D-Amino Acid Scan of γ-Melanocyte-Stimulating Hormone: Importance of Trp[8] on Human MC3 Receptor Selectivity

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Received May 18, 2000

In our search for potent and receptor-selective agonists and antagonists, we report here the results of D-amino acid substitution at each position of the short peptide γ-melanocyte-stimulating hormone (γ-MSH). The native γ-MSH shows weak binding at all three receptors (i.e., the human MC3, MC4, and MC5) and a selectivity of 1−2 orders of magnitude at the MC3R over the MC4R and MC5R. Sequential replacement of each residue in the γ-MSH sequence with the corresponding D-isomer results in analogues which mostly have weaker binding affinity than the native peptide, except for two analogues. For the DTrp[8] analogue, there is an increase in binding affinity by about 1 order of magnitude (IC_{50} = 6 nM) at the MC3R compared with that of the natural molecule and an increase in selectivity for the MC3R by 2 orders of magnitude compared with the activity at the MC4R and MC5R. The DPhe[6] analogue is about 10-fold more potent (IC_{50} = 8.8 nM) at the MC3R compared with the native peptide but lacks subtype selectivity. Measurement of the intracellular cAMP accumulation in human MC3R, MC4R, and MC5R revealed that the native peptide shows potent activity at the MC3R (EC_{50} = 5.9 nM) and is about 50−100-fold selective at this receptor compared with the MC4R and MC5R. The DArg[10] (EC_{50} = 35 nM) and DPhe[11] (EC_{50} = 11 nM) analogues are selective for the MC3R by 1 and 2 orders of magnitude compared with the MC4R and MC5R, respectively. The DTrp[8] compound (EC_{50} = 0.33 nM) shows about 300- and 250-fold increase in selectivity at the MC3R compared with the MC4R and MC5R, respectively. Finally, the DTyr[1] peptide is selective for the MC3R (EC_{50} = 12 nM) by 40−200-fold compared with the MC4R and MC5R. In general, the trend is that D-amino acid substitutions of the aromatic residues 1, 6, 8, and 11 and the basic residue Arg[10], but not Arg[7], result in an increase in MC3R selectivity over the MC4R and MC5R and only agonist activity is observed. Thus, the key residues of γ-MSH identified in this study include the aromatic residues 1, 6, 8, and 11 and the basic residue Arg[10] (but not Arg[7]), as important for MC3 selectivity over the MC4 and MC5 subtypes. Further, the study reveals the extreme importance of DTrp at position 8 in imparting potency and selectivity since this is the most selective analogue for the human MC3R reported thus far.

Introduction

The melanocortin receptors (MCRs) are members of the superfamily of G-protein coupled receptors (GPCRs). Until today, five melanocortin receptors (MC1R− MC5R) have been discovered and cloned.[1−9] The MC1R is expressed in melanocytes and leukocytes, and it is implicated in pigmentation and inflammation, respectively.[10,11] The MC2R is only expressed in the adrenal gland and mediates glucocorticoneogenesis.[12] The MC3R is mainly expressed in a few brain areas and some areas of the periphery, and its functional role is much less-defined.[5] The MC4R is exclusively localized in the brain where it is widely distributed.[13] Recently it has been demonstrated that this receptor plays an important role in weight homeostasis.[14,15] The MC5R is found in a variety of peripheral tissues and is believed to participate in regulation of exocrine gland function.[16]

The newly discovered MC3R, MC4R, and MC5R have comparatively lower affinity for the melanocortin peptides, and none of the natural hormones have enough selective properties which would be useful to distinguish between the physiological function of these subtypes of receptors. The natural melanocortin peptides (Table 1) are thus selective only for the MC1R, whereas ACTH is selective for the MC2R.[17−19] Consequently, potent and receptor-specific agonists and especially antagonists would be extremely valuable tools for determination of the physiological roles of MC3R, MC4R, and MC5R.

To develop effective agonist and antagonist ligands of the melanocortin receptors, it is necessary to know the structure−activity relationships (SARs) of the natural molecule in detail at each of the receptors. In the case of a short peptide like the γ-melanocyte-stimulating hormone (γ-melanotropin, γ-MSH), D-amino acid substitution can be a useful strategy to increase our knowledge about the SARs for this natural peptide. As a result, we have studied the effects of D-amino acid substitution at each position of the γ-MSH peptide

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10.1021/jm000211e CCC: $19.00
Published on Web 11/17/2000

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
NDP-MSH	Ac-Ser-**Tyr**-Ser-**Nle**-Glu-**His**-D**Phe-Arg-Trp**-Gly-Lys-Pro-Val-NH_2

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

		hMC3			hMC4			hMC5		
peptide	structure	IC_{50}[a] (nM)	EC_{50}[b] (nM)	% max 10 μM	IC_{50}[a] (nM)	EC_{50}[b] (nM)	% max 10 μM	IC_{50}[a] (nM)	EC_{50}[b] (nM)	% max 10 μM
1	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-D**Phe**-Gly-OH	340 ± 110	11 ± 3.5	94 ± 8.2	730 ± 220	190 ± 54	87 ± 6.7	1600 ± 270	770 ± 180	81 ± 11
2	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-D**Arg**-Phe-Gly-OH	610 ± 170	35 ± 13	96 ± 2.3	930 ± 98	190 ± 83	100 ± 4.1	1900 ± 41	810 ± 230	93 ± 4.9
3	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-D**Asp**-Arg-Phe-Gly-OH	1000 ± 220	250 ± 38	86 ± 3.5	1100 ± 180	940 ± 300	57 ± 3.3	540 ± 100	550 ± 100	88 ± 5.6
4	H-Tyr-Val-Met-Gly-His-Phe-Arg-D**Trp**-Asp-Arg-Phe-Gly-OH	6.7 ± 2.3	0.33 ± 0.13	100 ± 7.5	600 ± 57	100 ± 33	99 ± 4.3	340 ± 87	82 ± 13	97 ± 2.4
5	H-Tyr-Val-Met-Gly-His-Phe-D**Arg**-Trp-Asp-Arg-Phe-Gly-OH	7200 ± 2300	1500 ± 890	94 ± 5.2	>10000	NA[c]	NA	>10000	NA	NA
6	H-Tyr-Val-Met-Gly-His-D**Phe**-Arg-Trp-Asp-Arg-Phe-Gly-OH	8.8 ± 2.9	2.0 ± 0.83	96 ± 13	9.7 ± 1.0	3.6 ± 1.4	110 ± 13	77 ± 4.5	49 ± 10	65 ± 6.9
7	H-Tyr-Val-Met-Gly-D**His**-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	>10000	750 ± 100	71 ± 13	>10000	NA	NA	8500	NA	NA
8	H-Tyr-Val-D**Met**-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	280 ± 110	42 ± 18	97 ± 4.3	430 ± 47	190 ± 27	91 ± 4.2	350 ± 66	1800 ± 240	55 ± 2.6
9	H-Tyr-D**Val**-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	1900 ± 990	98 ± 53	100 ± 12	>5000	570 ± 69	86 ± 5.4	7100 ± 1800	5000 ± 500	25 ± 3.2
10	H-D**Tyr**-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	470 ± 180	12 ± 2	100 ± 18	2200 ± 410	460 ± 100	90 ± 3.7	5700 ± 2100	2700 ± 470	75 ± 7.9
γ-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	72 ± 4.0	5.9 ± 0.5	100	770 ± 160	260 ± 30	100	2500 ± 300	490 ± 110	100
NDP-MSH	Ac-Ser-Tyr-Ser-**Nle**-Glu-His-D**Phe**-Arg-Trp-Gly-Lys-Pro-Val-NH_2	2.9 ± 0.3	0.24 ± 0.08	100	3.3 ± 0.3	0.20 ± 0.05	100	0.89 ± 0.14	0.23 ± 0.07	100

[a] IC_{50} = concentration of peptide at 50% specific binding ($n = 3-4$). The peptides were tested in a range of concentrations ($10^{-10}-10^{-4}$ M). [b] EC_{50} = concentration of peptide at 50% maximal cAMP generation ($n = 4$). The peptides were tested in a range of concentrations ($10^{-10}-10^{-4}$ M). Both IC_{50} and EC_{50} values are reported with the respective SEM values as calculated: standard deviation divided by square root of number of samples. [c] NA = no activity.

sequence and report our results herein. Among other results, we have found that the Trp^8 residue of γ-MSH is critical for differentiating interactions of γ-MSH with the MC3R from other melanocortin receptors.

Results

Peptide Synthesis. The peptides (Table 2) were synthesized by standard Fmoc chemistry with an appropriate orthogonal protective strategy. Coupling was carried out with standard in situ activating reagents used in Fmoc SPPS, such as the uronium salts (HBTU) in the presence of a tertiary base (DIPEA), to generate HOBt esters. One concern was the oxidation of Met residues in the sequence during the synthesis and cleavage. This was dealt with by taking two precautions: (1) performing the entire synthesis under an atmosphere of argon; (2) using thiol-based scavengers in the cleavage cocktail. The other concern was that Trp residues are extremely susceptible to alkylation by cations produced during the cleavage process. Trialkylsilanes, such as Et_3SiH, have been shown to be effective, particularly for peptides containing Arg(Pbf) and Trp(Boc).[20,21] They also are very efficient at quenching those highly stabilized cations that are not irreversibly scavenged by thiols, e.g., Trt.[22] In our syntheses, cleavage of the peptide from the resin with a Et_3SiH-based TFA cocktail was adopted since the Trp residue had a Boc side chain protecting group.

The HPLC profile of the crude peptide obtained following cleavage from the resin showed a single peak by analytical RP-HPLC. Purification was accomplished by preparative RP-HPLC. The physicochemical properties and purity of the final peptides were assessed by FAB-MS, RP-HPLC, TLC in three solvent systems and amino acid analysis (see Experimental Section). These properties are complied in Tables 3 and 4.

Binding Assays. Table 2 summarizes the binding affinities of the D-amino acid analogues with that of the native peptide at cloned human MC3R, MC4R, and MC5R. 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., hMC3, hMC4, and hMC5) in the order of 72 nM to 2.5 μM with weak selectivity. Sequential replacement of each residue in the γ-MSH sequence with the corresponding D-isomer results in analogues which mostly have weaker binding affinity than the native peptide, except for two analogues. For the $D-Trp^8$ analogue, there is an increase in binding affinity by about 1 order of magnitude (IC_{50} = 6 nM) at the MC3R compared with that of the natural molecule. Also, this analogue shows an increase in selectivity for the MC3R by 2 orders of magnitude compared with the activity at the MC4R and MC5R. The $D-Phe^6$ analogue is equipotent in binding at the MC3R and MC4R and about 1 order of magnitude more potent than at the MC5R. Further, there is an increase in binding affinity at all three receptors, compared with that of the native peptide. This analogue is about 10-fold more potent at the MC3R and MC4R (IC_{50} = 8.8 and 9.7 nM, respectively) and about 2 orders of magnitude more potent than γ-MSH at the human MC5R compared with the native peptide.

cAMP Assays. Measurement of the intracellular cAMP accumulation (Table 2) in cloned human MC3R,

Table 3. Physicochemical Properties of the γ-Melanotropin Analogues

peptide	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	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-D**Phe**-Gly-OH	0.36	0.05	0.31	3.53	1571.0	1570.7
2	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-D**Arg**-Phe-Gly-OH	0.37	0.07	0.30	3.45	1569.9	1570.7
3	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-D**Asp**-Arg-Phe-Gly-OH	0.38	0.08	0.34	3.75	1571.0	1570.7
4	H-Tyr-Val-Met-Gly-His-Phe-Arg-D**Trp**-Asp-Arg-Phe-Gly-OH	0.35	0.05	0.35	3.68	1571.0	1570.7
5	H-Tyr-Val-Met-Gly-His-Phe-D**Arg**-Trp-Asp-Arg-Phe-Gly-OH	0.36	0.07	0.32	3.28	1571.0	1570.7
6	H-Tyr-Val-Met-Gly-His-D**Phe**-Arg-Trp-Asp-Arg-Phe-Gly-OH	0.38	0.06	0.33	3.37	1571.0	1570.7
7	H-Tyr-Val-Met-Gly-D**His**-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	0.37	0.08	0.34	3.60	1571.0	1570.7
8	H-Tyr-Val-D**Met**-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	0.39	0.07	0.33	3.92	1571.0	1570.7
9	H-Tyr-D**Val**-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	0.42	0.1	0.35	4.12	1571.0	1570.7
10	H-D**Tyr**-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH	0.38	0.06	0.36	3.93	1570.9	1570.7

[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); (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 mass spectra were determined by FAB-MS methods.

Table 4. Amino Acid Analysis[a] of the γ-Melanotropin Peptides

peptide	Tyr	Val	Met	Gly	His	Phe	Arg	Trp	Asp	Arg	Phe	Gly
1	0.95	0.98	1.05	1.01	0.98	0.98	0.97		0.90	0.92	1.03[b]	0.93
2	0.98	1.02	0.97	0.98	0.95	0.99	0.96		1.03	1.05[b]	0.97	0.95
3	0.72	1.20	0.80	1.05	0.90	1.0	0.96		1.10[b]	0.96	1.0	1.06
4	0.97	1.01	1.01	0.99	1.02	0.95	0.99		0.98	0.98	0.96	1.03
5	0.73	1.03	0.72	1.06	0.92	1.0	1.0[b]		1.05	1.0	1.0	1.06
6	0.90	0.80	0.60	1.07	1.03	1.0[b]	1.10		1.10	1.10	1.0	1.07
7	0.78	0.83	0.70	0.95	0.92[b]	1.0	1.01		0.94	1.01	1.0	0.94
8	0.75	0.79	0.87[b]	1.10	0.98	1.0	1.06		1.10	1.06	1.0	1.10
9	0.90	0.98[b]	0.73	0.96	1.08	1.0	1.07		1.20	1.07	1.0	0.96
10	0.70[b]	0.87	0.80	1.06	0.99	1.0	1.02		1.10	1.02	1.0	1.06

[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. Trp was not well-determined due to decomposition under these conditions. [b] Value for the corresponding D-amino acid.

MC4R, and MC5R in this functional assay revealed that the native peptide shows potent activity at the MC3R (EC_{50} = 5.9 nM) and is about 50−100-fold selective at this receptor compared with the MC4R and MC5R. The DPhe^{11} analogue (EC_{50} = 11 nM) is selective for the MC3R by 1 and 2 orders of magnitude compared with activity at the MC4R and MC5R, respectively. This selectivity is also seen with the DArg^{10} analogue. The DTrp^{8} compound (EC_{50} = 0.33 nM) shows about 300- and 250-fold increase in selectivity at the MC3R compared with the MC4R and MC5R, respectively. The DPhe^{6} analogue shows potent activity at all three receptors with loss in selectivity. Finally, the DTyr^{1} peptide is selective for the MC3R (EC_{50} = 12 nM) by 40−200-fold compared with activity at the MC4R and MC5R. All other analogues show very weak or almost no stimulation of intracellular cAMP accumulation. Thus, in general, the trend is that D-amino acid substitutions of the aromatic residues 1, 6, 8, and 11 and the basic residue Arg^{10}, but not Arg^{7}, result in an increase in MC3R selectivity over the MC4R and MC5R and only agonist activity is observed.

Discussion

Structure−affinity and structure−activity relationships have been traditionally used to characterize the interaction between a hormone or neurotransmitter and its receptor in the first phase of de novo peptidomimetic design. This phase usually involves the identification of key amino acid residues necessary for receptor recognition by (1) using shorter peptide segments to distinguish the essential segments for receptor recognition from nonessential ones and (2) by single-amino acid modifications in the peptide ligand. In the latter approach, the significance of each position can be assessed by the substitution of amino acid residues, e.g., with alanine or the corresponding D-amino acid. However, it should not be underestimated that each amino acid can have influence on the secondary structure, the dipole moment, and the hydrophobicity of the hormone. Consequently, it is difficult to estimate the quantitative importance of different effects and thus to identify the reason for an affinity loss. Nonetheless, however, SAR studies furnish valuable information for the development of small hormone analogues or even nonpeptidic lead structures, for the characterization of receptor subtypes, and for development of a peptide ligand−receptor model.

Replacement of each residue by its optical isomer provides useful information regarding the stereochemical requirements at each position in the sequence and the location of possible turn conformations as only certain turn types can accommodate both L- and D-residues and still place the amino acid side chains in the same relative position in three-dimensional space. Once probable turn positions are located, then cyclization can be used to confirm the turn location and constrain the peptide backbone to a particular turn type.

The MC3R, MC4R, and MC5R, so far, have not been adequately characterized in terms of their physiological roles and their implications in various CNS and peripheral pathologies. Further, for the native γ-MSH, the only information available is that it is characterized by the essential core sequence, the central tetrapeptide His-

Phe-Arg-Trp, found in all melanotropin peptides identified this far. However it has not been characterized in terms of the key residues that are required for molecular recognition. Thus, our D-amino acid substitution strategy was extended to all amino acids in the γ-MSH sequence to understand the possible stereochemical and topographical requirements of the side chain groups of each of the amino acids for interaction with the various melanocortin receptors.

Although the native γ-MSH shows weak binding affinity to all three new human melanocortin receptors (MC3R, MC4R, and MC5R) in the functional adenylate cyclase assay, it is selective for the MC3R (Table 2), with a low EC_{50} value. In general, D-amino acid substitution in the γ-MSH sequence leads to reduced affinities or weak binding to the MC3R, MC4R, and MC5R, compared to the native peptide and NDP-MSH. However, with the DTrp[8] substitution, compound **4** (Table 2) is more selective for the MC3R (over the MC4R and MC5R) by about 2 orders of magnitude in the binding assay and about 250−300-fold more selective in the cAMP assay. Further, the binding affinity and cAMP accumulation of the DTrp[8] analogue **4** at the human MC3R is significantly improved over that of the native peptide. This indicates the extreme importance of this optical isomer in imparting potency and selectivity.

The second important structural feature revealed in this study is the loss of binding selectivity with the substitution of Arg[10] with its D-isomer (analogue **2**). But, this analogue is still selective for the MC3R over the MC4R and MC5R in the functional assay. This pattern of selectivity (not in binding affinity but only in function) is also seen with the DTyr[1] analogue **10**. Finally, the study showed that substitution of Phe[6] with its D-isomer results in loss in binding affinity and selectivity between the human MC3R and MC4R. This is in contrast to the DPhe substitution in α-MSH (NDP-MSH, Table 1) which is highly potent and nonselective for the human MC3R and MC4R (Table 2). Thus, the key residues of γ-MSH identified in this study include the aromatic residues 1, 6, 8, and 11 and the basic residue Arg[10] (but not Arg[7]) as important for MC3 selectivity over the MC4 and MC5 subtypes. The DTrp[8] analogue is the most selective analogue for the human MC3R reported this far.

Experimental Section

Abbreviations. 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; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; *t*Bu, *tert*-butyl; cAMP, adenosine 3′,5′-cyclic monophosphate; 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*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HR-MS, high-resolution mass spectrometry; IBMX, 3-isobutyl-1-methylxanthine; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Trt, triphenylmethyl (trityl). Amino acid symbols denote L-configuration unless indicated otherwise.

Materials. N^{α}-Fmoc-protected amino acids and N^{α}-Fmoc-Gly-*O*-resin were purchased from Advanced ChemTech (Louisville, KY). 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*-*t*Bu), His(N^{im}-Trt), Trp(N^{in}-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 60 F_{254} plates using the following solvent systems: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4); (B) ethyl acetate/pyridine/acetic acid/water (5:5:1:3); (C) upper phase of 1-butanol/acetic acid/water (4:1:1). The peptides were detected on the TLC plates using iodine vapor. Amino acid analyses were performed at the University of Arizona Biotechnology Core Facility. The system used was an Applied Biosystems model 420A amino acid analyzer with automatic hydrolysis (vapor phase at 160 °C for 1 h 40 min using 6 N HCl) and a precolumn phenylthiocarbamyl-amino acid (PTC-AA) analysis. No corrections are made for amino acid decomposition. FAB-MS analyses were performed at the University of Arizona Core Facility. The instrument was custom-made in Breman, Germany, and consists of a LIQUID SIMS4 Sectors AMD mass spectrometer. The experimental conditions consisted of a glycerol matrix scan of 200−2000 Da in the positive ion mode. 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 (Table 3). 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 by FAB-MS (Table 3).

General Method for Peptide Synthesis and Purification. All peptides were synthesized by the solid-phase method of peptide synthesis and purification was accomplished by HPLC. The peptides were synthesized on 0.15 g each of N^{α}-Fmoc-Gly-*O*-resin (substitution 0.7 mmol/g) by automated methods using N^{α}-Fmoc chemistry and an orthogonal side chain protection strategy. The syntheses were carried out on a 16-well automated multiple peptide synthesizer ACT model 396 (Advanced ChemTech, Louisville, KY) using the DOS software. The entire synthesis was performed under an atmosphere of argon. The resin was first swollen in DCM/DMF (1:1) for 2 h. The peptides were prepared manually using Fmoc chemistry and an orthogonal side chain protection strategy. Briefly, the protected peptide-resin was prepared by first deblocking the N^{α}-Fmoc protecting group on the resin with 25% piperidine/DMF for 30 min. The following amino acids were then added to the growing peptide chain by stepwise addition of N^{α}-Fmoc-Phe-OH, N^{α}-Fmoc-Arg(N^{g}-Pbf)-OH, N^{α}-Fmoc-Asp(β-*O*-*t*Bu)-OH, N^{α}-Fmoc-Trp(*N*-Boc)-OH, N^{α}-Fmoc-Arg(N^{g}-Pbf)-OH, N^{α}-Fmoc-Phe-OH, N^{α}-Fmoc-His(N^{im}-Trt)-OH, N^{α}-Fmoc-Gly-OH, N^{α}-Fmoc-Met-OH, N^{α}-Fmoc-Val-OH, and N^{α}-Fmoc-Tyr(*O*-*t*Bu)-OH, using standard solid-phase methods. Each coupling reaction was achieved using a 3-fold excess each of the amino acid, HBTU, and HOBt in the presence of a 6-fold excess of DIPEA for 1 h. Amino acid solutions were in concentrations of 0.25 M in DMF, HBTU in a concentration of 0.4 M in DMF, and HOBt and DIPEA in a concentration of 0.5 M each in DMF. Deprotection of the N^{α}-Fmoc group was carried out by treating the protected peptide-resin with 25% piperidine solution in DMF (1 × 4 mL, 5 min, 1 × 4 mL, 20 min). After each coupling and deprotection, the peptide-resin was washed with DMF (3 × 4 mL), DCM (3 × 4 mL) and again with DMF. The peptide sequences were thus assembled by alternate cycles of coupling and deprotection. After each coupling, the Kaiser test (vide infra) was performed to determine the completeness of coupling, while after each deprotection, the test was performed to determine removal of the Fmoc 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 × 4 mL) and dried under an atmosphere of argon to yield dried peptide-resin.

Next, the peptide-resin was cleaved by treating with 4 mL of a solution of Et_3SiH (5%), water (5%), *p*-thiocresol/*p*-cresol (0.1%, 1:1) 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.

Final peptide purification was achieved using a preparative RP-HPLC Vydac C18 (218TP1520, 15 μm). 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, pooled, and lyophilized to yield the final peptides as pure (>95%) white solids. The analytical data for each compound is presented in Table 3 and the amino acid analysis data is in Table 4.

Kaiser Test.[23,24]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. Binding assays were carried out using membranes prepared from L-cells stably expressing human MC3R, MC4R, and MC5R by a procedure described previously.[25] The binding assay mixture contained 0.2 nM ^{125}I-[Tyr^2]-[Nle^4,D-Phe^7]α-MSH (^{125}I-NDP-α-MSH), varying concentrations of the peptide being tested, and an appropriate amount of membranes so that the total bound radioligand was less than 10% of the added radioligand. The above mixture in binding buffer (50 mM Tris, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, pH 7.2) was incubated at room temperature for 2 h, followed by filtration through GFC paper. The bound ligand was quantitated in a γ-counter. IC_{50} values were calculated as previously described. Assays were performed in triplicate and were repeated 2−4 times.

cAMP Assays. Intracellular cAMP concentration was measured by the New England Nuclear cAMP[^{125}I] Flash Plate assay. CHO cells stably expressing the human MC3R, MC4R, and MC5R were resuspended in Earle's balanced salt solution, 10 mM HEPES, 5 mM $MgCl_2$, 1 mg/mL BSA and 0.5 mM IBMX, incubated with the peptide being tested for 45 min, and lysed by 0.1 M HCl as described.[25] Assays were performed in triplicate and were repeated 2−4 times.

Acknowledgment. This research was supported in part by grants from the U.S. Public Health Service (DK-17420) and from Merck Research Laboratories. The opinions expressed are those of the authors and not necessarily of the U.S. Public Health Service.

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JM000211E