analogue (analogue **1**) is an order of magnitude more potent than the γ-MSH with K_i values of 68 nM, 22 nM, and 210 nM, respectively (Table 3). It also is more selective for the hMC4R vs the hMC3R and hMC5R than γ-MSH which is hMC3R selective. On the other hand, in the case of the cyclic D-Cys^3-Cys^9 analogue **2** and the Cys^3-D-Cys^9 analogue **3**, these analogues show almost no binding at the hMC3R, hMC4R, and hMC5R. The Cys^3-Pen^9 analogue (analogue **4**) shows weak binding at the hMC3R and hMC5R and no affinity for the hMC4R (Table 3). However the Pen^3-Cys^9 analogue (analogue **5**) shows strong binding affinity and selectivity for the hMC4R (K_i = 12. nM) vs the hMC3R and hMC5R by 1 and 2 orders of magnitude respectively. With a Cys^4-Cys^9 ring (analogue **6**, Table 3), there is an increase in binding at all three sites to low nanomolar binding affinity levels (K_i = 25 to 27 nM), but a loss of selectivity. The D-Cys^4-Cys^9 compound (analogue **7**) shows increased selectivity at the hMC5R although with moderate binding affinity (K_i = 210 nM). The Cys^4-D-Cys^9 analogue (analogue **8**) shows moderate binding at the hMC3R (K_i = 240 nM) and a moderate selectivity over the hMC4R and hMC5R. The Pen^4-Cys^9 analogue (analogue **9**) shows a loss of binding at the hMC4R, but no selectivity between the hMC3R and hMC5R (K_i = 150 nM). The Asp^4-Lys^9 lactam analogue (**10**, Table 3) shows considerable improvement in binding (K_i = 5.5−9.6 nM) but no selectivity.

Table 3 also summarizes the binding affinities of the D-naphthylalanine (D-Nal(2′))-containing analogues with that of the native γ-MSH and its Nle^3-C-terminal amidated analogue at cloned hMC3R, hMC4R, and hMC5R (Series **2**, Table 3). The D-Nal(2′)^6 analogue (analogue **11**) shows strong binding at the hMC3R and hMC5R (K_i = 0.81 and 1.0 nM) with selectivity of an order of magnitude over the MC4R (K_i = 5.7 nM, Table 3). On the other hand, the D-Nal(2′)^8 analogue (analogue **12**) shows a reversal of selectivity for the hMC4R (K_i = 1.5 nM) over the hMC3R and hMC5R by 1 order of magnitude. The Pro^5-D-Nal(2′)^6 analogue (analogue **13**) shows strong binding but poor selectivity between the hMC3R and hMC4R (K_i = 0.20 to 0.22 nM), though it is more selective over the hMC5R by one order. The D-Nal(2′)^6-D-Nal(2′)^8 analogue (analogue **14**) shows high affinity for the hMC4R (K_i = 4.6 nM) with weak selectivity over the hMC3R (K_i = 12. nM) and strong selectivity over the hMC5R (K_i = 4.8 μM). The Aib^5-D-Nal(2′)^8 analogue (analogue **15**) shows strong selectivity for the hMC5R over the hMC3R and hMC4R (30−40-fold). The methylphenylalanine (MePhe) analogue, MePhe^5-D-Nal(2′)^8 (analogue **16**), shows almost no binding at all three receptors.

cAMP Assay Data. Measurement of the intracellular cAMP accumulation (Table 3) in cloned human MC3R, MC4R, and MC5R (functional assay) revealed that the native peptide shows potent activity at the hMC3R (EC_{50} = 3.7 nM) and is about 50 to 100-fold more selective at this receptor compared with the hMC4R and hMC5R. Its C-terminal amidated Nle^3 analogue is slightly more potent at the hMC3R (EC_{50} = 1.6 nM) but shows a drop in selectivity (4 to 10-fold) over the hMC4R and hMC5R compared to the native peptide. The Cys^3-Cys^9 analogue (analogue **1**) shows potent activity at the hMC3R and hMC4R (EC_{50} = 9 to 36 nM) and selectivity over the MC5R by 1 to 2 orders of magnitude. The D-Cys^3-Cys^9, Cys^3-D-Cys^9, the Cys^3-Pen,^{9} and the Pen^3-Cys^9 analogues (analogues **2**, **3**, **4**, and **5**) show very weak stimulation of cAMP accumulation indicating antagonist activity at receptors where they have binding affinity for the receptors. The Cys^4-Cys^9 analogue **6** shows strong stimulation at the hMC3R and hMC5R (EC_{50} = 3.2−4.2 nM) with selectivity over the hMC4R by 2 orders of magnitude. The D-Cys^4-Cys^9 analogue **7** shows weak agonist potency at all three receptors, while the Cys^4-D-Cys^9 analogue **8** shows better cAMP stimulation at the hMC3R and hMC4R receptors. The Pen^4-Cys^9 analogue **9** has moderate selectivity at the hMC3R (EC_{50} = 19 nM) compared to the hMC4R and hMC5R by 1 order of magnitude. The Asp^4-Lys^9 cyclic amide analogue **10** does not demonstrate any selectivity between the three melanocortin receptors though this ligand binds very strongly (nanomolar) to its receptors.

The D-Nal(2′)^6 analogue **11** shows no stimulation of cAMP accumulation at the hMC3R and hMC5R, but moderate activation of the hMC4R (EC_{50} = 24 nM). It thus appears to be a highly selective hMC4R agonist since there is no measurable agonist activity at either the hMC3R or hMC5R. The D-Nal(2′)^8 analogue **12** shows potent cAMP activation (EC_{50} = 28 nM) at the hMC5R but no activation at the hMC4R, and only weak partial agonist activity at the hMC3R. The Pro^5-D-Nal(2′)^6 analogue (analogue **13**) shows very weak stimulation of cAMP accumulation at the hMC3R in comparison to hMC4R (EC_{50} = 50 nM) but no stimulation at the hMC5R. The D-Nal(2′)^6-D-Nal(2′)^8-analogue (analogue **14**) shows potent but nonselective cAMP activation at the hMC3R and hMC4R (EC_{50} = 12 and 44 nM, respectively, Table 3). While the MePhe analogue **16** displays no stimulation of cAMP accumulation at all three receptors, the Aib analogue **15** does not activate cAMP at the hMC4R or the hMC3R, but is a highly potent selective analogue for the hMC5R with strong activation (EC_{50} = 1.8 nM). Thus this analogue appears

Table 3. Binding and Intracellular cAMP Accumulation of the γ-Melanotropin Analogues at Human Melanocortin Receptors

		hMC3R			hMC4R			hMC5R		
peptide code	structure	K_i[a] (nM)	EC_{50}[b] (nM)	% act. at 10 μM	K_i[a] (nM)	EC_{50}[b] (nM)	% act. at 10 μM	K_i[a] (nM)	EC_{50}[b] (nM)	% act. at 10 μM
Series 1										
1	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	68 ± 11	36 ± 10	93	22 ± 11	9.0 ± 1.4	92	210 ± 40	450 ± 96	16
2	H-Tyr-Val-*c*[D-Cys-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	>10000	>10000	–[c]	460 ± 150	250	46	>3900	>10000	–
3	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-D-Cys]-Arg-Phe-Gly-NH_2	>10000	>10000	–	>10000	>10000	–	>3900	>10000	–
4	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-Pen]-Arg-Phe-Gly-NH_2	480 ± 94	>10000	–	>10000	>10000	–	480 ± 190	>10000	–
5	H-Tyr-Val-*c*[Pen-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	150 ± 58	400 ± 80	77	12 ± 4.8	>10000	–	1700 ± 120	>10000	–
6	H-Tyr-Val-Nle-*c*[Cys-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	6.8 ± 1.4	4.2 ± 0.8	74	2.5 ± 1.0	170 ± 30	90	27 ± 1.3	3.2 ± 1.0	93
7	H-Tyr-Val-Nle-*c*[D-Cys-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	1600 ± 96	210 ± 30	100	1200 ± 96	360 ± 88	89	210 ± 9	140 ± 15	91
8	H-Tyr-Val-Nle-*c*[Cys-His-Phe-Arg-Trp-D-Cys]-Arg-Phe-Gly-NH_2	240 ± 48	39 ± 2.0	72	>10000	83 ± 10	86	220 ± 37	270 ± 30	98
9	H-Tyr-Val-Nle-*c*[Pen-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	150 ± 14	19 ± 3	77	>10000	200 ± 33	80	150 ± 25	74 ± 8	100
10	H-Tyr-Val-Nle-*c*[Asp-His-Phe-Arg-Trp-Lys]-Arg-Phe-Gly-NH_2	5.5 ± 1.0	240 ± 33	100	9.6 ± 1	340 ± 56	100	8.8 ± 1.9	200 ± 20	100
Series 2										
11	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	0.81 ± 0.05	>10000	–	5.7 ± 0.9	24 ± 2	100	1.0 ± 0.08	>10000	–
12	H-Tyr-Val-Nle-Gly-His-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	15 ± 1.0	2400 ± 300	31	1.5 ± 0.6	>10000	–	17 ± 2	28 ± 3	80
13	H-Tyr-Val-Nle-Gly-Pro-D-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	0.20 ± 0.02	1700 ± 223	70	0.22 ± 0.03	50 ± 5	100	14 ± 1.4	>10000	–
14	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	12. ± 2.9	12 ± 1.5	40	4.6 ± 0.5	44 ± 4.5	100	>4800	>10000	–
15	H-Tyr-Val-Nle-Gly-Aib-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	3400 ± 96	>10000	–	4800	>10000	–	120 ± 21	1.8 ± 0.5	100
16	H-Tyr-Val-Nle-Gly-MePhe-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	1400 ± 145	150 ± 15	40	1300 ± 150	53 ± 5	80	3300	210 ± 30	40
γ-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	690[c]	3.7[c]	100[c]	730[c]	180[c]	101[c]	2100[c]	550[c]	88[c]
Nle^3-γ-MSH-NH_2	H-Tyr-Val-Nle-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	43[c]	1.6[c]	100[c]	62[c]	34[c]	105[c]	190[c]	99[c]	114[c]

[a] K_i = Computer analysis of competition curves, where IC_{50} is the 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 data was obtained from refs 12,13.

to be an exceptionally selective agonist ligand for the hMC5R, with no measurable cAMP agonist activity at the hMC3R or hMC4R.

Discussion

The preliminary phases of de novo peptidomimetic design involves the analysis of structure−affinity and structure−bioactivity relationships so as to characterize the interactions between a hormone or neurotransmitter and its target GPC receptor. In this approach,[35,36] the first step involves the identification of the key amino acid residues necessary for receptor recognition (the pharmacophore) by (1) using shorter peptide segments to distinguish the essential segments for receptor recognition from the nonessential ones; (2) single amino acid modifications (e.g., Ala and D-amino acid scans) in the peptide ligand. Once the core pharmacophoric residues are established, the next step is to further probe the conformation or topographical distribution of these residues in the sequence. Replacement of each residue with its optical isomer provides useful information regarding stereochemical requirements at each position in the sequence and the location of possible turn conformations. Once probable turns are located, the next step is cyclization of the key residues in the native sequence into different size rings to confirm the turn locations and then constrain the peptide backbone to a particular turn type. In addition, important information can be obtained by replacement of the key pharmacophore residues with the residues (such as Pro, D-Nal(2′), Aib, and MePhe) that are more hydrophobic in nature or less.

The hMC3R, hMC4R, and hMC5R so far have not been adequately characterized in terms of their physiological roles and the implications of their presence in various CNS and peripheral pathologies. Further, for native γ-MSH the only information available is that it is characterized by the essential core residues, the central tetrapeptide His-Phe-Arg-Trp, found in all melanotropin peptides identified thus far and the information obtained via an Ala scan[12] and a D-amino acid residue scan.[13] However no studies have been done in terms of the stereochemical, hydrophobic, and topographic requirements of γ-MSH in terms of molecular recognition and transduction at the hMC3R and hMC5R. The current SAR discussed in this paper is aimed at gaining some understanding of the above requirements interaction with the various melanocortin receptors.

Native γ-MSH shows modest binding affinity to all three human melanocortin receptors (hMC3R, hMC4R, and hMC5R), whereas in functional adenylate cyclase assay, it is a reasonably potent and selective ligand for the hMC3R (Table 3), with a low EC_{50} value. Interestingly, introducing a hydrophobic Nle^3 and amidating the C-terminal leads to increased ligand binding to all three human melanocortin receptors, and thus we have incorporated these changes into our current studies. In the 26-membered ring series (compounds **1** to **5**, Table 3), the Cys^3-Cys^9 analogue **1** is a potent selective agonist at the hMC4R while the Pen^3-Cys^9 analogue **5** (Table 3) is a potent and selective antagonist at this receptor. Also, the D-Cys^3-Cys^9 and Cys^3-D-Cys^9 analogues (**2** and **3,** Table 3) do not bind either to the hMC3R or the hMC5R. This is an indication that cyclizations of the native γ-MSH and highly selective sequences can lead to a large shift in potency and selectivity for the hMC4R instead of the hMC3R as seen in the native peptide. In the 23-membered ring series (compounds **6**−**10**, Table 3), there is a loss in potency and selectivity, suggesting that this ring size is not desirable for potent molecular recognition except in the cases of **6** and **10**. The cyclic Cys^4-Cys^9 (analogue **6**) is a very potent analogue with no selectivity, and this cyclic analogue is very similar in core structure to an earlier reported superpotent analogue c[Cys^4,Cys^{10}]-α-MSH with agonist activity.[35−37] Comparing analogue **6** to analogue **1**, we suggest that 23-membered ring is desirable for enhanced or decreased potency, whereas the 26-membered ring is more important for modulating selectivity. Similarly the Asp^4-Lys^9 analogue **10** with a lactam bridge has a very strong receptor recognition but it does not have any selectivity. In fact, analogue **10** is very much like MT-II[38] which has a comparable structure with superpotent agonist activity. Clearly the conformational effects of converting the linear γ-MSH to a cyclic structure must play a critical role in the large changes in potency and/or receptor subtype selectivity that accompany cyclization. The precise conformational effect will require comprehensive conformational analysis by 2D NMR and hopefully X-ray crystal structure analysis. At the same time the novel biological activity profiles of **1**, **5**, and **6** can be used as a starting point to obtain more selective agonist and antagonist analogues at the melanocortin receptor.

In the hydrophobic series, the D-Nal(2′)^6 analogue **11** (Table 3) is a potent antagonist at the hMC3R and hMC5R with no selectivity. The D-Nal(2′)^8 analogue **12**, on the other hand, is a potent and selective antagonist at the hMC4R and a moderate agonist at the hMC3R and at the hMC5R. The Pro^5-D-Nal(2′)^6 analogue **13** surprisingly show a potent agonist activity at the hMC4R compared to hMC3R (34-fold) while exhibiting no agonist activity at the hMC5R. This analogue is important for further evaluation in the feeding behavior studies as well as regulation of fat stores. The Aib^5-D-Nal(2′)^8 analogue **15** (Table 3) is the most potent agonist in the series and highly selective for the hMC5R over the hMC3R and hMC4R. These results suggest that replacement of His, Phe, or Trp can transform the peptide from an agonist to an antagonist in at least one of the melanocortin receptors and thus determines the role these amino acids play in structure−activity relationships. In the cases where Pro or Aib replaces the His residue (**12** and **15**, Table 3) the loss of the imidazole functional group and/or the local conformational restriction imposed by a Pro or Aib residue might lead to the unique biological profiles of each. In the other linear analogues the more bulky and hydrophobic D-Nal(2′) residue replaces either a Phe and/or the Trp residue, and as shown in our previous studies[20−22] this appears to be sufficient to convert a melanotropin peptide from an agonist to an antagonist.

Clearly these analogues provide interesting new departure points for developing highly potent and selective analogues for the human MC3R, MC4R, and MC5R.

Table 4. Physicochemical Properties of the γ-Melanotropin Analogues

peptide code	structure	TLC R_f[a]			HPLC k'[b]	HR-MS[c] obsd	HR-MS[c] calcd
Series 1							
1	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	0.77	0.90	0.03	2.39	1527.68	1527.81
2	H-Tyr-Val-*c*[D-Cys-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	0.80	0.87	0.04	2.33	1527.92	1527.81
3	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-D-Cys]-Arg-Phe-Gly-NH_2	0.79	0.88	0.04	2.39	1527.92	1527.81
4	H-Tyr-Val-*c*[Cys-Gly-His-Phe-Arg-Trp-Pen]-Arg-Phe-Gly-NH_2	0.80	0.88	0.04	2.42	1556.00	1555.86
5	H-Tyr-Val-*c*[Pen-Gly-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	0.82	0.89	0.22	2.44	1556.11	1555.86
6	H-Tyr-Val-Nle-*c*[Cys-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	0.84	0.79	0.07	2.77	1584.12	1583.91
7	H-Tyr-Val-Nle-*c*[D-Cys-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	0.84	0.79	0.08	2.99	1584.17	1583.91
8	H-Tyr-Val-Nle-*c*[Cys-His-Phe-Arg-Trp-D-Cys]-Arg-Phe-Gly-NH_2	0.84	0.80	0.36	2.68	1584.27	1583.91
9	H-Tyr-Val-Nle-*c*[Pen-His-Phe-Arg-Trp-Cys]-Arg-Phe-Gly-NH_2	0.86	0.82	0.33	2.81	1612.20	1611.97
10	H-Tyr-Val-Nle-*c*[Asp-His-Phe-Arg-Trp-Lys]-Arg-Phe-Gly-NH_2	0.72	0.84	0.03	2.78	1605.80	1604.91
Series 2							
11	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	0.82	0.79	0.12	2.77	1601.62	1601.84
12	H-Tyr-Val-Nle-Gly-His-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	0.82	0.79	0.07	2.84	1562.70	1562.81
13	H-Tyr-Val-Nle-Gly-Pro-D-Nal(2′)-Arg-Trp-Asp-Arg-Phe-Gly-NH_2	0.84	0.94	0.56	3.42	1561.75	1561.81
14	H-Tyr-Val-Nle-Gly-His-D-Nal(2′)-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	0.77	0.94	0.03	2.88	1612.82	1612.87
15	H-Tyr-Val-Nle-Gly-Aib-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	0.78	0.93	0.04	3.43	1510.78	1510.75
16	H-Tyr-Val-Nle-Gly-MePhe-Phe-Arg-D-Nal(2′)-Asp-Arg-Phe-Gly-NH_2	0.78	0.94	0.03	3.76	1586.82	1586.85

[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.

Table 5. Amino Acid Analysis[a] of the γ-Melanotropin Peptides (Series 1)[a]

peptide	Tyr	Val	Cys	Gly	His	Phe	Arg	Trp[b]	Asp	D-Cys	Pen	Nle	Lys
1	0.48 (1)	0.56 (1)	0.63 (2)	2.21 (2)	1.07 (1)	2 (2)	2.35 (2)	−	−	−	−	−	−
2	0.69 (1)	0.81 (1)	0.55 (0.5)	1.93 (2)	0.90 (1)	2 (2)	1.98 (2)	−	−	ND (1)	−	−	−
3	0.72 (1)	0.89 (1)	0.61 (0.5)	2.0 (2)	0.97 (1)	2 (2)	2.1 (2)	−	−	ND (1)	−	−	−
4	0.79 (1)	0.92 (1)	0.14 (1)	1.88 (2)	0.89 (1)	2 (2)	1.97 (2)	−	−	−	ND (1)	−	−
5	0.67 (1)	0.53 (1)	0.16 (1)	1.88 (2)	0.92 (1)	2 (2)	2.0 (2)	−	−	−	ND (1)	−	−
6	0.81 (1)	1.0 (1)	0.78 (1)	1.0 (1)	0.84 (1)	2 (2)	2.2 (2)	−	−	−	−	ND (1)	−
7	0.62 (1)	0.75 (1)	0.48 (0.5)	1.1 (1)	0.82 (1)	2 (2)	1.8 (2)	−	−	ND (1)	−	ND (1)	−
8	0.64 (1)	0.72 (1)	0.43 (0.5)	0.93 (1)	0.89 (1)	2 (2)	2.0 (2)	−	−	ND (1)	−	ND (1)	−
9	0.70 (1)	1.0 (1)	0.5 (0.5)	1.07 (1)	0.88 (1)	2 (2)	1.98 (2)	−	−	−	ND (1)	ND (1)	−
10	0.70 (1)	0.70 (1)	−	1.18 (1)	0.87 (1)	2 (2)	1.89 (2)	−	0.93 (1)	−	−	ND (1)	−

[a] The analyses were performed using an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (Vapor Phase at 160 °C for 1 h 40 min using 6 N HCl) and a precolumn phenylthiocarbamyl-amino acid (PTC-AA) analysis. No correction is made for amino acid decomposition. [b] Trp was not well determined due to decomposition under these conditions. Other notations: "−" refers to amino acid not present in peptide; "ND" (not determined) refers to the amino acid that could not be estimated due to unavailability of a standard sample.

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); 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 Facility. The system used was an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl) 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 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 high-resolution 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 by Amino Acid Analysis. This analytical data is given in Tables 4−6.

Linear Peptide Synthesis. The peptide series **1** was synthesized by 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 25% 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, and 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

Table 6. Amino Acid Analysis[a] of the γ-Melanotropin Peptides (Series 2)[a]

peptide	Tyr	Val	Gly	His	Phe	Arg	Trp[b]	Asp	Nle	D-Nal(2′)	Pro	MePhe
11	0.82 (1)	0.77 (1)	2.0 (2)	1.02 (1)	1.15(1)	2.41 (2)	−	1.15 (1)	ND (1)	ND (1)	−	−
12	0.69 (1)	0.60 (1)	2.0 (2)	0.95 (1)	2.0(2)	2.37 (2)	−	1.16 (1)	ND (1)	ND (1)	−	−
13	0.70 (1)	0.65 (1)	1.97 (2)	−	1.0(1)	2.20 (2)	−	1.04 (1)	ND (1)	ND (1)	1.04 (1)	−
14	0.75 (1)	0.71 (1)	1.89 (2)	1.0 (1)	1.09(1)	2.20 (2)	−	1.12 (1)	ND (1)	ND (2)	−	−
15	0.78 (1)	0.68 (1)	2.1 (2)	−	2.0(2)	2.22 (2)	−	1.19 (1)	ND (1)	ND (1)	−	−
16	0.74 (1)	0.73 (1)	2.07 (2)	−	2.0(2)	2.27 (2)	−	1.15 (1)	ND (1)	ND (1)	−	ND (1)

[a] The analyses were performed using an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (Vapor Phase at 160 °C for 1 h 40 min using 6 N HCl) and a precolumn phenylthiocarbamyl-amino acid (PTC-AA) analysis. No correction is made for amino acid decomposition. [b] Trp was not well determined due to decomposition under these conditions. Other notations: "−" refers to amino acid not present in peptide; "ND" (not determined) refers to the amino acid that could not be determined due to unavailability of a standard sample.

deprotection and coupling. After each coupling, the Kaiser test[26,27] 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 and dried under an atmosphere of argon to yield dried peptide-resin.

In the case of peptide series **2**, synthesis was performed batchwise on a manual peptide synthesizer. Rink amide resin was first swollen in DCM/DMF (1:1) for 2 h. Prior to the attachment of the first amino acid, the Fmoc group on the resin linker was deprotected with 25% piperidine in DMF. Then the first amino acid was coupled using 3 equiv each of the N^{α}-Fmoc-protected amino acid, HBTU, and HOBt, and 6 equiv of DIPEA in DMF for 1 h. Deprotection of the N^{α}-Fmoc protecting group was carried out using 25% piperidine in DMF for 20 min. Coupling of the penultimate amino acid in the sequence was accomplished by treating the peptide resin with 3 equiv each of the protected amino acid, HBTU, and HOBt and 6 equiv of DIEA in DMF for 1 h. The peptide sequence was thus assembled by alternate cycles of deprotection and coupling. After each coupling and deprotection, the Kaiser test was carried out. After coupling of the *N*-terminal amino acid, N^{α}-Fmoc protecting group was removed with 25% piperidine in DMF as before, and the peptide-resin was thoroughly washed with DCM and dried under vacuum for about 30 min to yield dried peptide-resin.

Cyclization to Lactam on Resin. After assembling the linear sequence, the orthogonal protecting groups on the aspartic acid 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), then DCM (1 mL/200 mg resin) and $PhSiH_3$ (24 equiv/g peptide-resin) were added and the resin suspension 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), 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. In the case of peptide series **1**, the peptide resin was cleaved by treating with 4 mL of a solution of TES (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.

In the case of peptide series **2**, the peptide resin were cleaved by treating with 6 mL/g peptide resin of a solution of TES (7%) and water (5%) in TFA on a shaker at room temperature. After 3 h, the solution, which contains the cleaved peptide, was filtered from the resin under vacuum on a fritted glass funnel 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. 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 μm) column. 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 pure (>95%) white solids.

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.[13] Transfected HEK293 cell line with hMCRs cells were seeded on 24-well plates, 48 h before assay, 50000 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 (pH7.4), 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, 100000cpm/ well, 0.1386nM) 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 NaOH and 500 μL of 1% Triton X-100. The lysed cells were 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 (50000 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 place 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. K_i and EC_{50} values represent the mean of duplicate experiments performed in triplicate. K_i 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, dichoromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; TES, triethylsilane; FAB-MS, fast-atom bombardment mass spectrometry; hMC3R, human melanocortin-3 receptor; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; HBTU, 2-(1*H*-benzotriazol-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; NMP, *N*-methylpyrrolidinone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; $Pd(PPh_3)_4$, palladium tetrakis triphenyl phosphine; $PhSiH_3$, 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; TLC, thin-layer chromatography; Trt, triphenylmethyl (trityl). Amino acid symbols denote L-configuration unless indicated otherwise.

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