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1,2,3-Triazole Rings as a Disulfide Bond Mimetic in Chimeric AGRP-Melanocortin Peptides: Design, Synthesis, and Functional Characterization

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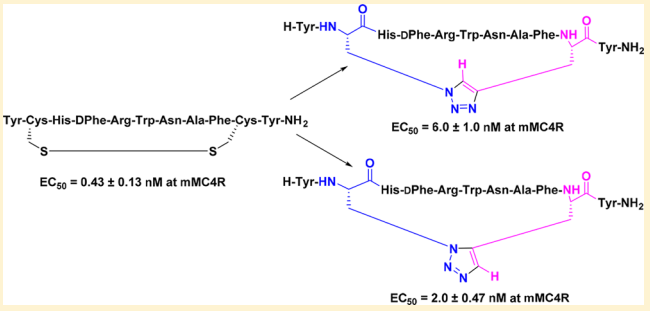
Supporting Information

ABSTRACT: The melanocortin system is involved in the regulation of complex physiological functions, including energy and weight homeostasis, feeding behavior, inflammation, sexual function, pigmentation, and exocrine gland function. The five melanocortin receptors that belong to the superfamily of G protein-coupled receptors (GPCRs) are regulated by endogenously expressed agonists and antagonists. The aim of this study was to explore the potential of replacing the disulfide bridge in chimeric AGRP-melanocortin peptide Tyr-c[Cys-His-D-Phe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH_2 (**1**) with 1,2,3-triazole moieties. A series of 1,2,3-triazole-bridged peptidomimetics were designed, synthesized, and pharmacologically evaluated at the mouse melanocortin receptors. The ligands possessed nanomolar to micromolar agonist cAMP signaling potency. A key finding was that the disulfide bond in peptide **1** can be replaced with the monotriazole ring with minimal effect on the functional activity at the melanocortin receptors. The 1,5-disubstituted triazole-bridged peptide **6** showed equipotent functional activity at the mMC3R and modest 5-fold decreased agonist potency at the mMC4R compared to those of **1**. Interestingly, the 1,4- and 1,5-disubstituted isomers of the triazole ring resulted in different selectivities at the receptor subtypes, indicating subtle structural features that may be exploited in the generation of selective melanocortin ligands. Introducing cyclic and acyclic bis-triazole moieties into chimeric AGRP template **1** generally decreased agonist activity. These results will be useful for the further design of neuronal chemical probes for the melanocortin receptors as well as in other receptor systems.



KEYWORDS: *Melanocortin receptors, AGRP, peptidomimetic, triazoles, disulfide bond*

INTRODUCTION

The melanocortin receptors (MCRs) are all G protein-coupled receptors (GPCRs) that signal mainly through the $G\alpha_s$ pathway to increase the amount of intracellular cAMP.[1] The melanocortin system includes five receptors (MC1−5R),[2−8] endogenous agonists [α-melanocyte-stimulating hormone (MSH), β-MSH, γ-MSH, and adrenocorticotropic hormone (ACTH)], and antagonists [agouti-signaling protein (ASP)[9] and agouti-related protein (AGRP)].[10] The melanocortin-1 receptor (MC1R) is expressed peripherally and is involved in skin and hair pigmentation.[2,3] The melanocortin-2 receptor (MC2R) is stimulated only by the ACTH and is expressed in the adrenal cortex to regulate steroidogenesis.[3] The melanocortin-3 receptor (MC3R) is expressed in several tissues, including the gut, placenta, heart, and brain, and is involved in metabolism and energy homeostasis.[4,6,11,12] The melanocortin-4 receptor (MC4R) is expressed primarily in the brain and regulates feeding behavior, energy homeostasis, and sexual function.[5,7,13,14] The melanocortin-5 receptor (MC5R) is expressed in a wide variety of tissues, both centrally and peripherally, and is involved in exocrine gland function in mice.[8,15]

The endogenous agonists α-MSH, β-MSH, γ-MSH, and ACTH are derived from posttranslational modifications of the proopiomelanocortin (POMC) protein.[16,17] All of these endogenous melanocortin linear peptides share a core "His-Phe-Arg-Trp" tetrapeptide sequence that has been hypothesized to be important for melanocortin receptor molecular recognition and stimulation.[18−20] The development of probes based on the His-Phe-Arg-Trp sequence targeting the neuronal MC3R and MC4R has been of specific interest to both the industrial and academic communities.[21] Specifically, melanocortin agonists produce anorexigenic effects via the neuronal MC3R and MC4R

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when administered intracerebroventricularly (icv) in mice and, therefore, may have utility in the treatment of metabolic disorders.[13,22]

In the study presented here, chimeric peptides based on the His-Phe-Arg-Trp sequence in an AGRP scaffold are studied. The MC3R and MC4R antagonist AGRP is produced in the brain and has been demonstrated to stimulate feeding and decrease energy expenditure.[23−27] Human AGRP is a 132-amino acid protein containing a cysteine-rich C-terminal domain.[10] Similar to the core sequence in the endogenous agonists, melanocortin antagonists possess a core "Arg-Phe-Phe" tripeptide sequence that has been demonstrated to be important for binding and function.[28,29] The C-terminal domain of AGRP has previously been shown to exhibit equipotent binding and activity compared with the full length peptide.[10,30] Further truncation typically results in diminished activity; however, a recent report of AGRP-derived peptides designed to mimic the active β-hairpin secondary structure resulted in subnanomolar antagonist activity at the mMC4R and was 160-fold selective for the mMC4R versus the mMC3R.[31]

The AGRP derivative, hAGRP(109−118) (Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-NH_2), was previously shown to have modest antagonist activity at the MC3R and MC4R.[29,32,33] However, replacing the Arg-Phe-Phe with the agonist scaffold His-D-Phe-Arg-Trp resulted in the subnanomolar potent agonist **1** (Tyr-c[Cys-His-D-Phe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH_2) at the melanocortin receptors.[34−37] Replacement of the disulfide bond with a lactam bridge, Tyr-c[β-Asp-His-D-Phe-Arg-Trp-Asn-Ala-Phe-Dpr]-Tyr-NH_2, resulted in a full agonist at the melanocortin receptors that was as potent as the endogenous α-MSH agonist.[34] Incorporation of a bioactive reverse-turn heterocycle into this template resulted in analogues with nanomolar potency at the mMC1R and MC3−5R,[38] a selective antagonist at MC3R,[39] and a selective and stable agonist of MC4R.[40] Hence, rational design of AGRP chimeric ligands may allow the development of potent and potentially selective ligands for the melanocortin receptors and may lead to novel chemical probes and possible therapeutic agents at the neuronal melanocortin receptors (i.e., MC3R and MC4R).

Disulfide bonds play important roles in the structure, stability, and biological function of endogenous proteins and peptides.[41] However, the disulfide bond is rather unstable in reducing environments or in the presence of certain enzymes, which can lead to structural rearrangements or low metabolic stability that lowers *in vivo* activity.[42,43] Stable covalent bond peptidomimetics such as lactam bridges,[44] thioethers,[45] olefins or carbon−carbon double bonds,[46,47] and diselenides[48] have been reported as replacements for disulfide bonds. However, some of these mimetic moieties result in lower bioactivity or include complex chemistry.[49] For example, the lactam bridge-cyclized variants require orthogonal protecting strategies to keep the N-terminus protected, while cyclization occurs through the side chains of aspartic acid and lysine residues. The multiple protection and deprotection steps result in an overall decrease in synthetic yield.

Utilization of the 1,2,3-triazole moiety as a disulfide bond replacement in peptide chemistry was first reported by Meldal et al.[50] The chemical orthogonality of the azide and alkyne building blocks, the robust synthetic methodology using click chemistry via either a copper-catalyzed (CuAAC)[51−53] or a ruthenium-catalyzed cycloaddition (RuAAC),[54,55] the hydrogen bonding capabilities, and the increased water solubility make the triazole moiety an attractive template for designing novel bioactive analogues. Moreover, 1,2,3-triazole-containing compounds have previously exhibited increased metabolic stability presumably through increased stability to isomerases or proteases.[56,57]

There have been various examples in the literature of the successful use of triazole-bridged peptides in ligand design. For example, a triazole-bridged peptide mimicking the epidermal growth factor receptor (EGFR) dimerization arm was reported to be resistant to proteolytic degradation while maintaining secondary structural characteristics.[58] Through the careful selection of triazole bridge length, β-hairpin peptides that inhibited acyl peptide hydrolase (APEH) enzyme activity were generated.[59,60] More specifically, there have been several reports of the utilization of 1,4-disubstituted and 1,5-disubstituted triazole bridges as a disulfide bond replacement.[50,61−63] Replacement of a disulfide bond with a 1,5-disubstituted triazole bridge was reported for the sunflower trypsin inhibitor-I.[63] The resulting 1,5-disubstituted triazole-bridged analogue retained nearly full biological activity in contrast to its 1,4-disubstituted triazole-bridged analogue. Importantly for the study presented here, incorporation of 4,1- or 1,4-disubstituted triazole bridges as replacements for lactam bridges in melanocortin agonist MT-II resulted in potent agonist ligands at the mMC1R, mMC3R, mMC4R, and mMC5R.[64]

The objective of this study was to investigate the effects of replacing the disulfide bond in AGRP chimeric peptide **1** (Figure 1a) with various triazole-based templates. It was hypothesized

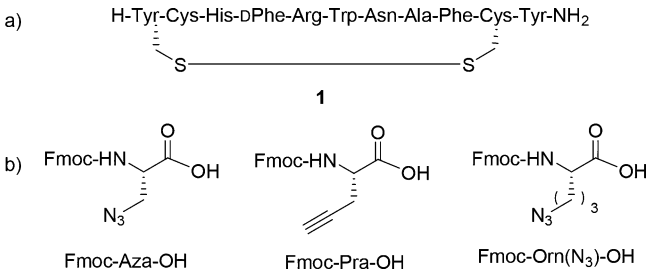

Figure 1. (a) Previously reported AGRP chimeric peptide template. (b) Non-natural amino acid building blocks Fmoc-L-azidoalanine (Fmoc-Aza-OH), Fmoc-L-propargylglycine (Fmoc-Pra-OH), and Fmoc-L-azidoornithine [Fmoc-Orn(N_3)-OH].

that the triazole bridge can mimic the disulfide bridge in **1** and may rigidify the backbone to result in potent and/or selective melanocortin ligands. Herein, the incorporation of 1,4- and 1,5-disubstituted triazole bridges as a replacement for the disulfide bond in the AGRP chimeric peptide template (**1**) is reported along with the synthesis of additional acyclic and cyclic bis-triazole-containing ligands. These compounds were pharmacologically assessed at the mMC1R, mMC3R, mMC4R, and mMC5R. These new chemical probes will be useful for studying the effects of the neuronal MC3R and MC4R on energy homeostasis. Also, the synthetic SAR lessons learned are broadly applicable to the future design of neuronal chemical probes for other receptor systems.

RESULTS AND DISCUSSION

Design and Synthesis of Triazole-Containing Peptides.

Previously, the AGRP chimeric peptide template Tyr-c[Cys-His-D-Phe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH_2 (**1**) was identified as a subnanomolar agonist for the melanocortin receptors.[34−37] Peptide **1** is derived from the active β-hairpin loop of the hAGRP(109−118) decapeptide sequence within the C-terminal region but replaces the key antagonist pharmacophore residues

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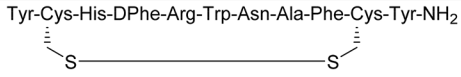
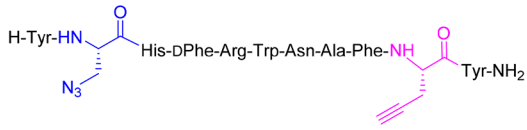
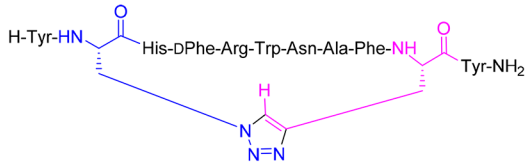
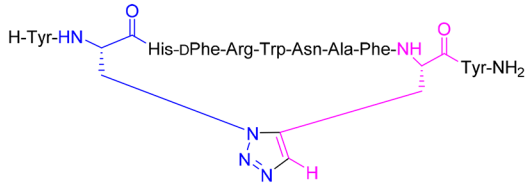
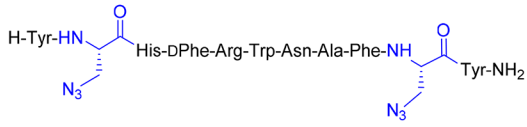
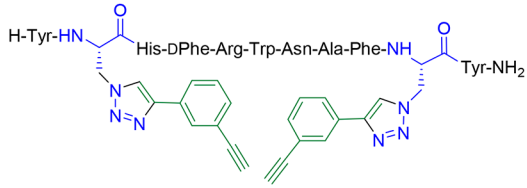
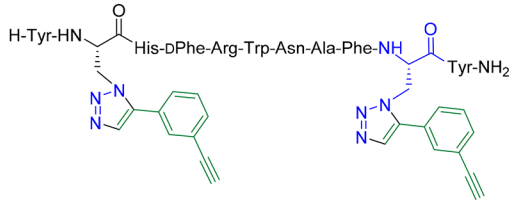
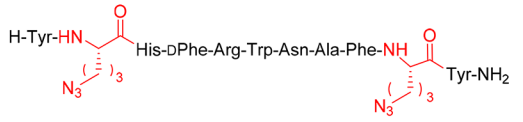
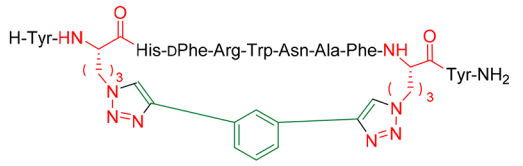
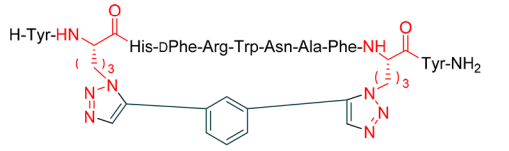
Table 1. Peptide Numbers and Sequences

Peptide	Sequence
1	
2	
3	
6	
7	
8	
12	
13	
14	
16	

Arg-Phe-Phe with the potent melanocortin agonist scaffold His-D-Phe-Arg-Trp.[34−37] This peptide is cyclized through a single disulfide bridge between two Cys side chains (Figure 1a). Peptide **1** is equipotent to the synthetic and potent agonist NDP-

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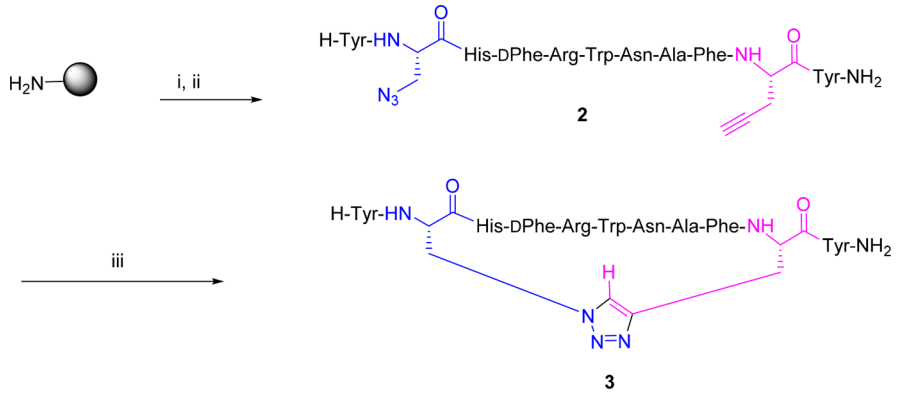
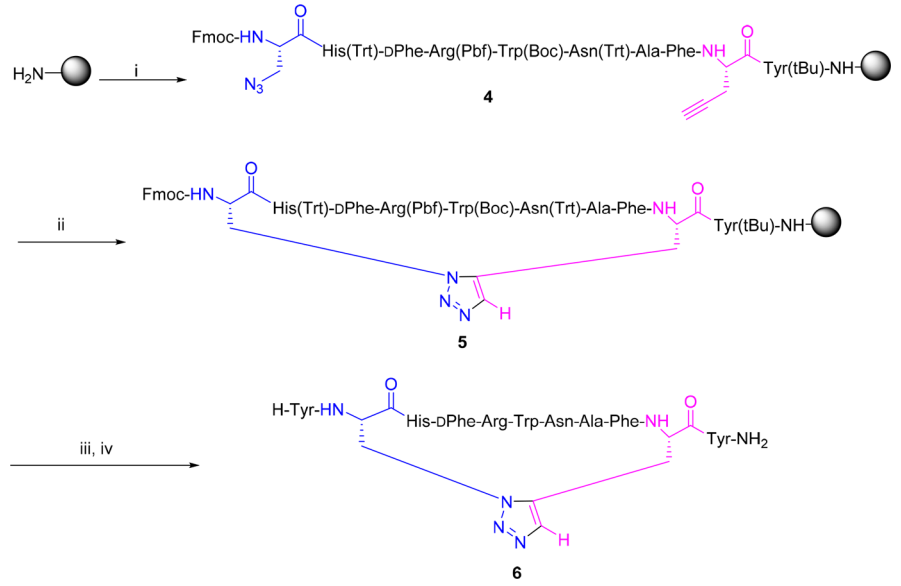
Scheme 1. Synthesis of 1,4-Disubstituted 1,2,3-Triazole-Bridged Peptide 3[a]



[a]Reagents and conditions: (i) Fmoc-SPPS; (ii) 91% TFA, scavengers; (iii) copper(II) sulfate pentahydrate, sodium ascorbate, H_2O-*t*BuOH (1:1), room temperature, overnight.

Scheme 2. Synthesis of 1,5-Disubstituted 1,2,3-Triazole-Bridged Peptide 6[a]



[a]Reagents and conditions: (i) Fmoc-SPPS; (ii) chloro(pentamethylcyclopentadienyl)(cyclooctadiene)ruthenium(II), microwave, 60 °C, 3 h; (iii) Fmoc-SPPS; (iv) 91% TFA, scavengers.

MSH at the centrally located mMC4R (Table 1).[34−37] NDP-MSH is a common melanocortin control peptide that decreases food intake upon central administration in mice. The similarity of the potency of peptide **1** to that of NDP-MSH suggests it may be a valuable scaffold for developing chemical probes for studying the neuronal melanocortin receptors.[13] In the study presented here, the possibility of a triazole bridge as a replacement for the disulfide bond in melanocortin chimeric peptide **1** is explored.

The 1,4-disubstituted and 1,5-disubstituted triazole-bridged cyclic peptides **3** and **6** were designed from the original chimeric peptide **1** by replacing the disulfide bond with triazole bridges (Schemes 1 and 2). First, linear peptide Tyr-Aza-His-D-Phe-Arg-Trp-Asn-Ala-Phe-Pra-Tyr-NH_2 (**2**) was synthesized, in which the N^{α}-Fmoc-Cys-OH residues were replaced by N^{α}-Fmoc-L-Pra-OH and N^{α}-Fmoc-L-Aza-OH amino acids during synthesis (Scheme 1 and Figure 1). This resulted in peptide **2** with side chains containing alkyne and azide groups. The stepwise solid-phase assembly of linear peptide **2** was performed following the Fmoc/*t*Bu SPPS strategy on Rink-amide MBHA resin.[65] Linear peptide **2** was then cyclized using Cu(I)-catalyzed intramolecular azide−alkyne cycloaddition in solution to afford 1,4-disubstituted triazole-bridged cyclic peptide **3** (Scheme 1).

The 1,5-disubstituted triazole-bridged cyclic peptide **6** was also synthesized using Fmoc-SPPS on Rink-amide MBHA resin (Scheme 2). First, amino acids were coupled on resin to give linear peptide resin **4**, which was then cyclized on resin using chloro(pentamethylcyclopentadienyl)(cyclooctadiene)-ruthenium(II) under microwave conditions (60 °C, 30 W, 3 h) to afford macrocyclized peptide **5** on resin. Further coupling of Tyr and cleavage yielded 1,5-substituted triazole-bridged cyclic peptide **6** (Scheme 2). The purity of peptides **2**, **3**, and **6** was confirmed by reverse-phase high-performance liquid chromatography (RP-HPLC), and the mass was determined by electrospray ionization mass spectrometry (ESI-MS) (Supporting Information). Because there was no change in the molecular mass between linear peptide **2** and triazole-bridged peptides **3** and **6**, infrared (IR) spectra was recorded for peptides **2**, **3**, and **6** to confirm the formation of the triazole bridge. Linear peptide **2** gave an IR absorption band around 2100 cm^{-1} supporting the presence of an azide group. The absence of this band in peptides

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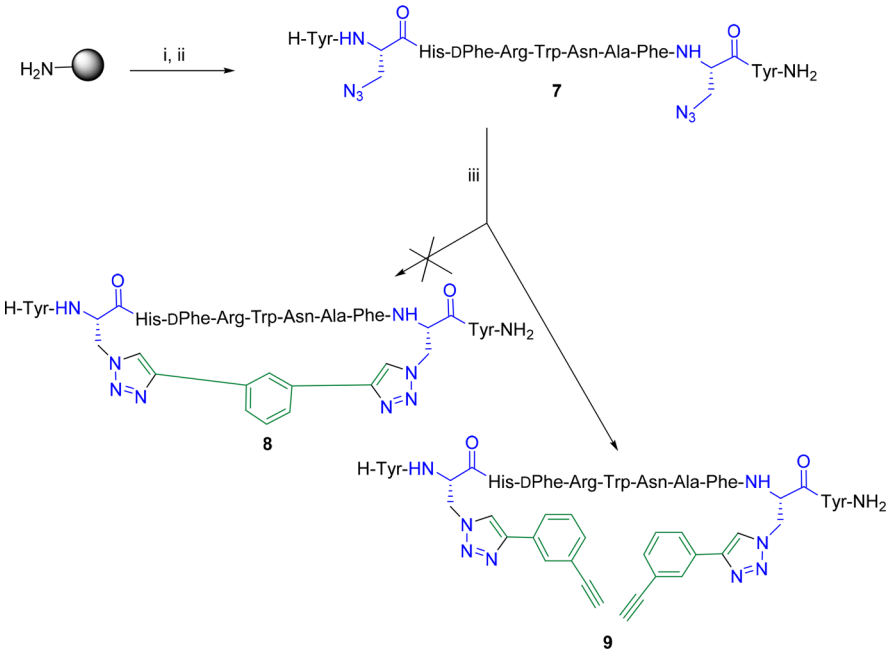
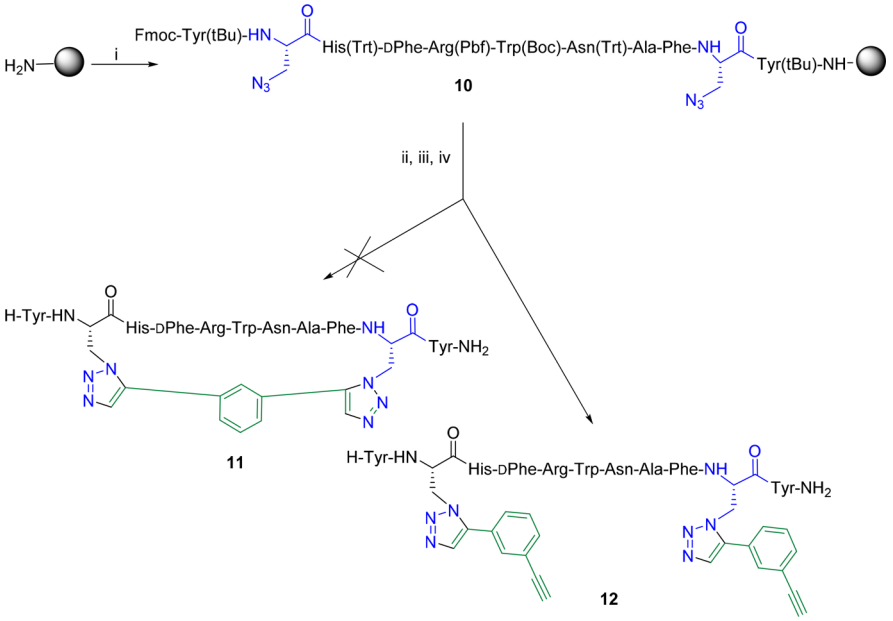
Scheme 3. Synthesis of 1,4-Disubstituted 1,2,3-Bis-Triazole-Containing Peptide 9[a]



[a]Reagents and conditions: (i) Fmoc-SPPS; (ii) 91% TFA, scavengers; (iii) 1,3-diethynylbenzene, copper(II) sulfate pentahydrate, tris(3-hydroxylpropyltriazolylmethyl)amine, sodium ascorbate, H_2O/*t*BuOH (1:1), room temperature, overnight.

Scheme 4. Synthesis of 1,5-Disubstituted 1,2,3-Bis-Triazole-Containing Peptide 12[a]



[a]Reagents and conditions: (i) Fmoc-SPPS; (ii) 1,3-diethynylbenzene, chloro(pentamethylcyclopentadienyl)(cyclooctadiene)ruthenium(II), microwave, 60 °C, 3 h; (iii) 20% piperidine in DMF; (iv) 91% TFA, scavengers.

3 and **6** supports the consumption of azide groups and formation of triazole bridges in these peptides (Supporting Information).[63,66,67]

The 1,4- and 1,5-substitution pattern in peptides **3** and **6** was confirmed using two-dimensional (2D) HSQC NMR spectroscopy studies by measuring the chemical shifts of the ^{1}H and ^{13}C coupling signal assigned to the unique carbon-bonded proton found in both heterocycles. In the ^{1}H NMR spectra, the carbon-bonded proton in the 1,4-disubstituted triazole appeared at 7.52 ppm, whereas this proton in the 1,5-disubstituted triazole appeared at 7.39 ppm (Supporting Information). The proton-bonded carbon in the 1,4-disubstituted triazole appeared at 124.6 ppm, whereas in 1,5-disubstituted triazole, it appeared at 132.6 ppm in the 2D HSQC NMR spectroscopic studies (Supporting Information). The measured ^{1}H and ^{13}C chemical shifts were in good agreement with previously reported data[63,68] and revealed a significant difference between the 1,4-disubstituted triazole and 1,5-disubstituted triazole patterns (Supporting Information).

Previously, bulky side chains were used to synthesize conformationally constrained analogues to modulate the potency

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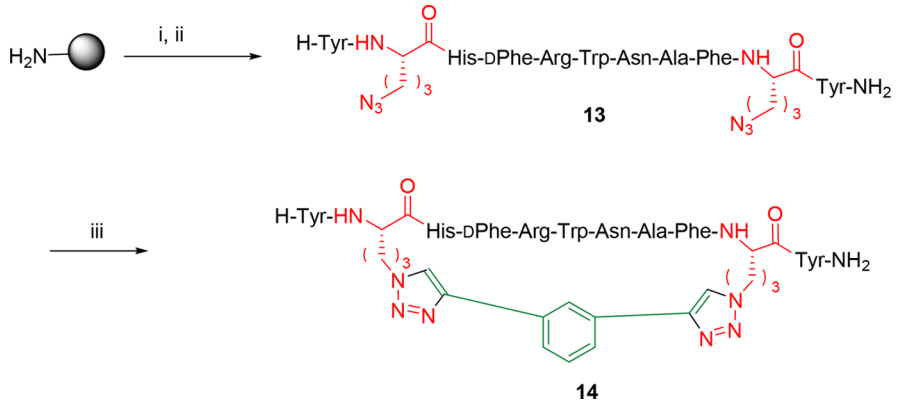
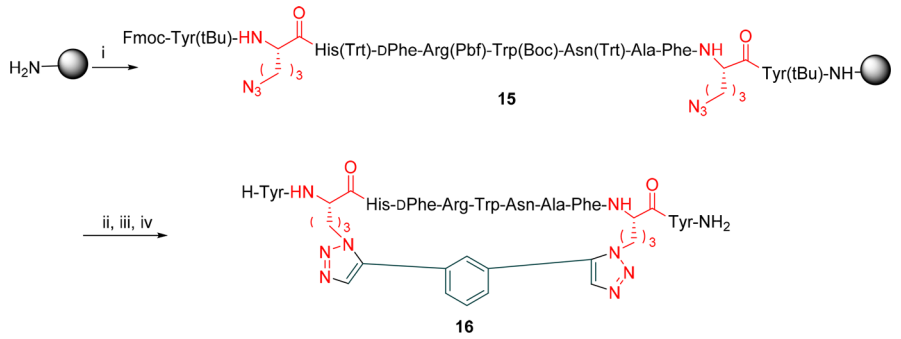
Scheme 5. Synthesis of 1,4-Disubstituted 1,2,3-Bis-Triazole-Bridged Peptide 14[a]



[a]Reagents and conditions: (i) Fmoc-SPPS; (ii) 91% TFA, scavengers; (iii) 1,3-diethynylbenzene, copper(II) sulfate pentahydrate, tris(3-hydroxylpropyltriazolylmethyl)amine, sodium ascorbate, H_2O/*t*BuOH (1:1), room temperature, overnight.

Scheme 6. Synthesis of 1,5-Disubstituted 1,2,3-Bis-Triazole-Bridged Peptide 16[a]



[a]Reagents and conditions: (i) Fmoc-SPPS; (ii) 1,3-diethynylbenzene, chloro(pentamethylcyclopentadienyl)(cyclooctadiene)ruthenium(II), microwave, 60 °C, 3 h; (iii) 20% piperidine in DMF; (iv) 91% TFA, scavengers.

and selectivity of the melanocortin receptor subtypes.[69] To modify the selectivity of triazole-bridged peptides at the melanocortin receptors, we designed bis-triazole-bridged macrocyclic peptides **8** and **11** (Schemes 3 and 4) that we envisioned would be more bulky and rigid than monotriazole-bridged macrocyclic peptides **3** and **6**. First, to synthesize 1,4-substituted bis-triazole-bridged peptide **8**, linear peptide **7** was synthesized using Fmoc-SPPS on Rink-amide MBHA resin in which both Cys amino acids were substituted with N^{α}-Fmoc-L-Aza-OH in lead chimeric peptide template **1**. Linear peptide **7** on reaction with 1,3-diethynylbenzene using a slightly modified Cu(I)-catalyzed intermolecular azide–alkyne cycloaddition previously reported did not afford the expected 1,4-disubstituted bis-triazole-bridged macrocyclic peptide **8**.[70] Instead, the 1,4-substituted bis-triazole-containing acyclic peptide **9** was isolated as the major product along with unreacted peptide **7** (Scheme 3). To synthesize the 1,5-substitited bis-triazole-containing acyclic peptide **12** on the same template, we first synthesized a peptide intermediate **10** on Rink-amide MBHA resin using the Fmoc/*t*Bu SPPS approach in which both Cys amino acids were again replaced with N^{α}-Fmoc-L-Aza-OH. Then the peptide on resin (**10**) was treated with 1,3-diethynylbenzene using chloro(pentamethylcyclopentadienyl)(cyclooctadiene)ruthenium(II) under microwave conditions (60 °C, 30 W, 3 h). Deprotection, cleavage, and purification of the peptide led to the purified 1,5-disubstituted bis-triazole-acyclic peptide **12** as the major product. Again in this case, we did not isolate the expected 1,5-disubstituted bis-triazole-bridged macrocyclic peptide **11**. These results suggested that the formation of the macrocyclic peptides is not feasible under current reaction conditions possibly because of the ring size and steric effects.

To study the effects of ring size on macrocyclization, N^{α}-Fmoc-L-Orn(N_3)-OH was utilized to replace the N^{α}-Fmoc-L-Aza-OH in the reaction (Figure 1). The use of N^{α}-Fmoc-L-Orn(N_3)-OH spaces the azide group from the peptide backbone by two extra methylene units compared to that seen with N^{α}-Fmoc-L-Aza-OH, which should reduce the steric hindrance during the desired reaction. First, linear peptide **13** was synthesized by replacing the two Cys amino acids in **1** with N^{α}-Fmoc-L-Orn(N_3)-OH using the Fmoc/*t*Bu SPPS approach (Scheme 5). Linear peptide **13** was reacted with 1,3-diethynylbenzene using Cu(I)-catalyzed intermolecular azide–alkyne cycloaddition to yield the macrocyclic 1,4-disubstituted bis-triazole-bridged peptide **14** as the major product (Scheme 5).

The 1,5-substituted bis-triazole peptide regioisomer of **14** was also synthesized (Scheme 6). Intermediate peptide resin **15** was treated with 1,3-diethynylbenzene using chloro(pentamethylcyclopentadienyl)(cyclooctadiene)ruthenium(II) under microwave conditions (60 °C, 30 W, 3 h). The deprotection, cleavage, and purification of the peptide resulted in the 1,5-disubstituted bis-triazole-macrocyclic peptide **16** as the major product (Scheme 6). These results suggest that the formation of the macrocyclic peptide under current reaction conditions is dependent on steric effects in which increased ring size or additional spacing to separate the azide from the peptide backbone is a requirement for efficient cyclization.

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Table 2. Summary of the Agonist Pharmacology of Peptides 1−3 and 6 Evaluated at the Mouse Melanocortin Receptors[a]

peptide	mMC1R EC_{50} (nM)	*x*-fold difference	mMC3R EC_{50} (nM)	*x*-fold difference	mMC4R EC_{50} (nM)	*x*-fold difference	mMC5R EC_{50} (nM)	*x*-fold difference
NDP-MSH	0.02 ± 0.008		0.24 ± 0.04		0.34 ± 0.09		0.39 ± 0.08	
1	0.14 ± 0.01	1	2.2 ± 0.58	1	0.43 ± 0.13	1	2.4 ± 0.31	1
2	58 ± 6.3	414	51 ± 9.3	23	29 ± 5.2	46	20 ± 3.8	8
3	5.8 ± 2.0	41	28 ± 5.4	13	6.0 ± 1.0	14	16 ± 0.66	6.6
6	4.0 ± 2.4	29	6.4 ± 2.0	3	2.0 ± 0.47	4.6	3.6 ± 1.1	1.5

[a]AlphaScreen technology was used to determine the relative potency of compounds to induce cAMP signaling. The indicated errors represent the standard error of the mean (SEM) determined from at least three independent experiments.

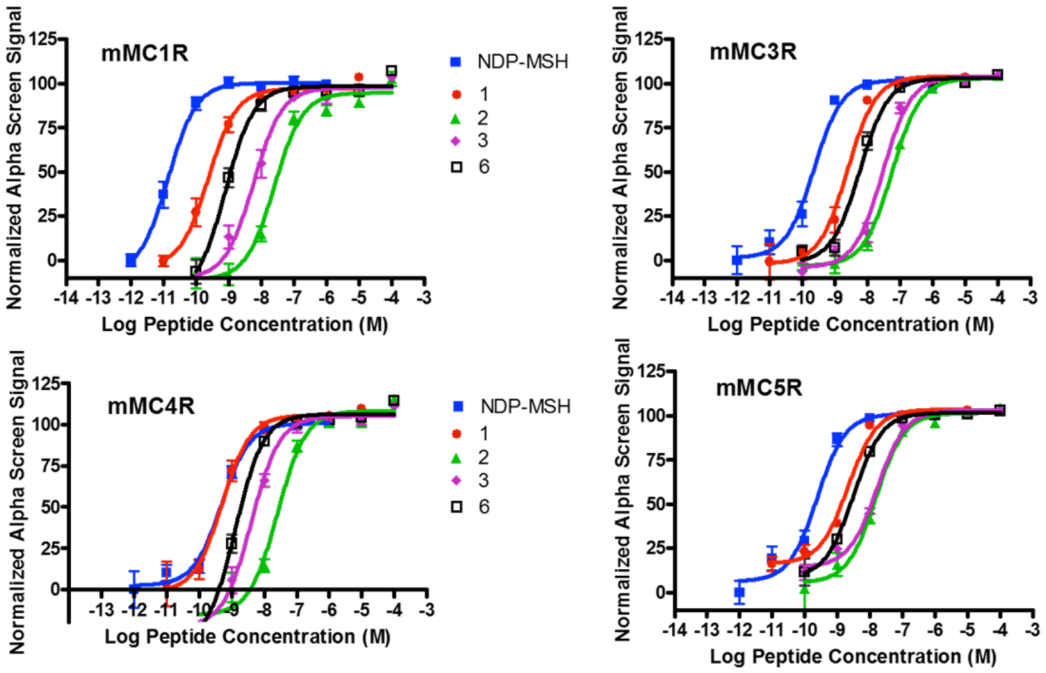

Figure 2. Illustration of the agonist pharmacology of compounds **1**−**3** and **6** at the mMC1R, mMC3R, mMC4R, and mMC5R. The AlphaScreen assay is a competition assay with a loss of signal resulting from increased agonist activity. Hence, the concentration−activity curves were normalized to the maximal response of NDP-MSH for the purpose of illustration.

Table 3. Summary of the Agonist Pharmacology of Peptides 7, 9, and 12 Evaluated at the Mouse Melanocortin Receptors[a]

peptide	mMC1R EC_{50} (nM)	*x*-fold difference	mMC3R EC_{50} (nM)	*x*-fold difference	mMC4R EC_{50} (nM)	*x*-fold difference	mMC5R EC_{50} (nM)	*x*-fold difference
NDP-MSH	0.02 ± 0.008		0.24 ± 0.04		0.34 ± 0.09		0.39 ± 0.08	
1	0.14 ± 0.01	1	2.2 ± 0.58	1	0.43 ± 0.13	1	2.4 ± 0.31	1
7	5.0 ± 0.24	36	59 ± 5.8	27	16.0 ± 5.0	37	20.0 ± 2.0	8
9	802 ± 227	5728	1462 ± 245	665	1522 ± 328	3540	294 ± 4.8	122
12	116 ± 40	829	404 ± 52	184	142 ± 30	330	107 ± 17	45

[a]AlphaScreen technology was used to determine the relative potency of compounds to induce cAMP signaling. The indicated errors represent the standard error of the mean (SEM) determined from at least three independent experiments.

Pharmacological Evaluation. The ability of the synthesized compounds (Table 1) to stimulate intracellular cAMP signaling was assayed in HEK293 cells stably expressing the cloned mouse (m)MC1R, mMC3R, mMC4R, and mMC5R using AlphaScreen cAMP Assay Technology (PerkinElmer).[71,72] The mMC2R is stimulated by only ACTH and was therefore excluded from this study. Because of the inherent error of the assay (in our hands), peptides that were within a 3-fold potency range were considered to be equipotent.

The agonist EC_{50} values at the mouse melanocortin receptors of compounds **2**, **3**, and **6** (as well as control compounds NDP-MSH and compound **1**) are summarized in Table 2 and Figure 2. Linear compound **2** containing azide and alkyne groups in place of thiols resulted in 400-, 23-, 46-, and 8-fold decreased agonist potency at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively, compared to that of control compound **1**. The covalent bond formation via click chemistry between the azide and alkyne group resulted in triazole-bridged compounds **3** and **6**. Cyclized peptide **3**, which contained a 1,4-disubstituted triazole ring, resulted in 41-, 13-, 14-, and 7-fold decreased activity at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively, when compared to that of cyclic disulfide compound **1**. Compound **3** had increased functional potency at the mMC1R and mMC4R in comparison to that of linear compound **2**, suggesting that cyclization is important for the activity at these two receptor isoforms. Cyclic 1,5-disubstituted

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Table 4. Summary of the Agonist Pharmacology of Peptides 13, 14, and 16 Evaluated at the Mouse Melanocortin Receptors[a]

peptide	mMC1R EC_{50} (nM)	x-fold difference	mMC3R EC_{50} (nM)	x-fold difference	mMC4R EC_{50} (nM)	x-fold difference	mMC5R EC_{50} (nM)	x-fold difference
NDP-MSH	0.02 ± 0.008		0.24 ± 0.04		0.34 ± 0.09		0.39 ± 0.08	
1	0.14 ± 0.01	1	2.2 ± 0.58	1	0.43 ± 0.13	1	2.4 ± 0.31	1
13	138 ± 18	986	674 ± 162	306	414 ± 54	963	36 ± 7.0	15
14	317 ± 9	2264	1944 ± 687	884	971 ± 213	2258	229 ± 52	95
16	570 ± 153	4071	2744 ± 706	1247	539 ± 154	1253	80 ± 26	33

[a]AlphaScreen technology was used to determine the relative potency of compounds to induce cAMP signaling. The indicated errors represent the standard error of the mean (SEM) determined from at least three independent experiments.

compound **6** resulted in 29- and 5-fold decreased activity at the mMC1R and mMC4R, respectively, compared to that of compound **1**. Compound **6** was within experimental error at the mMC3R and mMC5R compared to compound **1**. Interestingly, peptide **6** increased the potency at the mMC3R and mMC5R and maintained potent nanomolar agonist activity at the mMC1R and mMC4R when compared to that of compound **3**, suggesting the type of triazole linkage (i.e., 1,4-disubstituted vs 1,5-disubstituted) can modify functional activity.

In linear peptides **9** and **12**, both cysteine residues were replaced by either 1,4-disubstituted (3-ethenylphenyl) triazole or 1,5-disubstituted (3-ethenylphenyl) triazole groups and were derived from linear analogues **7** and **10**, respectively. Linear peptide **7** with azide side chains in place of the thiols showed 36-, 27-, 37-, and 8-fold decreased potency at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively, compared to that of control cyclic compound **1** (Table 3). The activities of compound **7** were similar at the mMC3R, mMC4R, and mMC5R compared to that of the similar linear compound **2**, which displays azide and alkenyl side chains. However, peptide **7** resulted in a 12-fold decreased potency at the mMC1R compared to that of peptide **2**. Linear peptides **9** and **12** both showed significantly decreased potencies compared to that of **1**. Peptide **9** resulted in 5728-, 665-, 3540-, and 144-fold decreased agonist potency compared to that of compound **1** at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively (Table 3). The 1,5-disubstituted analogue **12** was more potent than **9** but still had 829-, 184-, 330-, and 45-fold decreased potencies compared to that of compound **1** at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively (Table 3).

Macrocyclic peptides **14** and **16** were derived from linear analogues **13** and **15**, respectively, and showed improved potency compared to those of linear counterparts **8** and **9** (Table 4). Like the cyclic monotriazoles and linear bis-triazole, 1,5-disubstituted bis-triazole peptide **16** was equipotent or had increased potency compared to 1,4-disubstituted bis-triazole peptide **14**. Interestingly, peptides **14** and **16** showed modest selectivity for the mMC5R with EC_{50} values of 36 and 80 nM, respectively (Table 4).

CONCLUSIONS

In this study, we have designed and synthesized a series of click-generated triazole-containing cyclic or acyclic peptidomimetics to probe potency and selectivity at the melanocortin receptors. These peptidomimetics will be useful probes for studying the various physiological functions, including feeding behavior, energy homeostasis, sexual function, pigmentation, and cardiovascular function. Specifically, these probes can be used to study the neuronal MC3R and MC4R and their effects on energy homeostasis as their structure was based on AGRP, the endogenous antagonist of these receptors. The previously reported 11-mer peptide **1** was the starting scaffold of this study. Peptide **1** is based on the structure of hAGRP(109−118) containing one disulfide bond between the cysteine residues as well the substitution of the antagonist scaffold Arg-Phe-Phe with the agonist scaffold His-D-Phe-Arg-Trp.[34−37] This potent agonist peptide is not selective at the melanocortin receptors, and the disulfide bond may be a metabolic liability that reduces its utility as an *in vivo* probe. Triazoles were selected for replacing the disulfide bond because of their high dipole moment, chemical orthogonality, conformational stability, and ease of synthesis. Triazole rings are aromatic in nature and can participate in hydrogen bonding, which can facilitate additional putative ligand−receptor interactions and provide distinct functional properties.[50−64]

Of the novel chemical probes synthesized in this study, monotriazole compound **6** was the most potent compound with equipotent activity (within 3-fold experimental error) at the mMC3R and mMC5R and modest 5-fold decreased agonist potency at the mMC4R compared to that of the disulfide-containing peptide **1**. It also resulted in a 29-fold decrease in agonist potency at the mMC1R that may help give it increased utility as a chemical probe for the neuronal mMC3R and mMC4R. This reduced potency at the mMC1R was similar to the previous report of a 1,4-substituted triazole-containing MT-II analogue.[64] Disulfide peptide **1** showed some discrimination in potency, being 5−10-fold more potent at the mMC1R and mMC4R than at the mMC3R and mMC5R. This difference was lost when the 1,5-disubstituted triazole ring replaced the disulfide bridge in compound **6**. Compound **3** possessed 5-fold selectivity for the mMC4R versus the mMC3R but in general resulted in 7−40-fold decreased agonist potency compared to that of peptide **1**. Linear precursor **2** was less potent than cyclic peptides **3** and **6**, suggesting that the macrocyclization of the peptide via the triazole ring contributed to the stability of the bioactive conformation, resulting in improved agonist activity.

Acyclic peptides **9** and **12** resulted from unsuccessful cyclizations of precursors **7** and **10** with bulky 1,4-disubstituted (3-ethenylphenyl) triazole and 1,5-disubstituted (3-ethenylphenyl) triazole groups. Both **9** and **12** showed reduced potency compared to that of their monotriazole counterpart. The 1,5-disubstituted peptide **12** was more potent at all studied melanocortin receptors than the 1,4-disubstituted peptide **9**. This result can be explained by conformational studies of 1,4- and 1,5-substituted triazole rings previously reported.[73] The 1,4-disubstituted triazole moiety is flexible without a preference for a particular conformation, whereas the 1,5-disubstituted triazole exists in a number of stable and structurally diverse conformers, which may lead to several secondary structures, including turns.[73] Our data suggest that the 1,5-disubstituted triazole ring is preferred presumably because it is achieving a structural

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conformation that is better suited to activate the melanocortin receptors than those achieved by the 1,4-disubstituted triazole.

Peptides **14** and **16** are cyclic bis-triazoles based on a two-component system linked by a phenyl ring linker. Unlike linear precursor **7**, which had an azidomethyl group, peptide **13** had azidopropyl groups in place of the cysteine residues. This modification resulted in reduced agonist potency of linear peptide **13** at all melanocortin receptors. The mMC1R and mMC4R were more affected with ∼1000-fold decreased potency, while the mMC3R resulted in a 300-fold decreased agonist potency compared to that of **1**. However, at the mMC5R, both peptides **7** and **13** exhibited only 8- and 15-fold decreased agonist potency, respectively, compared to that of **1**. Cyclization of linear precursor **13** via the phenyl linker into the 1,4-disubstituted bis-triazole, **14**, and its 1,5-counterpart, **16**, did not improve activity. In fact, activity was reduced at all the melanocortin receptors except for compound **16** at the mMC5R, which was equipotent compared to linear precursor **13**. It may be hypothesized that the bis-triazole moiety with the phenyl linker altered the size of the ring and perturbed a favorable conformation inhibiting important ligand−receptor interactions at the mMC1R, mMC3R, and mMC4R.

In summary, we have designed, synthesized, and pharmacologically evaluated three series of triazole-containing peptides as possible replacements for the disulfide bridge in an AGRP chimeric peptide template as agonists at the mouse melanocortin receptors. The modular synthesis of 1,4-disubstituted triazole-bridged peptides was obtained using copper-catalyzed azide−alkyne "click" cycloaddition (CuAAC) in the solution phase. The 1,5-disubstituted triazole-bridged peptides were obtained using ruthenium(II)-catalyzed azide−alkyne cycloaddition (RuAAC) on the solid phase using microwave irradiation. Peptide **6** having a 1,5-disubstituted triazole bridge showed similar potency at the mMC3R and mMC5R and 5-fold decreased potency at the mMC4R compared to that of disulfide-bridged peptide **1**, supporting its further development as a neurochemical probe. The macrocyclization involving bis-triazoles resulted in compound **16**, which is a potent agonist of the mMC5R with an agonist potency of 80 nM. It can be postulated that the metabolic stability of these compounds will be improved by the replacement of the disulfide bridge with the triazole moiety because of the inherent redox stability of triazoles and the lack of specific enzymes to degrade the unnatural triazole linker. We demonstrated that disulfide bond replacement with a triazole bridge is a valid strategy for engineering melanocortin peptides and provided the field with novel chemical probes for studying the neuronal melanocortin receptors.

METHODS

Chemistry. All chemicals were obtained from commercial suppliers and used without further purification. The amino acids N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)-Cys(Trt)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-D-Phe-OH, Fmoc-Phe-OH, Fmoc-Asn(Trt)-OH, and Fmoc-Ala-OH, Rink amide *p*-methylbenzhydrylamine resin (*p*-MBHA resin, 0.35 mequiv/g substitution), and coupling reagent *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HBTU) were purchased from Peptides International (Louisville, KY). The amino acids N^{α}-(9-fluorenylmethyloxycarbonyl)-propargylglycine-OH (Fmoc-L-Pra-OH) and N^{α}-(9-fluorenylmethyloxycarbonyl)-3-azido-L-alanine (Fmoc-L-Aza-OH), tris(3-hydroxypropyltriazolylmethyl)amine, 1,3-diethynylbenzene, and chloro(pentamethylcyclopentadienyl)-(cyclooctadiene)ruthenium(II) were purchased from Sigma-Aldrich (St. Louis, MO). N^{α}-(9-Fluorenylmethyloxycarbonyl)-azido-L-ornithine [Fmoc-L-Orn(N_3)-OH] was synthesized according to a literature procedure.[70] Copper sulfate pentahydrate was purchased from Chem-Impex International Inc. (Wood Dale, IL). Sodium ascorbate and *tert*-butanol were purchased from Acros Organics.

Dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ). *N*,*N*-Dimethylformamide (DMF), trifluoroacetic acid (TFA), 1,3-diisopropylcarbodiimide (DIC), and piperidine were purchased from Sigma (St. Louis, MO). *N*,*N*-Diisopropylethylamine (DIEA) was purchased from Aldrich (Milwaukee, WI). All reagents and chemicals were ACS grade or better and used without further purification.

Solid-Phase Peptide Synthesis Using Microwave Irradiation. The Rink-amide MBHA resin was transferred into a 25 mL polypropylene reaction vessel (CEM) and swelled in dichloromethane for 1 h. The bottom cap of the vessel was removed, and the vessel was transferred onto a vacuum filtration manifold. Deprotection of the Fmoc group was achieved using 20% piperidine in DMF for 2 min at room temperature, followed by irradiation of the reaction mixture at 75 °C and 30 W for 4 min in a CEM Discover SPS instrument. After the resin had been cooled and washed with DMF (four times), a ninhydrin Kaiser test was performed.[74] The amino acid coupling step was performed under the microwave conditions (75 °C and 30 W) for 5 min. The Fmoc-protected amino acid (3 equiv) and HBTU (3 equiv) were dissolved in DMF and added to the reaction vessel followed by addition of DIEA (5 equiv). The coupling of the Cys and His amino acids was performed at a lower temperature (50 °C and 30 W) for 5 min. To ensure coupling of Arg, the number of equivalents were increased to 5 equiv of Arg and 7 equiv of DIEA and the reaction time was increased to 10 min. The reagents were mixed by bubbling nitrogen gas into the reaction vessel. The iterative process of the Fmoc deprotection/coupling cycle was performed to elongate the peptide chain. After the final deprotection, the peptide was cleaved from the resin using the TFA/anisole/1,2-ethanedithiol/phenol/H_2O cleavage cocktail [91:2.5:2.5:1.5:2.5 (v/v/v/v/v)] at room temperature for 3 h. After the simultaneous cleavage and side chain deprotection, the solution was concentrated. The peptide was precipitated from the solution and washed using cold (4 °C) anhydrous diethyl ether.

The crude peptides were purified by RP-HPLC (flow rate of 5 mL/min, acetonitrile/water with 0.1% TFA) 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). The purified peptides were >95% pure as determined by RP-HPLC in two diverse solvent systems (i.e., MeOH and ACN) (Supporting Information). ESI-MS on an ABI 3200Q TRAP was used to confirm masses of peptides. Full analytical data of the synthesized peptides are given in the Supporting Information.

Disulfide Bridge Formation in Peptide 1. Pure linear peptide H-Tyr-Cys-His-D-Phe-Arg-Trp-Asn-Ala-Phe-Cys-Tyr-NH_2 was dissolved in 20% DMSO in water (1.0 mg/mL) and stirred at room temperature for 24 h. The progress of the disulfide cyclization was monitored by RP-HPLC. The resulting solution was concentrated and purified by RP-HPLC to yield peptide **1** (H-Tyr-c[Cys-His-D-Phe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH_2).

Preparation of Peptides That Used Copper-Catalyzed Cyclization. The purified linear peptide of interest (1 equiv), $CuSO_4 \cdot 5H_2O$ (2 equiv), and sodium ascorbate (3 equiv) were added to a 6 mL deoxygenated solution mixture of H_2O and *t*BuOH (1:1), and the solution was mixed with nitrogen gas for 15 min. In the synthesis of peptides **9** and **14**, 1,3-diethynylbenzene (1 equiv) and tris(3-hydroxypropyltriazolylmethyl)amine (1 equiv) were also added. The mixture was stirred overnight at room temperature under a nitrogen atmosphere, concentrated, and then passed through a solid-phase extraction (SPE) RP (C18) column with ACN in water. Peptides were purified using semipreparative HPLC as described above to afford pure peptides.

Preparation of Ruthenium-Catalyzed 1,5-Disubstituted Triazole Peptides. The appropriate peptide resin was placed in a 25 mL polypropylene reaction vessel (CEM) and swelled in DMF (10 mL) for 30 min while argon gas was being bubbled through the solution. Then the chloro(pentamethylcyclopentadienyl)(cyclooctadiene)ruthenium-

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(II) (20 mol % of initial loading of the resin) was added to the reaction mixture. In the synthesis of peptides **12** and **16**, 1,3-diethynylbenzene (1 equiv) was also added to the reaction mixture. The resulting mixture was heated under microwave irradiation for 3 h at 60 °C and 30 W under an argon atmosphere. The resin was cooled and washed with methanol, 2% sodium dithiocarbamate in DMF (v/v), and DMF. Final peptides were cleaved and purified by semipreparative HPLC to afford purified peptides.

NMR Spectroscopy. The ligand NMR samples were prepared by dissolving 1.6 mg of the purified peptides in a 700 μL solution containing 480 μL of CD_3CN and 220 μL of H_2O. The NMR data were collected at 32 °C with a Bruker Avance II 600 MHz spectrometer and a 5 mm CryoProbe in the McKnight Brain Institute's Advanced Magnetic Resonance and Imaging Spectroscopy (AMRIS) facility at the University of Florida. Standard 2D $^{1}H-^{13}C$ HSQC NMR data were collected, processed, and analyzed.

AlphaScreen cAMP Bioassay Studies. Pharmacological characterization of the synthesized peptides at the mMC1R, mMC3R, mMC4R, and mMC5R was performed using the AlphaScreen cAMP assay (catalog no. 676062M, PerkinElmer Life Sciences). It was performed as described by the manufacturer and described previously by our lab,[71,72] but it is also described briefly below.

The peptide ligands were dissolved in DMSO at a stock concentration of 10^{-2} M and stored at −20 °C until they were assayed. HEK-293 cells were stably transfected with the mouse melanocortin receptors using a pCDNA3 expression vector using a calcium phosphate method and G418 selection as previously reported.[22,36,71,72,75] These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum (NCS) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Cells were 70−90% confluent in 10 cm plates on the day of the assay. Stimulation buffer [Hank's Balanced Salt Solution (HBSS 10× [-] sodium bicarbonate and [-] phenol red, Gibco), 0.5 mM isobutylmethylxanthine (IBMX), a 5 mM HEPES buffer solution (1 M, Gibco), and 0.1% bovine serum albumin (BSA) in Milli-Q water (pH 7.4)] and lysis buffer {5 mM HEPES buffer solution (1 M, Gibco), 0.1% BSA, and 0.3% Tween 20 [10% (w/v) aqueous solution] in Milli-Q water (pH 7.4)} were prepared on the day of the assay. The cells were dislodged with Versene (Gibco) at 37 °C followed by centrifugation at 800 rpm for 5 min at room temperature (Sorvall Super T21 high-speed centrifuge, swinging bucket rotor). The medium was aspirated, and cells were suspended in Dulbecco's phosphate-buffered saline solution (DPBS) [1× [-] calcium chloride and [-] magnesium chloride (Gibco)]. A 10 μL aliquot of cell suspension was mixed with 10 μL of a Trypan blue dye solution (Bio-Rad), and 10 μL of this solution was counted manually by hemocytometer. The cells were centrifuged again at 800 rpm for 5 min at room temperature. The cells were resuspended in stimulation buffer at a concentration of 10×10^6 cells/mL. Anti-cAMP acceptor beads (1.0 unit/well, AlphaScreen) were added to the cells, and 10000 cells were added to each well of 384-well plate (Optiplate, PerkinElmer). The cells were stimulated with the peptide at the desired in-well concentration in stimulation buffer ranging from 10^{-4} to 10^{-12} M as appropriate or a forskolin control (10^{-4} M). The plate was then incubated in the dark at room temperature for 2 h.

A three-component mixture of lysis buffer, biotinylated cAMP (1 unit/well, AlphaScreen), and a streptavidin donor bead working solution (1 unit/well, AlphaScreen) was prepared in the dark at least 1 h prior to the addition. Following the 2 h incubation period, the three-component solution was added and mixed under green light in each well. The plate was incubated for 2 h in the dark at room temperature and then read on the Enspire Alpha plate reader using a prenormalized assay protocol (set by the manufacturer). The assays were performed using duplicate data points and repeated in at least three independent experiments. The potencies and their associated standard errors of mean (SEM) were determined by fitting the data to a nonlinear regression method using the PRISM program (version 4.0, GraphPad Inc.).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.7b00422.

Analytical data of the peptides and NMR spectra of the 1,4- and 1,5-disubstituted triazole-bridged peptides **3** and **6** (PDF)

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S.R.T. and C.H.-L. designed the research. S.R.T., A.S., S.M.S., and K.T.F. performed the experiments. J.R.R. acquired NMR spectra. S.R.T., A.S., and C.H.-L. analyzed the data. S.R.T. wrote the manuscript with the help of A.S., C.J.L., and C.H.-L.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACTH, adrenocorticotropic hormone; AGRP, agouti-related protein; ASIP, agouti-signaling protein; cAMP, cyclic 5′-adenosine monophosphate; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; Fmoc, N^{α}-9-fluorenylmethoxycarbonyl; GPCR, G protein-coupled receptor; MC1R, melanocortin-1 receptor; MC2R, melanocortin-2 receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; MC5R, melanocortin-5 receptor; MCR, melanocortin receptor; MeOH, methanol; MSH, melanocyte-stimulating hormone; POMC, proopiomelanocortin; SEM, standard error of the mean; TFA, trifluoroacetic acid; α-MSH, α-melanocyte-stimulating hormone; β-MSH, β-melanocyte-stimulating hormone; γ-MSH, γ-melanocyte-stimulating hormone; NDP-MSH (4-norleucine-7-D-phenylalanine), Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

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