Structure−Activity Relationships of the Melanocortin Tetrapeptide Ac-His-DPhe-Arg-Trp-NH₂ at the Mouse Melanocortin Receptors. 1. Modifications at the His Position

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The melanocortin pathway is an important participant in obesity and energy homeostasis. The centrally located melanocortin-3 and melanocortin-4 receptors (MC3R, MC4R) are involved in the metabolic and food intake aspects of energy homeostasis and are stimulated by melanocortin agonists such as α-melanocyte stimulation hormone (α-MSH). The melanocortin agonists contain the putative message sequence "His-Phe-Arg-Trp", and it has been well documented that inversion of chirality of the Phe to DPhe results in a dramatic increase in melanocortin receptor potency. Herein, we report a tetrapeptide library based on the template Ac-His-DPhe-Arg-Trp-NH₂, consisting of 17 members that have been modified at the His[6] position (α-MSH numbering) and pharmacologically characterized for agonist activity at the mouse melanocortin receptors MC1R, MC3R, MC4R, and MC5R. These studies provide further experimental evidence that the His[6] position can determine MC4R versus MC3R agonist selectivity and that chemically nonreactive side chains may be substituted for the imidazole ring (generally needs to be side chain protected in synthetic schemes) in the design of MC4R-selective, small-molecule, non-peptide agonists. Specifically, the tetrapeptide containing the amino-2-naphthylcarboxylic acid (Anc) amino acid at the His position resulted in a potent agonist at the mMC4R (EC_{50} = 21 nM), was a weak mMC3R micromolar antagonist (pA_2 = 5.6, K_i = 2.5 μM), and possessed >4700-fold agonist selectivity for the MC4R versus the MC3R. Substitution of the His[6] amino acid in the tetrapeptide template by the Phe, Anc, 3-(2-thienyl)alanine (2Thi), and 3-(4-pyridinyl)alanine (4-Pal) resulted in equipotency or only up to a 7-fold decrease in potency, compared to the His[6]-containing tetrapeptide at the mMC4R, demonstrating that these amino acid side chains may be substituted for the imidazole in the design of MC4R-selective non-peptide molecules.

Introduction

The melanocortin receptors belong to the superfamily of seven transmembrane-spanning G-protein-coupled receptors (GPCRs) and stimulate the cAMP signal transduction pathway.[1] The endogenous agonist ligands for these melanocortin receptors are derived from post-translational processing of the pro-opiomelanocortin (POMC) gene transcript, which upon differential processing results in the generation of the α-, β-, and γ-melanocyte stimulating hormones (MSH) and adrenocorticotropin (ACTH). All these melanocortin peptide agonists contain a core His-Phe-Arg-Trp tetrapeptide sequence that has been attributed to the ligand selectivity and stimulation of the melanocortin receptors.[2−4] The melanocortin receptor family also has two endogenous antagonists, agouti[5] and the agouti-related protein (AGRP),[6,7] which are the only known naturally occurring antagonists of GPCRs discovered to date. The centrally located melanocortin-3 and -4 receptors (MC3R, MC4R) have been identified in knockout mice to be involved in feeding behavior, obesity, metabolism, and energy homeostasis.[8−10] The most well-studied melanocortin receptor ligands are for the skin melanocortin-1 receptor (MC1R), which are involved in pigmentation

and animal coat coloration.[11−13] Additionally, the melanocortin-5 receptor has been deleted from the mouse genome and identified as playing a role in exocrine gland function.[14]

The role of the His amino acid at the 6 position of α-MSH has not been previously explored in extensive detail throughout the literature, although an invention disclosure has been issued detailing modifications at the His position.[15] A peptide modified at the His position of the SHU9119 template (Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH₂)[16] with (1-Me)His resulted in conversion of the SHU9119 mMC5R agonist into an antagonist.[17] Modification of the His[6] by Pro in the MTII peptide template (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH₂)[18,19] resulted in the identification of modifications that might lead to increased MC4R selectivity versus the MC3R.[20−22] More recently, modification of the MTII lactam cyclization ring size of peptides containing the His-DPhe-Arg-Trp sequence resulted in the identification of 50-fold[23] and 90-fold[24] MC4 versus MC3 receptor selectivity. Incorporation of the unusual amino acid Atc in its racemic form (Figure 1) at the 6 position in the peptide c[Asp-(racemic)Atc-DPhe-Arg-Trp-Lys]-NH₂ resulted in a peptide possessing 65 nM agonist activity at the human MC4R while possessing only slight agonist activity at the hMC3R, resulting in the most MC4R versus MC3R selective compound disclosed to date.[25]

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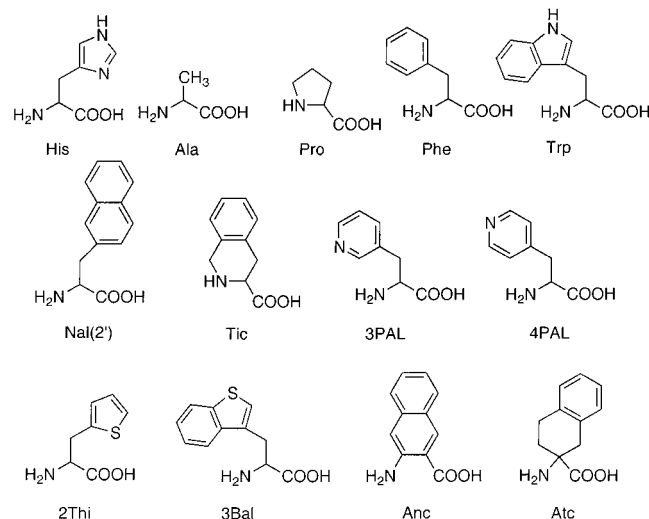
Figure 1. Structures of the amino acids used to replace His in the peptide template Ac-Xaa-DPhe-Arg-Trp-NH$_2$.

This latter report also made several other amino acid substitutions and Atc derivatives at the His position, resulting in interesting pharmacological properties at the melanocortin receptors.[25] Substitution of the His amino acid by Pro, DPro, Glu, Gly, and Ala in various cyclic peptide templates has been reported by different research laboratories, generally resulting in decreased peptide potency for the melanocortin receptors.[20−22,24,26,27] Interestingly, upon deletion of the His amino acid in the Ac-His-DPhe-Arg-Trp-NH$_2$ tetrapeptide, at the human MC4R only 2-fold decreased potency was observed[28] while at the mouse MC4R 220-fold decreased potency was observed,[29] with a loss of full agonist activity at the mMC3R and 170- and 480-fold decreased potency at the mMC1R and mMC5R, respectively.[29] These latter results demonstrate that the 6 position of the melanocortin peptides (α-MSH numbering) may be important for receptor selectivity and potency in the Ac-His-DPhe-Arg-Trp-NH$_2$ tetrapeptide template. This study was undertaken to examine the role of various aromatic, natural, and unnatural amino acids in the His position of the tetrapeptide Ac-His-DPhe-Arg-Trp-NH$_2$ for structure−activity-relationships and selectivity properties at the mouse melanocortin receptors.

Results

Chemical Synthesis and Characterization. The peptides reported herein were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc)[29,30] chemistry and a parallel synthesis strategy on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY). The peptides were purified to homogeneity using semipreparative reversed-phase high-pressure liquid chromatography (RP-HPLC). The purities of these peptides were assessed by mass spectrometry (Table 2), analytical RP-HPLC in two diverse solvent systems (Table 2), and one-dimensional ^{1}H NMR (NMR data in Supporting Information). Previously, we have synthesized and reported[29] the Ac-His-DPhe-Arg-Trp-NH$_2$ (included herein), Ac-His-Phe-Arg-Trp-NH$_2$, Ac-His-Phe-Arg-DTrp-NH$_2$, and Ac-His-DPhe-Arg-DTrp-NH$_2$ diastereomeric peptides as having distinct analytical RP-HPLC k' values in two diverse solvent systems.

Biological Evaluation. Table 1 summarizes the His^6-substituted tetrapeptide agonist pharmacology at the mouse melanocortin receptors MC1R, MC3R, MC4R, and MC5R.

Mouse Melanocortin-1 Receptor. The peripheral skin melanocortin receptor, MC1R, is involved in human skin pigmentation[12,31] and animal coat coloration.[13] The lead tetrapeptide **1**, Ac-His-DPhe-Arg-Trp-NH$_2$, has been previously reported to possess 25 nM stimulatory activity at the mMC1R,[29] an EC$_{50}$ value of 200 nM in the classical *Rana pipiens* frog skin assay (putative MC1R),[4] and possesses an mMC1R EC$_{50}$ of 20 nM reported herein. Substitution at the His^6 position within this tetrapeptide by Pro, Phe, DPhe, Trp, DTrp, Nal-(2′), DNal(2′), Tic, DTic, 3PAL, 4PAL, 2Thi, 3Bal, Anc, Atc, and DHis (Figure 1) all resulted in 9- to 570-fold decreased agonist activity at the mMC1R. Removal of the imidazole His side chain and replacement by the methyl side chain of Ala (**2**) resulted in 90-fold decreased potency. The Pro^6-containing tetrapeptide **3** resulted in 211-fold decreased potency, while the Phe^6- and DPhe^6-containing tetrapeptides (**4** and **5**, respectively) possessed 25- and 570-fold decreased potencies, respectively, compared with the His^6 peptide **1**. Substitution of the imidazole His^6 side chain with the Trp^6 indole in either the L or D stereochemical configurations resulted in 269- and 328-fold decreased potencies of peptides **6** and **7**, respectively. In tetrapeptides **8** and **9**, containing Nal(2′)^6 and DNal(2′)^6, 473- and 353-fold decreased potencies were observed at the mMC1R, respectively, compared with peptide **1**. Replacement of the imidazole side chain at the 6 position with the topographically constrained χ side chain derivatives of the Phe amino acid, Tic and DTic (**10** and **11**), resulted in 129- and 562-fold decreased potencies at the mMC1R, respectively. Substitution at the 6 position with phenyl derivatives containing nitrogen at either the 3 or 4 positions of the phenyl ring, 3PAL peptide **12** and 4PAL peptide **13**, resulted in 57- and 9-fold decreased potencies at the mMC1R compared with the His^6-containing peptide **1**. Interestingly, for substitution of the imidazole ring with the sulfur-containing 2Thi (Figure 1) peptide **14**, a 10-fold decreased potency was observed at the mMC1R. Incorporation of an additional benzyl ring (3Bal, **15**) onto the peptide **14** position 6 side chain resulted in 219-fold decreased potency compared with the imidazole side chain (**1**) and in a 22-fold decreased potency compared to peptide **14**, at the mMC1R. The Anc- and racemic Atc-containing peptides **16** and **17**, respectively, are equipotent to each other and resulted in 393- and 384-fold decreased potency compared with peptide **1** at the mMC1R. Stereochemical inversion of His^6 to DHis^6 (**18**) resulted in 14-fold decreased potency at the mMC1R.

Mouse Melanocortin-3 Receptor. The MC3R is expressed both peripherally and centrally and appears to be involved in metabolism and energy homeostasis.[8,9,32,33] The lead tetrapeptide **1**, Ac-His-DPhe-Arg-Trp-NH$_2$, has been previously reported to possess a 195 nM agonist EC$_{50}$ at the mMC3R[29] and a 1000 nM EC$_{50}$ at the hMC3R[34] and to possess a 156 nM EC$_{50}$ herein. Substitution at the His^6 position within this tetrapeptide by Pro, Phe, DPhe, Trp, DTrp, Nal(2′), DNal(2′), Tic, DTic, 3PAL, 4PAL, 2Thi, 3Bal, Anc, Atc, and DHis (Figure 1) all resulted in 15- to 127-fold decreased

**Table 1.** Functional Activity of the His[6]-Modified Tetrapeptides at the Mouse Melanocortin Receptors[a]

peptide	structure	mMC1R		mMC3R		mMC4R		mMC5R	
		EC_{50} (nM)	fold difference	EC_{50} (nM)	fold difference	EC_{50} (nM)	fold difference	EC_{50} (nM)	fold difference
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂	0.55 ± 0.09		0.79 ± 0.14		5.37 ± 0.62		0.44 ± 0.09	
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂	0.038 ± 0.012		0.098 ± 0.013		0.21 ± 0.03		0.071 ± 0.012	
MTII	Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH₂	0.020 ± 0.003		0.16 ± 0.03		0.087 ± 0.008		0.16 ± 0.03	
1	Ac-**His**-DPhe-Arg-Trp-NH₂	20.1 ± 0.57	1.0	156 ± 9.2	1.0	17.2 ± 2.80	1.0	3.96 ± 0.94	1.0
2	Ac-**Ala**-DPhe-Arg-Trp-NH₂	1,800 ± 370	90	9000 ± 2500	58	1000 ± 280	58	300 ± 7	76
3	Ac-**Pro**-DPhe-Arg-Trp-NH₂	4,250 ± 1400	211	15000 ± 1600	96	1500 ± 660	87	684 ± 142	173
4	Ac-**Phe**-DPhe-Arg-Trp-NH₂	503 ± 100	25	11900 ± 1800	76	70.6 ± 13.8	4	143 ± 5	36
5	Ac-**DPhe**-DPhe-Arg-Trp-NH₂	11500 ± 1800	570	>100000		2170 ± 390	126	2980 ± 1800	753
6	Ac-**Trp**-DPhe-Arg-Trp-NH₂	5400 ± 1800	269	10300 ± 1500	66	528 ± 150	31	507 ± 96	128
7	Ac-**DTrp**-DPhe-Arg-Trp-NH₂	6600 ± 1200	328	>100000		6200 ± 200	260	1260 ± 300	319
8	Ac-**Nal(2')**-DPhe-Arg-Trp-NH₂	9500 ± 5100	473	14700 ± 2000	94	2900 ± 2400	169	606 ± 360	153
9	Ac-**DNal(2')**-DPhe-Arg-Trp-NH₂	7100 ± 980	353	>100000		1500 ± 300	87	2240 ± 900	566
10	Ac-**Tic**-DPhe-Arg-Trp-NH₂	2600 ± 600	129	11800 ± 1600	76	2900 ± 1600	169	369 ± 140	93
11	Ac-**DTic**-DPhe-Arg-Trp-NH₂	11300 ± 3100	562	>100000		11700 ± 420	680	12000 ± 500	3030
12	Ac-**3PAL**-DPhe-Arg-Trp-NH₂	1150 ± 240	57	slight agonist		887 ± 99	52	400 ± 147	101
13	Ac-**4PAL**-DPhe-Arg-Trp-NH₂	176 ± 80	9	2270 ± 770	15	128 ± 33	7	92.5 ± 22.3	23
14	Ac-**2Thi**-DPhe-Arg-Trp-NH₂	203 ± 40	10	2950 ± 250	19	91.2 ± 39	5	40.5 ± 13	10
15	Ac-**3Bal**-DPhe-Arg-Trp-NH₂	4400 ± 3000	219	19800 ± 3200	127	211 ± 62	12	381 ± 123	96
16	Ac-**Anc**-DPhe-Arg-Trp-NH₂	7900 ± 4200	393	slight agonist pA₂ = 5.60 ± 0.11	antagonist (K_i = 2500 nM)	21.1 ± 6.0	1	45.6 ± 6.9	12
17	Ac-**rac(Atc)**-DPhe-Arg-Trp-NH₂	7730 ± 1200	384	slight agonist		714 ± 150	42	2400 ± 1030	606
18	Ac-**DHis**-DPhe-Arg-Trp-NH₂	289 ± 107	14	6190 ± 2460	40	506 ± 58	29	138 ± 24	35

[a] The indicated errors represent the standard error of the mean determined from at least four independent experiments. Slight agonist denotes that some stimulatory response was observed but not enough to determine an EC_{50} value. The compounds not demonstrating full agonism were assayed for antagonism using the Schild pA₂ analysis and the MTII peptide as the agonist; $K_i = -\log pA_2$.

Table 2. Analytical Data for the Peptides Synthesized in this Study[a]

peptide	structure	HPLC k' (system 1)	HPLC k' (system 2)	m/z (M + 1, calcd)	m/z (M + 1, exptl)	purity, %
1	Ac-His-DPhe-Arg-Trp-NH$_2$	3.9	6.9	686.8	686.3	>98
2	Ac-**Ala**-DPhe-Arg-Trp-NH$_2$	4.9	8.2	620.7	620.3	>97
3	Ac-**Pro**-DPhe-Arg-Trp-NH$_2$	8.1	9.0	646.8	646.1	>99
4	Ac-**Phe**-DPhe-Arg-Trp-NH$_2$	6.8	10.6	696.8	696.3	>99
5	Ac-**DPhe**-DPhe-Arg-Trp-NH$_2$	6.5	9.9	696.8	696.4	>99
6	Ac-**Trp**-DPhe-Arg-Trp-NH$_2$	6.7	10.5	735.9	735.9	>99
7	Ac-**DTrp**-DPhe-Arg-Trp-NH$_2$	6.5	10.0	735.9	735.1	>99
8	Ac-**Nal(2′)**-DPhe-Arg-Trp-NH$_2$	7.7	12.0	746.9	746.2	>99
9	Ac-**DNal(2′)**-DPhe-Arg-Trp-NH$_2$	7.5	11.4	746.9	746.1	>97
10	Ac-**Tic**-DPhe-Arg-Trp-NH$_2$	6.9	10.3	708.8	708.2	>99
11	Ac-**DTic**-DPhe-Arg-Trp-NH$_2$	6.9	10.3	708.8	708.3	>99
12	Ac-**3PAL**-DPhe-Arg-Trp-NH$_2$	4.0	6.7	697.8	697.3	>99
13	Ac-**4PAL**-DPhe-Arg-Trp-NH$_2$	3.9	6.6	697.8	697.3	>99
14	Ac-**2Thi**-DPhe-Arg-Trp-NH$_2$	6.4	10.4	702.8	702.3	>99
15	Ac-**3Bal**-DPhe-Arg-Trp-NH$_2$	5.3	12.1	752.9	752.1	>99
16	Ac-**Anc**-DPhe-Arg-Trp-NH$_2$	7.3	11.1	717.8	717.7	>99
17	Ac-**Atc**-DPhe-Arg-Trp-NH$_2$	7.2, 7.3	11.2, 11.4	721.9	722.2	>99
18	Ac-**DHis**-DPhe-Arg-Trp-NH$_2$	4.1	6.7	685.8	686.0	>99

[a] HPLC k' = [(peptide retention time − solvent retention time)/(solvent retention time)] in solvent system 1 (10% acetonitrile in 0.1% trifluoroacetic acid/water and a gradient to 90% acetonitrile over 35 min) or solvent system 2 (10% methanol in 0.1% trifluoroacetic acid/water and a gradient to 90% methanol over 35 min). An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.5 mL/min. The peptide purity was determined by HPLC at a wavelength of 214 Å.
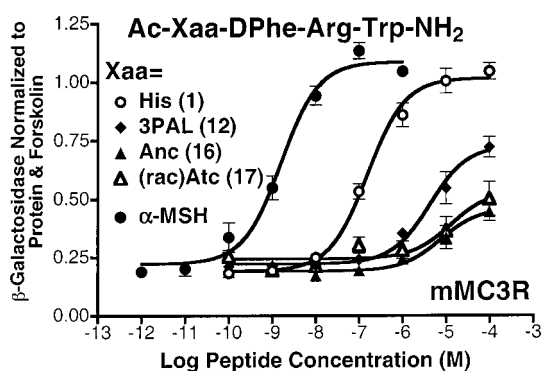
Figure 2. Illustration of the tetrapeptides possessing slight agonist activity (Table 1) at the mMC3R. The amino acid in the Xaa position is listed, and the number in parentheses represents the compound number. The peptides α-MSH and **1** are included as controls to illustrate the maximal response observed for full agonists using this assay.
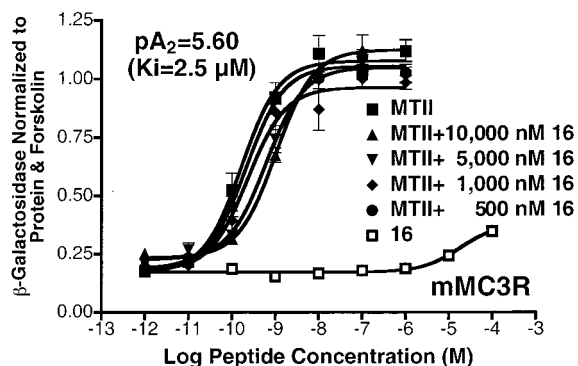
Figure 3. Illustration of weak antagonism of the tetrapeptide **16**, Ac-Anc-DPhe-Arg-Trp-NH$_2$, at the mouse MC3R.

potency, slight agonism at 100 μM (but not enough to determine an EC$_{50}$ value; Figure 2), a complete loss of agonist activity (up to 100 μM), or for a single peptide (**16**), a weak micromolar antagonist (Figure 3) at the mMC3R. The peptides that did not demonstrate agonist activities were tested for antagonism at up to 10 μM and did not result in any observable antagonistic properties (data not shown). Peptides **2** and **3**, contain-

ing Ala^6 and Pro^6, resulted in 58- and 96-fold decreased mMC3R potency, respectively, compared with the His^6 lead peptide **1**. Phenyl side chain substitution of the imidazole at the 6 position with Phe- and DPhe-containing peptides **4** and **5** resulted in 76-fold decreased potency and a loss of stimulatory activity at up to 100 μM, respectively, at the mMC3R. Substitution of the His^6 imidazole side chain with the Trp indole in either the L or D stereochemical configuration resulted in 66-fold decreased potency for the Trp^6-containing peptide **6** and loss of stimulatory activity at up to 100 μM for the DTrp^6-containing tetrapeptide **7** at the mMC3R. Tetrapeptide **8**, containing Nal(2′)^6, resulted in 94-fold decreased potency compared with the His^6 peptide **1**, while the DNal(2′)^6 tetrapeptide **9** resulted in a loss of stimulatory activity at up to 100 μM at the mMC3R. Replacement of the imidazole side chain at the 6 position with Tic^6 (**10**) resulted in 76-fold decreased potency compared with **1**, while the DTic^6-containing tetrapeptide **11** resulted in loss of stimulatory activity at up to 100 μM at the mMC3R. At the mMC3R substitution at the 6 position with phenyl derivatives containing nitrogen at either the 3 or 4 position of the phenyl ring, 3PAL peptide **12** possessed agonist activity (at 100 μM, Figure 2) but not enough for determining an EC$_{50}$ value, whereas 4PAL peptide **13** resulted in only 15-fold decreased potency compared with peptide **1**. Tetrapeptide **14**, containing 2Thi at the 6 position, resulted in 19-fold decreased mMC3R potency, whereas the 3Bal^6-containing tetrapeptide **15** resulted in 127-fold decreased potency at the mMC3R compared with the His^6 peptide **1**. The Anc^6-containing tetrapeptide **16** possessed only slight agonist activity, and when tested for antagonism, it resulted in a weak micromolar antagonist with a pA$_2$ of 5.6 (K_i = 2.5 μM = $-$log pA$_2$) (Figure 3). Interestingly, peptides **16** and **17** possessed only slight agonist activity at the mMC3R at up to 100 μM (Figure 2). Finally, inversion of chirality from His^6 to DHis^6, peptide **18**, resulted in 40-fold decreased potency at the mMC3R.

Mouse Melanocortin-4 Receptor. The central MC4R has been identified as physiologically participating in

food consumption[35] and obesity in mice[10] with several polymorphisms of the MC4R observed in obese humans.[36–41] The lead tetrapeptide in this study, Ac-His-DPhe-Arg-Trp-NH$_2$ (**1**), was previously reported to possess a 10 nM agonist EC$_{50}$ value at the mMC4R[29] and 8[28] and 47[34] nM agonist EC$_{50}$ values at the hMC4R, with a potency at the mMC4R of 17 nM reported herein. Substitution at the His[6] position within this tetrapeptide by Pro, Phe, DPhe, Trp, DTrp, Nal(2′), DNal(2′), Tic, DTic, 3PAL, 4PAL, 2Thi, 3Bal, Anc, Atc, and DHis (Figure 1) resulted in 4- to 680-fold decreased potency at the mMC4R. Tetrapeptides **2** (Ala[6]) and **3** (Pro[6]) resulted in 58- and 87-fold decreased potencies, respectively, compared to the His[6]-containing tetrapeptide **1** at the mMC4R. Substitution of the His[6] imidazole with either Phe (**4**) or DPhe (**5**) resulted in 4- and 126-fold decreased potencies, respectively, at the mMC4R. The Trp substitution of the His at the 6 position resulted in 31-fold (**6**, Trp[6]) and 260-fold (**7**, DTrp[6]) decreased potencies at the mMC4R. The Nal(2′)[6]-containing peptide **8** possessed 169-fold decreased potency and the DNal(2′)[6]-containing peptide **9** resulted in 87-fold decreased potency at the mMC4R, compared with the His[6]-containing peptide **1**. Substitution of the imidazole amino acid with the Phe derivatives Tic[6] (**10**) and DTic[6] (**11**) resulted in 169- and 680-fold decreased potencies at the mMC4R. Insertion of a nitrogen into the phenyl side chain of Phe in peptide **12** (3PAL[6]) and peptide **13** (4PAL[6]) resulted in 52- and 7-fold decreased potencies at the mMC4R compared with peptide **1**. Finally, peptide **14** (2Thi[6]) resulted in 5-fold decreased potency whereas the 3Bal[6]-containing tetrapeptide **15** resulted in 12-fold decreased potency at the mMC4R compared with the His[6]-containing tetrapeptide **1**. Peptide **16**, containing Anc in the 6 position, resulted in equipotency with the His[6] peptide **1** at the mMC4R. Peptide **17** (racemic Atc) resulted in 42-fold decreased potency, while the DHis[6] peptide **18** resulted in 29-fold decreased potency at the mMC4R compared with **1**.

Melanocortin-5 Receptor. The peripheral MC5R is expressed in a variety of tissues and has been implicated as physiologically participating in the role of exocrine gland function.[1,14,42] The lead tetrapeptide **1**, Ac-His-DPhe-Arg-Trp-NH$_2$, has been previously reported to possess a 3.4 nM agonist EC$_{50}$ at the mMC5R,[29] a 17% response at 5 μM at the hMC5R,[34] and a 3.9 nM EC$_{50}$ at the mMC5R reported herein. Substitution at the His[6] position within this tetrapeptide by Pro, Phe, DPhe, Trp, DTrp, Nal(2′), DNal(2′), Tic, DTic, 3PAL, 4PAL, 2Thi, 3Bal, Anc, Atc, and DHis (Figure 1) resulted in 10- to 3030-fold decreased potency at the mMC5R. Replacement of the His side chain at the 6 position with Ala (**2**) or Pro (**3**) resulted in a 76- or 173-fold decrease in potency, respectively, at the mMC5R. Substitution of the His imidazole side chain with Phe (**4**) or DPhe (**5**) resulted in 36- or 753-fold decreased potencies, respectively, at the mMC5R. The indole Trp substitution of the His imidazole at the 6 position in peptides **6** (Trp) and **7** (DTrp) resulted in 128- and 319-fold decreased potencies, respectively, at the mMC5R. The Nal(2′)[6]-containing peptide **8** possessed 153-fold decreased potency compared to peptide **1**, while the DNal(2′)[6] peptide **9** possessed 566-fold decreased potency compared to peptide **1**, at the mMC5R. Substitution of the His[6]

amino acid by Tic (**10**) or DTic (**11**) resulted in 93- and 3030-fold decreased potencies at the mMC5R compared to peptide **1**. Peptides **12** (3PAL[6]) and **13** (4PAL[6]) possessed 101- and 23-fold decreased potencies, respectively, compared with the His[6] peptide **1** at the mMC5R. Peptides **14** (2Thi[6]) and **15** (3Bal[6]) resulted in 10- and 96-fold decreased potencies, respectively, compared with peptide **1** at the mMC5R. The Anc[6]-containing peptide (**16**) resulted in 12-fold decreased mMC5R potency, the racemic Atc[6]-containing peptide (**17**) resulted in 600-fold decreased mMC5R potency, while the DHis[6] peptide (**18**) resulted in 35-fold decreased mMC5R potency, compared with peptide **1**.

Discussion

Modification of the His[6] Side Chain Imidazole by Other Functional Groups. Figure 4 summarizes the effect on potency of modifying the His side chain of the template peptide Ac-Xaa-DPhe-Arg-Trp-NH$_2$ with the amino acids examined in this study, at the melanocortin receptors. Substitution of the His[6] side chain (α-MSH numbering) with Ala (**2**) in the lead tetrapeptide Ac-His-DPhe-Arg-Trp-NH$_2$ (**1**) resulted in decreased receptor potencies in the order of mMC5R > mMC4R > mMC1R $\gg$ mMC3R. Replacement of the His[6] residue of MTII with Ala resulted in 1- to 5-fold decreased potencies at the hMC3, hMC4, and hMC5 receptors,[27] and His[6] to Ala in NDP-MSH resulted in only a 4-fold decreased potency at the hMC4R.[28] Interestingly, substitution of His[6] with Ala in a 23-membered lactam cyclic agonist peptide template (c[COCH$_2$CH$_2$CO-His[6]-DPhe-Arg-Trp-Lys]-NH$_2$) resulted in a lack of cAMP response (up to 5000 nM) at the hMC3R and hMC5R, while resulting in only a 48-fold decreased potency at the hMC4R.[24] These data suggest that in the tetrapeptide template the peptide backbone at the 6 position is less important for biological activity than the presence of the imidazole side chain, while in longer, more potent peptides such as MTII and NDP-MSH the His side chain appears to be less important for agonist potency.

When Pro replaces His at the 6 position of tetrapeptide **1**, an 87- to 211-fold decrease in ligand potency is observed at the mMC1 and mMC3–5 receptors, which results in approximately the same order of magnitude (within experiment error) reduction in ligand potency at these receptors compared with **1** and results in the same trend in potency as the Ala at these receptors (mMC5R > mMC4R > mMC1R > mMC3R). When Pro is substituted for His[6] in MTII, a 2- to 4-fold decreased potency is observed at the hMC4R and hMC3R, while a 2.5-fold increased potency is observed for the Pro[6]-MTII peptide at the hMC5R.[22] Interestingly, substitution of His[6] with Pro in the cyclic agonist peptide template, c[COCH$_2$CH$_2$CO-His[6]-DPhe-Arg-Trp-Lys]-NH$_2$, resulted in 10- and 76-fold decreased potency at the hMC3R and hMC4R while increasing potency 2-fold at the hMC5R.[24] In this latter study by Bednarek et al., inverting the stereochemistry of the Pro[6] to DPro[6] in their peptide template resulted in 3–5% agonist stimulation at 10 μM. Modification of the His in the 6 position by Pro in the MTII peptide template resulted in the identification of modifications that might lead to increased MC4R selectivity versus that of the MC3R.[20–22]

Replacement of the His[6] imidazole by the phenyl side chain of Phe (**4**) resulted in only a 4-fold decrease in
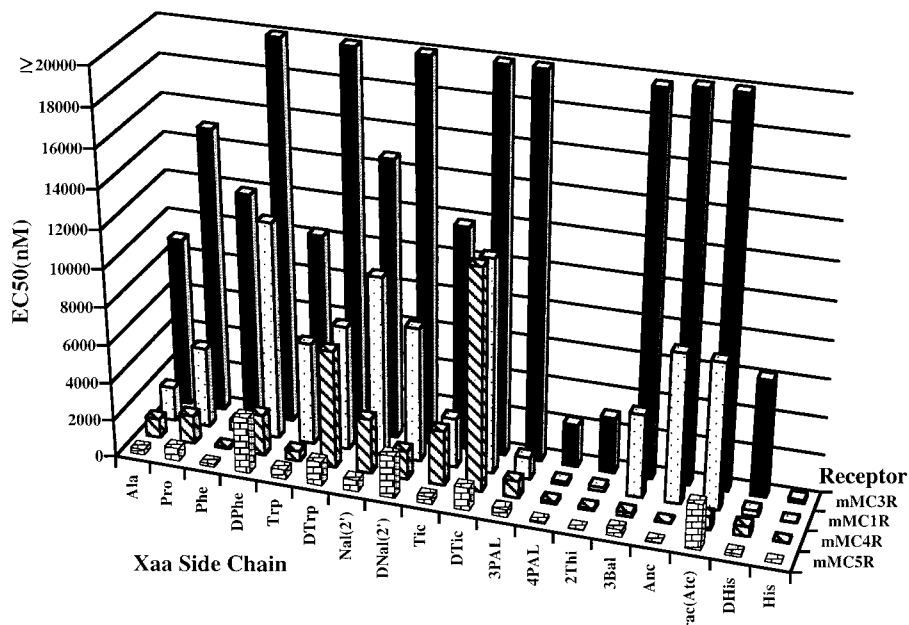
Figure 4. Graphical representation summarizing the effect on melanocortin receptor (*Y* axis) agonist EC_{50} values (*Z* axis) of the indicated amino acid substitution (*X* axis) of the His residue in the tetrapeptide template Ac-His-DPhe-Arg-Trp-NH$_2$.

potency at the mMC4R while possessing 25-, 76-, and 36-fold decrease in potencies at the mMC1R, mMC3R, and mMC5R, respectively, compared with **1**. This information suggests that substitution of a His by Phe for the design of non-peptide, MC4R-selective, small-molecule agonists may be a viable option. When this Phe amino acid is used for the development of non-peptide molecules, the side chain does not need to possess a protecting group like the His residue may during synthesis, facilitating an easier synthetic chemistry strategy. Inversion of stereochemistry to the DPhe[6]-containing tetrapeptide **5** resulted decreased potencies compared to the His[6] peptide **1** (mMC4R > mMC5R > mMC1R) while losing the ability to stimulate the mMC3R at up to 100 μM. When tetrapeptides **4** (Phe[6]) and **5** (DPhe[6]) are compared, a 2-, 31-, and 21-fold decrease in potency of the DPhe[6] peptide compared with the Phe[6] tetrapeptide was observed at the mMC1R, mMC4R, and mMC5R, respectively (Figure 4).

The Tic amino acid[43] (Figure 1) is a topographically constrained derivative of Phe that severely restricts rotation about the χ_1 and χ_2 side chain torsional angles and has been previously reported to result in increased biological activities and potencies.[44] In the tetrapeptides **10** (Tic[6]) and **11** (DTic[6]), the L-configured compound possessed full agonism at the melanocortin receptors with 76- to 169-fold decreased potencies compared with the His[6] tetrapeptide **1** while the D-configured molecule (**11**) resulted in a lack of observable mMC3R stimulation (was not an mMC3R antagonist) at up to 100 μM and resulted in 562- to 3000-fold decreased potencies at the mMC1R, mMC4R, and mMC5R. When the Tic[6] tetrapeptide (**10**) was compared with the corresponding Phe[6] (**4**) peptide, the L configuration resulted in 5-fold decreased potency, equipotency, and 41- and 3-fold (equipotent within experimental error) decreased potencies at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively. Comparing the DTic[6] tetrapeptide (**11**) with their corresponding DPhe[6] (**5**) peptide, the D-configuration resulted in equipotency at the mMC1R,

lack of stimulation at up to 100 μM at the mMC3R, 5- and 4-fold decreased potency at the mMC4R and mMC5R, respectively.

Modification of the Phe[6] benzyl ring by the insertion of a nitrogen in either the meta (3PAL, **12**) or para (4 PAL, **13**) position generally resulted in decreased melanocortin receptor potency compared with peptide **1**. Tetrapeptide Ac-4PAL-DPhe-Arg-Trp-NH$_2$ (**13**) resulted in only 7- to 23-fold decreased melanocortin receptor potencies compared with **1** and was equipotent at the mMC1R, mMC4R, and mMC5R within experimental error but was 5-fold more potent at the mMC3R than the Phe[6]-containing tetrapeptide **4**. Thus, incorporation of a nitrogen into the Phe side chain at the meta or para position generally resulted in decreased melanocortin receptor potency except for the 4PAL derivative at the mMC3R.

Naphthyl(2′) side chain replacement of the His[6] (**1**), tetrapeptide **8**, resulted in decreased potencies at the melanocortin receptors compared with **1**. Inversion of stereochemistry to DNal(2′)[6] in tetrapeptide **9** resulted in a loss of agonist activity at the mMC3R (up to 100 μM) and 87- to 566-fold decreased potencies at the mMC1R, mMC4R, and mMC5R compared with **1**. Comparison of the Nal(2′)[6] peptide **8** with the DNal-(2′)[6] peptide **9** resulted in nearly equipotent activities at the mMC1R, mMC4R, and mMC5R.

Substitution of the imidazole by an indole to Trp (**6**) at the 6 position resulted in decreased potencies at the mMC1 and mMC3−5 receptors compared with tetrapeptide **1**. Converting Trp to the DTrp (**7**) stereochemistry at the 6 position resulted in a loss of agonist activity at the mMC3R (up to 100 μM) and 260- to 328-fold decreased potencies at the mMC1 and mMC4−5 receptors compared with **1**. Comparing tetrapeptides **6** (Trp[6]) and **7** (DTrp[6]) resulted in equipotent activities at the mMC1R and mMC5R (within experimental error) and an 8-fold decreased potency for the DTrp[6] analogue at the mMC4R. Upon substitution of His[6] with Trp in the SHU9119 peptide (Ac-Nle-c[Asp-His-DNal(2′)-Arg-

Trp-Lys]-NH$_2$), 51-, 25-, and 16-fold decreased potencies were observed for the mMC1R agonist and the mMC3R and mMC4R antagonists, respectively, while the compound was converted from a full agonist (His^6 EC$_{50}$ = 2.3 nM) to a compound resulting in only slight agonist activity up to 1 μM (Trp^6).[17] Upon substitution of the indole nitrogen of Trp^6 (**6**) with a sulfur (3Bal,^{6} **15**), nearly equipotency was observed at all the melanocortin receptors examined, suggesting that the heteroatom of the indole ring at the 6 position may not be particularly important for ligand potency. However, comparison of the 2Thi^6 (**14**) and 3Bal^6 (**15**) peptides resulted in 22- and 7-fold decreased potencies, equipotency, and 10-fold decreased potency at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively, suggesting that the three-dimensional location of the benzyl portion of the indole-like structure does not effect the mMC4R ligand–receptor interactions as much as the other receptor isoforms.

Comparison of the 2Thi^6 amino acid derivative (Figure 1) with the His^6-containing tetrapeptides resulted in 5- to 19-fold decreased melanocortin receptor potencies, suggesting that the electronic and basic features of the imidazole ring may be important for receptor potency, perhaps through salt bridge or hydrogen-bonding interactions.

MC4 Versus MC3 Receptor Selectivity. Identification of ligands selective for either of the centrally located melanocortin receptors, MC3R or MC4R, is highly sought because both these receptors have been identified as physiologically participating in the neuroendocrine process of energy homeostasis using a combination of nonspecific melanocortin agonists and antagonists and knockout mice.[8–10,35,45] As mentioned previously, substitution of His with Pro in the MTII agonist template resulted in the identification of this amino acid position for brain MC4 versus MC3 receptor selectivity.[20–22] The recent public disclosure by Hoffmann-La Roche, Inc. indicated the His position as the most critical position for the identification of MC4 versus MC3 receptor compounds, as demonstrated by the peptide c[Asp-(D,L)-Atc-DPhe-Arg-Trp-Lys]-NH$_2$ possessing 65 nM hMC4R potency and only possessing slight agonist activity at the hMC3R at micromolar concentrations.[25] The studies presented herein support this hypothesis by the identification of compounds that possess full agonist activity at the mMC4R while lacking mMC3R full agonist activity at up to 100 μM (Figure 2). The most potent and mMC4R-selective (versus mMC3R) tetrapeptide is Ac-Anc-DPhe-Arg-Trp-NH$_2$ (**16**), which is equipotent to the His-containing tetrapeptide **1** and only 4-fold less potent than the endogenous agonist α-MSH at the mMC4R (Table 1) while only possessing slight agonist activity (<50% maximal stimulation at 100 μM) at the mMC3R (Figure 2).[46] Unexpectantly, Ac-Anc-DPhe-Arg-Trp-NH$_2$ (**16**) resulted in a micromolar mMC3R antagonist (Figure 3), but even with this pharmacological profile, if the compound is administered in the high nanomolar range, stimulation of the MC4R should result, with a postulated absence of activity at the mMC3R. The tetrapeptide containing the same amino acid reported by Danho and colleagues,[25] Ac-(racemic)-Atc-DPhe-Arg-Trp-NH$_2$ (**17**), was 34-fold less potent than peptide **16** at the mMC4R and only 11-fold less
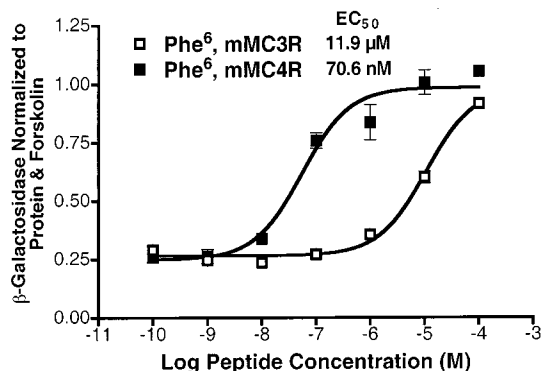
Figure 5. Comparison of the Phe^6-containing tetrapeptide **4** EC$_{50}$ values at the centrally located mMC3R and mMC4 receptors. This figure illustrates that this compound possesses 160-fold receptor selectivity for the MC4R versus the MC3R.

potent than the previously identified cyclic hexapeptide.[25] Similar to the Roche cyclic hexapeptide (although it was not mentioned if it was tested for MC3R antagonist activity), the Atc^6-containing peptide **17** only possessed slight agonist activity (<50% maximal stimulation at 100 μM) at the mMC3R. Figure 5 illustrates the MC4 versus MC3 receptor selectivity for the Phe^6-containing tetrapeptide (**4**). Interestingly, the aromatic side chains in the D configuration at the 6 position resulted in decreased micromolar potencies at the mMC4R but did not stimulate the mMC3R at up to 100 μM. These data and the fact that the Atc peptide **17** is actually a racemic mixture might suggest that the DAtc^6-containing tetrapeptide might be more potent and selective for the MC4R than the Anc peptide reported herein. Thus, these data support the hypothesis that the His^6 position is important for MC4 versus MC3 receptor selectivity[20–22,25] and extend this hypothesis to include an aromatic benzyl ring in the proper topographical three-dimensional space as an additional consideration. The information gained by the studies reported herein are extremely valuable and applicable for the design of small-molecule, non-peptide, MC4R-selective agonists.

Conclusions

This study reports modifications of the His^6 side chain imidazole ring with various aromatic and nonaromatic substitutions in the tetrapeptide template Ac-His-DPhe-Arg-Trp-NH$_2$. The data reported herein support the hypothesis that this His position of melanocortin agonists is an important residue for the design of compounds selective for the centrally located MC4 versus MC3 receptors. We have identified melanocortin tetrapeptide agonists with MC4R versus MC3R selectivity ranging from >4700 to 9-fold with a trend for this selectivity observed for aromatic D-configured amino acids. The most potent MC4R agonist and MC4R- versus MC3R-selective compound identified from this study is the Ac-Anc-DPhe-Arg-Trp-NH$_2$ tetrapeptide that possesses an MC4R EC$_{50}$ of 21 nM with only micromolar antagonism and a slight agonist activity (<50% maximal stimulation at 100μM) at the MC3R, demonstrating >4700-fold MC4R versus MC3R agonist selectivity. Additionally, the data reported herein provide experimental evidence that the His amino acid may be replaced by other amino acids for the design of MC4R-

selective non-peptide molecules. Substitution of the His side chain in non-peptide molecules by amino acids such as Phe will potentially dramatically simplify the chemistry required to synthesize molecules possessing potent MC4R agonist activity.

Experiemental Section

Peptide Synthesis. Peptide synthesis was performed using standard Fmoc methodology[30] on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY). The amino acids Fmoc-Tyr(tBu), Fmoc-His(Trt), Fmoc-Arg(Pbf), Fmoc-DPhe, Fmoc-Trp(Boc), Fmoc-Pro, Fmoc-Ala, Fmoc-Phe, Fmoc-DPhe, Fmoc-3-(2-naphthyl)-alanine [Nal(2′)], and Fmoc-3-(2-Naphthyl)-D-alanine [DNal(2′)] were purchased from Peptides International (Louisville, KY). Fmoc-DTrp(Boc) was purchased from Advanced ChemTech (Louisville, KY). Fmoc-3-(2-thienyl)-alanine (2-Thi), Fmoc-3-(3-pyridinyl)alanine (3-Pal), and Fmoc-3-(4-pyridinyl)alanine (4-Pal) were purchased from Bachem (Torrance, CA). Fmoc-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) and Fmoc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (DTic) were purchased from Synthetech (Albany, OR). Fmoc-(racemic)-aminotetrahydro-2-naphthyl-carboxylic acid (Atc) and Fmoc-amino-2-naphthylcarboxylic acid (Anc) were purchased from Pharma Core (High Point, NC). The coupling reagents 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from Peptides International. Glacial acetic acid (HOAc), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ). N,N-Dimethylformamide (DMF) was purchased from Burdick and Jackson (McGaw Park, IL). Trifluoroacetic acid (TFA), 1,3-diisopropylcarbodiimide (DIC), pyridine, piperidine, and acetic anhydride were purchased from Sigma (St. Louis, MO). N,N-Diisopropylethylamine (DIEA) and triisopropylsilane (Tis) were purchased from Aldrich (Milwaukee, WI). All reagents and chemicals were ACS grade or better and were used without further purification.

The peptides were assembled on Rink amide MBHA resin (0.44 mequiv/g substitution) purchased from Peptides International. The synthesis was performed using a 40-well Teflon reaction block with a coarse Teflon frit. Approximately 100 mg of resin (0.044 mmol) was added to each reaction block well. The resin was allowed to swell for 2 h in DMF and deprotected using 25% piperidine in DMF for 5 min followed by a 20 min of 25% piperidine incubation at 450 rpm. A positive Kaiser[46] test resulted, indicating free amine groups on the resin. The growing peptide chain was added to the amide resin using the general amino acid cycle as follows: 500 μL DMF is added to each reaction well to "wet the frit"; 3-fold excess amino acid starting from the C terminus is added (275 μL of 0.5M amino acid solution containing 0.5 M HOBt in DMF) followed by the addition of 275 μL of 0.5 M DIC in DMF; the reaction well volume is brought up to 3 mL using DMF. The coupling reaction is mixed for 1 h at 450 rpm, followed by emptying of the reaction block by positive nitrogen gas pressure. A second coupling reaction is performed by the addition of 500 μL of DMF to each reaction vessel, followed by the addition of 275 μL of the respective amino acid (3-fold excess), 275 μL of 0.5 M HBTU, and 225 μL of 1 M DIEA. The reaction-well volume is brought up to 3 mL with DMF and mixed at 450 rpm for 1 h. After the second coupling cycle, the reaction block is emptied and the Nα-Fmoc-protected peptide resin is washed with DMF (4.5 mL, 4 times). Nα-Fmoc deprotection is performed by the addition of 4 mL of 25% piperidine in DMF and mixed for 5 min at 450 rpms followed by a 20 min deprotection at 450 rpm. The reaction well is washed with DMF (4.5 mL, 4 times), and the next coupling cycle is performed as described above. After Nα-Fmoc deprotection of the final amino acid, acetylation of the Nα-amine was performed by addition of 2 mL of acetic anhydride, 1 mL of pyridine, and 1 mL of DMF to the reaction block wells and mixing for 30 min at 450 rpm. The acetylated peptide resin

was washed with DCM (4 mL, 5 times) and dried thoroughly prior to cleavage from the resin. Deprotection of the amino acid side chains and cleavage of the acetylated peptide from the resin was performed by incubating the peptide resin with 3 mL of cleavage cocktail (95% TFA, 2.5% water, 2.5% Tis) for 3 h at 450 rpm. The cleavage product was emptied from the reaction block into a cleavage block containing 7 mL collection vials under positive nitrogen gas pressure. The resin was washed with 1.5 mL of cleavage cocktail for 5 min and 450 rpm and added to the previous cleavage solution. The peptides were transferred to preweighed 50 mL conical tubes and precipitated with cold (4 °C) anhydrous ethyl ether (up to 50 mL). The flocculent peptide was pelleted by centrifugation (Sorval Super T21 high-speed centrifuge using the swinging bucket rotor) at 4000 rpm for 5 min, the ether was decanted off, and the peptide was washed one time with cold anhydrous ethyl ether and again pelleted. The crude peptide was dried *in vacuo* for 48 h. The crude peptide yields ranged from 60% to 90% of the theoretical yields. A 15−30 mg sample of crude peptide was purified by RP-HPLC using a Shimadzu chromatography system with a photodiode array detector and a semipreparative RP-HPLC C18 bonded silica column (Vydac 218TP1010, 1.0 cm × 25 cm) and was lyophilized. The purified peptides were at least >95% pure as determined by analytical RP-HPLC and had the correct molecular mass (University of Florida protein core facility) (Table 2).

One-Dimensional ¹H Nuclear Magnetic Resonance Spectroscopy (NMR Data in Supporting Information). Peptides were analyzed for purity and structural integrity by nuclear magnetic resonance (NMR). Peptides were dissolved in 600 μL of DMSO-d_6 that contained 0.1% TMS, with an approximate final concentration of 2mM. ¹H NMR spectra were obtained at 27 °C on a Bruker Avance 500 MHz spectrometer in the Advanced Magnetic Resonance Imaging and Spectroscopy facility at the McKnight Brain Institute, University of Florida. One-dimensional ¹H data were collected using the decoupler coil of a Bruker 5 mm BBO probe with 128 scans, 26 684 total time domain points, a tip angle of 45°, an acquisition time of 2 s, and a delay time of 3 s. The spectral widths were 12 ppm, and TMS was referenced to 0.0 ppm. To correctly determine the integral values of peaks that occasionally occurred in the region around 3.3 ppm, a standard presaturation procedure (Bruker zgf2pr) for H_2O in DMSO was used. Prior to Fourier transformation, the FID was apodized with an exponential line broadening of 0.5 Hz and transformed with minimal zero-filling to 16K data points. The data were processed and analyzed using Bruker XWINNMR and XWINPLOT software. In the ¹H NMR spectra of peptides **3**, **10**, and **11**, doubling of the resonances was observed in an approximately 60:40 ratio. These peptides contain either a Pro or a "proline-like" Tic amino acid residue (Figure 1). The doubling can be explained by rotational isomerization about the Pro−DPhe, Tic−DPhe, and DTic−DPhe amide bonds of peptides **3**, **10**, and **11**, respectively. In these cases, the total integration of both resonances is reported.

Cell Culture and Transfection. Briefly, HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and seeded 1 day prior to transfection at $(1−2) × 10^6$ cells per 100 mm dish. Melanocortin receptor DNA in the pCDNA₃ expression vector (20 μg) was transfected using the calcium phosphate method. Stable receptor populations were generated using G418 selection (1 mg/mL) for subsequent bioassay analysis.

Functional Bioassay. HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described.[29,47,48] Briefly, 5000−15 000 posttransfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours after transfection, the cells were stimulated with 100 μL of peptide ($10^{-4}−10^{-12}$ M) or forskolin (10^{-4} M) control 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 cell 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. To the cell lysate plates, 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 Bio Rad 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 to both the relative protein content and nonreceptor-dependent forskolin stimulation. The antagonistic properties of these compounds were evaluated by the ability of these ligands to competitively displace the MTII agonist (Bachem) in a dose-dependent manner, at up to 10 μM.[48] The pA_2 values were generated using the Schild analysis method.[49]

Data Analysis. EC_{50} and pA_2 values represent the mean of duplicate experiments performed in quadruplet or more independent experiments. EC_{50} and pA_2 estimates, and their associated standard errors, were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (version 3.0, GraphPad, Inc.). The results are not corrected for peptide content, although all the peptides examined in this study were determined to have approximately equal peptide content as determined by using Beer's Law.

Supporting Information Available: NMR spectra of peptides **1**–**18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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