De Novo Design, Synthesis, and Pharmacology of α-Melanocyte Stimulating Hormone Analogues Derived from Somatostatin by a Hybrid Approach†

Guoxia Han,‡ Carrie Haskell-Luevano,‖ Laura Kendall,‡ Gregg Bonner,‡ Mac E. Hadley,§ Roger D. Cone,⊥ and Victor J. Hruby*,‡

Departments of Chemistry and Anatomy, University of Arizona, Tucson, Arizona 85721; Department of Medicinal Chemistry, University of Florida, Gainesville, Florida 32610; and Vollum Institute, Oregon Health and Sciences University, Portland, Oregon 97201

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A number of α-melanotropin (α-MSH) analogues have been designed de novo, synthesized, and bioassayed at different melanocortin receptors from frog skin (*f*MC1R) and mouse/rat (*m*MC1R, *r*MC3R, *m*MC4R, and *m*MC5R). These ligands were designed from somatostatin by a hybrid approach, which utilizes a modified cyclic structure (H-D-Phe-c[Cys---Cys]-Thr-NH_2) related to somatostatin analogues (e.g. sandostatin) acting at somatostatin receptors, CTAP which binds specifically to μ opioid receptors, and the core pharmacophore of α-MSH (His-Phe-Arg-Trp). Ligands designed were H-D-Phe-*c*[XXX-YYY-ZZZ-Arg-Trp-AAA]-Thr-NH_2 [XXX and AAA = Cys, D-Cys, Hcy, Pen, D-Pen; YYY = His, His(1′-Me), His(3′-Me); ZZZ = Phe and side chain halogen substituted Phe, D-Phe, D-Nal(1′), and D-Nal(2′)]. The compounds showed a wide range of bioactivities at the frog skin MC1R; e.g. H-D-Phe-*c*[Hcy-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**6**, EC_{50} = 0.30 nM) and H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**, EC_{50} = 0.10 nM). In addition, when a lactam bridge was used as in H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2 (**7**, EC_{50} = 0.10 nM), the analogue obtained is as potent as α-MSH in the frog skin MC1R assay. Interestingly, switching the bridge of **6** to give H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2 (**5**, EC_{50} = 1000 nM) led to a 3000-fold decrease in agonist activity. An increase in steric size in the side chain of D-Phe^7 reduced the bioactivity significantly. For example, H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-D-Cys]-Thr-NH_2 (**24**) is 2000-fold less active than **9**. On the other hand, H-D-Phe-*c*[Cys-His-D-Phe(*p*-I)-Arg-Trp-D-Cys]-Thr-NH_2 (**23**) lost all agonist activity and became a weak antagonist ($IC_{50} = 1 \times 10^{-5}$ M). Furthermore, the modified CTAP analogues with a D-Trp at position 7 all showed weak antagonist activities ($EC_{50} = 10^{-6}$ to 10^{-7} M). Compounds bioassayed at mouse/rat MCRs displayed intriguing results. Most of them are potent at all four receptors tested (*m*MC1R, *r*MC3R, *m*MC4R, and *m*MC5R) with poor selectivities. However, two of the ligands, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**9**, $EC_{50} = 6.9 \times 10^{-9}$ M, 6.4×10^{-8} M, 2.0×10^{-8} M, and 1.4×10^{-10} M at *m*MC1R, *r*MC3R, *m*MC4R, and *m*MC5R, respectively) and H-D-Phe-*c*[Cys-His(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**16**, $EC_{50} = 3.5 \times 10^{-8}$ M, 3.1×10^{-8} M, 8.8×10^{-9} M, and 5.5×10^{-10} M at *m*MC1R, *r*MC3R, *m*MC4R, and *m*MC5R, respectively) showed significant selectivities for the *m*MC5R. Worthy of mention is that neither of these two ligands is potent in the frog skin MC1R assay ($EC_{50} = 10^{-7}$ M for **9** and $EC_{50} = 10^{-5}$ M for **16**). These results clearly demonstrated that binding behaviors in rodent MCRs are quite different from those in the classical frog skin (*R pipiens*) assay.

Introduction

α-Melanocyte stimulating hormone (α-melanotropin, α-MSH), Ac-Ser-Tyr-Ser-Met-Glu^5-His-Phe-Arg-Trp-Gly^{10}-Lys-Pro-Val-NH_2, is one of the products generated by posttranslational synthesis from proopiomelanocortin (POMC) located in the brain, pituitary gland, skin, and elsewhere.[1] It was discovered decades ago for its function in pigmentation,[2] exerted at the melanocortin-1 receptor (MC1R).[3,4] More recent research has revealed that this peptide and its analogues have several other profound biological activities,[1,5−24] including roles in cardiovascular functions, erectile function, the regulation of energy homeostasis, and immune response. These findings have prompted us to seek an understanding of how these ligands interact differently with these receptors and to correlate structure to biological activity relationships at these different receptors.

Since the discovery of α-MSH, many α-MSH analogues have been designed and tested for their biological activity.[2,25−32] However, it was not until the early 1980s that the rational design of α-MSH analogues became successful with the discovery of Ac-Ser-Tyr-Ser-Nle^4-Glu-His-D-Phe^7-Arg-Trp-Gly-Lys-Pro-Val-NH_2 ([Nle,^{4}D-Phe^7]α-MSH, NDP-α-MSH, melanotan-I, MT-I, the numbering scheme for α-MSH is used throughout),[33] which showed enhanced metabolic stability and greatly improved potency compared with α-MSH. Since then,

* Corresponding author. Phone: 520-621-6332. Fax: 520-621-8407. E-mail: hruby@u.arizona.edu.

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‡ Department of Chemistry, University of Arizona.
§ Department of Anatomy, University of Arizona.
‖ University of Florida.
⊥ Oregon Health and Sciences University.

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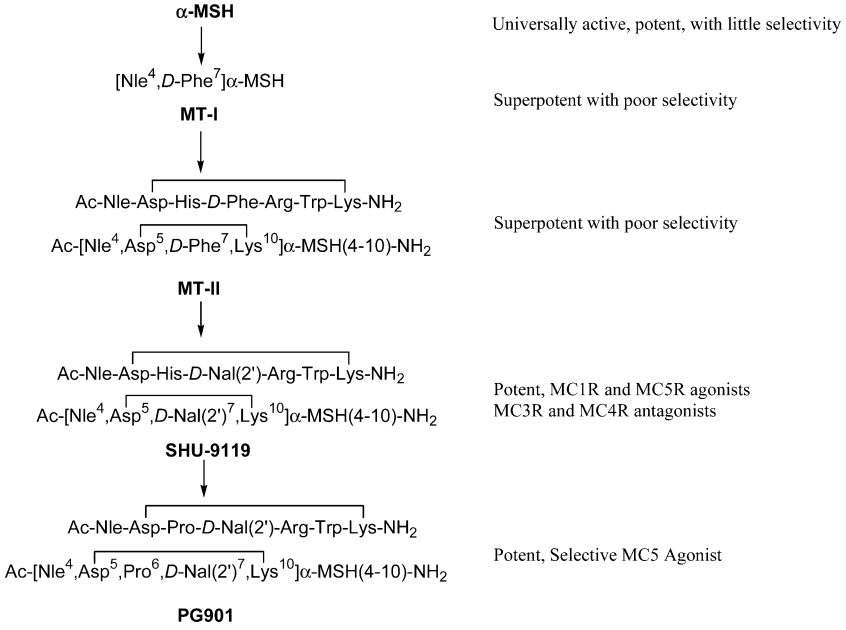
Scheme 1. Brief History of Conformational Constraints in α-MSH Analogues



considerable efforts were made to constraining MT-I (Scheme 1).

Efforts to obtain short sequences of α-MSH analogues with super potent bioactivities paid off most successfully with the discovery of Ac-Nle^4-*c*[Asp^5,D-Phe^7,Lys^{10}]α-MSH(4−10)-NH_2 (melanotan-II, MT-II).[34,35] More recently, the search for potent and more conformationally constrained α-MSH analogues was intensified when it was discovered that there are other types of melanocortin receptors (MCRs) besides the melanocortin 2 receptor (the traditional adrenocorticotropin receptor) and the melanocortin 1 receptor (MC1R, the traditional melanocortin receptor for pigmentation). These were named the melanocortin 3 receptor (MC3R), melanocortin 4 receptor (MC4R), and melanocortin 5 receptor (MC5R) based on the order of their discovery.[17,23,36,37] Neither α-MSH, MT-I, nor MT-II showed strong selectivity for these different receptors. Hence, the focus on the search for potent α-MSH analogues shifted to discovering selective ligands for each of these new MCRs.[17,24,38,39] By replacing D-Phe^7 of MT-II with D-Nal(2′), the resulting ligand, Ac-Nle,^{4} *c*[Asp^5,D-Nal(2′),^{7}Lys^{10}]α-MSH(4−10)-NH_2 (SHU-9119),[38] an antagonist at the *h*MC3R and *h*MC4R, was obtained and showed weak selectivities among the newly discovered MCRs, while potency at the MC1R was not compromised. In addition, replacing His^6 with the hydrogen bonding deprived His(1′-Me)[40] in its side chain, led to the discovery of Ac-Nle,^{4} *c*[Asp^5,His(1′-Me),^{6}D-Nal(2′),^{7}Lys^{10}]α-MSH(4−10)-NH_2 (WY012), a relatively potent mouse MC5R (*m*MC5R) antagonist ($pA_2 = 7.1$). On the other hand, modification of α-MSH with a S−S disulfide bridge at positions 4 and 11[41] led to Ac-*c*[Cys^4-Glu-His-D-Nal(2′)-Arg-Trp-Gly-Cys^{11}]-NH_2 (HS014),[39] which is a ligand with fair selectivity for the MC4R.

Though significant achievements in designing somewhat selective and potent ligands for the new MCRs have been made in recent years,[38−40,42−55] many questions remain to be answered. First, almost all of the MSH analogues designed so far are based on α-MSH itself and MI-I and MT-II.[27,38−40,42,46−56] The discovery of more potent and highly selective ligands through this traditional manner has been difficult. Hence, we began to look for other possible starting points to provide design paradigms for selectivity and potency, but which also would give us new insights into how the ligands interact with the MCRs and the chemical basis for structure−activity relationships.

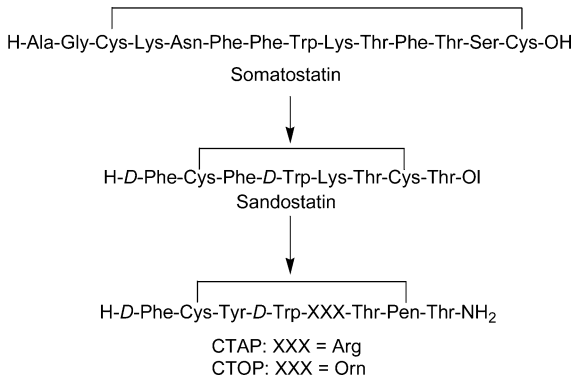


Figure 1. Diagram of designed opioids from somatostatin.

About 18 years ago, our laboratory reported somatostatin-related analogues (Figure 1) specifically designed to possess potent and selective binding to the μ-opioid receptor.[57−63] Among those constrained cyclic peptides (cyclized by formation of a S−S bond), H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Thr-Cys]-Thr-NH_2 (CTAP) and H-D-Phe-*c*[Cys-Tyr-D-Trp-Orn-Thr-Cys]-Thr-NH_2 (CTOP) were found to be highly selective super-potent antagonists at the μ-opioid receptor, with little or no affinity for κ and δ opioid receptors. Most interestingly, both of these peptides showed very little affinity for somatostatin receptors.

Since the somatostatin, opioid, and melanocortin receptors are structurally related and belong to the seven transmembrane G-protein-coupled receptor (GPCR) super-family, we reasoned that their ligands also may share some topographical structural similarities. Hence, a straightforward hypothesis, which is consistent with the discovery of the conversion of somatostatin analogues specific for opioid receptors, is that it should be

Table 1. Frog Skin Bioassay Results for Chimeric Cyclic Analogues*

no.	serial no.	compound	EC_{50} (nM)[b]	IC_{50} (nM)[b]
	CTOP	H-D-Phe-*c*[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH_2	100	IA[a]
	CTAP	H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2	IA[a]	500
1	GH-11b	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2	10	IA[a]
2	GH-4c	H-D-Phe-*c*[Cys-His-D-Tic-Arg-Trp-Cys]-Thr-NH_2	IA[a]	1,000
3	GH-6b	H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2	30	IA[a]
4	GH-6d	H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-Cys]-Thr-NH_2	10 000	IA[a]
5	GH-13	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2	1000	IA
6	GH-4d	H-D-Phe-*c*[Hcy-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2	0.30	IA[a]
7	GH-15	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2	100	IA[a]
8	GH-14a	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2	0.10	IA[a]
9	GH-5a	H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2	0.10	IA[a]
10	GH-6c	H-D-Phe-*c*[Cys-His(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2	10 000	IA[a]
11	GH-7b	H-D-Phe-*c*[Glu-His(3′-Me)-D-Phe-Arg-Trp-Lys]-Thr-NH_2	1.0	IA[a]
12	GH-6a	H-D-Phe-*c*[Cys-D-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2	3000	IA
13	GH-3a	H-D-Phe-*c*[Cys-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2	100	IA[a]
14	GH-3b	H-D-Phe-*c*[Pen-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2	IA[a]	antagonist
15	GH-21	H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Ser-Pen]-Thr-NH_2	IA[a]	1000
16	GH-22	H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Trp-Pen]-Thr-NH_2	IA[a]	1000
17	GH-20	H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-D-Trp-Pen]-Thr-NH_2	IA[a]	500
18	GH-19	H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-Trp-Pen]-Thr-NH_2	IA[a]	1,000
19	GH-17	H-D-Phe-*c*[Cys-Phe-D-Phe-Arg-Thr-Pen]-Thr-NH_2	IA[a]	IA[a]
20	GH-18	H-D-Phe-*c*[Cys-Phe(*p*-I)-D-Trp-Arg-Thr-Pen]-Thr-NH_2	IA[a]	100
21	GH-16	H-D-Phe-*c*[Cys-Phe(*p*-I)-D-Trp-Arg-Thr-Cys]-Thr-NH_2	IA[a]	500
22	GH-62	H-D-Phe-*c*[Cys-His-D-Phe(*p*-F)-Arg-Trp-D-Cys]-Thr-NH_2	1000	IA[a]
23	GH-63	H-D-Phe-*c*[Cys-His-D-Phe(*p*-I)-Arg-Trp-D-Cys]-Thr-NH_2	IA[a]	IA[a]
24	GH-61	H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-D-Cys]-Thr-NH_2	5000	IA[a]
25	GH-60	H-D-Phe-*c*[Cys-His-D-Nal(2′)-Arg-Trp-D-Cys]-Thr-NH_2	IA[a]	IA[a]

[a] No activity up to 10^{-5} M (IA). *For α-MSH, $EC_{50} = 0.10$ nM. [b] EC_{50}: concentration of ligand (nM) required for 50% maximum agonist response; IC_{50}: concentration of ligand (nM) required for 50% inhibition of 1 nM α-MSH.

possible to design somatostatin analogues possessing potent activities for receptors other than the somatostatin or opioid receptors. To achieve this for melanocortin receptors, we envision that the β-turn tetrapeptide (Phe-D-Trp-Lys-Thr), so important for binding to somatostatin receptors,[64] could be utilized as a conformational template on which to build the structural and conformational features necessary for interaction with the MCRs. We then used another β-turn tetrapeptide (His-Phe-Arg-Trp),[35,65] the minimum active sequence for α-MSH,[66] as the template for design of somatostatin related analogues. Similar to MT-I, MT-II, and sandostatin (Octreotide), the incorporation of a D-amino acid[7,33,35,67,68] in the $i + 1$ position of the β-turn would help to design more potent ligands. Thus we chose the N-terminus of both CTOP and CTAP, H-D-Phe, as the N-terminus of all designed peptides, and Thr-NH_2 as the C-terminus. To make constrained peptides, we chose the disulfide bond scaffold by the incorporation of cysteine and related amino acids for the designed peptides and prepared a number of analogues with substitution at position 7 and variations at the disulfide bridge.[69]

Results

Peptide Synthesis. Solid-phase peptide synthesis (SPPS) was used for the synthesis of all the designed peptide analogues. None of the syntheses were optimized for yields. For lactam-bridged peptides, the cyclizations were carried out on the resins before the peptides were cleaved from the resin. For disulfide-bridged peptides, the cyclizations were carried out in water/methanol or water/acetonitrile solution mixtures after the linear peptides were cleaved from the resins. In both cases, cyclized peptides were subsequently purified by preparative RP-HPLC.

Peptides synthesized were characterized by RP-HPLC, mass spectroscopy, and amino acid analysis (see Experimental Section). The peptides all were demonstrated to be pure (>98%) as determined by RP-HPLC. The mass spectra results all agreed with the calculated values, as did the results from amino acid analysis for those amino acids which could be unambiguously assigned and quantitatively determined.

Frog Skin Melanocortin 1 Receptor (MC1R) Assays. Assays were performed by measuring the darkening of *Rana pipiens* frog skin after treatment with the designed analogues (see Experimental Section). This classical method provided a rapid assay to determine whether compounds possess agonist or antagonist activities. It also provides an evaluation of the potential of analogues functioning at other melanocortin receptors (MC3R, MC4R, and MC5R).

As can be seen from Table 1, the new analogues in this study exhibited a wide variety of activities at the classical *R. pipiens* frog skin's MC1R.

As shown in Table 1, for H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**, GH-11b), which has a Cys^5 and Cys^{10} disulfide bridge with the core sequence of His-D-Phe-Arg-Trp, the EC_{50} is about 10 nM, about 1% the potency of α-MSH. Past research has shown that increased steric bulkiness at the D-Phe^7 position of melanotropin analogues could modify bioactivity. Replacing D-Phe^7 with the more bulky D-Tic gave an analogue (Figure 2), H-D-Phe-*c*[Cys-His-D-Tic-Arg-Trp-Cys]-Thr-NH_2 (**2**, GH-4c) which has no agonist activity, and instead is an antagonist, with an IC_{50} value of 1 μM. However, replacing D-Phe with D-Phe(*p*-Cl) which has a larger substituent in the para position of phenyl group in the aromatic side chain than D-Phe gave **3**, H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2 which was a less potent agonist compared with **1**. Furthermore, H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-Cys]-Thr-

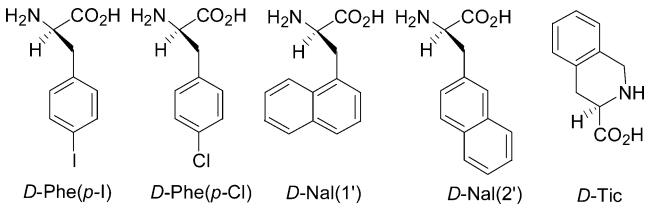


Figure 2. Structures of some substituted phenylalanine used at position 7.

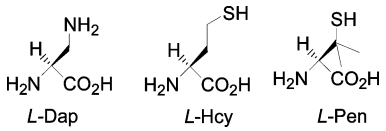


Figure 3. Structures of special amino acids.

NH_2 (**4**, GH-6d, Table 1) with D-Nal(1′) as a replacement for D-Phe in **1** almost lost agonist activity completely (EC_{50} = 10 μM).

Previously it was shown that restriction of the lactam bridge of MT-II led to dramatic changes in bioactivity. For example, replacing Lys^{10} in MT-II with a 2,3-diaminopropanoic acid (Dap, Figure 3), three CH_2 units shorter in the side chain compared with Lys, gave Ac-Nle^4-*c*[Asp^5,D-Phe^7,Dap^{10}]α-MSH(4−10)-NH_2, which lost nearly 2 orders of magnitude in agonist activity compared with MT-II.[35] Hence, modifying the bridge in the cyclic compounds could be very interesting. Replacement of Cys^{10} in H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (GH-11b) with a Hcy^{10} residue (Figure 3) led to H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2 (**5**, GH-13, Table 1), which showed very weak potency (EC_{50} = 1 μM). However, replacing Cys^5 in **1** with Hcy^5 led to **6**, H-D-Phe-*c*[Hcy-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (GH-4d), which was nearly as potent as MT-II (EC_{50} = 0.3 nM) in the *R. pipiens* frog skin bioassay. On the other hand, replacing Cys^{10} in **1** with the more constrained Pen^{10} residue (*β*,*β*-dimethylcysteine, Figure 3) to give H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**, GH-15) which has an EC_{50} of 100 nM in the same assay. Interestingly, when the Cys^{10} in **1** is replaced with a D-Cys^{10}, the analogue H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**, GH-14a) showed the same potency as MT-II in the frog skin bioassay. In addition, by replacing the disulfide bridge in **1** with a lactam bridge to give **9** (H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2), an analogue essentially equipotent as MT-II was obtained.

Of the amino acid residues in the core sequence, His-Phe-Arg-Trp, of α-melanotropin, His^6 has only recently been studied for structure−activity relationships. A few years ago, during the studies of the hydrogen bonding properties of imidazole ring of His^6, a decision was made to replace the hydrogen with methyl group so that hydrogen bonding could be eliminated. In earlier studies we showed, using SHU-9119 as a model, that if substituted by His(1′-Me) to give Ac-Nle-*c*[Asp-His(1′-Me)-D-Nal(2′)-Arg-Trp-Lys]-NH_2, a selective *m*MC5R antagonist[40] could be obtained. Hence, the His^6 position plays a significant role not only in MCR selectivity but also in function. When His^6 was replaced by Pro^6 to give Ac-Nle-*c*[Asp-Pro-D-Nal(2′)-Arg-Trp-Lys]-NH_2, a selective and super potent agonist at human MC5R (*h*MC5R) was obtained.[53,70] We thus turned to modifications, where the His^6 of **1** was replaced by His(3′-Me), to give the analogue **10** (GH-6c, Table 1), which had very weak agonist activity (EC_{50} = 0.10 μM). Interestingly, by replacing the S−S bridge c[Cys^5---Cys^{10}] with a lactam bridge c[Glu^5---Lys^{10}] to give H-D-Phe-*c*[Glu-His(3′-Me)-D-Phe-Arg-Trp-Lys]-Thr-NH_2 (**11**), we obtained a potent agonist in the frog skin assay with an EC_{50} of 1 nM. On the other hand, replacing His^6 of **1** with D-His gave H-D-Phe-*c*[Cys-D-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**12**, GH-6a) which was much less potent (EC_{50} = 3 μM).

In the past, replacement of Arg^8 led to decreased potency of α-MSH analogues suggesting that the side chain's guanidino group of Arg^8 plays a significant role in interacting with the receptor. Replacement of Arg^8 with Orn (Table 1) led to H-D-Phe-*c*[Cys-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**13**, Table 1) with EC_{50} of 100 nM. Interestingly, further replacing Cys^5 with Pen^5 gave H-D-Phe-*c*[Pen-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**14**), a very weak antagonist in the frog skin assay with no agonist activity (Table 1).

Examination of the activities of the starting compounds, CTAP and CTOP, in the frog skin assay are quite interesting (Table 1). CTOP with Orn at position 8 is a sub-micromolar agonist in the *R. pipiens* frog skin assay (Table 1), while CTAP with an Arg at position 8 is a weak antagonist in the same assay. By replacing the Thr^9 of CTAP with a Ser to give H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Ser-Pen]-Thr-NH_2 (**15**, GH-21, Table 1), a weak antagonist was obtained. In previous α-MSH research, Trp^9 was found to be an important residue for the activities of potent α-MSH analogues.[40,71,72] Replacement of the Thr^9 of CTAP with a Trp led to H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Trp-Pen]-Thr-NH_2 (**16**, GH-22), whose bioactivity (IC_{50} = 1 μM) was essentially the same as CTAP (Table 1). Nor did H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-D-Trp-Pen]-Thr-NH_2 (**17**, GH-20), with a D-Trp^9, lead to any significant change in activity (EC_{50} = 5 μM). To check whether the phenolic OH group is important, Tyr^6 was replaced by Phe^6 in which an H replaces the phenolic OH in CTAP. As can be seen in Table 1, H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-Trp-Pen]-Thr-NH_2 (**18**, GH-19) possessed antagonist activity similar to that of CTAP, while H-D-Phe-*c*[Cys-Phe-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**19**, GH-17) which has a D-Phe^7 residue has no agonist or antagonist activity at 10^{-5} concentration. Further modification of position 6 with a Phe(*p*I) residue gives H-D-Phe-*c*[Cys-D-Phe(*p*I)-D-Trp-Arg-Thr-Pen]-Thr-NH_2 (**20**, [Phe(*p*-I)^6-CTAP]), an antagonist analogue with an IC_{50} of 100 nM. Replacing the c[Cys^5---Pen^{10}] bridge on **20** with a c[Cys^5---Cys^{10}] bridge led to **21** (GH-16, H-D-Phe-*c*[Cys-D-Phe(*p*I)-D-Trp-Arg-Thr-Cys]-Thr-NH_2), which had the same antagonist potency as CTAP (Table 1). Replacing D-Phe^7 of MT-II with more bulky aromatic side chain amino acids has generated potent and selective antagonist analogues, such as SHU-9119, Ac-Nle-*c*[Asp-His-D-Nal(2′)^7-Arg-Trp-Lys]-NH_2.[38] Therefore, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**, Table 1), which was equipotent to MT-II in the *R. pipiens* frog skin assay, was chosen for modification. Replacing D-Phe^7 with a D-Phe(*p*-F) to give H-D-Phe-*c*[Cys-His-D-Phe(*p*-F)-Arg-Trp-D-Cys]-Thr-NH_2 (**22**, Table 1) led to a weak agonist with an EC_{50} = 100 nM. Increasing the size of the halogen from F to I led to H-D-Phe-*c*[Cys-His-D-Phe(*p*-I)-Arg-Trp-D-Cys]-Thr-NH_2 (**23**, GH-63), which had no agonist activity (Table 1). A more

Table 2. Bioassay Results at *m*/*r* MCRs for Chimeric Analogues with Structural Variation at the S−S Bridge

	compound	structure	EC_{50} (nM) *m*MC1R	*r*MC3R	*m*MC4R	*m*MC5R
	NDP-α-MSH	Ac−Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.046 ± 0.45	0.10 ± 0.094	0.29 ± 0.048	0.064 ± 0.0056
1	GH-11b	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2	1.5 ± 1.1	3.0 ± 2.9	1.4 ± 001	0.12 ± 0.022
5	GH-13	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2	5.1 ± 0.86	75 ± 7.2	51 ± 2.0	2.2 ± 0.24
8	GH-14a	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2	0.23 ± 0.012	0.23 ± 0.094	0.20 ± 0.034	0.10 ± 0.068
9	GH-5a	H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2	0.047 ± 0.0013	0.23 ± 0.15	0.38 ± 0.026	0.074 ± 0.0040
7	GH-15	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2	6.9 ± 1.3	64 ± 5.6	20 ± 16	0.14 ± 0.014
3	GH-6b	H-D-Phe-*c*[Cys-His-**D-Phe(*p*-Cl)**-Arg-Trp-Cys]-Thr-NH_2	0.37 ± 0.080	7.30 ± 1.20	0.67 ± 0.025	0.11 ± 0.015
2	GH-4c	H-D-Phe-*c*[Cys-His-**D-Tic**-Arg-Trp-Cys]-Thr-NH_2	IA[a]	IA	IA	IA
4	GH-6d	H-D-Phe-*c*[Cys-His-**D-Nal(1′)**-Arg-Trp-Cys]-Thr-NH_2	22 ± 2.2	5.2 ± 1.2	IA	4.30 ± 0.88
13	GH-3a	H-D-Phe-*c*[Cys-His-D-Phe-**Orn**-Trp-Cys]-Thr-NH_2	47 ± 12	660 ± 110	180 ± 20	8.5 ± 0.050
14	GH-3b	H-D-Phe-*c*[Pen-His-D-Phe-**Orn**-Trp-Cys]-Thr-NH_2	490 ± 400	59 ± 11	IA[a]	46 ± 13
10	GH-6c	H-D-Phe-*c*[Cys-**His(3′-Me)**-D-Phe-Arg-Trp-Cys]-Thr-NH_2	35 ± 16	31 ± 1.5	8.9 ± 0.10	0.55 ± 0.082

[a] No activity up to 10^{-5} M (IA).

bulky side chain group can be implemented by introducing a D-Nal(1′) or D-Nal(2′) residue for D-Phe[7]. The D-Nal(1′) derivative, H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-D-Cys]-Thr-NH_2 (**24**, Table 1), showed much reduced agonist potency in the *R. pipiens* frog skin bioassay with EC_{50} of 5 μM, whereas the D-Nal(2′) derivative, H-D-Phe-*c*[Cys-His-D-Nal(2′)-Arg-Trp-D-Cys]-Thr-NH_2 (**25**), completely lost agonist and antagonist activity.

Mouse Melanocortin Receptors Assays. The cloned rodent MC1R, MC3R, MC4R, and MC5R have served as model mammalian species for the roles of MCRs in physiology. These receptors have been used widely for evaluating melanotropin analogues.[3,38,40,47] As can be seen from Table 2, the new analogues in these studies exhibited a wide range of potencies and selectivities at the mouse MC1R (*m*MC1R), rat MC3R (*r*MC3R), mouse MC4R (*m*MC4R), and mouse MC5R (*m*MC5R).

In the in vitro *R. pipiens* frog skin assay, the parent molecule of the newly designed analogues, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**, Table 1), functioned as an agonist with an EC_{50} of 10 nM (Table 1). In the mouse MCRs assays, **1** displayed much more potent activities, especially at the *m*MC5R (EC_{50} = 0.12 nM) where it is almost 100-fold more potent than its activity in the *R. pipiens* frog skin assay. At the other *m*MCRs, this compound also displayed agonist activities and EC_{50}s ranging from 1.4 to 3.0 nM (Table 2).

H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2 (**6**), though not very potent in the *R. pipiens* frog skin MC1R assay, showed potent agonist activities at all rodent MCRs with EC_{50}s around 2−5 nM at the *m*MC5R and *m*MC1R, and 50−70 nM at *m*MC4R and *r*MC3R (Table 2). H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**) is highly potent in the *R. pipiens* frog MC1R assay, displayed highly potent but nonselective agonist activity at all rodent MCRs with subnanomolar EC_{50}s [EC_{50}s (nM): 0.22 at *m*MC1R; 0.23 at *r*MC3R; 0.20 at *m*MC4R; and 0.10 at *m*MC5R]. On the other hand, H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2 (**9**), a lactam version (side chain cyclized Asp^5---Lys^{10} bridge) of **8** (c[Cys^5---D-Cys^{10}] side chain S−S bridge), displayed almost the same potency as **8** in the *R. pipiens* frog skin MC1R assay, but it showed particularly high potency at the *m*MC1R (EC_{50} ∼ 0.047 nM) and the *m*MC5R (EC_{50} ∼ 0.074 nM). However, **9** displayed relatively weak agonist activities at the *r*MC3R (EC_{50} ∼ 0.23 nM) and *m*MC4R (EC_{50} ∼ 0.04 nM) and thus was selective for the *m*MC1R and *m*MC5R versus the *r*MC3R and *m*MC4R. H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**), a weak agonist ($EC_{50} = 1 \times 10^{-5}$ M) in the *R. pipiens* frog skin MC1R assay, displayed high potency and selectivity at the *m*MCRs with exceptional potency at the *m*MC5R (EC_{50} ∼ 0.015 nM). It is 50-, 500-, and 150-fold more potent at the *m*MC5R than at the *m*MC1R (EC_{50} ∼ 7 nM), *r*MC3R (EC_{50} ∼ 70 nM), and *m*MC4R (EC_{50} ∼ 25 nM), respectively, making it the most selective MC5R agonist reported thus far.

In the *R. pipiens* frog skin assay, increases in the bulkiness of the side chain groups of D-Phe in H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) led to decreased agonist activity to varying degrees (Table 1). In assays at the *m*MCRs, the situation is different. H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2 (**3**) with a D-Phe(*p*-Cl) at position 7 (Table 2) was highly potent at most of the *m*MCRs (EC_{50}s were 0.4, 0.7, and 0.1 nM at *m*MC1R, *m*MC4R, and *m*MC5R, respectively) except at the *r*MC3R where the EC_{50} for **3** (GH-6b) was about 7 nM. H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-Cys]-Thr-NH_2 (**4**, GH-6d), which incorporated a D-Nal(1′) at position 7, showed relatively weaker agonist activity at the *m*MC1R, *r*MC3R, and *m*MC5R compared with **1**, while at the *m*MC4R, **4** was inactive even at 1 μM. Interestingly, H-D-Phe-*c*[Cys-His-D-Tic-Arg-Trp-Cys]-Thr-NH_2 (**2**, GH-4c), which has a D-Tic at position 7, was inactive at all *m*MCRs. Replacing Arg^8 in **1** with Orn gave H-D-Phe-*c*[Cys-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**13**, GH-3a, Table 2), which showed decreased potencies at all *m*MCRs compared with **1**. The most dramatic change in activity is at the *m*MC4R, where **4** (EC_{50} ∼ 180 nM) is more than 100-fold weaker than **1** (EC_{50} = 1.4 nM) at that receptor.

Constraining the disulfide bridge of **13** c[Cys^5---Cys^{10}] with a c[Pen^5---Cys^{10}] disulfide bridge gave H-D-Phe-*c*[Pen-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**14**, GH-3b), which showed decreased potency at the *m*MC1R (EC_{50} ∼ 500 nM). The activity at the *m*MC5R also decreased to an EC_{50} of about 50 nM. However, the potency at *r*MC3R increased 10-fold compared with that of **13**, but lost potency at the *m*MC4R. As mentioned earlier, replacing His^6 with a His(3′-Me) eliminates the possibility of hydrogen bonding at the side chain, which might affect biological activity. Indeed, H-D-Phe-*c*[Cys-His(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**10**, GH-6c, Table 2) displayed a broad spectrum of potencies compared with **1**. Though less potent at the *r*MC3R,

*m*MC4R, and *m*MC5R than **1**, the selectivities of **10** for the *m*MC5R versus *m*MC1R or *m*MC3R improved (Table 2).

Discussion

Activities of Somatostatin-Like Analogues in Frog Skin Assays. In Table 1 we have listed the agonist and antagonist activities of the new designed ligands. The starting compound, which can be viewed as CTOP (Table 2), has weak agonist activity ($EC_{50} = 10^{-7}$ M), about 300-fold less potent than α-MSH in the same assay. Interestingly, the chimeric peptide, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**, GH-15, Table 1), has the same potency ($EC_{50} = 10^{-7}$ M) as CTOP and is very similar to the tetrapeptide Ac-His-D-Phe-Arg-Trp-NH_2, which has a reported EC_{50} of 2×10^{-7} M. Hence, just the bioactive sequence His-D-Phe-Arg-Trp^{60} does not ensure an increase in potency (CTOP vs **7**). Clearly, the proper conformation also is important, and hence we synthesized several analogues to examine this question. Interestingly, a 10-fold increase in agonist potency for **8** can be obtained by replacing the Pen residue in **8** with a Cys residue, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**, Table 1), and a further 30-fold increase in potency is obtained by replacing the Cys^5 in **1** by a homocysteine (Hcy) residue, H-D-Phe-*c*[Hcy-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**6**, Table 1). Analogue **6** is essentially equipotent to α-MSH. It is interesting to note that replacement of the exocyclic moieties in the highly potent and prolonged acting cyclic agonist analogue Ac-Nle-*c*[Asp^5,D-Phe^7,Lys^{10}]α-MSH(4−10)-NH_2 (MT-II) by the exocyclic moieties of CTOP (i.e. Ac-Nle by H-D-Phe at the N-terminal and carboxyamide by Thr-NH_2) gives compound H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2 (**9**, Table 1), which is slightly more potent than **6** (Table 1). Finally, it should be noted that none of the new analogues with a disulfide bridge have substantial prolonged activity as the lactam bridged analogue **10** and MT-II do.

Ring Size. It has been reported that the ring size[35,41,73,74] can change the potency for α-MSH analogues. For example, MT-II (Ac-Nle-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2) with a 23-membered ring is about 70 times more potent than its 20-membered ring counterpart, Ac-Nle-*c*[Asp-His-D-Phe-Arg-Trp-Dpr]-NH_2, where α,*β*-diaminopropanic acid (Dpr) replaced Lys in position 10 of MT-II.[35] Such a dramatic change was attributed to a change in the active conformation and changes in the orientation of functional groups in side chains which are essential for ligand−receptor binding.[74]

In our current study, it was discovered that the ring sizes of our new chimeric analogues are important to bioactivities. H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2 (**9**), with the same ring size as MT-II, displayed a similar potency ($EC_{50} = 0.10$ nM) as that of MT-II. To further investigate the importance of ring sizes, it was decided to replace this lactam bridge with a disulfide bridge. Changing Asp^5 to Hcy^5 and Lys^{10} to Cys^{10} in **9** gives H-D-Phe-*c*[Hcy-His-D-Phe-Arg-Trp-Cys]-NH_2 (**6**) with a 21-membered ring, and it has about one-third the potency of **9**. However, on replacing Asp^5 with Cys^5 and Lys^{10} with Hcy^{10} in **9**, the resulting analogue H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-NH_2 (**5**, $EC_{50} = 1 \times 10^{-6}$ M) is about 5000 times less potent than **6**, though both analogues have 21-membered rings with a disulfide bridge (Table 1).

This dramatic change in the bioactivities between **6** and **5** apparently resulted from the position shift of the disulfide bond between these two molecules, because this physical difference (Hcy^5---Cys^{10} in **6** versus Cys^5---Hcy^{10} in **5**) is the only difference in the structures of the two molecules. Such a difference in structure might result in a significantly different conformation for the two ligands. One hypothesis is that the highly polarizable sulfur atoms might interact with one or more of the key aromatic side chain groups through lone pair electrons-π (S-π) interactions[75] which subsequently alters the orientations of side chain groups of the ligands which are essential for binding. Once the orientations of side chains are changed, the backbone conformation could be changed. Such a hypothesis needs to be confirmed by conformational analysis via NMR and computational studies.

Further reducing the size of the ring in **6** by replacing Hcy^5 with Cys^5 led to H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) which has a 20-membered ring. Compound **1** displayed a potency ($EC_{50} = 10^{-8}$ M) which is about 50 times less than **6**. On the basis of the above discussion, it is apparent that the ring size of the cyclized α-MSH analogues plays a significant role in potency.

Configuration of Amino Acids at the Cyclic Bridges. Previously it has been shown that the configurations of key amino acid residues in melanotropins are very important for the potency of designed analogues.[25,33,72] Such changes in potency might be derived directly from conformational changes including either loss or enhancement of secondary structures, such as *β*-turns and *γ*-turns. Earlier studies of α-MSH analogues found that D-Phe replacement at position 7 of α-MSH enhanced the *β*-II′ turn structure, and that this enhancement dramatically improve the potency and also prolonged the bioactivity relative to the parent compound, α-MSH.[31,33,76,79] On the other hand, changing the configuration (L → D) at other positions (excluding position 7) in MT-II or SHU-9119 often leads to analogues with decreased potency.[42,72]

The present study provides additional evidence that changes in the configuration at certain amino acids result in large changes in bioactivity for α-MSH analogues. Changing the naturally occurring His^6 in H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**, $EC_{50} = 10$ nM) to the nonnatural D configuration (D-His^6) gives H-D-Phe-*c*[Cys-D-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**12**, Table 1) which provides a less potent ligand with an EC_{50} of 3×10^{-6} M. On the other hand, when the L configuration of Cys^{10} of **1** was replaced by D-Cys^{10} to give H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**, Table 1), the analogue had an increased potency of almost 2 orders of magnitude with an EC_{50} of 0.10 nM. Further experiments are needed to understand how these configuration changes, such as position 6 to D-His in **12**, and position 10 to D-Cys in **8**, affects the conformations of these ligands.

Bulky Side Chain Groups at Position 7. In the past, we have demonstrated that substitutions in position 7 are important for agonist/antagonist activity in

melanotropin analogues.[38] For example, Ac-Nle-*c*[Asp-His-D-Nal(2′)[7]-Arg-Trp-Lys]-NH_2 (SHU-9119), with a bulky 2′-naphthyl side chain group at position 7, is a potent antagonist at both *m*MC3R and *m*MC4R, but is a potent agonist at both the *m*MC1R and the *m*MC5R. Interestingly, if the bulky side chain is not properly oriented, completely different results are obtained. For example, if the D-2′-naphthylalanine residue was replaced by a D-1′-naphthylalanine residue, the corresponding α-MSH analogue, Ac-Nle-*c*[Asp-His-D-Nal(1′)-Arg-Trp-Lys]-NH_2, is an agonist at all *m*MCRs.[38] In our present study, we also found that incorporation of substituted phenylalanine derivatives at position 7 affect the potency and bioactivity of α-MSH analogues but differently than in the MT-II series. Substituting the *p*-hydrogen of D-Phe[7]with a chlorine in H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2 (**1**) gave the analogue H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2 (**3**, Table 1) which showed slightly reduced potency (EC_{50} = 30 nM) compared with its parent compound (**1**, 10 nM). However, replacing D-Phe[7] with D-Nal(1′), which has a more bulky aromatic side chain than phenyl, gave the analogue H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-Cys]-Thr-NH_2 (**4**, EC_{50} = 10 μM, Table 1) which is 3 orders of magnitude less active than its parent compound **1** in the *R. pipiens* frog skin bioassay.

Bulky Side Chain Groups at Residues Other Than Position 7. The replacement of amino acid residues at positions other than position 7 with bulky side chain amino acids have not been closely investigated until recently. Modification of His[6] in Ac-Nle-*c*[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (SHU-9119) with methyl-substituted imidazole rings, a hydrogen bonding deprived thiazole ring, or a tryptophan residue yielded analogues with profiles similar to that of SHU-9119 in the *R. pipiens* frog skin MC1R bioassay.[40] Substitution of His[6] in H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) with a 3′-Me-His gave H-D-Phe-*c*[Cys-His(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**10**, Table 1) which is about 1000 times less potent (EC_{50} = 10^{-5} M) than **1** (EC_{50} = 10^{-8} M) and possessed no antagonist activity in the frog skin assay. It has been assumed that Arg[8] is critical for bioactivity. Replacement of Arg[8] of **1** with an ornithine gave H-D-Phe-*c*[Cys-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**13**) which has a 10-fold decreased potency with an EC_{50} of 10^{-7} M (Table 1). Interestingly, when Cys[4] in **1** was replaced by β,β-dimethyl-substituted cysteine (Pen) to give H-D-Phe-*c*[Pen-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**14**), the analogue was inactive and became a weak antagonist. This suggests that modifications in the degree of constraint in the disulfide bridge might shed light on our understanding of structure−activity relationships for α-MSH analogues. Thus, it was decided to modify H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) for this purpose. Cys[10] was then replaced by Pen, resulting in H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**, Table 1) which showed a 10-fold decrease in agonist activity (EC_{50} = 10^{-7} M) compared with the parent compound GH-11b.

Antagonist Analogues Derived from Chimeric Somatostatin. Bulky Side Chain Groups at Positions 5, 6 and 7. Early studies[38] showed that certain bulky side chain groups in position 7 of constrained α-MSH derivatives, such as Ac-Nle-*c*[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (SHU-9119), display potent antagonist activity at both the *m*MC3R and the *m*MC4R. Further modification[40] of positions 6 with substituted His(1′-Me) led to Ac-Nle-*c*[Asp-His(1′-Me)-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (WY012), a potent *m*MC5R antagonist (pA_2 = 7.1), while substitution of His[6] with His(3′-Me) showed only minor changes in activity. In addition, replacing His[6] in Ac-Nle-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2 (MT-II) with other amino acids, including the hydrophobic neutral residue Ala, the acidic residue Glu, and the basic residue Lys, led to analogues[42,48] with significantly decreased potencies at all receptors bioassayed (*h*MC3R, *h*MC4R, and *h*MC5R), with the greatest loss at the *h*MC3R.

We have found that it also is possible to obtain antagonists for *R. pipiens* frog melanocortin 1 receptor (MC1R) starting with the potent μ opioid receptor antagonist CTOP. As discussed earlier, the Pen[5]-substituted analogue H-D-Phe-*c*[Pen-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**14**, Table 1) has weak antagonist activity. We realized that increasing the steric bulk of appropriate side chain groups might give antagonist activity for α-MSH derivatives. As can be seen in Table 1, we found that H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2 (CTAP), the Arg[8] modified version of CTOP, acted as a weak antagonist (IC_{50} = 10^{-6} to 10^{-7} M) in the frog skin bioassay. It is striking that such a small modification (primary amino group to a guanidino group) of a potent μ opioid receptor antagonist leads to a weak MC1R antagonist. Replacement of the Thr[9] residue in CTAP by a Ser residue, a less hindered and more hydrophilic residue, led to H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Ser-Pen]-Thr-NH_2 (**15**, Table 1) which has the same antagonist activity (IC_{50} = 10^{-6} M) as CTAP. Interestingly, when the Thr[9] residue in CTAP was replaced by the larger hydrophobic amino acid Trp to give H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Trp-Pen]-Thr-NH_2 (**16**), an antagonist equipotent to CTAP was obtained. Further replacement of the Tyr residue in **16** by a Phe residue led to H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-Trp-Pen]-Thr-NH_2 (**18**) whose antagonist potency (IC_{50} = 10^{-6} M) was essentially the same as **16**. By changing the L configuration of Trp[9] in **18** to a D configuration to give H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-D-Trp-Pen]-Thr-NH_2 (**17**), an improved antagonist potency was obtained. In addition, when the Tyr[6] of CTAP was replaced by a sterically hindered residue Phe(*p*-I) to give H-D-Phe-*c*[Cys-Phe(*p*-I)-D-Trp-Arg-Thr-Pen]-Thr-NH_2 (**20**), slightly improved potency (IC_{50} = 10^{-7} M) was obtained. Furthermore, replacing the Pen[10] of **20** with a Cys residue gave H-D-Phe-*c*[Cys-Phe(*p*-I)-D-Trp-Arg-Thr-Cys]-Thr-NH_2 (**21**) which displayed almost the same antagonist activity (IC_{50} = 10^{-6} to 10^{-7} M) as **20**. This demonstrated that steric hindrance at position 10 for CTAP analogues might not be important in terms of antagonist activity in the frog skin.

Activities of the New Analogues at Mouse Melanocortin Receptors. In the past, analogues containing the α-MSH-related sequence, His-D-Phe-Arg-Trp,[33−35,38] generally displayed potent activities in the frog skin MC1R and lizard skin MC1R assays. Later, after other subtypes of MCRs were discovered, these analogues displayed high potency at the MC3, MC4, and MC5 receptors as well. High selectivities were not

realized until modification of the D-Phe[7] in the core sequence, His-D-Phe-Arg-Trp, of MT-II by the more sterically hindered D-Nal(2′) to give Ac-Nle-*c*[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (SHU-9119), a very potent antagonist at both *m*MC3R and *m*MC4R and agonist at *m*MC1R and *m*MC5R.[38] Since this discovery, we have speculated that further modification of the core sequence at other positions (6, 8, and 9) might afford more selective ligands for the various MCRs. Recently, it was discovered that modification of His[6] with side chain substitution in SHU-9119 led to the first *m*MC5R antagonist ($pA_2 = 7.1$), Ac-Nle-*c*[Asp-His(1′-Me)-D-Nal-(2′)-Arg-Trp-Lys]-NH_2 (WY012), which is an antagonist at both *m*MC3R ($pA_2 = 9.5$) and *m*MC4R ($pA_2 = 9.8$) and an agonist at only the *m*MC1R ($EC_{50} = 0.39$ nM).[40] This suggested that position 6 is critical for ligands to have antagonist activity at the *m*MC5R. Further modification of this lead compound at Trp[9] with a more bulky and hydrophobic amino acid Nal(2′) led to Ac-Nle-*c*[Asp-His(1′-Me)-D-Nal(2′)-Arg-Nal(2′)-Lys]-NH_2, which had a similar bioactivity profile (*m*MC1R: $EC_{50} = 0.54$ nM; *m*MC3R: $pA_2 = 9.0$; *m*MC4R: $pA_2 = 10.3$; *m*MC5R: $pA_2 = 7.2$).[40]

Interestingly, modification of Arg[8] in MT-II with the bulky, hydrophobic amino acid Nal(2′) led to Ac-Nle-*c*[Asp-His-D-Phe-D-Nal(2′)-Trp-Lys]-NH_2, which was less potent than MT-II at all *m*MCRs (*m*MC1R: $EC_{50} = 1.8$ nM; *r*MC3R: $EC_{50} = 38.$ nM; *m*MC4R: $EC_{50} = 920$ nM; *m*MC5R: $EC_{50} = 3.70$ nM).[40] Thus, this ligand has become more selective at the *m*MC1R where it is about 20 times more potent than at the *r*MC3R and 500 more potent than at the *m*MC4R. Interestingly, it also is very potent at the *m*MC5R.

Hence, these studies have revealed that positions 6 to 9 in the core sequence of His-D-Phe/D-Nal(2′)-Arg-Trp are important for selectivities for the various *m*MCRs. On the other hand, positions at 5 and 10 have not been studied well due to the general unavailability of side chain-constrained aspartic acid, glutamic acid, ornithine, and lysine amino acid analogues. The current study was undertaken to further examine structure−activity relationships by constraining α-MSH ligands with smaller ring sizes and varying the substitutions at positions 5 through 8 and position 10, so as to characterize the substructural components of these ligands which are responsible for agonist and antagonist properties at the MCRs.

***m*MC1R.** As shown in Table 2, variations in the disulfide bridge generally yielded ligands with EC_{50}s in the *m*MC1R assay in the nanomolar range, except for H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**, $EC_{50} \approx 0.20$ nM). When the D-Cys[10] of **8** was substituted with an L-Cys[10] residue, the resulting ligand H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) showed a 10-fold decreased potency ($EC_{50} = 1.5$ nM, Table 2). Further replacing Cys[10] with either a more bulky Pen[10] or with a Hcy[10] gave H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**) and H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2 (**5**), respectively, both of which showed similar activities at the *m*MC1R (EC_{50} for **7** is around 7 nM, while for **5** it is about 5 nM). Interestingly, when changing the disulfide bridge back to a lactam bridge with a 23-membered ring to give **7** an analogue with the highest potency with an $EC_{50} = 0.047$ nM (47 pM) at the *m*MC1R (**9**, Table 2) was obtained.

Modification at position 7 with D-Nal(1′) H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-Cys]-Thr-NH_2 (**4**) gave an analogue which is less potent ($EC_{50} = 22$ nM) than the parent ligand H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**). However, **3** with D-Phe(*p*-Cl)[7] showed improved bioactivity ($EC_{50} = 0.37$ nM) at the *m*MC1R. Since both D-Nal(1′) and D-Phe(*p*-Cl) are substituted for D-Phe, the results suggest that χ-space topography in position 7 is important for ligand bioactivity, as discovered earlier when modifications were made on the phenyl ring of D-Phe[7] in MT-II analogues.[38] When D-Tic, a constrained version of D-Phe, was substituted at position 7 to give H-D-Phe-*c*[Cys-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**2**), an inactive analogue was obtained. It is worth mentioning that H-D-Phe-*c*[Cys-His-D-Nal-(1′)-Arg-Trp-Cys]-Thr-NH_2 (**4**, Table 2) is about 500 times more potent in the *m*MC1R assay than in the frog skin assay. Other position 7 substituted ligands also generate significantly greater potencies. H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2 (**3**): 90 times; H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**): 8 times. This suggests that residue 7 critically affects potency at MC1Rs in different ways in different species which has not been apparent from any previous studies.

Modification of position 8 (Arg) in **1** (GH-11b) by an Orn residue led to H-D-Phe-*c*[Cys-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**13**, Table 2) which showed agonist activity with an EC_{50} of 4.7×10^{-8} M (30 times less potent than the parent ligand **1**, and another analogue, H-D-Phe-*c*[Pen-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2 (**14**) with a Pen−Cys disulfide bridge and Orn at position 8, displayed weak agonist activity in the *m*MC1R assay ($EC_{50} = 490$ nM, Table 2).

***r*MC3R.** As shown in Table 2, the potency of H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) at the *r*MC3R ($EC_{50} \sim 3$ nM) was about the same as at the *m*MC1R ($EC_{50} \sim 1.5$ nM). In addition, for ligand H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**), the bioactivities are similar ($EC_{50} = 2.3$ nM at both *m*MC1R and *r*MC3R). The only difference between these two ligands is the configuration at position 10. Hence, it seems that changes in configuration (D or L) at position 10 are not critical for the *m*MC1R or the *m*MC3R in this cyclic disulfide family. On the other hand, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**) with a Pen at position 10 is about 10-fold less potent at the *r*MC3R than at the *m*MC1R. In addition, it was found that H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2 (**5**) with a Hcy at the position 10 is almost 10-fold less potent at the *r*MC3R than at the *m*MC1R. Hence, increases in steric hindrance or hydrophobicity (Pen[10] in **7** versus Cys[10] in **1**) or flexibility (Hcy[10] in **5** versus Cys[10] in **1**) of position 10 can help improve selectivities.

Modification of position 7 in H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) to give H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-Cys]-Thr-NH_2 (**4**, Table 2) and H-D-Phe-*c*[Cys-His-D-Tic-Arg-Trp-Cys]-Thr-NH_2 (**2**) did not show any significant differences in potency at the *r*MC3R and the *m*MC1R. However, H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2 (**3**) with a D-Phe(*p*-Cl) at position 7 is 20-fold less potent at the *r*MC3R than at the *m*MC1R. That suggests that substituents at the

para-position 7 can affect ligand selectivities at the *m*MC1R and the *r*MC3R. Modification of Arg^8 showed interesting results. Replacement of Arg^8 in H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**) by an Orn residue led to compound **13** (Table 2) with a loss of more than 200-fold in potency at the *r*MC3R. Hence Arg at position 8 is very important for the *r*MC3R. On the other hand, for **1** with an Arg at position 8, its potency at the *r*MC3R was virtually the same as that at the *m*MC1R, while in **13** it is about 15-fold less potent at the *r*MC3R than at the *m*MC1R. These results suggest that the residue at position 8 can play a role for the selectivities at the *m*MC1R and the *r*MC3R. As seen in Table 2, modification at position 6 also had some effects on potency at the *r*MC3R. Replacement of His^6 in **1** by a His(3′-Me) led to H-D-Phe-*c*[Cys-His(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**10**, EC_{50} = 31 nM) with a loss of about 10-fold in potency. However, the potency at the *m*MC1R (EC_{50} = 35 nM) for **10** is virtually the same as that at the *r*MC3R.

***m*MC4R.** As shown in Table 2, there were virtually no differences in potencies at the *r*MC3R and *m*MC4R for all the analogues tested. This suggests that other strategies will be needed to design selective ligands for these receptors.

***m*MC5R.** As shown in Table 2, H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2 (**9**) with a 23-membered ring and a lactam bridge had almost the same potency at the *m*MC1R (EC_{50} = 46 pM) as at the *m*MC5R (EC_{50} = 74 pM). When this 23-membered ring lactam was replaced by a 20-membered ring disulfide derivative H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**1**), its potency (EC_{50} = 0.12 nM) remained high at the *m*MC5R. Moreover, **1** is more potent (at least 10-fold) at the *m*MC5R than at any other of the MCRs tested (EC_{50} = 1.5, 3.0 and 1.4 nM at *m*MC1R, *r*MC3R, and *m*MC4R, respectively). Also notable, this ligand is >80-fold more potent at the *m*MC1R than at the frog MC1R (EC_{50} = 10 nM). Replacing Cys^{10} in **1** with a Hcy led to **5** with a 21-membered ring. Compound **5** is about 20-fold less potent (EC_{50} = 2.2 × 10^{-9} M) than **1** at the *m*MC5R, though these two ligands have comparable activity at the *m*MC1R (EC_{50} = 1.5 nM for **1** and 5.1 for **5**). Furthermore replacing Cys^{10} in **1** with a D-Cys^{10} led to H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**), which had virtually the same EC_{50} as **1** at the *m*MC5R. When Cys^{10} was replaced by a highly constrained Pen (*β*,*β*-dimethylcysteine residue), the resulting analogue, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**), was as potent (EC_{50} = 0.14 nM) as **1** at the *m*MC5R. However, the biggest difference between **7** and all other ligands in Table 2 is that the former is highly selective for the *m*MC5R. The potency of **7** was 50 times better at the *m*MC5R than at the *m*MC1R, while it was 500 times more potent at the *m*MC5R than at the *r*MC3R, and more than 2 orders of magnitude more potent at the *m*MC5R than at the *m*MC4R. Furthermore, **7** was at least 750 times more potent in the *m*MC5R assay than in the frog MC1R assay. Thus, **7** appears to be the most selective agonist compound for the *m*MC5R identified so far. It also suggests that hydrophobic and sterically hindered residues at position 10 may be a key for ligand selectivity for the *m*MC5R. This opens an avenue for design of more potent and selective ligands for the *m*MC5R.

Further replacing Arg^8 in **1** with an Orn gave H-D-Phe-*c*[Cys-His-D-Phe-Orn^8-Trp-Cys]-Thr-NH_2 (**13**, EC_{50} = 8.5 nM, Table 2) which had a 70-fold loss of potency. However, its selectivity at the *m*MC5R versus the *r*MC3R (EC_{50} = 660 nM) was about 80-fold, and its selectivity for the *m*MC5R versus the *m*MC4R (EC_{50} = 180 nM) was more than 20-fold. These selectivities are much better than its counterpart **1** that has an Arg at position 8. Hence, despite the lower potency, **13** had higher selectivity. Replacing Arg^8 with other amino acids might provide another route to discover selective *m*MC5R ligands.

With an Orn at position 8, modification of Cys^5 of **13** with a Pen led to H-D-Phe-*c*[Pen-His-D-Phe-Orn^8-Trp-Cys]-Thr-NH_2 (**14**) which had a lower potency (EC_{50} = 8.5 nM) at the *m*MC5R. Selectivity for the *m*MC5R versus other MCRs also was lost. Modification of His at position 7 in **1** with substitution at position 3′ of the side chain imidazole ring led to H-D-Phe-*c*[Cys-His(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**10**, GH-6c, Table 2), which lost about 4-fold agonist activity compared with that of GH-11b at the *m*MC5R. However, **10** was relatively more selective for the *m*MC5R versus all the other MCRs. It is about 60-fold more potent at the *m*MC5R than at either the *m*MC1R or the *r*MC3R, while it is about 16-fold more potent at the *m*MC5R than at the *m*MC4R.

Conclusions

By adopting the modified peripheral structure (H-D-Phe-c[Cys---Cys]-Thr-NH_2) of somatostatin-related analogues with high *μ* opioid potency, CTOP and CTAP, and the core sequence of α-MSH, 26 ligands have been designed and synthesized. These ligands have the following substitution patterns: H-D-Phe-*c*[XXX-YYY-ZZZ-Arg-Trp-AAA]-Thr-NH_2 {XXX and AAA = Cys, D-Cys, Pen, D-Pen; YYY = His, His(1′-Me), His(3′-Me); ZZZ = Phe and side chain halogen substituted Phe, D-Phe, D-Nal(1′), and D-Nal(2′)}.

In the *R. pipiens* frog MC1R assay, ligands, such as H-D-Phe-*c*[Cys-His-D-Tic-Arg-Trp-Cys]-Thr-NH_2 (**2**), displayed no activity at 10^{-5} M, while others, such as H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2 (**8**), exhibited agonist activity with potencies the same as α-MSH with an EC_{50} of 1 × 10^{-10} M. It is interesting to note that changes of the S−S bridges often lead to dramatic changes in bioactivity. For example, H-D-Phe-*c*[Hcy-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**6**, EC_{50} = 3 × 10^{-10} M) has about half the potency as α-MSH, but when the S−S bridge was switched around, H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2 (**5**) lost 3000-fold of potency (EC_{50} = 1 × 10^{-6} M) compared with **6** (Table 1). We also were able to develop antagonists at the frog MC1R based on CTAP, though most of the ligands displayed relatively weak antagonist potencies with IC_{50}s around 1 × 10^{-6} to 1 × 10^{-7} M.

In the mouse melanocortin receptors assays, the potencies ranged from 10^{-7} M to nearly 10^{-12} M, and none of the ligands tested possess any antagonist activity. Several of the ligands tested displayed very significant selectivities at various MCRs. For example, modifying the S−S bridge to give H-D-Phe-*c*[Cys-His-

Table 3. Analytical Data for Somatostatin Analogues

no.	serial no.	sequence	HPLC k'	MF[a]	MS $[M+H]^+$ calcd	MS $[M+H]^+$ found	amino acid analysis Phe	His	Arg	Thr
1	GH-11b	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2	3.36	$C_{51}H_{63}N_{15}O_9S_2$	1097.29	1097.4	2.00	0.93	0.98	0.95
2	GH-4c	H-D-Phe-*c*[Cys-His-D-Tic-Arg-Trp-Cys]-Thr-NH_2	4.12	$C_{52}H_{65}N_{15}O_9S_2$	1109.30	1108.9	0.96	0.95	1.10	0.94
3	GH-6b	H-D-Phe-*c*[Cys-His-D-Phe(*p*-Cl)-Arg-Trp-Cys]-Thr-NH_2	3.59	$C_{51}H_{64}ClN_{15}O_9S_2$	1131.73	1131.5	0.91	1.03	1.04	1.00
4	GH-6d	H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-Cys]-Thr-NH_2	3.68	$C_{55}H_{67}N_{15}O_9S_2$	1147.35	1146.5	1.00	0.97	1.11	1.00
5	GH-13	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Hcy]-Thr-NH_2	3.51	$C_{52}H_{69}N_{15}O_9S_2$	1111.30	1110.7	2.00	0.90	1.13	1.07
6	GH-4d	H-D-Phe-*c*[Hcy-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2	3.40	$C_{52}H_{67}N_{15}O_9S_2$	1111.31	1110.6	2.00	0.92	1.03	0.97
7	GH-15	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-Pen]-Thr-NH_2	3.63	$C_{53}H_{69}N_{15}O_9S_2$	1125.36	1124.9	1.84	0.85	1.10	0.86
8	GH-14a	H-D-Phe-*c*[Cys-His-D-Phe-Arg-Trp-D-Cys]-Thr-NH_2	3.29	$C_{51}H_{65}N_{15}O_9S_2$	1097.29	1197.4	1.90	0.90	1.09	1.08
9	GH-5a	H-D-Phe-*c*[Asp-His-D-Phe-Arg-Trp-Lys]-Thr-NH_2	3.35	$C_{55}H_{72}N_{16}O_{10}$	1118.26	1117.6	1.94	0.92	1.09	0.93
10	GH-6c	H-D-Phe-*c*[Cys-His(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2	3.33	$C_{52}H_{67}N_{15}O_9S_2$	1111.32	1111.4	1.94	-	1.09	1.00
11	GH-7b	H-D-Phe-*c*[Glu-His(3′-Me)-D-Phe-Arg-Trp-Lys]-Thr-NH_2	3.24	$C_{57}H_{76}N_{16}O_{10}$	1146.35	1145.7	1.90	-	1.06	1.00
12	GH-6a	H-D-Phe-*c*[Cys-D-His-D-Phe-Arg-Trp-Cys]-Thr-NH_2	3.34	$C_{51}H_{65}N_{15}O_9S_2$	1097.29	1096.7	2.00	0.91	1.16	1.01
13	GH-3a	H-D-Phe-*c*[Cys-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2	2.94	$C_{50}H_{63}N_{13}O_9S_2$	1055.25	1054.6	1.80	1.05	-	1.00
14	GH-3b	H-D-Phe-*c*[Pen-His-D-Phe-Orn-Trp-Cys]-Thr-NH_2	3.49	$C_{52}H_{67}N_{13}O_9S_2$	1083.32	1082.4	1.90	0.90	-	1.07
15	GH-21	H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Ser-Pen]-Thr-NH_2	3.79	$C_{50}H_{67}N_{13}O_{11}S_2$	1091.30	1090.8	0.94	-	1.11	0.90
16	GH-22	H-D-Phe-*c*[Cys-Tyr-D-Trp-Arg-Trp-Pen]-Thr-NH_2	4.43	$C_{58}H_{72}N_{14}O_{10}S_2$	1190.44	1189.6	0.97	-	1.10	0.94
17	GH-20	H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-D-Trp-Pen]-Thr-NH_2	4.81	$C_{58}H_{72}N_{14}O_9S_2$	1174.44	1173.5	1.94	-	1.10	0.88
18	GH-19	H-D-Phe-*c*[Cys-Phe-D-Trp-Arg-Trp-Pen]-Thr-NH_2	5.02	$C_{58}H_{72}N_{14}O_9S_2$	1174.44	1173.6	1.88	-	1.10	0.88
19	GH-17	H-D-Phe-*c*[Cys-Phe-D-Phe-Arg-Thr-Pen]-Thr-NH_2	4.03	$C_{49}H_{68}N_{12}O_{10}S_2$	1050.29	1049.5	2.76	-	1.10	1.97
20	GH-18	H-D-Phe-*c*[Cys-Phe(*p*-I)-D-Trp-Arg-Thr-Pen]-Thr-NH_2	4.72	$C_{51}H_{68}IN_{13}O_{10}S_2$	1215.23	1214.5	1.00	-	1.05	1.01
21	GH-16	H-D-Phe-*c*[Cys-Phe(*p*-I)-D-Trp-Arg-Thr-Cys]-Thr-NH_2	4.54	$C_{49}H_{64}IN_{13}O_{10}S_2$	1187.17	1186.4	0.90	-	1.06	1.08
22	GH-62	H-D-Phe-*c*[Cys-His-D-Phe(*p*-F)-Arg-Trp-D-Cys]-Thr-NH_2	NA	$C_{55}H_{664}FN_{15}O_9S_2$	1115.30	1114.8	1.00	0.98	1.08	0.95
23	GH-63	H-D-Phe-*c*[Cys-His-D-Phe(*p*-I)-Arg-Trp-D-Cys]-Thr-NH_2	NA	$C_{55}H_{664}IN_{15}O_9S_2$	1223.21	1222.5	1.00	0.94	1.10	1.03
24	GH-61	H-D-Phe-*c*[Cys-His-D-Nal(1′)-Arg-Trp-D-Cys]-Thr-NH_2	NA	$C_{55}H_{67}N_{15}O_9S_2$	1147.37	1146.6	1.00	0.93	1.10	1.05
25	GH-60	H-D-Phe-*c*[Cys-His-D-Nal(2′)-Arg-Trp-D-Cys]-Thr-NH_2	NA	$C_{55}H_{67}N_{15}O_9S_2$	1147.37	1146.5	1.00	0.96	1.06	0.98

[a] Molecular formula. [b] Not available.

D-Phe-Arg-Trp-Pen]-Thr-NH_2 (**7**) provided an analogue with 50- to 500-fold selectivities for the *m*MC5R (EC_{50} = 0.14 nM) versus the other *m*MCRs. To our knowledge, this is one of the most selective and potent agonists found so far for the *m*MC5R. In addition, modification of the His^6 with $His(3'\text{-Me})^6$ to give H-D-Phe-*c*[Cys-His-(3′-Me)-D-Phe-Arg-Trp-Cys]-Thr-NH_2 (**10**) produced an analogue which also displayed selectivities (range from 15 to 50-fold) at the *m*MC5R. Recent discoveries also demonstrate that position 5 (His) is crucial for binding at the MC5R selectively.[49] It is worth mentioning that neither **7** nor **10** displayed potent bioactivities at frog skin MC1R ($EC_{50} = 10^{-7}$ M for **7** and $EC_{50} = 10^{-5}$ M for **10**).

Overall, these results provide strong evidence for our hypothesis that peptide ligand scaffolds for one family of G-protein-coupled receptors (GPCRs) can be used to design ligands for other families of GPCRs.

Experimental Section

General Methods of Peptide Synthesis. All peptides were synthesized in a stepwise fashion via the solid-phase method, using manual peptide synthesis and 1 or 2% cross-linked, *p*-methylbenzhydrylamine (*p*MBHA) resin (0.30−0.45 mequiv/g, Peptides International, Louisville, KY). The protected amino acids incorporated in positions other than the amino terminus were purchased from commercial sources (BaChem California, Torrance, CA; Advanced ChemTech, Louisville, KY; PeptechCorp, Cambridge, MA; BaChem Science, King of Prussia, PA). The following amino acids were used in the synthesis: N^{α}-Boc-Arg(N^{G}-Tos)-OH, N^{α}-Boc-Asp-(OFm)-OH, N^{α}-Boc-Cys(pMb)-OH, N^{α}-Boc-His(Bom)-OH, N^{α}-Boc-Lys(Fmoc)-OH, N^{α}-Boc-Pen(pMb)-OH, N^{α}-Boc-Ser(Bn)-OH, N^{α}-Boc-Thr(Bn)-OH, N^{α}-Boc-Trp(CHO)-OH, and N^{α}-Boc-Tyr(2′,6′-Cl_2Bn)-OH. N^{α}-Boc protected amino acids were coupled to this resin using a 3-fold excess of the *N*-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (Dic), and the couplings were monitored by the Kaiser test.[80] Each natural amino acid was coupled in this fashion, while nonnatural amino acids were coupled using a 1.2−2-fold excess of *O*-(1-benzotriazolyl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU),[81−85] with HOBt (1 equiv) and diisopropylethylamine (DIPEA, DIEA). For side chain lactam bridge cyclization, the OFm and Fmoc side chain protecting groups were removed by Pd-$(PPh_3)_4$,[86] and a 3−4-fold excess of HBTU with HOBt (1−2 equiv) and DIPEA was used for cyclization.[87] The final N^{α}-Boc-peptide resin was directly cleaved by HF without removing the N-terminal Boc protecting group.[88] All the side chain protecting groups were cleaved by HF in the presence of scavengers [commonly used: thioanisole and *p*-cresol (1:1 mixture)],[89] as was the N-terminal Boc group. The crude peptide, scavengers and resin mixture was washed three times with anhydrous ether which was discarded. The filter cake, crude peptide, and resin mixture was washed successively with glacial acetic acid three times to dissolve the peptides followed by deionized water once. The solution mixture was then lyophilized.

A. Cyclization of Peptides with Two Free Thiol Groups (SH). The lyophilized linear crude peptide mixture was directly cyclized using potassium ferric(III) hexacyanide [K_3-$Fe(CN)_6$] following published procedures.[90] The cyclized crude peptide was subject to purification by HPLC using a preparative C-18 column (reverse phase), with a gradient, 90−10% B in 40 min [Component A: acetonitrile, Biograde, Fisher, Pittsburgh, PA; Component B: deionized water containing 0.1% (v/v) trifluoroacetic acid (TFA)]. The absorbance of the eluents was detected by a Rainin UVD detector, monitored at 230 and 280 nm. The fractions collected were then checked for purity by an analytical reverse HPLC column using a Hewlett-Packard HPLC 1090II with a diode array detector monitored at various wavelengths, typically at 230, 254, and 280 nm. The pure fractions were then pooled and lyophilized. Yields varied from 10% to 60% depending on the sequences (none of the syntheses were optimized for better yields).

Amino acid analysis was used to confirm the identity of the synthesized peptides and was performed at the University of Arizona Biotechnology Core Facility using an Applied Biosystems 420A Amino Acid Analyzer with automatic hydrolysis (vapor phase hydrolysis at 160 °C for 1 h using 6 N HCl) or with prior hydrolysis (110 °C for 24 h, using 6 N HCl), with precolumn phenylthiocarbamoyl-amino acid (PTA-AA) analysis. However, some amino acids are not reliably analyzed quantitatively under these conditions, such as Cys, Pen, and Trp residues all of which were destroyed to varying degrees. Positive ion fast atom bombardment mass spectroscopy (FAB-MS) was performed at the College of Pharmacy or Department of Chemistry at the University of Arizona. The analytical data for all compounds is given in Table 3.

B. For Peptides with No Thiol Groups. These peptides were directly purified following the protocol described above.

Bioassays at *Rana pipiens* Frog MC1R. The frogs, *R. pipiens*, were obtained from Nasco, Fort Atkinson, WI. Animals were killed by decapitation, and the skins from each animal were prepared for photo reflectance measurements following the methods of Shizume, Lerner, and Fitzpatrick,[91] Wright and Lerner,[92] and Huntington and Hadley.[93] Skins were placed in 50 mL beakers containing amphibian Ringer (NaCl, 111 mmol/L; $NaHCO_3$, 2 mmol/L; KCl, 2 mmol/L; $CaCl_2$, 1 mmol/L) at pH 7.3−7.5 for a 2 h pre-experimental equilibration period. During this time there was a slow perinuclear aggregation of melanosomes within melanophores which resulted in the skins becoming quite light in color. The skins were then placed in control or experimental solutions, and after a number of fresh changes of the respective solutions, 20 mL of solution were allowed to remain in each beaker to cover the skins.

Reflectance measurements were obtained from the outer surface of the skin and involved color changes, lightening (melanosome aggregation), or darkening (melanosome dispersion), resulting from intracellular melanin granule movements within integumental dermal melanophores in response to hormonal or pharmacological stimulation. The initial mean reflectance value for each group of skins was taken as the basal (zero) value, and succeeding average values were recorded as the percent change (or response) above or below the initial reading. Each value represented the mean (± SEM, of selected values) response of the skins under each experimental condition. In each experiment, the melanotropins were added at time zero. At various times thereafter the skins were transferred to fresh Ringer without the melanotropins. In most experiments, a control group of skins was maintained in Ringer without melanotropin. The noradrenaline and melatonin used in these studies were obtained from Sigma Chemical Company, St Louis, MO.

Bioassays at Mouse Melanocortin Receptors (*m*MCRs). A. Galactosidase Bioassay.[94] Cells stably expressing wild-type receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene. Briefly, 5000 to 15 000 post-transfection cells were plated into 96-well Primeria plates (Falcon) and incubated overnight. At 48 h post-transfection, the cells were stimulated with compound or peptide at concentrations indicated, or forskolin (10^{-4} M), in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated, and 50 μL of lysis buffer (250 mM Tris-HCl pH = 8.0 and 0.1% Triton X-100) was added. The plates were stored at −80 °C overnight. The plates containing the cells lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. The 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 200 mg of ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, OD_{405}, was measured using a 96-well plate reader (Molecular Devices). The relative protein was determined by adding 200 μL of 1:5 dilution BioRad G250 protein dye:water to the 10 μL cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and nonreceptor dependent forskolin stimulation. Data analysis and EC50 values were determined using nonlinear regression analysis with the PRISM program (v2.0, GraphPad Inc.).

Data Analysis. All data points are the means of duplicates of triplicate determinations if not defined otherwise, and standard deviations were determined. Data were plotted using Prism from GraphPad (San Diego, CA). Curves were fitted and EC_{50} values were determined by nonlinear regression analysis.

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