Novel Cyclic Templates of α-MSH Give Highly Selective and Potent Antagonists/Agonists for Human Melanocortin-3/4 Receptors

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In an effort to develop highly selective and potent agonists and/or antagonists for the hMC3 and hMC4 receptors, a new approach involving the use of linker arms and a backbone to side chain cyclization strategy was employed. Three key analogues were identified to have the required selectivity and potency at the hMC3 or hMC4 receptors, implicated to play pivotal roles in energy homeostasis and other biological effects. The novel cyclic peptide (O)C-CH_2-CH_2-C(O)-c-[His^6-*D*-Phe^7-Arg^8-Trp^9-Lys^{10}]-NH_2 (**1**) was found to be a highly selective and potent agonist of the hMC4 receptor. Structure−activity studies have shown that replacing the succinyl linker arm of **1** by an *o*-phthalic acid group and substituting a *D*-Nal(2′)^7 residue in place of *D*-Phe^7 results in a potent antagonist **7** at the hMC4 receptor. Furthermore, increasing the 23-membered lactam ring of **1** by one carbon atom (succinyl → glutaric acid linker) gives a highly selective and potent antagonist **9** for the hMC3 receptor. Analogues **1**, **7**, and **9** therefore represent the first examples of a class of cyclic melanotropin ligands with high selectivity and defined biological activities at the physiologically important hMC3 and hMC4 receptors.

Introduction

α-, β-, and γ-melanocyte-stimulating hormone (MSH) together with the adrenocorticotropic hormone form a group of endogenous neuropeptides that are derived by posttranslational processing and modification of the pro-opiomelanocortin (POMC) prohormone.[1] Several key physiological functions are attributed to this class of neuropeptides including skin pigmentation, erectile function,[2−5] blood pressure and heart rate,[6] and control of feeding behavior,[7−10] among others. The physiological activity of the melanocortins is modulated via five transmembrane G-protein-coupled receptors (GPCRs). The MC1 receptor is mainly expressed in melanocytes and leukocytes and has been implicated to play a role in skin pigmentation and inflammatory response.[11,12] The MC2 receptor is expressed only in the adrenal gland and mediates glucocorticoneogenesis.[13] The MC3 and MC4 receptors are both found in the brain especially in the arcuate nucleus although at different loci.[14] Both of these receptors have been implicated to play a role in the control of feeding behavior and energy homeostasis. Finally, the MC5 receptor, found in a variety of peripheral tissues, plays a role in regulating exocrine gland function.[15]

α-MSH was among the first peptide hormones to be isolated and its structure determined. Extensive studies of this hormone have confirmed its role in skin pigmentation as well as in various central nervous system (CNS) activities. Structure−activity relationships (SAR) of α-MSH resulted in the development of various potent, prolonged acting, and enzymatically stable analogues, such as [Nle,^{4}D-Phe^7]α-MSH (NDP-α-MSH, MT-I)[16] and the cyclic lactam analogue Ac-Nle^4-c[Asp^5,*D*-Phe^7, Lys^{10}]α-MSH-(4-10)NH_2 (MT-II).[17−19] Although these compounds have been widely used in biological studies of the melanocortin receptors, a drawback of these analogues is that they do not show significant selectivity for the different melanocortin receptors. Given the complexity of the biological functions controlled by these receptors, it has been difficult to draw simple correlations between the biological activities of various α-MSH analogues and the physiological functions of the melanocortin receptors. Thus, potent and more selective agonists and/or antagonists could serve as valuable tools for determining the role of the melanocortin receptors in humans.[20]

Recently, there has been a heightened interest in designing highly selective and potent agonists/antagonists at the human melanocortin 3 and 4 receptors (hMC3R/hMC4R), since these receptors play an important part in controlling feeding behavior[7,21−23] in humans. The observations that agonists at the hMC4R produce a feeling of satiety while antagonists at this receptor promote feeding has led to the hypothesis that highly selective and potent agonists at the hMC4 receptor might find therapeutic applications for treating obesity. Furthermore, it has been suggested that the hMC3 receptor is also physiologically involved in the control of energy partitioning;[23] therefore, selective ligands at this receptor in conjunction with a hMC4R agonist might provide a novel approach in the treatment of metabolic diseases.

This paper describes the design and development of the first highly selective and potent agonist at the hMC4R versus the hMC1R and versus other hMC receptors as well. It also describes the development of the first selective and potent antagonist at the hMC3 and hMC4 receptors. The novel cyclic lactams **1** (Figure 1),[24] **7**, and **9** (Figure 2) were designed as a modification of the (Figure 2) nonselective but potent agonist MT-II.

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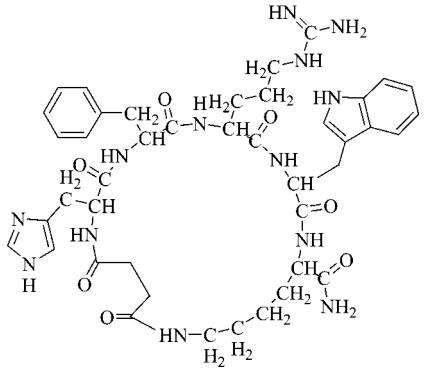
Table 1. Biological Activities of the Cyclic α-MSH Analogues at Various Human Melanocortin Receptors[a]

peptide		hMC3R			hMC4R			hMC5R		
serial no.	designation	K_i (nM)	EC_{50} (nM)	% act	K_i (nM)	EC_{50} (nM)	% act	K_i (nM)	EC_{50} (nM)	% act
1	VJH-085	77 ± 10	70 ± 5	95	5.7 ± 0.9	1.6 ± 0.1	100	660 ± 58	560 ± 100	67
2	MK-2	370 ± 48	1000 ± 100	53	0	>1000	0	0	>10 000	0
3	MK-3	0	>10 000	0	0	>10 000	0	0	>10 000	0
4	MK-4	1400 ± 190	42 ± 1.3	19	1700 ± 94	22 ± 2	35	0	>10 000	0
5	MK-5	12.1 ± 1.9	70 ± 10	88	87 ± 1.4	260 ± 20	35	140 ± 19	150 ± 10	100
6	MK-6	280 ± 27	13 ± 0.60	76	58 ± 9.4	70 ± 13	98	0	>5000	0
7	MK-7	7.8 ± 1.4	18 ± 4	36	57 ± 9.4	>10 000	0	5700 ± 19	640 ± 100	56
8	MK-8	110 ± 8.7	75 ± 0.01	100	670 ±14	50 ± 2	100	3100 ± 95	>10 000	0
9	MK-9	5.9 ± 0.19	>10 000	0	220 ± 13	1200 ± 200	22.8	26 ± 1.4	1.01 ± 0.090	81
10	MK-10	390 ± 19	790 ± 20	21	190 ± 22	2300 ± 100	60.5	990 ± 9.4	1300 ± 22	100
11	MK-11	140 ± 6.7	68 ± 0.50	100	630 ± 29	3000 ± 36	26.5	880 ± 130	1100 ± 86	100
	MT-II	1.2 ± 0.2	1.88 ± 0.2	100	1.01 ± 0.2	2.9 ± 0.52	100	6.96 ± 0.2	3.3 ± 0.70	100

[a] Cyclic analogues of MT-II (**1**) (O)C-$(CH_2)_2$-C(O)-c[His-D-Phe-Arg-Trp-Lys]-NH_2; (**2**) (O)C-$(CH_2)_2$-C(O)-c[His-D-Phe-Ala-Trp-Lys]-NH_2; (**3**) (O)C-$(CH_2)_2$-C(O)-c[His-Ala-Arg-Trp-Lys]-NH_2; (**4**) (O)C-$(CH_2)_2$-C(O)-c[Ala-D-Phe-Arg-Trp-Lys]-NH_2; (**5**) (O)C-$(CH_2)_2$-C(O)-c[His-D-Nal(2′)-Arg-Trp-Lys]-NH_2; (**6**) (O)C-(C_6H_4)-C(O)-c[His-D-Phe-Arg-Trp-Lys]-NH_2; (**7**) (O)C-(C_6H_4)-C(O)-c[His-D-Nal(2′)-Arg-Trp-Lys]-NH_2; (**8**) (O)C-$(CH_2)_3$-C(O)-c[His-D-Phe-Arg-Trp-Lys]-NH_2; (**9**) (O)C-$(CH_2)_3$-C(O)-c[His-D-Nal(2′)-Arg-Trp-Lys]-NH_2; (**10**) (O)C-$(CH_2)_2$-C(O)-c[Pro-D-Phe-Arg-Trp-Lys]-NH_2; (**11**) HO_2C-$(CH_2)_2$-C(O)-[His-D-Phe-Arg-Trp-Lys]-NH_2, (linear).



c[(O)C-CH_2-CH_2-C(O)-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10}]-NH_2

(1)

Figure 1. Full structure of the highly selective and potent agonist of the human melanocortin-4-receptor **1** is a modified cyclic lactam analogue of the nonselective and potent agonist.

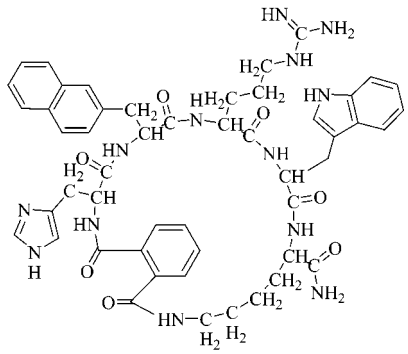
Interestingly, we found that the nature and size of the cyclic lactam ring plays an important role in selectivity at these receptors.

Results and Discussion

A. Analogues of α-MSH Cyclized Via Aliphatic Linker Arms. This study describes the development of a novel class of cyclic α-MSH analogues that are highly selective and potent agonist and/or antagonist ligands at the hMC3 and hMC4 receptors. Figure 1 shows the structure of analogue **1**, and Table 1 summarizes the biological activities of cyclic lactam derivatives (**1**−**10**) and of MT-II, a potent but nonselective agonist,[17,18] at various cloned human melanocortin receptors (hMC3R−hMC5R). It should be noted that **1** was the only analogue tested at the hMC1 receptor. Binding studies showed that **1** is 700-fold more selective at the hMC4R vs the hMC1R (IC_{50} hMC1R = 4200 nM and IC_{50} hMC4R = 6.0 nM). Additionally, this analogue is 55- and 120-fold more selective at the hMC4R vs the hMC3 and hMC5 receptors, respectively (Table 1). Intracellular cAMP accumulation assays showed that **1** is a superpotent agonist of the hMC4R with an EC_{50} value similar to MT-II, which was used as a control in these experiments (EC_{50} **1** = 1.5 nM; EC_{50} MT-II = 2.87 nM). Furthermore, these studies also showed that **1** is a weak agonist at the hMC3 and hMC5 receptors, with EC_{50} values 38- and 170-fold greater than that of MT-II at the hMC3 and hMC5 receptors, respectively (Table 1). Interestingly, this analogue is able to stimulate intracellular cAMP to its near maximum level at a concentration greater than 10 μM. Similar results were reported by Bednarek et al. for this compound.[25] In an attempt to elucidate the energetic contributions toward binding due to cyclization of the peptide, the linear analogue **11** (Table 1) was synthesized and tested for its ability to bind and stimulate cAMP in vitro. The biological activity results indicate that the linear analogue binds approximately 2 orders of magnitude weaker at all three hMC receptors suggesting a substantial entropic contribution due to cyclization of the peptide toward binding.

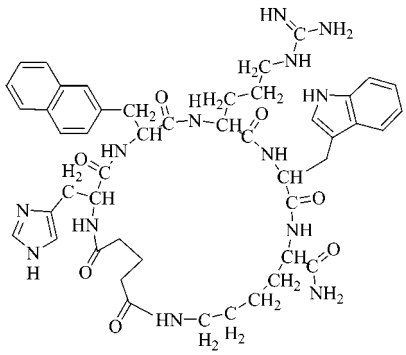
To elucidate the structural requirements for binding and bioactivity at the hMC receptors for **1**, an alanine scan was performed. The alanine-substituted analogues **2**−**4** displayed very weak binding interactions at all three hMC receptors (Table 1). However, **2** and **4** were found to be weak partial agonists at the hMC3 receptor with EC_{50} values of 1000 and 42 nM, respectively. In addition, these two analogues showed no detectable cAMP stimulation at the hMC4 and hMC5 receptors, even at concentrations greater than 10 μM. Taken together, these results suggest that the requirements for tight binding are different from those required for partial agonist activity. The weak binding affinities of **2** and **4** at the hMC3R receptor (K_i values in the micromolar range) are presumably due to the loss in either electrostatic or hydrophobic interactions between these ligands and the hMC3 receptor, when Arg^8 and His^6 residues are replaced with an aliphatic group. However, both peptides stimulate only low levels of intracellular cAMP in CHO cells expressing the hMC3 receptors. This suggests that the topographical requirements for receptor activation differ from those for binding of ligands to the receptor and that only a few percent of the receptors need to be occupied for partial agonist/antagonist activity. These findings further reinforce[26] the importance of the core amino acid message sequence, His-*D*-Phe-Arg-Trp, in the formation of stable ligand−receptor complexes.

Finally, to test the importance of hydrophobic interactions at position seven of the message sequence, ana-



c[(O)C-C_6H_4-C(O)-His^6-DNal(2')^7-Arg^8-Trp^9-Lys^{10}]-NH_2

(7)



c[(O)C-CH_2-CH_2--CH_2-C(O)-His^6-DNal(2')^7-Arg^8-Trp^9-Lys^{10}]-NH_2

(9)

Figure 2. Key analogues of **1**: (A) **7** synthesized by introducing an *o*-phthalic acid in the cyclization linker of **1**; (B) **9** synthesized by replacing D-Phe^7 with *D*-Nal(2′)^7 and by replacing the cyclization linker of **1** by a glutaric acid linker.

logue **5** was synthesized by replacing D-Phe^7 in **1** with a bulky hydrophobic residue *D*-Nal(2′).[7] This substitution resulted in a loss of binding affinity at the hMC4R by a factor of 15-fold when compared to **1** (Table 1). Interestingly, **5** shows a 7−12-fold increased selectivity for the hMC3 receptor (K_i = 12.1 nM) vs the hMC4 (K_i = 87 nM) and the hMC5 (K_i = 140 nM) receptors. This loss in binding at the hMC4 receptor may be due to steric hindrance between the larger side chain group of *D*-Nal(2′)^7 in **5** and the amino acid residues that form the receptor pocket. This suggests that hydrophobic interactions are more important in binding at the hMC3R than at the hMC4 or hMC5 receptors. Alternatively, it might suggest that the hMC3 receptor pocket is more flexible and is better able to accommodate bulky substituents as compared to the other hMC receptors. Whatever may be the combination of factors that explain this change in receptor selectivity, it is evident that steric bulk promotes hMC3R selectivity and suggests that a rational approach toward the development of hMC3R selective ligands is to further increase the hydrophobic nature of these cyclic peptides.

B. Analogues of α-MSH Cyclized Via Novel Hydrophobic Linker Arms. Peptides **6** and **7** are analogues of **1** and **5**, respectively. These were synthesized by replacing the succinic acid linker with a hydrophobic *o*-phthalic acid linker (Figure 2). This modification resulted in greater binding affinity for both peptides **6** and **7** at the hMC3 receptor vs the hMC4 and hMC5 receptors (Table 1).

Table 2. Selectivity of the Cyclic α-MSH Analogues to hMC4 and hMC3 Receptors

pept	hMC1R/ hMC4R	hMC3R/ hMC4R	hMC5R/ hMC4R	hMC1R/ hMC3R	hMC4R/ hMC3R	hMC5R/ hMC3R
1	700	13.3	120	52.5	0.07	8.9
7	nd	0.13	103	n.d.	7.5	755
9	nd	0.03	0.12	n.d.	38	4.5

Analogue **6** with a *D*-Phe^7 substitution and an *o*-phthalic acid linker arm has a similar binding affinity as that of **1**, cyclized via a succinic acid linker (K_i of **6** at hMC3R = 280 nM and K_i of **1** at hMC3R = 77 nM). Interestingly, when a *D*-Nal(2′)^7 residue and *o*-phthalic acid linker are both introduced together into the cyclic motif (analogue **7**), a 35-fold increase in binding affinity at the hMC3 receptor is observed (K_i of **7** at hMC3R = 7.8 nM and K_i of **6** at hMC3R = 280 nM). Compound **7** also showed an 8-fold increase in selectivity (Table 2) for the hMC3 vs the hMC4 receptor (K_i **7** hMC3R = 7.8 nM; K_i **7** hMC4R = 57 nM), whereas its selectivity for the hMC3 receptor is approximately 3 orders of magnitude greater than that observed for the hMC5 receptor (K_i **7** hMC3R = 7.8 nM; K_i **7** hMC5R = 5700 nM). Thus, it appears that tighter binding at the hMC3 receptor with the *o*-phthalic group in the linker arm is probably due to a combination of two factors: (i) an increase in the hydrophobicity of the ligand and (ii) a gain in binding energy associated with restricting the rotation of the carbon−carbon bond in the succinyl linker by an aromatic ring (CH_2−CH_2 → C_6H_4).

In vitro cAMP studies with **6** and **7** showed that both peptides were partial agonists at the hMC3 receptor. Interestingly, it was observed that substituting the *D*-Phe^7 residue in **6** by *D*-Nal(2′)^7 does not make any difference in the efficacy of analogue **7** (Table 1). With regards to the partial agonist activity, **7** was found to be leaning more toward antagonism when compared to **6**, which further indicates that increasing the hydrophobic nature of residues at position seven promotes hMC3 receptor antagonism. In the case of the hMC4 receptor, a similar substitution [*D*-Phe^7 → *D*-Nal(2′)^7] leads to a potent antagonist (pA_2 **7** = 9.5; K_i = 57 nM). The above findings suggest that this hydrophobic substitution at position seven promotes partial agonist/antagonist behavior at the hMC3 and hMC4 receptors as was the case in the MT-II series.[20] However, at the hMC5 receptor, a similar substitution (*D*-Phe^7 → *D*-Nal(2′)^7) leads to the development of an analogue with reduced biological activity.

C. Ring-Expanded Lactam Analogues of α-MSH. The size of the cyclic lactam ring can play an important role in determining the three-dimensional solution conformation of the peptide ligand and also can contribute in fixing the topography of the side chain group in the ligand presented to the receptor. To elucidate the role of lactam ring size in receptor selectivity and efficacy, the succinyl linker was replaced by a glutaric acid linker, which increases the ring size from a 23-membered lactam to a 24-membered lactam (analogues **8** and **9**), respectively. Analogue **9** differs from **8** with the substitution of a *D*-Nal(2′) residue at position seven in place of *D*-Phe. Binding potencies of peptides **8** and **9** at the hMC3, hMC4, and hMC5 receptor parallel the

general trend observed above (analogues **6** and **7**) of improved binding with increasing hydrophobicity at position seven. In addition, both of the peptides had greater affinity for the hMC3 receptor versus the hMC4 and hMC5 receptors, indicating that the 24-membered ring is preferred for hMC3 receptor selectivity (Table 1) with **9** being 40-fold more selective at the hMC3R versus the hMC4R (Table 2).

With regards to the in vitro biological activity at the hMC3R, it was found that analogue **8** is a full agonist (EC_{50} = 75 nM), whereas analogue **9** is a potent antagonist (K_i = 5.9 nM; pA_2 = 10.6). In the case of the hMC4 receptor, analogue **8** shows full agonist activity (EC_{50} = 50 nM) while **9** is a partial agonist. Interestingly, **8** did not stimulate cAMP at the hMC5 receptor up to a concentration of 10 μM suggesting it could be a weak antagonist. However, analogue **9**, a potent antagonist of the hMC3 receptor, was found to be a full agonist at the hMC5R (EC_{50} = 1 nM; Table 1). This compound (**9**) represents the first example of a selective and potent antagonist at the hMC3 receptor.

Generally, proline substitution in peptides is known to induce a turnlike structure. Grieco et al. have shown that substituting His^6 for Pro^6 in the MT-II template (side chain to side chain cyclization) greatly improves its selectivity for the hMC5 receptor.[27] To test if a similar substitution in the cyclic lactam motif would also enhance receptor selectivity, analogue **10**, having backbone to side chain cyclization via a succinyl linker, was synthesized. However, this compound showed poor binding at all three hMC receptors with K_i values in the low micromolar range. Although the molecular basis for poor selectivity and binding potency of analogue **10** is unclear, one can speculate that in **10** the aliphatic linker arm provides a conformational constraint that may, in turn, hinder receptor−ligand interaction. Next, the ability of **10** to stimulate intracellular cAMP at the hMC3 and hMC4 receptors was determined and it was observed that this compound had weak agonist activity at both of these receptors. In contrast, this analogue is able to fully stimulate the hMC5 receptor, at the high concentration of 10 μM (EC_{50} = 1285 nM, Table 1).

Conclusions

In an effort to develop highly selective and potent agonists and/or antagonists for the hMC3 and hMC4 receptors, a series of novel cyclic analogues with backbone to side chain cyclization were developed and their biological activities were evaluated. It was found that lactam ring size and the nature of the linking arm are both important for selectivity and potency at the hMC3 and hMC4 receptors. The cyclic lactam **1** (23-membered ring) was designed by modifying the nonselective but highly potent agonist MT-II. Although the molecular basis for the selectivity of **1** at the hMC4R is not clearly understood, this study reveals several key structural features in the **1** template that may play an important role in its selectivity and potent agonist activity at the hMC4 receptor. The above SAR studies demonstrate that using a (succinyl) linker arm to cyclize between the α-amino group of His^6 (N-terminus) and the ϵ-amino group of Lys^{10} (C-terminus) greatly improves hMC4R selectivity. It is noteworthy that introducing a *D*-Nal(2′)^7 residue in place of *D*-Phe^7 lowers hMC4R binding affinity. This result is in contrast to our earlier finding[20] that a similar substitution in MT-II converts this potent agonist to a potent antagonist (SHU9119).

Modifying the succinyl linker to a hydrophobic *o*-phthalic acid moiety and maintaining the 23-membered lactam ring (analogues **6** and **7**) changed receptor selectivity from hMC4 to hMC3. In terms of the biological activity of these two analogues, it was observed that the combination of a bulky *D*-Nal(2′)^7 residue and a *o*-phthalic acid group in the linker arm gives a relatively potent antagonist of the hMC4 receptor (**7**; K_i = 57 nM; pA_2 = 9.5). On the other hand, increasing the ring size to a 24-membered lactam (**9**) resulted in a highly selective and potent antagonist of the hMC3 receptor (K_i = 5.9 nM; pA_2 = 10.6). Thus, it appears that modifying the ring size alters the topography of the peptide sufficiently to change its interactions with the hMC3 receptor as well as its antagonist activity.

In conclusion, this strategy illustrates a general method for developing new selective and potent ligands for human melanocortin receptors as well as other physiologically important peptide hormones. These newly developed melanotan analogues may find therapeutic use for treating metabolic diseases such as anorexia and obesity and can also be used as biochemical tools for determining the physiological role played by the hMC3 and hMC4 receptors in feeding disorders and erectile function.

Experimental Section

Materials. All peptides designed in this investigation were synthesized manually by solid phase methods. N^{α}-Fmoc-protected amino acids and Rink amide resin were purchased from Nova Biochem (U.S.A.). HBTU and HOBt were purchased from Quantum Biotechnologies (Montreal, Canada). The following side chain protecting groups were used, $Lys(N^{\epsilon}$-Alloc), $Trp(N^{in}$-Boc), $Arg(N^{\epsilon}$-Pbf), and $His(N^{im}$-Trt). Peptide synthesis solvents and reagents were purchased from the following: Trifluoroacetic acid was purchased from Chem-Impex International (Wood Dale, IL); piperidine, tetrakis(triphenyphosphine)palladium(0), and phenylsilane were purchased from Aldrich (Milwaukee, WI); DCM, DMF, and high-pressure liquid chromatography (HPLC) quality acetonitrile were purchased from J. T. Baker (Phillipsburg, NJ) and used without further purification unless otherwise noted. All amino acids are in the *L*-configuration unless otherwise noted. The purity of the peptides was checked by thin-layer chromatography (TLC) in three different solvent systems, by analytical reverse-phase HPLC using Vydac C_{18} column (catalog no. 218TP104) monitored at 220, 254, 280, and 350 nm and by characterization by electrospray mass spectroscopy (Table 3).

TLC was performed using Merck silica gel 60 F-254 plates (0.25 mm layer thickness), and solvent systems were used as follows: (A) 1-butanol/acetic acid/pyridine/water (5:4:1:4); (B) 1-butanol/acetic acid/pyridine/water (4:1:1:3); and (C) ethyl acetate/acetic acid/pyridine/water (6:2:2:1). The peptides were detected on the TLC plate using iodine vapor. The purification of the peptides was achieved using a Hewlett-Packard 1100 Series HPLC fitted with a Hewlett-Packard 1100 Series variable wavelength UV detector for semipreparative HPLC on C_{18}-bonded silica columns (Vydac, Hesperia, CA; catalog no. 218TP1010). The peptides were eluted using a linear gradient of acetonitrile in 0.1% aqueous TFA (10:90 to 100% acetonitrile over 40 min) at a flow rate of 15 mL/min. The separations were monitored using a dual wavelength detector at 230 and 280 nm.

Peptide Synthesis. General Protocol for N^{α}-Fmoc Chemistry. The cyclic melanotropin analogues used in this investigation were synthesized manually using N^{α}-Fmoc chemistry. Rink amide resin (4-(2′,4′-dimethoxyphenyl-Fmoc-ami-

Table 3. Analytical Data for New Melanotropin Ligands

structure	TLC R_f[a] A	TLC R_f[a] B	TLC R_f[a] C	HPLC k'[b]	HRMS[c] obsd	HRMS[c] calcd
1, (o)C-$(CH_2)_2$-C(O)-c[His-D-Phe-Arg-Trp-Lys]NH_2	0.78	0.625	0.36	3.87	854.4	853.43
2, (o)C-$(CH_2)_2$-C(O)-c[His-D-Phe-Ala-Trp-Lys]NH_2	0.8	0.66	0.56	2.8	769.3	768.37
3, (o)C-$(CH_2)_2$-C(O)-c[His-Ala-Arg-Trp-Lys]NH_2	0.71	0.6	0.1	1.64	778.5	777.4
4, (o)C-$(CH_2)_2$-C(O)-c[Ala-D-Phe-Arg-Trp-Lys]NH_2	0.8	0.72	0.65	3.06	788.4	787.41
5, (o)C-$(CH_2)_2$-C(O)-c[His-D-Nal (2′)-Arg-Trp-Lys]NH_2	0.81	0.67	0.4	3.23	904.7	903.45
6, (o)C-C_6H_4-C(O)-c[His-D-Phe-Arg-Trp-Lys]NH_2	0.78	0.65	0.42	4.21	902.7	901.43
7, (o)C-C_6H_4-C(O)-c[His-D-Nal (2′)-Arg-Trp-Lys]NH_2	0.79	0.68	0.46	4.35	952.46	951.45
8, (o)C-$(CH_2)_3$-C(O)-c[His-D-Phe-Arg-Trp-Lys]NH_2	0.77	0.63	0.35	3.45	868.5	867.45
9, (o)C-$(CH_2)_3$-C(O)-c[His-D-Nal(2′)-Arg-Trp-Lys]NH_2	0.787	0.648	0.361	2.95	918.47	917.47
10, (o)C-$(CH_2)_2$-C(O)-c[Pro-D-Phe-Arg-Trp-Lys]NH_2	0.80	0.696	0.59	3.38	814.5	813.43
11, (o)C-$(CH_2)_2$-C(O)-[His-D-Phe-Arg-Trp-Lys]NH_2	0.81	0.769	0.75	3.51	996.1	996.5

[a] R_f values using thin-layer silica gel chromatography in the following three solvent systems: (A) 1-butanol/pyridine/acetic acid/water (5:1:4:4); (B) 1-butanol/pyridine/acetic acid/water (4:1:1:3); and (C) ethyl acetate/pyridine/acetic acid/water (6:2:2:1). [b] HPLC $k' = [$(peptide retention time − solvent retention time)/solvent retention time] in a solvent of 10% CH_3CN in 0.1% TFA and a gradient to 90% CH_3CN over 40 min. An analytical Vydac C_{18} column was used with a flow rate of 1 mL/min. [c] High-resolution MS.

nomethyl)phenoxy resin, substitution 0.4−0.7 mmol/g) was swollen in DCM:DMF (1:1) overnight. The resin was washed with DMF (3 × 10 mL), and the N^{α}-Fmoc protecting group was removed by 25% piperidine in DMF (1 × 5 min and 1 × 20 min). The resin was washed with DMF (3 × 5 mL × 1 min) and then with DCM (3 × 5 mL × 1 min), and the first N^{α}-Fmoc amino acid was coupled using preactivated ester (3 equiv of N^{α}-Fmoc amino acid, 3 equiv of HOBt, and 3 equiv of HBTU) in DMF solution containing 6 equiv of DIEA and stirred at room temperature for 60 min under an inert atmosphere. At the end of the coupling, the resin was washed with DMF (3 × 10 mL) and DCM (3 × 10 mL), and a Kaiser ninhydrin test was done to check the extent of coupling. If the test was positive, the coupling was repeated for another hour. If double coupling did not result in a negative Kaiser test, the resin was washed with DMF and DCM and the unreacted amino groups were capped using acetic anhydride in DMF for 30 min. When coupling was complete, the resin was once again washed with DMF followed by DCM and the same procedure was repeated to couple the remaining amino acids in the desired sequence.

General Procedure for Cyclization of Peptides Via Lactam Bridge. The fully protected resin-bound peptide containing an Alloc protecting group for the C-terminal lysine ϵ-amino group and an Allyl ester organic linker attached to the N-terminal residue was placed in a glass vessel fitted with a sintered glass filter and argon inlet. The peptide-bound resin was constantly agitated with argon gas as an inert atmosphere within the reaction vessel was critical for the catalytic deprotection of the Allyl and Alloc groups.[28] The mouth of the reaction vessel was closed via a Teflon-coated cap, and the used solvents and reagents were removed from the reaction vessel by filtration.

In a typical experiment, cyclization was carried out on the resin-bound peptide through the use of various organic dicarboxylic acid linkers to connect the α-amino group of the N-terminal residue to the ϵ-amino group of lysine at the C-terminal end of the peptide (backbone to side chain strategy). The organic dicarboxylic acid linkers had previously been derivatized to their mono allyl esters as mentioned above. To the resin-bound peptide in DMF (∼3 mL) was added a DMF solution of the appropriate mono allyl ester (3 mol equiv), HOBt (3 mol equiv), HBTU (3 mol equiv), and DIPEA (6 mol equiv). After coupling was complete (monitored via Kaiser test), the Allyl and Alloc groups were simultaneously removed by treating the peptide resin with tetrakis(triphenyphosphine)-palladium(0) (0.2 mol equiv) and triphenylsilane (25 mol equiv) in DCM for 30 min at room temperature.[28] Then, the resin was washed with DCM (3 × 5 mL), then DMF (3 × 5 mL), and finally DCM, and the deprotection was repeated.

The free carboxyl group of the linker and ϵ-amino group of Lys were then coupled to give the cyclic peptide using HOBt/HBTU/DIPEA in DMF as mentioned above. After cyclization, the peptide resin was washed with DMF (3 × 15 mL), followed by DCM (3 × 15 mL) and diethyl ether (3 × 10 mL). The resin was dried overnight under vacuum.

General Procedure for Cleavage and Deprotection of Peptides. The cleavage cocktail consisting of TFA (8.5 mL), Et_3SiH (0.7 mL), and water (0.8 mL) was chilled on ice.[29] The cold solution was then added to the resin-bound protected peptide (1 g, 0.4 mmol) placed in a clean glass vial having a Teflon-coated screw cap. The glass vial was capped, and the reaction mixture was gently agitated for 2.5 h at room temperature using a mechanical shaker. The reddish solution was then filtered through a Pasteur pipet plugged with some cotton wool. The resin was washed with TFA (4−5 mL), and the solution was filtered off. The volume of the combined cleavage cocktail was reduced to 4 mL using a stream of argon gas, and the crude peptide was precipitated out by the addition of diethyl ether (40 mL) to give a white solid. The organic layer was decanted off after centrifugation of the peptide for 5 min at 12,000 rpm. The precipitate was washed with diethyl ether (3 × 40 mL), and after the final centrifugation, the peptide was dried in vacuo overnight.

General Procedure for Purification of Peptides. The crude peptide was first dissolved in a 1:1 mixture of acetonitrile:0.1% aqueous TFA, and the insoluble impurities were removed by passing the solution through a syringe filter (Gelman Laboratory, Acrodisc, 25 mm Syringe Filter with 1 μM Glass Fiber Membrane). The crude peptides were purified by reverse-phase HPLC using a Vydac (C_{18}-bonded, 300 Å; 10 mm × 25 cm) column and a gradient elution at a flow rate of 3 mL/min. The gradient used was 10−30% acetonitrile in 0.1% aqueous TFA over 30 min followed by 30−60% acetonitrile in 0.1% aqueous TFA from 30 to 40 min, and then 60−100% acetonitrile in 0.1% aqueous TFA from 40 to 50 min. Approximately 7 mg of crude peptide was injected each time, and the fractions containing the purified peptide were collected and lyophilized to dryness. The extent of purity was monitored by analytical RP HPLC using an analytical Vydac (C_{18}-bonded) column monitored at 220, 254, 280, and 350 nm, flow rate 1 mL/min, using a gradient of 10−90% acetonitrile in 0.1% aqueous TFA over 40 min. The analytical data for each compound are given in Table 3. The purity of all of the final peptides was found to be >95%.

Receptor Binding Assay. Competition binding experiments were performed on whole cells. Transfected HEK293 cell lines with hMCRs[30,31] were seeded on 24 well plates, 48 h before assay, 50 000 cells/well. For the assay, the medium was removed and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated with different concentrations of unlabeled peptide and labeled ^{125}I-[Tyr^2]-[Nle,^{4}D-Phe^7]α-MSH (PerkinElmer Life Science, 100 000cpm/ well, 0.1386 nM) for 40 min at 37 °C. The medium was subsequently removed, and each well was washed twice with the assay buffer. The cells were lysed by the addition of 500 μL of 0.1 NaOH and 500 μL of 1% Triton X-100. The lysed cell was transferred to the 12 mm × 75 mm

glass tubes and counted by Wallac 1470 WIZARD Gamma Counter. Data were analyzed using Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

Adenylate Cyclase Assay. HEK293 cells transfected with human melanocortin receptors were grown to confluence in MEM medium (GIBCO) containing 10% fatal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 24 well plates 48 h before assay (50 000 cells/well). For the assay, the medium was removed and cells were rinsed with 1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with isobutylmethylxanthine (IBMX; 5 μL; 0.5 mM) for 1 min at 37 °C. Varying concentrations of melanotropins (0.1 mL) were added, and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the buffer and adding ice cold Tris/EDTA buffer to each well (0.15 mL). The 24 well plates were covered and placed on ice. After the cells were dislodged with the help of a cell scrapper, the suspension of cells was transferred to polypropylene microcentrifuge tubes, capped, and placed in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min (6500 rpm), and 50 μL of the supernatant was aliquoted into a clean Eppendorf tube. The cAMP content was measured by competitive binding assay according to the assay kit instructions (TRK 432, Amersham Corp.).

Data Analysis. K_i and EC_{50} values represent the mean of duplicate experiments performed in triplicate. K_i and EC_{50} estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis,[32] with the help of Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

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. Additional abbreviations are used as follows: Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; 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; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyl-uronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; Pbf, 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl; RP HPLC, reverse phase high-performance liquid chromatography; TFA, trifluoroacetic acid; Trt, triphenylmethyl (trityl). Amino acid symbols represent the *L*-configuration unless denoted otherwise. All amino acids used are of the *L*-configuration unless otherwise noted.

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Supporting Information Available: Proton NMR spectra and list of chemical shifts and coupling constants for compounds **1**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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