

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

Jerry Ryan Holder, Rayna M. Bauzo, Zhimin Xiang, and Carrie Haskell-Luevano*

Department of Medicinal Chemistry, University of Florida, Gainesville, Florida 32610

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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 His⁶ 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 His⁶ 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 (EC₅₀ = 21 nM), was a weak mMC3R micromolar antagonist (pA₂ = 5.6, K_i = 2.5 μ M), and possessed >4700-fold agonist selectivity for the MC4R versus the MC3R. Substitution of the His⁶ 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 His⁶-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, agouti⁵ 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 His⁶ 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-fold²³ and 90-fold²⁴ 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

* To whom correspondence should be addressed. Phone: (352) 846–2722. Fax: 352-392-8182. E-mail: Carrie@cop.ufl.edu.

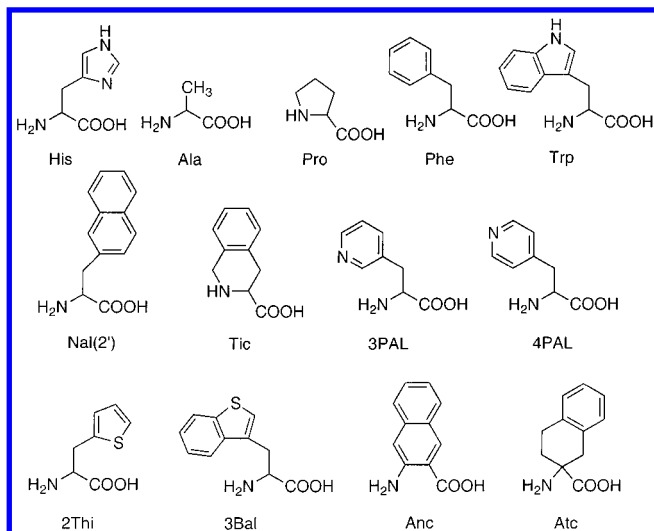


Figure 1. Structures of the amino acids used to replace His in the peptide template Ac-Xaa-DPhe-Arg-Trp-NH₂.

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-NH₂ tetrapeptide, at the human MC4R only 2-fold decreased potency was observed²⁸ 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-NH₂ 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-NH₂ 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 ¹H NMR (NMR data in Supporting Information). Previously, we have synthesized and reported²⁹ the Ac-His-DPhe-Arg-Trp-NH₂ (included herein), Ac-His-Phe-Arg-Trp-NH₂, Ac-His-Phe-Arg-DTrp-NH₂, and Ac-His-DPhe-Arg-DTrp-NH₂ diastereomeric peptides as having distinct analytical RP-HPLC *K'* values in two diverse solvent systems.

Biological Evaluation. Table 1 summarizes the His⁶-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-NH₂, has been previously reported to possess 25 nM stimulatory activity at the mMC1R,²⁹ an EC₅₀ value of 200 nM in the classical *Rana pipiens* frog skin assay (putative MC1R),⁴ and possesses an mMC1R EC₅₀ of 20 nM reported herein. Substitution at the His⁶ 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 Pro⁶-containing tetrapeptide **3** resulted in 211-fold decreased potency, while the Phe⁶- and DPhe⁶-containing tetrapeptides (**4** and **5**, respectively) possessed 25- and 570-fold decreased potencies, respectively, compared with the His⁶ peptide **1**. Substitution of the imidazole His⁶ side chain with the Trp⁶ 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 His⁶-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 His⁶ to DHis⁶ (**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-NH₂, has been previously reported to possess a 195 nM agonist EC₅₀ at the mMC3R²⁹ and a 1000 nM EC₅₀ at the hMC3R³⁴ and to possess a 156 nM EC₅₀ herein. Substitution at the His⁶ 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 His⁶-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-NH ₂ | 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-NH ₂ | 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]-NH ₂ | 0.020 ± 0.003 | | | 0.16 ± 0.03 | | | 0.087 ± 0.008 | | | 0.16 ± 0.03 | | |
| 1 | Ac-His-DPhe-Arg-Trp-NH ₂ | 20.1 ± 0.57 | 1.0 | | 156 ± 9.2 | 1.0 | | 17.2 ± 2.80 | | | 3.96 ± 0.94 | | |
| 2 | Ac-Ala-DPhe-Arg-Trp-NH ₂ | 1.800 ± 370 | 90 | | 9000 ± 2500 | 58 | | 1000 ± 280 | | | 300 ± 7 | | 76 |
| 3 | Ac-Pro-DPhe-Arg-Trp-NH ₂ | 4.250 ± 1400 | 211 | | 15000 ± 1600 | 96 | | 1500 ± 660 | | | 684 ± 142 | | 173 |
| 4 | Ac-Phe-DPhe-Arg-Trp-NH ₂ | 503 ± 100 | 25 | | 11900 ± 1800 | 76 | | 70.6 ± 13.8 | | | 143 ± 5 | | 36 |
| 5 | Ac-DPhe-DPhe-Arg-Trp-NH ₂ | 11500 ± 1800 | 570 | | > 100000 | | | 2170 ± 390 | | | 2980 ± 1800 | | 753 |
| 6 | Ac-Trp-DPhe-Arg-Trp-NH ₂ | 5400 ± 1800 | 269 | | 10300 ± 1500 | 66 | | 528 ± 150 | | | 507 ± 96 | | 128 |
| 7 | Ac-DTrp-DPhe-Arg-Trp-NH ₂ | 6600 ± 1200 | 328 | | > 100000 | | | 6200 ± 200 | | | 1260 ± 300 | | 319 |
| 8 | Ac-Nal(2)-DPhe-Arg-Trp-NH ₂ | 9500 ± 5100 | 473 | | 14700 ± 2000 | 94 | | 2900 ± 2400 | | | 606 ± 360 | | 153 |
| 9 | Ac-DNal(2)-DPhe-Arg-Trp-NH ₂ | 7100 ± 980 | 353 | | > 100000 | | | 1500 ± 300 | | | 2240 ± 900 | | 566 |
| 10 | Ac-Tic-DPhe-Arg-Trp-NH ₂ | 2600 ± 600 | 129 | | 11800 ± 1600 | 76 | | 2900 ± 1600 | | | 369 ± 140 | | 93 |
| 11 | Ac-DTic-DPhe-Arg-Trp-NH ₂ | 11300 ± 3100 | 562 | | > 100000 | | | 11700 ± 420 | | | 12000 ± 500 | | 3030 |
| 12 | Ac-3PAL-DPhe-Arg-Trp-NH ₂ | 1150 ± 240 | 57 | | slight agonist | | | 887 ± 99 | | | 400 ± 147 | | 101 |
| 13 | Ac-4PAL-DPhe-Arg-Trp-NH ₂ | 176 ± 80 | 9 | | 2270 ± 770 | 15 | | 128 ± 33 | | | 92.5 ± 22.3 | | 23 |
| 14 | Ac-2Thi-DPhe-Arg-Trp-NH ₂ | 203 ± 40 | 10 | | 2950 ± 250 | 19 | | 91.2 ± 39 | | | 40.5 ± 13 | | 10 |
| 15 | Ac-3Bal-DPhe-Arg-Trp-NH ₂ | 4400 ± 3000 | 219 | | 19800 ± 3200 | 127 | | 211 ± 62 | | | 381 ± 123 | | 96 |
| 16 | Ac-Anc-DPhe-Arg-Trp-NH ₂ | 7900 ± 4200 | 393 | | slight agonist pA ₂ = 5.60 ± 0.11 | antagonist (K _i = 2500 nM) | | 21.1 ± 6.0 | | | 45.6 ± 6.9 | | 12 |
| 17 | Ac-rac(Atc)-DPhe-Arg-Trp-NH ₂ | 7730 ± 1200 | 384 | | slight agonist | | | 714 ± 150 | | | 2400 ± 1030 | | 606 |
| 18 | Ac-DHis-DPhe-Arg-Trp-NH ₂ | 289 ± 107 | 14 | | 6190 ± 2460 | 40 | | 506 ± 58 | | | 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 EC₅₀ 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 pA₂.

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

| peptide | structure | HPLC <i>K'</i> (system 1) | HPLC <i>K'</i> (system 2) | <i>m/z</i> (<i>M</i> + 1, calcd) | <i>m/z</i> (<i>M</i> + 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.

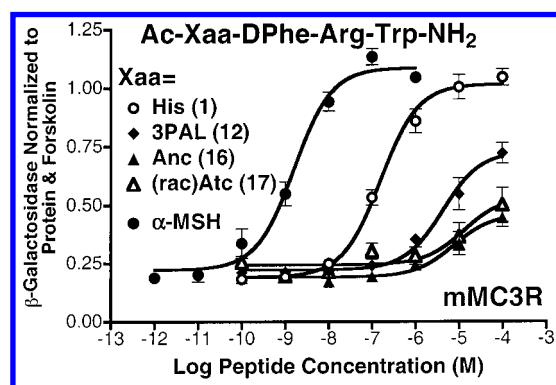


Figure 2. Illustration of the tetrapeptides possessing slight agonist activity (Table 1) at the mMC3R. The amino acid in the Xaa position is listed, and the number in parentheses represents the compound number. The peptides α-MSH and **1** are included as controls to illustrate the maximal response observed for full agonists using this assay.

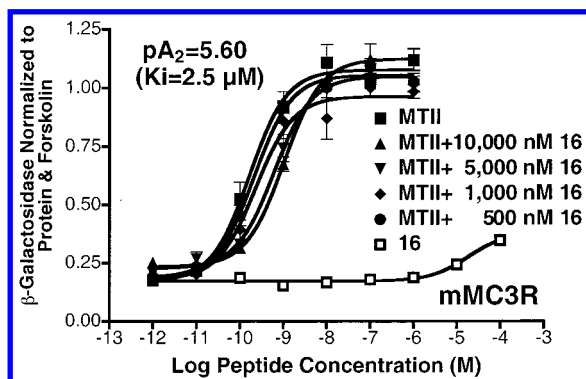


Figure 3. Illustration of weak antagonism of the tetrapeptide **16**, Ac-Anc-DPhe-Arg-Trp-NH₂, at the mouse MC3R.

potency, slight agonism at 100 μM (but not enough to determine an EC₅₀ 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 Ala⁶ and Pro⁶, resulted in 58- and 96-fold decreased mMC3R potency, respectively, compared with the His⁶ 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 His⁶ imidazole side chain with the Trp indole in either the L or D stereochemical configuration resulted in 66-fold decreased potency for the Trp⁶-containing peptide **6** and loss of stimulatory activity at up to 100 μM for the DTrp⁶-containing tetrapeptide **7** at the mMC3R. Tetrapeptide **8**, containing Nal(2')⁶, resulted in 94-fold decreased potency compared with the His⁶ 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 Tic⁶ (**10**) resulted in 76-fold decreased potency compared with **1**, while the DTic⁶-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 EC₅₀ 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 3Bal⁶-containing tetrapeptide **15** resulted in 127-fold decreased potency at the mMC3R compared with the His⁶ peptide **1**. The Anc⁶-containing tetrapeptide **16** possessed only slight agonist activity, and when tested for antagonism, it resulted in a weak micromolar antagonist with a pA₂ of 5.6 (*K*_i = 2.5 μM = –log pA₂) (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 His⁶ to DHis⁶, 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 consumption³⁵ and obesity in mice¹⁰ with several polymorphisms of the MC4R observed in obese humans.^{36–41} The lead tetrapeptide in this study, Ac-His-DPhe-Arg-Trp-NH₂ (**1**), was previously reported to possess a 10 nM agonist EC₅₀ value at the mMC4R²⁹ and 8²⁸ and 47³⁴ nM agonist EC₅₀ values at the hMC4R, with a potency at the mMC4R of 17 nM reported herein. Substitution at the His⁶ 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** (Ala⁶) and **3** (Pro⁶) resulted in 58- and 87-fold decreased potencies, respectively, compared to the His⁶-containing tetrapeptide **1** at the mMC4R. Substitution of the His⁶ 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**, Trp⁶) and 260-fold (**7**, DTrp⁶) 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 His⁶-containing peptide **1**. Substitution of the imidazole amino acid with the Phe derivatives Tic⁶ (**10**) and DTic⁶ (**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** (3PAL⁶) and peptide **13** (4PAL⁶) resulted in 52- and 7-fold decreased potencies at the mMC4R compared with peptide **1**. Finally, peptide **14** (2Thi⁶) resulted in 5-fold decreased potency whereas the 3Bal⁶-containing tetrapeptide **15** resulted in 12-fold decreased potency at the mMC4R compared with the His⁶-containing tetrapeptide **1**. Peptide **16**, containing Anc in the 6 position, resulted in equipotency with the His⁶ peptide **1** at the mMC4R. Peptide **17** (racemic Atc) resulted in 42-fold decreased potency, while the DHis⁶ 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-NH₂, has been previously reported to possess a 3.4 nM agonist EC₅₀ at the mMC5R,²⁹ a 17% response at 5 μ M at the hMC5R,³⁴ and a 3.9 nM EC₅₀ at the mMC5R reported herein. Substitution at the His⁶ 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 His⁶

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** (3PAL⁶) and **13** (4PAL⁶) possessed 101- and 23-fold decreased potencies, respectively, compared with the His⁶ peptide **1** at the mMC5R. Peptides **14** (2Thi⁶) and **15** (3Bal⁶) resulted in 10- and 96-fold decreased potencies, respectively, compared with peptide **1** at the mMC5R. The Anc⁶-containing peptide (**16**) resulted in 12-fold decreased mMC5R potency, the racemic Atc⁶-containing peptide (**17**) resulted in 600-fold decreased mMC5R potency, while the DHis⁶ peptide (**18**) resulted in 35-fold decreased mMC5R potency, compared with peptide **1**.

Discussion

Modification of the His⁶ 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-NH₂ with the amino acids examined in this study, at the melanocortin receptors. Substitution of the His⁶ side chain (α -MSH numbering) with Ala (**2**) in the lead tetrapeptide Ac-His-DPhe-Arg-Trp-NH₂ (**1**) resulted in decreased receptor potencies in the order of mMC5R > mMC4R > mMC1R \gg mMC3R. Replacement of the His⁶ residue of MTII with Ala resulted in 1- to 5-fold decreased potencies at the hMC3, hMC4, and hMC5 receptors,²⁷ and His⁶ to Ala in NDP-MSH resulted in only a 4-fold decreased potency at the hMC4R.²⁸ Interestingly, substitution of His⁶ with Ala in a 23-membered lactam cyclic agonist peptide template (c[COCH₂CH₂CO-His⁶-DPhe-Arg-Trp-Lys]-NH₂) 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 His⁶ 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 Pro⁶-MTII peptide at the hMC5R.²² Interestingly, substitution of His⁶ with Pro in the cyclic agonist peptide template, c[COCH₂CH₂CO-His⁶-DPhe-Arg-Trp-Lys]-NH₂, 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 Pro⁶ to DPro⁶ in their peptide template resulted in 3–5% agonist stimulation at 10 μ M. 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 His⁶ imidazole by the phenyl side chain of Phe (**4**) resulted in only a 4-fold decrease in

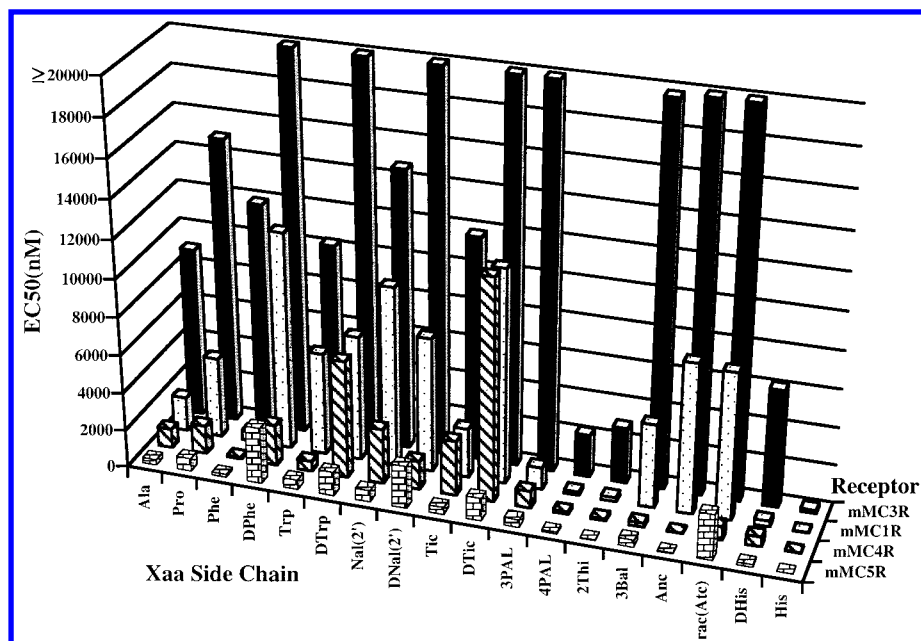


Figure 4. Graphical representation summarizing the effect on melanocortin receptor (Y axis) agonist EC_{50} values (Z axis) of the indicated amino acid substitution (X axis) of the His residue in the tetrapeptide template Ac-His-DPhe-Arg-Trp-NH₂.

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 DPhe⁶-containing tetrapeptide **5** resulted decreased potencies compared to the His⁶ peptide **1** (mMC4R > mMC5R > mMC1R) while losing the ability to stimulate the mMC3R at up to 100 μ M. When tetrapeptides **4** (Phe⁶) and **5** (DPhe⁶) are compared, a 2-, 31-, and 21-fold decrease in potency of the DPhe⁶ peptide compared with the Phe⁶ tetrapeptide was observed at the mMC1R, mMC4R, and mMC5R, respectively (Figure 4).

The Tic amino acid⁴³ (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** (Tic⁶) and **11** (DTic⁶), the L-configured compound possessed full agonism at the melanocortin receptors with 76- to 169-fold decreased potencies compared with the His⁶ 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 μ M and resulted in 562- to 3000-fold decreased potencies at the mMC1R, mMC4R, and mMC5R. When the Tic⁶ tetrapeptide (**10**) was compared with the corresponding Phe⁶ (**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 DTic⁶ tetrapeptide (**11**) with their corresponding DPhe⁶ (**5**) peptide, the D-configuration resulted in equipotency at the mMC1R,

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

Modification of the Phe⁶ benzyl ring by the insertion of a nitrogen in either the meta (3PAL, **12**) or para (4PAL, **13**) position generally resulted in decreased melanocortin receptor potency compared with peptide **1**. Tetrapeptide Ac-4PAL-DPhe-Arg-Trp-NH₂ (**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 Phe⁶-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 His⁶ (**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 μ M) 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 μ M) and 260- to 328-fold decreased potencies at the mMC1 and mMC4–5 receptors compared with **1**. Comparing tetrapeptides **6** (Trp⁶) and **7** (DTrp⁶) resulted in equipotent activities at the mMC1R and mMC5R (within experimental error) and an 8-fold decreased potency for the DTrp⁶ analogue at the mMC4R. Upon substitution of His⁶ with Trp in the SHU9119 peptide (Ac-Nle-c[Asp-His-DNal(2')-Arg-

Trp-Lys]-NH₂), 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 (His⁶ EC₅₀ = 2.3 nM) to a compound resulting in only slight agonist activity up to 1 μ M (Trp⁶).¹⁷ Upon substitution of the indole nitrogen of Trp⁶ (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 2Thi⁶ (14) and 3Bal⁶ (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 2Thi⁶ amino acid derivative (Figure 1) with the His⁶-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]-NH₂ 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 μ M (Figure 2). The most potent and mMC4R-selective (versus mMC3R) tetrapeptide is Ac-Anc-DPhe-Arg-Trp-NH₂ (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 μ M) at the mMC3R (Figure 2).⁴⁶ Unexpectedly, Ac-Anc-DPhe-Arg-Trp-NH₂ (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-NH₂ (17), was 34-fold less potent than peptide 16 at the mMC4R and only 11-fold less

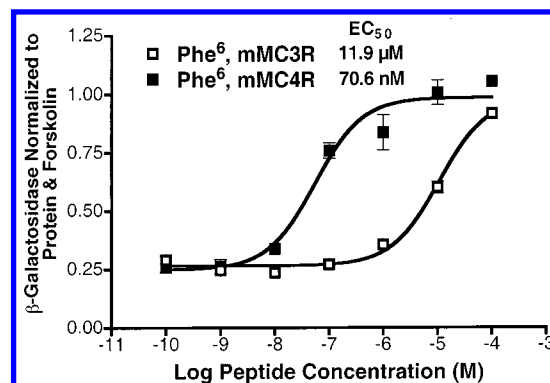


Figure 5. Comparison of the Phe⁶-containing tetrapeptide 4 EC₅₀ values at the centrally located mMC3R and mMC4R receptors. This figure illustrates that this compound possesses 160-fold receptor selectivity for the MC4R versus the MC3R.

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 Atc⁶-containing peptide 17 only possessed slight agonist activity (<50% maximal stimulation at 100 μ M) at the mMC3R. Figure 5 illustrates the MC4 versus MC3 receptor selectivity for the Phe⁶-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 μ M. These data and the fact that the Atc peptide 17 is actually a racemic mixture might suggest that the DAtc⁶-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 His⁶ 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 His⁶ side chain imidazole ring with various aromatic and nonaromatic substitutions in the tetrapeptide template Ac-His-DPhe-Arg-Trp-NH₂. 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-NH₂ tetrapeptide that possesses an MC4R EC₅₀ of 21 nM with only micromolar antagonism and a slight agonist activity (<50% maximal stimulation at 100 μ M) 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.

Experimental Section

Peptide Synthesis. Peptide synthesis was performed using standard Fmoc methodology³⁰ 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-naphthylcarboxylic acid (Anc) and Fmoc-amino-2-naphthylcarboxylic acid (Anc) were purchased from Pharma Core (High Point, NC). The coupling reagents 2-(1*H*-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 Kaiser⁴⁶ 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 μ L DMF is added to each reaction well to "wet the frit"; 3-fold excess amino acid starting from the C terminus is added (275 μ L of 0.5M amino acid solution containing 0.5 M HOBt in DMF) followed by the addition of 275 μ L 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 μ L of DMF to each reaction vessel, followed by the addition of 275 μ L of the respective amino acid (3-fold excess), 275 μ L of 0.5 M HBTU, and 225 μ L 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 Na-Fmoc-protected peptide resin is washed with DMF (4.5 mL, 4 times). Na-Fmoc deprotection is performed by the addition of 4 mL of 25% piperidine in DMF and mixed for 5 min at 450 rpm 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 Na-Fmoc deprotection of the final amino acid, acetylation of the Na-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 \times 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 μ L 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 zgpg30) for H₂O 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 $(1-2) \times 10^6$ cells per 100 mm dish. Melanocortin receptor DNA in the pCDNA3 expression vector (20 μ g) 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 μ g 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 μ L 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 μ L 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 $10\ \mu\text{L}$ were taken from each well and transferred to another 96-well plate for relative protein determination. To the cell lysate plates, $40\ \mu\text{L}$ of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, $150\ \mu\text{L}$ of substrate buffer ($60\ \text{mM}$ sodium phosphate, $1\ \text{mM}$ MgCl_2 , $10\ \text{mM}$ KCl , $5\ \text{mM}$ β -mercaptoethanol, $200\ \text{mg}$ 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 $200\ \mu\text{L}$ of 1:5 dilution Bio Rad G250 protein dye/water to the $10\ \mu\text{L}$ 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 $10\ \mu\text{M}$.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.

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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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