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ARTICLE

Trigonal scaffolds for multivalent targeting of melanocortin receptors

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Melanocortin receptors can be used as biomarkers to detect and possibly treat melanoma. To these ends, molecules bearing one, two, or three copies of the weakly binding ligand MSH(4) were attached to scaffolds based on phloroglucinol, tripropargylamine, and 1,4,7-triazacyclononane by means of the copper-assisted azide-alkyne cyclization. This synthetic design allows rapid assembly of multivalent molecules. The bioactivities of these compounds were evaluated using a competitive binding assay that employed human embryonic kidney cells engineered to overexpress the melanocortin 4 receptor. The divalent molecules exhibited 10- to 30-fold higher levels of inhibition when compared to the corresponding monovalent molecules, consistent with divalent binding, whereas the trivalent molecules were only statistically (~2-fold) better than the divalent molecules, still consistent with divalent binding but inconsistent with trivalent binding. Possible reasons for these behaviors and planned refinements of the multivalent constructs targeting melanocortin receptors based on these scaffolds are discussed.

Introduction

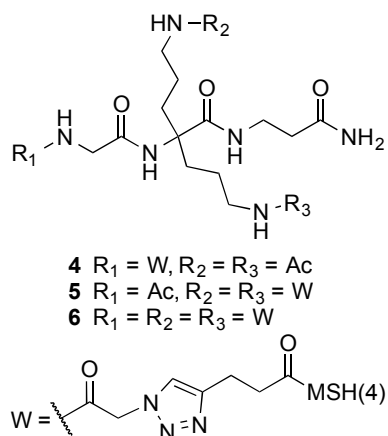
Melanoma is the most aggressive form of skin cancer in the world and accounts for over 75% of skin cancer-related deaths.¹ As with many serious cancers, melanoma has a good prognosis when detected in its early stages.² Malignant cells often overexpress characteristic receptors on their cell surfaces when compared to normal cells,³⁻⁵ affording a potential means of detection. For example, the melanocortin 1 receptor, a G-protein coupled receptor (GPCR), is overexpressed in most forms of melanoma.^{6,7} Among other research groups,⁸⁻¹⁰ we are pursuing detection strategies that employ *multivalent interactions* by two or more weakly binding ligands attached to a scaffold. Such *multivalent molecules* can selectively bind with high avidity to cells overexpressing the targeted ligand receptors.¹¹⁻¹²

Factors that can influence the interactions of multivalent molecules with cell surface receptors include the potency of the ligands utilized, the inter-ligand distances, and the possible geometries of ligand display permitted by the scaffold. Peptide ligands that are known to bind to melanocortin receptors include Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂ (**1**, NDP- α -MSH, high affinity),¹³ Ser-Nle-Glu-His-DPhe-Arg-Trp-NH₂ (**2**, MSH(7), medium affinity),¹⁴ and His-DPhe-Arg-Trp-NH₂ (**3**, MSH(4), low affinity).¹⁴ Because cooperative binding leading to enhanced avidity is more evident when weakly binding ligands are utilized,^{11,12} we (and others¹⁵⁻¹⁹) have used MSH(4) in our multivalent constructs.

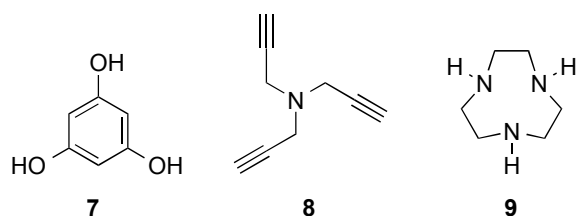
Control of ligand number, spacing, and geometry depends on the scaffold used to display the MSH(4) ligand. In the absence of crystallographic data on the melanocortin receptors,

a homology model based on the crystal structure of the GPCR rhodopsin suggested distances ranging from 20-50 Å between the binding sites of abutted receptors.²⁰ In previous articles, we described multivalent constructs based on linear²¹⁻²⁴ and spherical²⁵ scaffolds, typically with ligand spacing in this range. Using a competitive binding assay and a human embryonic kidney cell line, HEK293, genetically engineered to overexpress the human melanocortin 4 receptor (hMC4R),⁹ these constructs were shown to exhibit enhanced binding consistent with statistical effects, but inconsistent with simultaneous binding of two or more ligands to receptors on the cell surface.

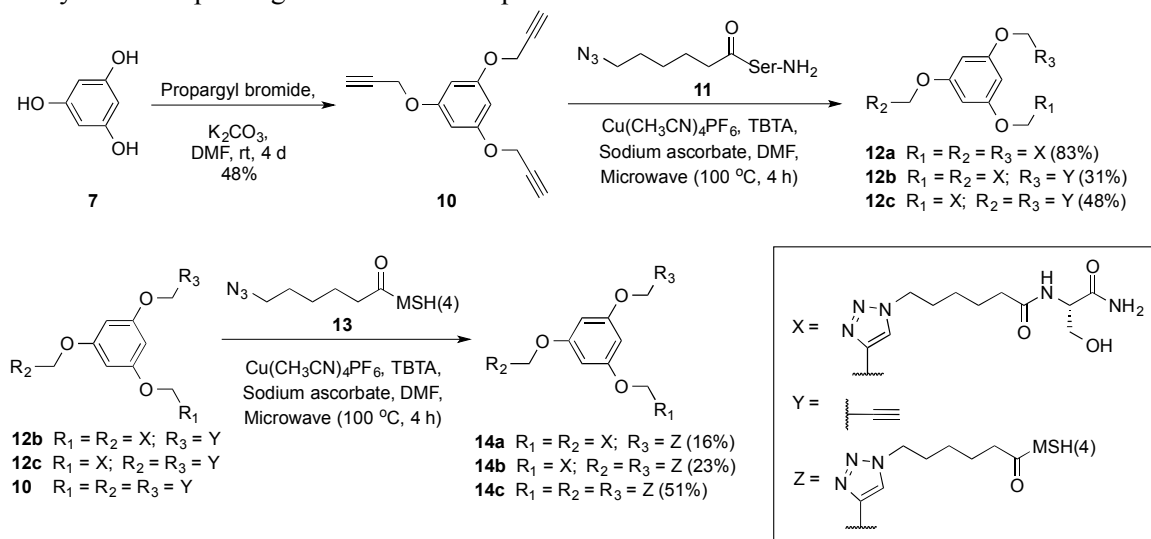
Recently, multivalent binding of molecules that incorporate MSH(4) ligands was demonstrated using mono-, di-, and trivalent compounds **4-6**.¹⁸ The divalent and trivalent constructs **5** and **6** exhibited 16-fold and 350-fold enhancements, respectively, in the measured IC₅₀ values compared to the monovalent construct **4** in a competitive binding assay against an NDP- α -MSH-based fluorescent probe. The inter-ligand distances in **5** and **6** were estimated to be 24 \pm 5 Å by molecular modeling. Structurally related dendrimeric constructs bearing six or nine MSH(4) ligands exhibited somewhat reduced inhibitory potencies.¹⁹ These studies strongly suggest that narrow limits on ligand spacing exist for observation of true multivalent binding to hMC4 receptors, at least for the engineered HEK293 cell line used in the competitive binding assays.



In light of these observations, we wished to more fully evaluate the effects of ligand spacing and orientation on avidity while simplifying the scaffold to enhance the economy and versatility of the synthetic approach to useful multivalent molecules. In this article, we present syntheses and bioassay results from MSH(4)-bearing multivalent molecules based on phloroglucinol (**7**), tripropargylamine (**8**), and 1,4,7-triazacyclononane (**9**) scaffold cores. As a part of this study, we also describe new protocols that improve the signal-to-noise ratio for *in vitro* binding assays when using the engineered HEK293 cells.



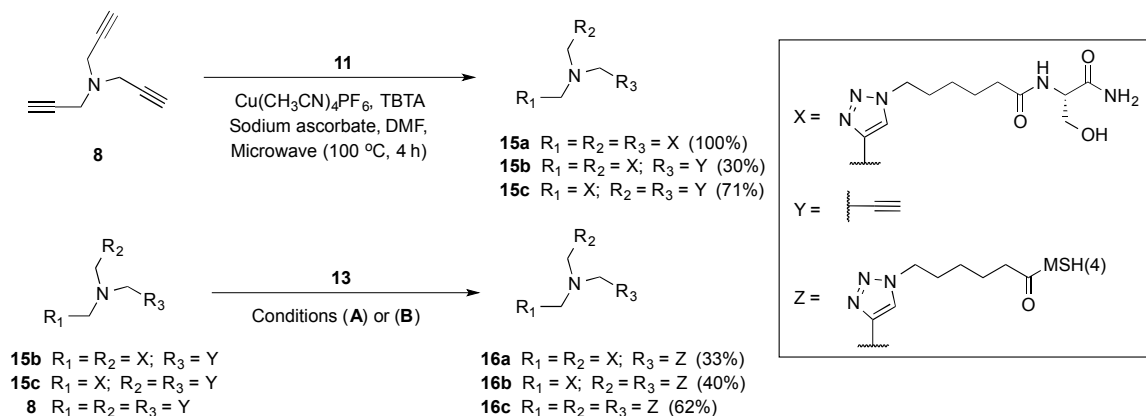
Scheme 1. Synthesis of phloroglucinol-based compounds **5a** and **14a-c**.



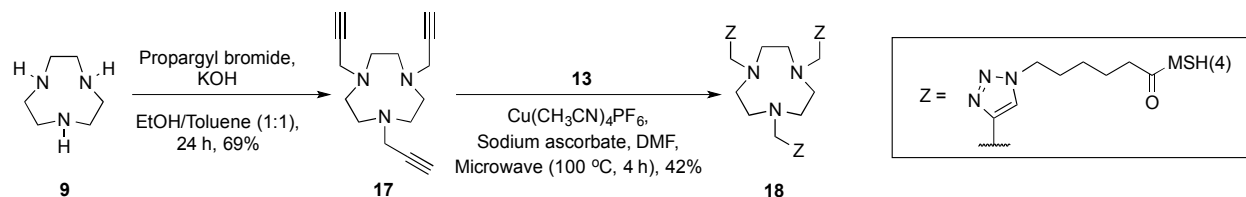
Results

Chemistry. Commercially available phloroglucinol (**7**) was alkylated according to a reported method²⁶ to produce trisalkyne **10** (Scheme 1). Using microwave-assisted copper-catalyzed azide-alkyne cycloaddition (CuAAC) reactions, the known serinamide azide **11**²⁴ was attached to the phloroglucinol-derived hub, leading to **12a-c**. CuAAC reactions of **12b**, **12c**, and **10** with the known MSH(4) azide **13**²⁴ afforded mono-, bis-, and tris-MSH(4) constructs **14a-c**, respectively. Although the reaction vials and solvents were purged with argon to exclude oxygen from the system, if air is introduced during handling oxidation of the reactive Cu(I) species can occur. We observed that inclusion of sodium ascorbate in the reaction mixtures enhanced the repeatability of the product yields.

Serinamide-containing constructs **15a-c** were prepared from commercially available tripropargylamine (**8**) and azide **11** in a similar manner (Scheme 2). However, CuAAC reactions of **15b**, **15c**, and **8** with MSH(4) azide **13** under the conditions used to prepare **14a-c** were unsatisfactory. For the synthesis of **16a** and **16b**, acceptable yields were obtained when tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) was omitted from the reaction mixture. Normally TBTA stabilizes Cu(I),²⁷ but in these reactions the product triazoles might serve this function. Along with added TBTA, the products might complex Cu(I) ions, reducing the reaction rate. Reaction of **8** with azide **13** to produce **16c** was more challenging. Microwave-assisted reactions with different reagent compositions, solvents, and reaction times failed. However, reaction of **8** with azide **13** in the presence of tetrakis(acetonitrile)copper(I) hexafluorophosphate (TACP) catalyst and 2,6-lutidine in a mixture of water and acetonitrile (MeCN) for 4 days at room temperature provided **16c** in 62% yield.

Scheme 2. Synthesis of tripropargylamine-based compounds **15a** and **16a-c**.^a

^aConditions **A**: TACP, sodium ascorbate, DMF, microwave irradiation to maintain 100 °C, 4 h; used in the synthesis of **16a** and **16b**. Conditions **B**: TACP, 2,6-lutidine, H₂O/MeCN (1:1), rt, 4d; used in the synthesis of **16c**.

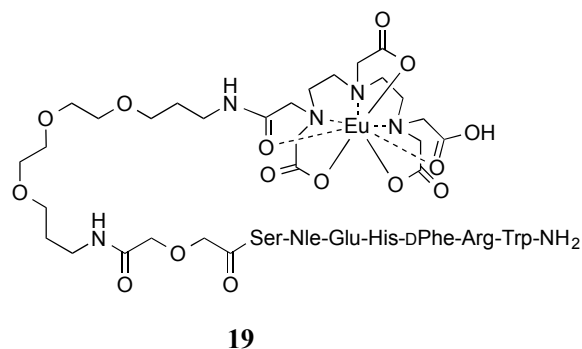
Scheme 3. Synthesis of 1,4,7-triazacyclononane-based compound **18**.

Reaction of 1,4,7-triazacyclononane (**9**) with propargyl bromide using a reported method²⁸ gave **17** along with quaternary ammonium salts due to over-alkylation (Scheme 3). By reducing the number of equivalents of propargyl bromide and controlling the reaction temperature during its addition, **17** was produced in 69% yield. The CuAAC reaction between **17** and azide **13** under the conditions used for preparation of **14a-c** but without TBTA afforded compound **18** in 42% yield. Removal of copper ions from the crude product **18** was especially difficult, possibly due to metal ion chelation by the side arms and the 1,4,7-triazacyclononane core. It was necessary to remove the copper ions by extraction,^{29,30} because preparative HPLC does not remove them. Fortunately, prolonged contact with dithizone in chloroform gave complete removal of the copper ions.³¹

Compounds **14a-c**, **16a-c**, and **18** used in biological assays were $\geq 95\%$ pure as determined by HPLC analysis (see Electronic Supplementary Information).[†]

Biological Assays. Using a new protocol (see the Discussion and the Experimental Section for details), saturation binding

assays were performed using the known time-resolved fluorescence (TRF) probe, Eu-DTPA-PEGO-MSH(7)-NH₂ (**19**)^{14,32} and HEK293 cells engineered to overexpress hM4R (approximately 640,000 copies per cell) and human cholecystinin 2 receptor (hCCK2R, approximately 1,100,000 copies per cell).⁹ Saturation binding curves are depicted in Figure 1. The K_d for **19** calculated from these assays was 21 ± 3 nM, which is consistent with the value of 27 ± 4 nM reported from saturation binding assays using an older protocol.¹⁴



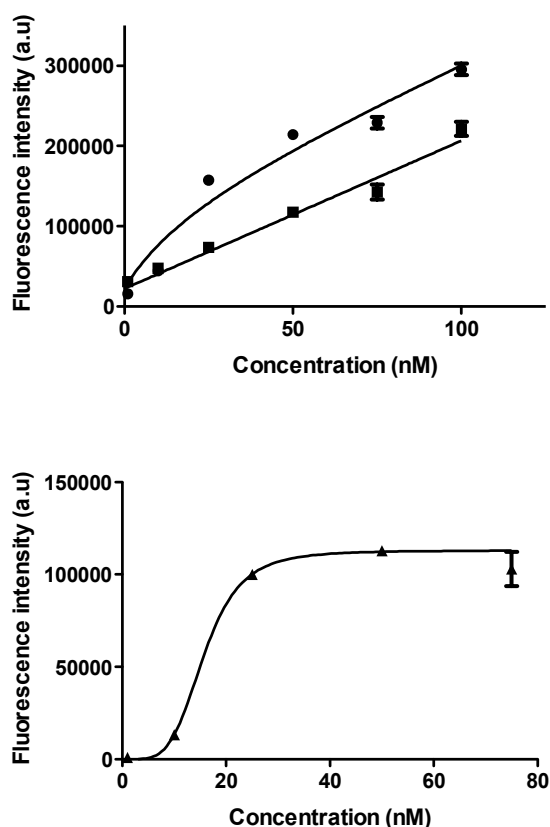


Figure 1. Saturation binding curves for probe **19** generated using the six-well plate assay method. *Top:* Total binding (●) and non-specific binding (◻). *Bottom:* Specific binding (▲). The calculated $K_d = 21 \pm 3$ nM ($n = 5$).

Competitive binding assays employed the new protocol, HEK293 cells engineered to overexpress hMC4R and hCCK2R, and the TRF probe **19**. Representative binding curves are depicted in Figure 2, and the results for all compounds are numerically summarized in Table 1. Control compounds **12a** and **15a** did not inhibit the binding and uptake³³ of **19** over the concentration range tested. The mono-, di-, and trivalent constructs **14a-c** exhibited K_i values of 990, 91, and 45 nM, respectively. A similar trend was observed for constructs **16a-c**, which exhibited K_i values of 2210, 69, and 36 nM, respectively. Finally, the trivalent construct **18** exhibited a K_i value of 25 nM.

Discussion

Previously we prepared MSH(4)-bearing multivalent molecules based on linear²¹⁻²⁴ and spherical²⁵ scaffolds, most with inter-ligand distances in the 20-50 Å range. In competitive binding assays, these constructs generally exhibited the inhibitory potency of the MSH(4) ligand statistically amplified by the number of ligands present in the construct. In no case was potency clearly attributable to multivalent binding observed. Divalent and trivalent binding to hMC4 receptors

was finally demonstrated by Brabez, *et al.*, using the MSH(4)-bearing mono-, di-, and trivalent compounds **4-6**.¹⁸ The inter-ligand distances in **5** and **6** were estimated to be 24 ± 5 Å by molecular modeling, suggesting that the limits on ligand spacing for multivalent binding to hMC4 receptors had been overestimated by the prior homology model,²⁰ at least for the engineered HEK293 cell line used in the competitive binding assays. To confirm this observation, we elected to pursue multivalent molecules based on the “disc-like” cores afforded by phloroglucinol (**7**), tripropargylamine (**8**), and 1,4,7-triazacyclononane (**9**). These compounds have been used as cores for dendrimeric molecules³⁴⁻³⁷ and in syntheses of multivalent molecules.³⁸⁻⁴⁰ Their simplicity and commercial availability suggest synthetic versatility and economy, important considerations for implementation of a general strategy for ligand multimerization. The kind, number, and spacing of the ligands can easily be controlled, and in the case of trivalent molecules, the ligands would be prearranged in a trigonal planar fashion.

Table 1. Results of competitive binding assays.^a

Compound	$K_i \pm \text{SEM}^b$ (nM)	Relative Potency ^c
4^d	330±52	1
5^d	37±10	9
6^d	4.3±0.4	77
12a	NB ^e	NA ^f
14a	990 ± 140	1
14b	91 ± 13	11
14c	45 ± 11	22
15a	NB ^e	NA ^f
16a	2210 ± 460	1
16b	69 ± 12	32
16c	36 ± 4	61
18	25 ± 4	NA ^f

^aCompetitive binding experiments were carried out against probe **19** ($K_d = 21$ nM, $[\mathbf{19}] = 20$ nM) using HEK293 cells overexpressing hMC4R and CCK2R. ^bSEM = standard error of the mean; $n = 5$ independent determinations. ^cRelative potency compared to the monovalent MSH(4) construct with the same scaffold core. ^dResult taken from reference 14. ^eNB = no competitive binding observed. ^fNA = not applicable.

In the event, assembly of **12a**, **14a-c**, **15a**, **16a-c**, and **18** by alkylations of **7** and **9** with propargyl bromide, followed by CuAAC attachment of the placeholder serinamide ligand **11** and/or the MSH(4) ligand **13** to trialkynes **8**, **10**, and **17** required short reaction sequences that were reasonably straightforward and efficient.

Standard high throughput TRF assays used to screen for functional binding are normally conducted with cells grown in 96-well plates (Costar 3603).^{41,42} In the present study with HEK293 cells, these assays exhibited relatively poor signal-to-noise ratios due to cell detachment during the multiple wash and reagent addition steps. The use of more adherent A375

cells bearing melanocortin 1 receptors⁴³ also gave poor signal-to-noise ratios due to the lower receptor expression levels of these cells. While the engineered HEK293 cells express about 640,000 MC4 receptors per cell,⁹ the A375 cells express about 75,000 MC1 receptors per cell.⁴³

To improve the signal-to-noise ratios of the assays to provide more robust data for calculation of binding and inhibition constants, we shifted the assays from 96-well plates to 6-well plates in order to work with much larger populations of cells in a given sample well. While the 96-well plates were seeded at 20,000 cells per well and grew to 90% confluence (~40,000-50,000 cells per well) over about three days, the 6-well plates were seeded at 240,000 cells per well and required

five days to grow to 90% confluence (~1 × 10⁶ cells per well). The significantly larger cell population meant that small losses of cells during assay manipulations would have a lesser effect on the repeatability of the TRF data. To minimize the loss of cells during binding assays, solutions were carefully added down the sides of the well walls. To reduce non-specific signal due to adherence of the probe to the wells, the cells were scraped from the wells after the binding incubation and transferred to micro-centrifuge tubes for post-assay processing. These changes led to a robust assay with high repeatability and reproducibility.⁴⁴

To test the consistency of a result from the new protocol in 6-well plates with a published result from a previous protocol in 96-well plates, a saturation binding assay was performed for the known TRF probe **19**^{14,32} using the new protocol and HEK293 cells engineered to overexpress hMC4R and hCCK2R.⁹ The K_d for **19** calculated from this assay was 21 ± 3 nM, while the reported K_d for **19** from use of the previous protocol was 27 ± 4 nM.¹⁴ This level of agreement was taken as a validation of the new protocol.

In the competitive binding assays, control compounds **12a** and **15a** did not inhibit the binding and uptake³³ of the TRF probe **19** over the concentration range tested. The monovalent constructs **14a** and **16a** exhibited K_i values consistent with monovalent MSH(4) binding (approximately 1 μM).²³ Impressively, the divalent constructs **14b** and **16b** exhibited K_i values of 91 and 69 nM, respectively, 11-fold and 32-fold lower than the corresponding monovalent molecules **14a** and **16a**. These increases in potency are consistent with the 9-fold enhancement observed when **5** was compared to **4** using probe **19**¹⁴ and are indicative of divalent binding.⁴⁵

Compounds **14c** and **16c** possess three copies of MSH(4) with what were presumed to be appropriate inter-ligand spacings for trivalent binding, and yet these compounds exhibited only two-fold enhancements in potency when compared with the corresponding divalent compounds **14b** and **16b**. These results, attributable to statistical effects, stand in contrast with the 9-fold enhancement in potency observed for trivalent **6** (K_i = 4.3 nM) compared to divalent **5** (K_i = 37 nM) when competed against probe **19**.¹⁴ Trivalent compound **18** exhibited a K_i of 25 nM, a value reasonably consistent with those of **14c** (K_i = 45 nM) and **16c** (K_i = 36 nM).

The possibility that these results might have been due to a limit to the dynamic range of the competitive binding assay was excluded by competing NDP- α -MSH (**1**) against probe **19**. The K_i observed for **1** using the 6-well plate binding assay was 3.2 ± 0.4 nM,⁴⁶ a value consistent with earlier observations in 96-well plate binding assays. Thus, the greater avidity of **6**, *i.e.* its ability to bind trivalently, must be rooted in structural differences with **14c**, **16c**, and **18**.

In the design phase of this work, the maximum inter-ligand distances for **6**, **14c**, **16c**, and **18**, as measured by the distances between the *N*-terminal nitrogen atoms of the histidine residues, were estimated to be 33, 33, 28, and 32 Å, respectively, assuming full extension of all chain segments.⁴⁷ To better address issues that concern structure and conformation, molecular dynamics studies were performed using Molecular Operating Environment (MOE).^{48,49} Views of representative conformations of **6**, **14c**, **16c**, and **18** are displayed in Figure 3. Given a sufficiently long molecular dynamics run, the side arms of the C₃ symmetric molecules **14c**, **16c**, and **18** would display conformational equivalence.⁵⁰ However, at most points in time the spatial relationships between the ligands will vary. Over the 2 ns time courses of our molecular dynamics runs, the inter-

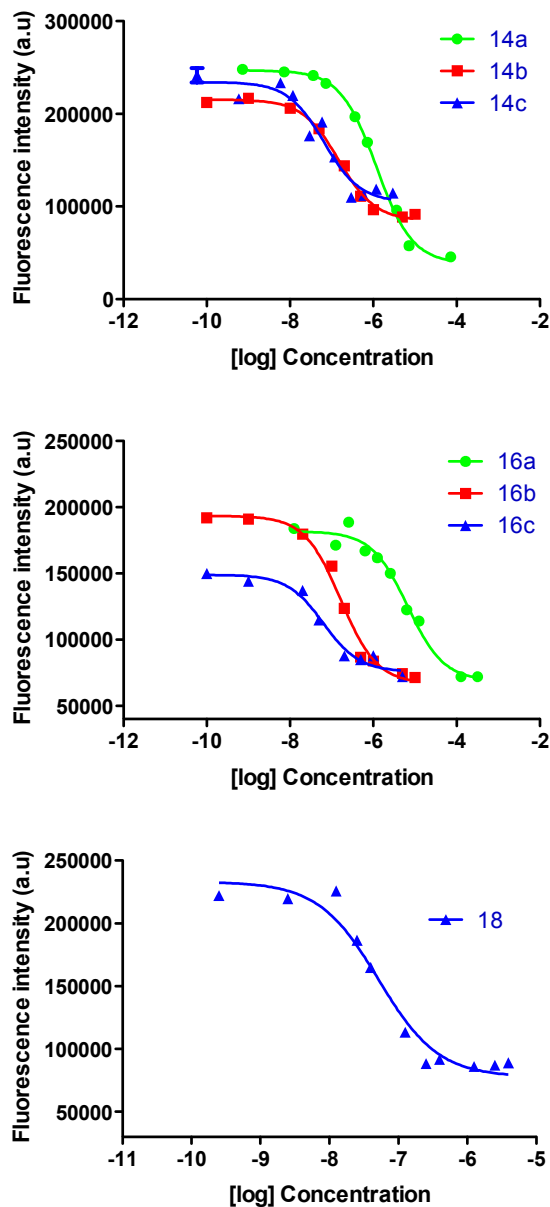


Figure 2. Competitive binding curves for the compounds **14a-c** (top), **16a-c** (middle), and **18** (bottom) against probe **19** (20 nM) generated using the six-well plate assay method. Control compounds **12a** and **15a** were not competitive inhibitors of **19** over the concentration ranges tested.

ligand distances lay between 17.0-28.0 Å for compound **6**, 19.5-24.5 Å for **14c**, 16.5-21.0 Å for **16c**, and 16.5-23.0 Å for **18**. These distances are at the low end of the 24 ± 5 Å range previously suggested for compound **6**.¹⁸

It is generally held that, like other melanocortin receptors, MC4R forms constitutive dimers on the cell surface.⁵¹ If a third MC4 receptor approaches, a trimer may form, but it need not be symmetrical. If the trimer is asymmetric, different inter-ligand distances may be required in order to simultaneously bridge to three ligand binding sites. Since the ligand binding sites of dimeric MC4Rs can be bridged by any of the divalent or trivalent molecules we have studied here, it seems reasonable to postulate that an optimum distance lies between 17 and 23 Å. Furthermore, we postulate that while compound **6** has sufficient "reach" to permit binding to a more remote third ligand binding site of a receptor trimer, compounds **14c**, **16c**, and **18** are unable to do so.

Given the ready adaptability of the synthetic method we have described, we plan to examine these postulates by construction and testing of sets of divalent molecules with inter-ligand distances ranging from 17-23 Å and sets of trivalent molecules possessing the optimal short inter-ligand distance and one longer inter-ligand distance (from the optimal short distance to 32 Å).

Additionally, assuming the order of magnitude difference in avidity afforded by divalency is sufficient for distinguishing healthy from abnormal cells by discernment of receptor overexpression, medically useful compounds might result from attachment of imaging or therapeutic agents to scaffolds derived from **7-9**. We plan to pursue this line of investigation by replacement of the serinamide in **14b** and **16b** with radiolabels and fluorescent tags for use in *in vitro* saturation binding and uptake studies and *in vivo* imaging studies.

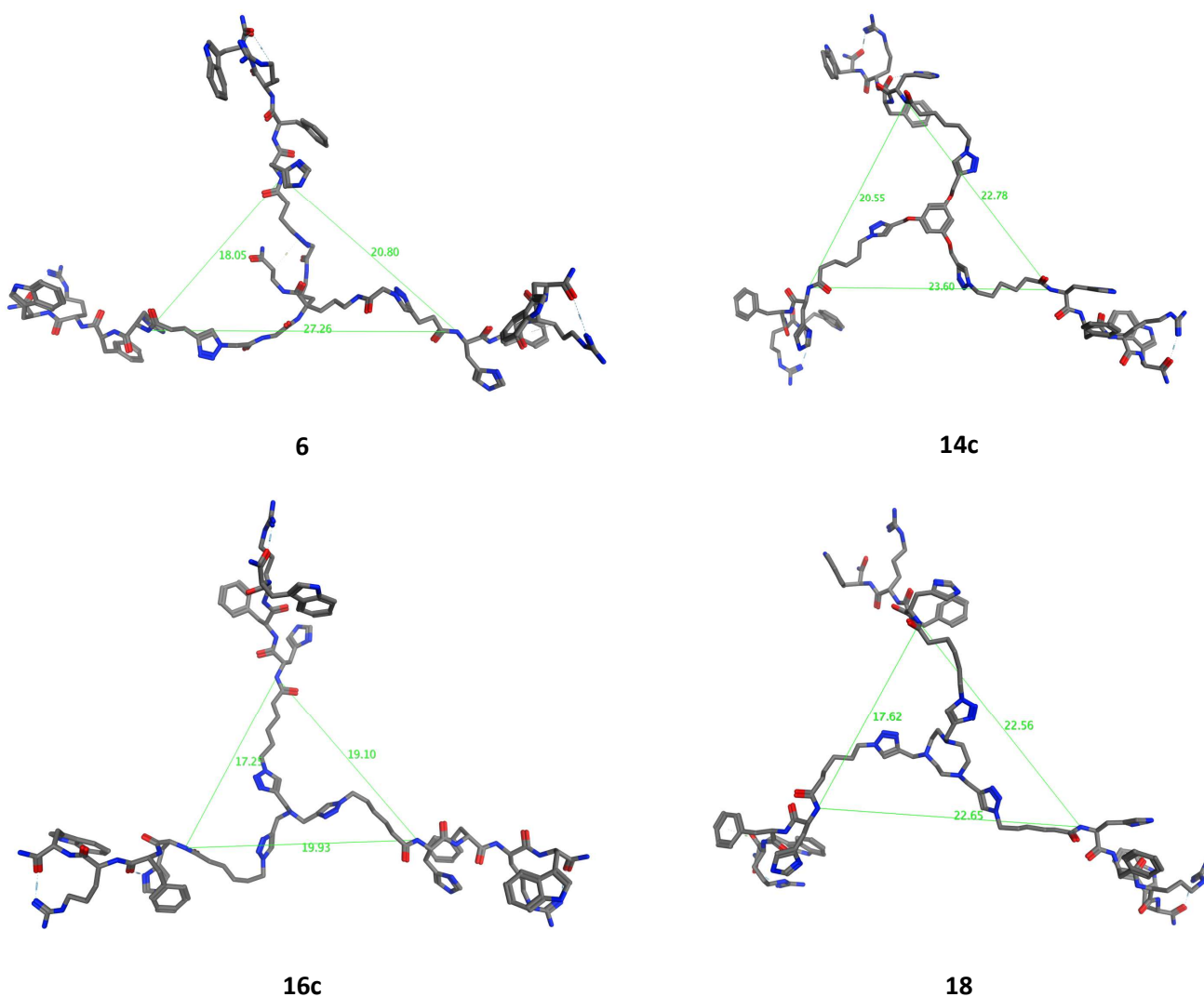


Figure 3. Views of representative conformations observed for compounds **6**, **14c**, **16c**, and **18**. The distances from the *N*-terminal nitrogen atoms of the histidine residues are given in Angstroms as a measure of the three inter-ligand distances. The average distances between ligands for **6**, **14c**, **16c**, and **18** are 22, 22, 19, and 21 Å, respectively.

Experimental

Chemical Synthesis

General Materials and Methods. Commercial reagents were used as supplied unless otherwise noted. Dichloromethane (DCM) and tetrahydrofuran (THF) were dried by passage through activated alumina. Dimethylsulfoxide (DMSO) and *N,N*-dimethylformamide (DMF) were dried by contact with activated 4 Å molecular sieves, followed by distillation under reduced pressure. Analytical thin-layer chromatography (TLC) was carried out on pre-coated silica gel 60 F-254 plates with staining by 10% phosphomolybdic acid (PMA) solution in ethanol or potassium permanganate in aqueous acetone and heat. Column chromatography was performed using silica gel 60 (200–400 mesh). Melting points were recorded on an Electrothermal Mel-Temp apparatus (Model 1001) and are uncorrected. IR spectra were recorded on a Thermo Nicolet iS5 FT-IR Spectrophotometer as KBr pellets. NMR spectra were recorded at 500 or 600 MHz for ¹H NMR and at 125 MHz for ¹³C NMR on Bruker DRX-500 and DRX-600 NMR instruments. Chemical shifts (δ) are expressed in ppm and are internally referenced for ¹H NMR (CDCl₃ 7.26 ppm, methanol-*d*₄ 3.31 or 4.87 ppm, and DMSO-*d*₆ 2.50 ppm) and ¹³C NMR (CDCl₃ 77.16 ppm, methanol-*d*₄ 49.00 ppm, and DMSO-*d*₆ 39.52 ppm). Reactions done under microwave conditions utilized a Biotage Initiator 2.0 microwave reactor. Preparative scale reversed phase HPLC was performed using a 19 × 250 mm Waters XBridge 10 μm OBD C₁₈ preparative HPLC column. A linear gradient of mobile phase was used over 45 min from 0–90% MeCN/water containing 0.1% TFA. The flow rate was 10 mL/min and a dual channel UV detector was used at 230 and 280 nm. Analytical HPLC was performed on a 3.0 × 150 mm Waters XBridge 3.5 μm C₁₈ analytical HPLC column. A linear gradient of mobile phase was used over 30 min from 10–90% MeCN/water containing 0.1% TFA. The flow rate was 0.3 mL/min and a dual channel UV detector was used at 220 and 280 nm. A VWR SympHony pH meter (Model SB20) equipped with a Ag/AgCl electrode was used for pH measurements. ESI experiments were performed on a Bruker 9.4 T Apex-Qh hybrid Fourier transform ion-cyclotron resonance (FT-ICR) instrument using standard ESI conditions. The samples were dissolved in MeCN/water 1:1 containing 0.1% formic acid in a concentration range of 1–30 μM. Optical rotations were measured on a Rudolph Research Autopol III polarimeter using a 50 mm sample cell (1 mL volume). Engineered HEK293 cells⁹ overexpressing both hMC4R and hCCK2R were used to measure the affinity of the probe for binding to hMC4R by means of saturation binding assays. Unless otherwise specified, all cell incubations were done in a Fisher Scientific Isotemp CO₂ incubator (Model 3530) maintained at 37 °C and 5% CO₂ atmosphere. Europium-based time-resolved fluorescence (TRF) competitive binding assays were employed to study the binding of all multivalent constructs and controls. The probe used for binding assays, Eu-DTPA-PEGO-MSH(7)-NH₂ (**19**), was prepared by a published procedure.³² Centrifugations were performed on a VWR Galaxy 7 microcentrifuge or a Fischer Scientific Model 59A microcentrifuge. TRF was measured using a VICTOR X4 2030 Multilabel Reader (PerkinElmer) employing the standard Eu TRF measurement settings (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm).

Solid phase synthesis. NDP- α -MSH (**1**) and the MSH(4) derivative azide **13** were synthesized manually via an N^α-Fmoc

solid-phase peptide synthesis strategy. Tentagel S resin (0.24 mmol/g loading) was used for the synthesis of **1** and Rink amide AM resin (200–400 mesh, 0.68 mmol/g loading) was used for the synthesis of **13**. Resin (1 g) was allowed to swell in THF for 1 h in a polypropylene syringe equipped with a polypropylene frit. THF was removed, a solution of 20% piperidine in DMF (15 mL) was added, and the tube was shaken for 2 min. This solution was removed, 20% piperidine in DMF (15 mL) was again added, and the mixture was shaken for another 18 min. After removal of the solution, the resin was washed sequentially with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), 0.5 M 1-hydroxybenzotriazole (HOBt) in DMF (15 mL), 0.5 M HOBt in DMF + a drop of 0.01 M bromophenol blue solution in DMF (15 mL), DMF (2 × 15 mL), and DCM (15 mL) in that order. The amino acid (3 eq) to be coupled was activated by reaction in DMF (15 mL) in a glass vial with 1-hydroxy-6-chlorobenzotriazole (Cl-HOBt, 3 eq) and diisopropyl carbodiimide (DIC, 6 eq) over two min. This solution was then added to the resin and the syringe shaken for 1 h. The coupling solution was removed and the resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). Free amine groups were capped by shaking the resin with acetic anhydride/pyridine (1:1, 6 mL) for 20 min. The resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). The coupling cycle was then repeated for each of the remaining amino acids in the sequence. The Kaiser test⁵² was used to determine coupling completion at each attachment step.

For the synthesis of **1**, half (approximately 0.12 mmol) of the resin-bound, side chain-protected Fmoc-NDP- α -MSH was treated as follows. A solution of 20% piperidine in DMF (8 mL) was added and the tube shaken for 2 min. The solution was removed, 20% piperidine in DMF (8 mL) was added, and the mixture shaken for 18 min. After the removal of the solution, the resin was washed sequentially with DMF (3 × 8 mL), DCM (3 × 8 mL), and DMF (3 × 8 mL). *N*-terminal acylation was accomplished by treatment with a mixture of acetic anhydride/pyridine (1:1, 1 mL) in DMF (2 mL) for 1 h. The resin was then washed with DMF (3 × 8 mL), DCM (3 × 8 mL), DMF (3 × 8 mL), THF (8 mL), and DCM (8 mL), then left to dry for 1 h.

For the synthesis of azide **13**, the *N*-terminal histidine residue was acylated using activated 6-azidohexanoic acid (2.04 mmol) in 15 mL of DCM with Cl-HOBt (345 mg, 2.04 mmol, 3 eq) and DIC (512 mg, 4.08 mmol, 6 eq).

A cleavage cocktail (6 mL for **1** and 10 mL for **13** and) consisting of TFA, thioanisole, triisopropylsilane, and H₂O (9.1:0.3:0.3:0.3) was added to the resin and the syringe was shaken for 4 h at rt. The solution was transferred to a 15 mL centrifuge tube and the resin washed with further aliquots of TFA (2 × 2 mL, 2 min). The combined TFA solutions were concentrated in the centrifuge tube under a stream of argon, and the product precipitated by the addition of cold ether (8 mL). The tube was centrifuged and the supernatant removed. The pellet was washed with cold ether (3 × 6 mL), air dried, dissolved in 1 M acetic acid, and lyophilized. The resultant solid was subjected to reversed-phase preparative HPLC, product-containing fractions combined, and the solutions lyophilized.

NDP- α -MSH (1**).**^{13,23} White solid; yield 53% (83 mg, 0.050 mmol); HRMS (FT-ICR) *m/z* calcd. for C₇₈H₁₁₂N₂₁O₁₉ [M+H]⁺ 1646.8438, found 1646.8487; Analytical HPLC *t_R* = 13.13 min.

MSH(4) Azide (13).²⁴ Off-white solid; yield 72% (384 mg, 0.49 mmol); HRMS (FT-ICR) m/z calcd. for $C_{38}H_{51}N_{14}O_5$ $[M+H]^+$ 783.41614, found 783.41606; Analytical HPLC t_R = 13.61 min.

1,3,5-Tris(prop-2-yn-1-yloxy)benzene (10).^{26,53} To a round bottomed flask purged with argon was added DMF (20 mL), propargyl bromide (80% in toluene, 8.020 mL, 72 mmol, 4.5 eq), and K_2CO_3 (8.95 g, 64.8 mmol, 4.0 eq). A solution of phloroglucinol (**7**, 2.02 g, 16.0 mmol, 1.0 eq) in DMF (12 mL) was added at rt dropwise with stirring over 15 min. After 4 d the solids were removed by filtration and washed with DCM. The filtrate and washings were concentrated *in vacuo* to yield a reddish slurry. To this material was added DCM (100 mL) and the mixture washed with water (3 x 100 mL) and brine (2 x 100 mL). The resultant red organic layer was dried over sodium sulfate, filtered, and volatiles removed *in vacuo* to yield 4.1 g of a red slurry. This material was subjected to column chromatography on silica gel 60 with a mobile phase of 20% ethyl acetate in hexanes. The product-containing fractions were combined, volatiles removed *in vacuo*, and the resultant solid recrystallized from hexanes to give **10** as an off-white solid. Yield 1.85 g (7.6 mmol, 48%); R_f 0.47 (20% ethyl acetate/hexanes, visualization PMA); mp 82–84 °C (lit⁵³ mp 83 °C); IR (KBr, cm^{-1}) 3278, 3268, 3257, 2907, 2133, 2114, 1616; ¹H NMR (500 MHz, $CDCl_3$) δ 6.27 (s, 3H, Ar C-H), 4.65 (d, J = 2.4 Hz, 6H, CH_2), 2.53 (t, J = 2.4 Hz, 3H, C≡C-H); ¹³C NMR (125 MHz, $CDCl_3$) δ 159.48 (Ar C-O), 95.61 (Ar C=C-O), 78.39 (C≡C-H), 75.87 (C≡C-H), 56.11 (CH_2).

***N*-(1-Amino-3-hydroxy-1-oxopropan-2-yl)-6-azidohexanamide (11).**²⁴ To a stirred solution of 6-azidohexanoic acid⁵⁴ (6.00 g, 36.2 mmol, 1.0 eq) and *N*-hydroxysuccinamide (4.73 g, 39.9 mmol, 1.1 eq) in $CHCl_3/DMF$ (9:1, 15 mL) was added *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (7.80 g, 39.9 mmol, 1.1 eq). The mixture was stirred under argon overnight at rt. Chloroform was removed under vacuum and the resultant liquid partitioned between 1 N HCl (50 mL) and ether (50 mL). The organic layer was washed with 1 N HCl (3 x 50 mL), 5% $NaHCO_3$ (2 x 50 mL), water (2 x 50 mL), and brine (2 x 50 mL). The organic layer was then dried using anhydrous sodium sulfate, filtered, and concentrated under vacuum to produce **3**. Yield 8.20 g (32.3 mmol, 89%); ¹H NMR (500 MHz, $CDCl_3$) δ 3.26 (t, J = 6.9 Hz, 2H), 2.84–2.72 (m, 4H), 2.59 (t, J = 7.4 Hz, 2H), 1.81–1.67 (m, 2H), 1.64–1.54 (m, 2H), 1.52–1.40 (m, 2H); ¹³C NMR (125 MHz, $CDCl_3$) δ 169.21, 168.39, 51.10, 30.78, 28.38, 25.87, 25.61, 24.15.

To a solution of serinamide hydrochloride (3.30 g, 23.0 mmol, 1.1 eq) in DMF (50 mL) was added triethylamine (3.21 mL, 23.0 mmol, 1.1 eq). To the resultant white suspension was added the succinamide ester from above (5.32 g, 20.9 mmol, 1.0 eq) and the reaction mixture stirred overnight. Volatiles were removed under vacuum and the resultant slurry subjected to flash column chromatography using a mobile phase of 10% MeOH/ $CHCl_3$. Product-containing fractions were combined and solvents removed *in vacuo* to give **11** as a white solid, mp 94–96 °C (lit²⁴ mp 94–96 °C). Yield 3.81 g (15.7 mmol, 75%); R_f 0.38 (10% MeOH/ $CHCl_3$, visualization $KMnO_4$); ¹H NMR (500 MHz, methanol- d_4) δ 4.47 (t, J = 5.3 Hz, 1H, CH), 3.96–3.70 (m, 2H, O- CH_2), 3.33 (t, J = 6.9 Hz, 2H, N_3 - CH_2), 2.34 (t, J = 7.5 Hz, 2H, CH_2), 1.67 (m, 4H, overlapped CH_2), 1.56–1.35 (m, 2H, CH_2); ¹³C NMR (125 MHz, methanol- d_4) δ 175.98

(CO), 175.09 (CO), 63.11, 56.43, 52.27, 36.62, 29.61, 27.35, 26.21.

6,6',6''-(4,4',4''-((Benzene-1,3,5-triyl)tris(oxy))tris(methylene))tris(1*H*-1,2,3-triazole-4,1-diyl))tris(*N*-((*S*)-1-amino-3-hydroxy-1-oxopropan-2-yl)hexanamide) (12a). DMF and a microwave vial (0.5–2 mL) were purged with argon for 30 min. To the vial were added tris(alkyne) **10** (49 mg, 204 μ mol, 1.0 eq), serinamide azide **11** (304 mg, 1.25 mmol, 6.0 eq), TBTA (66.2 mg, 124.9 μ mol, 0.6 eq), and TACP (46.6 mg, 124.9 μ mol, 0.6 eq) in that order. To this mixture was added 600 μ L of argon-purged DMF, whereupon a green solution was obtained. Sodium ascorbate (27.2 mg, 137.4 μ mol, 1.1 eq per TACP) was added in a single portion and the solution color changed to light brown. The walls of the vial were washed down with 400 μ L of argon-purged DMF, the vial sealed, and irradiated in a microwave reactor to maintain a temperature of 100 °C for 4 h. The reaction mixture was then cooled, volatiles removed *in vacuo*, and the residue subjected to flash column chromatography using DCM/MeOH/conc NH_4OH (5:2:0.5) as the eluent. Product-containing fractions were combined and volatiles were removed *in vacuo*, leaving an oily yellow residue. This was dissolved in H_2O (10 mL) and the minimum amount of MeCN necessary to effect solution and the mixture lyophilized, yielding 161 mg (166 μ mol, 81%) of **12a** as a white solid; R_f 0.29 (DCM/MeOH/conc NH_4OH 5:2:0.5, visualization $KMnO_4$); mp 83–85 °C; $[\alpha]_D^{25} +43.90$ (c 0.66, MeCN/ H_2O 1:1); IR (KBr, cm^{-1}) 3321 (br), 2932, 2863, 1675 (CO); ¹H NMR (500 MHz, $DMSO-d_6$) δ 8.23 (s, 3H), 7.73 (d, J = 8.1 Hz, 3H), 7.25 (s, 3H), 7.03 (s, 3H), 6.34 (s, 3H), 5.09 (s, 6H), 4.83 (t, J = 5.5 Hz, 3H), 4.35 (t, J = 7.1 Hz, 6H), 4.20 (dt, J = 8.1, 5.4 Hz, 3H), 3.54 (hept, J = 5.5 Hz, 6H), 2.22–2.10 (m, 6H), 1.82 (p, J = 7.3 Hz, 6H), 1.52 (p, J = 7.5 Hz, 6H), 1.23 (p, J = 7.7 Hz, 6H); ¹³C NMR (125 MHz, $DMSO-d_6$) δ 172.25, 171.99, 159.88, 142.42, 124.40, 94.54, 61.80, 61.22, 54.86, 49.28, 34.90, 29.49, 25.49, 24.48; HRMS (FT-ICR) m/z calcd. for $C_{42}H_{64}N_{15}O_{12}$ $[M+H]^+$ 970.4853, found 970.4864.

6,6'-(4,4'-(((5-(Prop-2-yn-1-yloxy)-1,3-phenylene)bis(oxy))bis(methylene))bis(1*H*-1,2,3-triazole-4,1-diyl))bis(*N*-((*S*)-1-amino-3-hydroxy-1-oxopropan-2-yl)hexanamide) (12b). DMF and a microwave vial (0.5–2 mL) were purged with argon for 30 min. To the vial were added tris(alkyne) **10** (72.3 mg, 301 μ mol, 1.0 eq), serinamide azide **11** (146.4 mg, 602 μ mol, 2.0 eq), TBTA (99.2 mg, 187 μ mol, 0.6 eq), and TACP (69.8 mg, 187 μ mol, 0.6 eq) in that order. To this mixture was added 600 μ L of argon-purged DMF, whereupon a green solution was obtained. Sodium ascorbate (40.8 mg, 206 μ mol, 1.1 eq per TACP) was added in a single portion, and the solution color changed to a light brown. The walls of the vial were washed down with 400 μ L of argon-purged DMF, the vial sealed, irradiated in a microwave reactor to maintain a temperature of 100 °C for 4 h. The reaction mixture was then cooled, volatiles removed *in vacuo*, and the residue subjected to flash column chromatography using DCM/MeOH/conc NH_4OH (5:2:0.25) as the eluent. The product-containing fractions were combined and volatiles were removed *in vacuo*, leaving an oily yellow residue. This was dissolved in H_2O (5 mL) and the minimum amount of MeCN necessary to effect solution and the mixture lyophilized, giving 68 mg (93.6 μ mol, 31%) of **12b** as a white solid; R_f 0.42 (DCM/MeOH/conc NH_4OH 5:2:0.25, visualization $KMnO_4$); mp 72–74 °C; IR (KBr, cm^{-1}) 3295 (br), 2937, 2865, 2118

(C≡C), 1676 (CO); ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.23 (s, 2H), 7.74 (d, *J* = 8.1 Hz, 2H), 7.25 (s, 2H), 7.03 (s, 2H), 6.38 (s, 1H), 6.27 (d, *J* = 1.9 Hz, 1H), 5.09 (s, 4H), 4.84 (t, *J* = 5.4 Hz, 2H), 4.75 (d, *J* = 2.2 Hz, 2H), 4.35 (t, *J* = 7.1 Hz, 4H), 4.20 (dt, *J* = 7.9, 5.4 Hz, 2H), 3.60–3.46 (m, 4H), 2.15 (t, *J* = 7.1 Hz, 4H), 1.89–1.73 (m, 4H), 1.60–1.44 (m, 4H), 1.32–1.17 (m, 4H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 172.25, 171.99, 159.83, 158.99, 142.37, 124.40, 94.76, 94.69, 79.19, 78.21, 61.80, 61.26, 55.55, 54.87, 49.28, 34.90, 29.49, 25.48, 24.48; HRMS (FT-ICR) *m/z* calcd. for C₃₃H₄₇N₁₀O₉ [M+H]⁺ 727.35220, found 727.35184.

(S)-N-(1-Amino-3-hydroxy-1-oxopropan-2-yl)-6-(4-((3,5-bis(prop-2-yn-1-yloxy)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)hexanamide (12c). DMF and a microwave vial (0.5–2 mL) were purged with argon for 30 min. To the vial were added tris(alkyne) **10** (292.7 mg, 1.22 mmol, 2.0 eq), serinamide azide **11** (148.2 mg, 609.1 μmol, 1.0 eq), TBTA (64.6 mg, 121.8 μmol, 0.2 eq), and TACP (45.4 mg, 121.8 μmol, 0.2 eq) in that order. To this mixture was added 600 μL of argon-purged DMF, whereupon a green solution was obtained. Sodium ascorbate (26.5 mg, 113 μmol, 1.1 eq per TACP) was added in a single portion, and the solution color changed to a light brown. The walls of the vial were washed down with 400 μL of argon-purged DMF, the vial sealed, and irradiated in a microwave reactor to maintain a temperature of 100 °C for 4 h. The reaction mixture was then cooled, volatiles removed *in vacuo*, and the residue subjected to flash column chromatography using DCM/MeOH/conc NH₄OH (5:0.5:0.1) as the eluent. The product-containing fractions were combined and volatiles were removed *in vacuo*, leaving an oily yellow residue. This was dissolved in H₂O (5 mL) and the minimum amount of MeCN necessary to effect solution and the mixture lyophilized, giving 141 mg (292 μmol, 48%) of **12c** as a white solid; R_f 0.25 (DCM/MeOH/conc NH₄OH 5:0.5:0.1, visualization KMnO₄); mp 58–59 °C; IR (KBr, cm⁻¹) 3419, 3289 (br), 2938, 2862, 2120 (C≡C), 1659 (CO), 1602 (phenyl); ¹H NMR (600 MHz, DMSO-*d*₆) δ 8.22 (s, 1H), 7.71 (d, *J* = 8.1 Hz, 1H), 7.26–7.22 (m, 1H), 7.03 (s, 1H), 6.31 (d, *J* = 2.1 Hz, 2H), 6.23 (t, *J* = 2.1 Hz, 1H), 5.08 (s, 2H), 4.82 (t, *J* = 5.6 Hz, 1H), 4.75 (d, *J* = 2.5 Hz, 3H), 4.35 (t, *J* = 7.1 Hz, 2H), 4.21 (dt, *J* = 8.2, 5.4 Hz, 1H), 3.57–3.50 (m, 4H), 2.15 (td, *J* = 7.3, 2.7 Hz, 2H), 1.82 (p, *J* = 7.4 Hz, 2H), 1.53 (p, *J* = 7.5 Hz, 2H), 1.27–1.19 (m, 2H); ¹³C NMR (125 MHz, DMSO-*d*₆) δ 172.23, 171.97, 159.76, 158.95, 142.31, 124.37, 94.98, 94.92, 79.12, 78.25, 61.80, 61.30, 55.58, 54.83, 49.28, 34.89, 29.48, 25.48, 24.48; HRMS (FT-ICR) *m/z* calcd. for C₂₄H₃₀N₅O₆ [M+H]⁺ 484.2191, found 484.2191.

6,6'-(4,4'-(((5-((1-((6S,9R,12S)-12-((1H-Imidazol-4-yl)methyl)-1-amino-6-(((S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)-9-benzyl-1-imino-8,11,14-trioxo-2,7,10,13-tetraazanadecan-19-yl)-1H-1,2,3-triazol-4-yl)methoxy)-1,3-phenylenebis(oxy))bis(methylene))bis(1H-1,2,3-triazole-4,1-diyl))bis(N-(1-amino-3-hydroxy-1-oxopropan-2-yl)hexanamide) (14a). To a microwave vial (0.2–0.5 mL) purged with argon were added **12b** (28.2 mg, 38.8 μmol, 1.0 eq), MSH(4) azide **13** (48.5 mg, 61.9 μmol, 1.5 eq), TBTA (4.4 mg, 8.3 μmol, 0.2 eq), and TACP (3.1 mg, 8.3 μmol, 0.2 eq). To this mixture were added 150 μL of argon-purged DMF and sodium ascorbate (1.8 mg, 9.1 μmol, 1.1 eq per TACP). The vial walls were washed down with 50 μL of argon-purged DMF, the vial sealed, and irradiated in a microwave reactor to

maintain a temperature of 100 °C for 4 h. Volatiles were removed *in vacuo* and the residue dissolved in H₂O (50 mL). The aqueous solution was extracted with a solution of dithizone in chloroform (0.5 mM, 3 × 30 mL) and chloroform (2 × 30 mL). The resulting aqueous solution was lyophilized, giving 56.2 mg of residue. Preparative reversed phase HPLC gave 9.3 mg (6.2 μmol, 16%) of **14a** as a white solid; HRMS (FT-ICR) *m/z*, calcd. for C₇₁H₉₈N₂₄O₁₄ [M+2H]²⁺ 755.3842, found 755.3849; Analytical HPLC t_R 12.32 min.

6,6'-(4,4'-(((5-((1-((6-((1-Amino-3-hydroxy-1-oxopropan-2-yl)amino)-6-oxohexyl)-1H-1,2,3-triazol-4-yl)methoxy)-1,3-phenylenebis(oxy))bis(methylene))bis(1H-1,2,3-triazole-4,1-diyl))bis(N-(((S)-1-(((R)-1-(((S)-1-(((S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-5-guanidino-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-3-(1H-imidazol-4-yl)-1-oxopropan-2-yl)hexanamide) (14b). To a microwave vial (0.2–0.5 mL) purged with argon were added **12c** (28.5 mg, 58.9 μmol, 1.0 eq), MSH(4) azide **13** (145.8 mg, 186.1 μmol, 3 eq), TBTA (13.1 mg, 24.8 μmol, 0.4 eq), and TACP (9.2 mg, 24.8 μmol, 0.4 eq). To this mixture was added 300 μL of argon-purged DMF and sodium ascorbate (5.4 mg, 27.3 μmol, 1.1 eq per TACP). The vial walls were washed down with 50 μL of argon-purged DMF, the vial sealed, and irradiated in a microwave reactor to maintain a temperature of 100 °C for 4 h. Volatiles were removed *in vacuo* and the residue dissolved in H₂O (50 mL). The aqueous solution was extracted with a solution of dithizone in chloroform (1.2 mM, 3 × 30 mL) and chloroform (2 × 30 mL). The resulting yellow aqueous solution was lyophilized, giving 136 mg of residue. Preparative reversed phase HPLC gave 27.4 mg (13.4 μmol, 23%) of **14b** as a white solid; HRMS (FT-ICR) *m/z* calcd. for C₁₀₀H₁₃₁N₃₃O₁₆ [M+2H]²⁺ 1025.02203, found 1025.02205; Analytical HPLC t_R 13.06 min.

6,6',6''-(4,4',4''-((Benzene-1,3,5-triyltris(oxy))tris(methylene))tris(1H-1,2,3-triazole-4,1-diyl))tris(N-(((S)-1-(((R)-1-(((S)-1-(((S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-5-guanidino-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-3-(1H-imidazol-4-yl)-1-oxopropan-2-yl)hexanamide) (14c). To a microwave vial (0.2–0.5 mL) purged with argon were added MSH(4) azide **13** (87 mg, 111.1 μmol, 6.0 eq), TBTA (11.7 mg, 22 μmol, 1.2 eq), and TACP (8.2 mg, 22 μmol, 1.2 eq). To this mixture was added 125 μL (18.5 μmol, 1.0 eq) of a solution of **10** in DMF (35.6 mg/mL). Sodium ascorbate (4.8 mg, 24 μmol, 1.1 eq per TACP) was added followed by 75 μL of argon-purged DMF. The vial was sealed and irradiated in a microwave reactor to maintain a temperature of 100 °C for 4 h. The reaction mixture was then cooled, volatiles removed *in vacuo*, and the residue dissolved in H₂O (60 mL). The aqueous solution was extracted with a solution of dithizone in chloroform (0.5 mM, 3 × 30 mL) and chloroform (3 × 30 mL). The resulting yellow aqueous phase was lyophilized, giving 80 mg of residue. Preparative reversed phase HPLC gave 24.5 mg (9.5 μmol, 51%) of **14c** as a fluffy white solid; HRMS (FT-ICR) *m/z* calcd. for C₁₂₉H₁₆₅N₄₂O₁₈ [M+3H]³⁺ 863.77661, found 863.77676; Analytical HPLC t_R 13.41 min.

6,6',6''-(4,4',4''-(Nitrilotris(methylene))tris(1H-1,2,3-triazole-4,1-diyl))tris(N-(((S)-1-amino-3-hydroxy-1-oxopropan-2-yl)hexanamide) (15a). Using the procedure outlined for **12a**, compound **15a** was prepared from **8** (28.9 μL,

200 μmol , 1.0 eq), serinamide azide **11** (292 mg, 1.2 mmol, 6.0 eq), TBTA (95.4 mg, 180 μmol , 0.9 eq), and TACP (67.1 mg, 180 μmol , 0.9 eq), sodium ascorbate (39.2 mg, 198 μmol , 1.1 eq per TACP), and DMF (1 mL). Purification by flash chromatography (mobile phase DCM/MeOH/conc NH_4OH 5:2:0.5) afforded 172 mg (200 μmol , 100%) of **15a** as a yellow oily residue; R_f 0.24 (DCM/MeOH/conc NH_4OH 5:2:0.5, visualization KMnO_4); $[\alpha]_D^{25} +1.18$ (c 0.56, MeCN/ H_2O 1:1); IR (KBr, cm^{-1}) 3313 (br), 2930, 2861, 1654 (CO); ^1H NMR (500 MHz, methanol- d_4) δ 8.01 (s, 3H), 4.44–4.39 (m, 9H), 3.81–3.73 (m, 12H), 2.29 (t, $J = 7.4$ Hz, 6H), 1.93 (p, $J = 7.1$ Hz, 6H), 1.67 (p, $J = 7.5$ Hz, 6H), 1.39–1.28 (m, 6H); ^{13}C NMR (125 MHz, methanol- d_4) δ 175.89, 175.10, 145.19, 125.54, 63.11, 56.51, 51.16, 36.47, 30.89, 26.97, 25.97; HRMS (FT-ICR) m/z calcd. for $\text{C}_{36}\text{H}_{61}\text{N}_{16}\text{O}_9$ $[\text{M}+\text{H}]^+$ 861.48019, found 861.47968.

6,6'-(4,4'-((Prop-2-yn-1-ylazanediyl)bis(methylene))bis(1H-1,2,3-triazole-4,1-diy))bis(N-((S)-1-amino-3-hydroxy-1-oxopropan-2-yl)hexanamide) (15b). Using the procedure outlined for **12b**, compound **15b** was prepared from **8** (59.2 μL , 410 μmol , 1.0 eq), serinamide azide **11** (200 mg, 820 μmol , 2.0 eq), TBTA (84.8 mg, 160 μmol , 0.4 eq), and TACP (59.6 mg, 160 μmol , 0.4 eq), sodium ascorbate (34.9 mg, 180 μmol , 1.1 eq per TACP), and DMF (2 mL). Purification by flash chromatography (mobile phase DCM/MeOH/conc NH_4OH 5:2:0.5) afforded 76.1 mg (123 μmol , 30%) of **15b** as a yellow oily residue; R_f 0.34 (DCM/MeOH/conc NH_4OH 5:2:0.5, visualization KMnO_4); ^1H NMR (500 MHz, methanol- d_4) δ 7.95 (s, 2H), 4.41 (m, 6H), 3.84 (s, 4H), 3.82–3.72 (m, 4H), 3.36–3.33 (m, 2H), 2.73 (t, $J = 2.4$ Hz, 1H), 2.29 (t, $J = 7.4$ Hz, 4H), 1.92 (p, $J = 7.2$ Hz, 4H), 1.66 (p, $J = 7.5$ Hz, 4H), 1.39–1.27 (m, 4H); ^{13}C NMR (125 MHz, methanol- d_4) δ 175.87, 175.09, 145.32, 125.35, 78.70, 75.64, 63.09, 56.48, 51.14, 48.72, 42.58, 36.45, 30.89, 26.94, 25.94; HRMS (FT-ICR) m/z calcd. for $\text{C}_{27}\text{H}_{44}\text{N}_{11}\text{O}_6$ $[\text{M}+\text{H}]^+$ 618.3471, found 618.3471.

(S)-N-(1-amino-3-hydroxy-1-oxopropan-2-yl)-6-(4-((di(prop-2-yn-1-yl)amino)methyl)-1H-1,2,3-triazol-1-yl)hexanamide (15c). Using the procedure outlined for **12c**, compound **15c** was prepared from **8** (178 μL , 1.23 mmol, 3.0 eq), serinamide azide **11** (100 mg, 410 μmol , 1.0 eq), TBTA (43.5 mg, 82 μmol , 0.2 eq), and TACP (30.6 mg, 82 μmol , 0.2 eq), sodium ascorbate (17.9 mg, 90 μmol , 1.1 eq per TACP), and DMF (700 μL). Purification by flash chromatography (mobile phase DCM/MeOH/conc NH_4OH 5:1:0.1) afforded 108.4 mg (290 μmol , 71%) of **15c** as a yellow oily residue; R_f 0.37 (DCM/MeOH/conc NH_4OH 5:1:0.1, visualization KMnO_4); ^1H NMR (500 MHz, methanol- d_4) δ 7.91 (s, 1H), 4.44–4.38 (m, 3H), 3.84 (s, 2H), 3.81–3.73 (m, 2H), 3.45 (d, $J = 2.4$ Hz, 4H), 3.35 (s, 1H), 2.69 (t, $J = 2.4$ Hz, 2H), 2.29 (t, $J = 7.4$ Hz, 2H), 1.93 (p, $J = 7.2$ Hz, 2H), 1.67 (p, $J = 7.5$ Hz, 2H), 1.35 (tt, $J = 9.7, 6.6$ Hz, 2H); ^{13}C NMR (125 MHz, methanol- d_4) δ 175.84, 175.04, 144.89, 125.32, 79.00, 75.18, 63.08, 56.42, 51.14, 48.47, 42.48, 36.44, 30.90, 26.94, 25.93; HRMS (FT-ICR) m/z calcd. for $\text{C}_{18}\text{H}_{27}\text{N}_6\text{O}_3$ $[\text{M}+\text{H}]^+$ 375.2139, found 375.2139.

6,6'-(4,4'-((((1-((6S,9R,12S)-12-((1H-Imidazol-4-yl)methyl)-1-amino-6-((S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl)carbamoyl)-9-benzyl-1-imino-8,11,14-trioxo-2,7,10,13-tetraazanadecan-19-yl)-1H-1,2,3-triazol-4-yl)methyl)azanediyl)bis(methylene))bis(1H-1,2,3-triazole-

4,1-diy))bis(N-((S)-1-amino-3-hydroxy-1-oxopropan-2-yl)hexanamide) (16a). Using the procedure given for the synthesis of **14a**, compound **16a** was prepared from **15b** (183 μL of a 0.41 M solution in DMF, 75 μmol , 1.0 eq), MSH(4) azide **13** (88.1 mg, 112.5 μmol , 1.5 eq), TACP (5.6 mg, 15 μmol , 0.2 eq), sodium ascorbate (3.3 mg, 16.5 μmol , 1.1 eq per TACP), and DMF (117 μL). No TBTA was used in this reaction. Preparative reversed phase HPLC gave 20.9 mg (14.9 μmol , 33%) of **16a** as an off-white solid; HRMS (FT-ICR) m/z calcd. for $\text{C}_{65}\text{H}_{94}\text{N}_{25}\text{O}_{11}$ $[\text{M}+\text{H}]^+$ 1400.7559, found 1400.7562; Analytical HPLC t_R 10.41 min.

6,6'-(4,4'-((((1-((6-((S)-1-Amino-3-hydroxy-1-oxopropan-2-yl)amino)-6-oxohexyl)-1H-1,2,3-triazol-4-yl)methyl)azanediyl)bis(methylene))bis(1H-1,2,3-triazole-4,1-diy))bis(N-((S)-1-(((R)-1-(((S)-1-(((S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-5-guanidino-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-3-(1H-imidazol-4-yl)-1-oxopropan-2-yl)hexanamide) (16b). Using the procedure given for the synthesis of **14b**, compound **16b** was prepared from **15c** (120 μL of a 0.50 M solution in DMF, 60 μmol , 1.0 eq), MSH(4) azide **13** (141 mg, 180 μmol , 3 eq), TACP (8.9 mg, 24 μmol , 0.4 eq), sodium ascorbate (5.2 mg, 26.4 μmol , 1.1 eq per TACP), and DMF (130 μL). No TBTA was used in this reaction. Preparative reversed phase HPLC gave 46.3 mg (23.9 μmol , 40%) of **16b** as a white solid; HRMS (FT-ICR) m/z calcd. for $\text{C}_{94}\text{H}_{128}\text{N}_{34}\text{O}_{13}$ $[\text{M}+2\text{H}]^{2+}$ 970.51946, found 970.51975; Analytical HPLC t_R 11.88 min.

6,6',6''-(4,4',4''-(Nitrilotris(methylene))tris(1H-1,2,3-triazole-4,1-diy))tris(N-((S)-1-(((R)-1-(((S)-1-(((S)-1-amino-3-(1H-indol-3-yl)-1-oxopropan-2-yl)amino)-5-guanidino-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-3-(1H-imidazol-4-yl)-1-oxopropan-2-yl)hexanamide) (16c). A solution of **8** in MeCN (571 μL of 0.035 M solution, 20 μmol , 1.0 eq) was added to a microwave vial (0.5–2 mL) purged with argon. To this mixture were added H_2O (500 μL), MSH(4) azide **13** (94 mg, 120 μmol , 6.0 eq), 2,6-lutidine (470 μL of 0.085 M solution in MeCN, 40 μmol , 2.0 eq), and TACP (29 mg, 78 μmol , 3.9 eq) in that order. The walls of the vial were rinsed down with 540 μL of H_2O , the vial sealed, and left to stir at rt for 4 days. The reaction mixture was then diluted with H_2O (40 mL), extracted with a solution of dithizone in chloroform (2.0 mM, 3 \times 30 mL) and chloroform (3 \times 30 mL), and lyophilized to give 95 mg of residue. Preparative reversed phase HPLC gave 31.0 mg (12.5 μmol , 62%) of **16c** as an off-white solid; HRMS (FT-ICR) m/z calcd. for $\text{C}_{123}\text{H}_{162}\text{N}_{43}\text{O}_{15}$ $[\text{M}+3\text{H}]^{3+}$ 827.10730, found 827.44156; Analytical HPLC t_R 12.29 min.

1,4,7-Tri(prop-2-yn-1-yl)-1,4,7-triazonane (17).²⁸ To a stirred solution of 1,4,7-triazacyclononane hydrochloride (**9**, 250 mg, 1.02 mmol, 1.0 eq) in toluene/ethanol (1:1, 5 mL) was added potassium hydroxide (355 mg, 6.3 mmol, 6.3 eq). After 30 min, the flask was cooled in an ice-water bath and propargyl bromide (80% in toluene, 0.317 mL, 2.13 mmol, 2.1 eq) was added dropwise as a solution in toluene/ethanol (1:1, 5 mL). The reaction mixture was allowed to attain rt and was stirred for 24 h. The resultant suspension was filtered to remove the solid matter and volatiles removed *in vacuo* to give a brownish solid. Flash chromatography on silica gel initially buffered with 1% triethylamine in hexanes using MeOH/DCM/conc NH_4OH (0.5:9.5:0.05) as the eluent afforded 171 mg (0.70

mmol, 69%) of **17** as a yellowish oil which solidified upon standing overnight; R_f 0.38 (MeOH/DCM/conc NH_4OH 0.5:9.5:0.05, visualization KMnO_4); mp 52-53 °C; IR (KBr, cm^{-1}) 3292, 3276, 3262, 3133, 2922, 2804, 2082 ($\text{C}\equiv\text{C}$); ^1H NMR (500 MHz, CDCl_3) δ 3.42 (d, $J = 2.4$ Hz, 6H, $\text{N-CH}_2\text{-C}\equiv$), 2.79 (s, 12H, $\text{N-CH}_2\text{-CH}_2\text{-N}$), 2.14 (t, $J = 2.3$ Hz, 3H, $\text{C}\equiv\text{C-H}$); ^{13}C NMR (125 MHz, CDCl_3) δ 80.34 ($\text{-C}\equiv\text{C-H}$), 71.63 ($\text{-C}\equiv\text{C-H}$), 53.77 ($\text{N-CH}_2\text{-CH}_2\text{-N}$), 46.82 ($\text{N-CH}_2\text{-C}\equiv$); HRMS (FT-ICR) m/z calcd. for $\text{C}_{15}\text{H}_{22}\text{N}_3$ ($\text{M}+\text{H}$) $^+$ 244.18082, found 244.18044.

6,6',6''-(4,4',4''-((1,4,7-Triazonane-1,4,7-triyl)tris(methylene))tris(1*H*-1,2,3-triazole-4,1-diyl))tris(*N*-((*S*)-1-(((*R*)-1-(((*S*)-1-((*S*)-1-amino-3-(1*H*-indol-3-yl)-1-oxopropan-2-yl)amino)-5-guanidino-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-3-(1*H*-imidazol-4-yl)-1-oxopropan-2-yl)hexanamide) (18**). Using the procedure given for the synthesis of **14c**, compound **18** was prepared from MSH(4) azide **13** (75.0 mg, 95.7 μmol , 4.5 eq), TACP (4.7 mg, 12.7 μmol , 0.6 eq), **17** (5.2 mg, 21.2 μmol , 1.0 eq), sodium ascorbate (2.8 mg, 14 μmol , 1.1 eq per TACP), and DMF (300 μL). No TBTA was used in this reaction. Additionally, at each of the dithizone extractions, the aqueous and chloroform layers were stirred rapidly together in an Erlenmeyer flask for 30 min. Extra contact time is necessary to make sure any copper chelated by the macrocyclic ring is removed. Preparative reversed phase HPLC gave 23.1 mg (8.9 μmol , 42%) of **18** as a fluffy white solid; HRMS (FT-ICR) m/z calcd. for $\text{C}_{129}\text{H}_{175}\text{N}_{45}\text{O}_{15}$ [$\text{M}+4\text{H}$] $^{4+}$ 648.6073, found 648.6074; Analytical HPLC t_R 12.43 min.**

Biological Assays

Preparation of Solutions. Stock solutions of the MSH(4) constructs **14a-c**, **16a-c**, and **18**, control compounds **12a** and **15a**, and the Eu-DTPA-PEGO-MSH(7)- NH_2 probe (**19**) were made up in DMSO at a nominal concentration of 2.0 mM based on measured weights of solutes. Except for the control compounds, concentrations were refined by comparison to a DTrp standard solution (0.50 mM) using analytical HPLC. *Selective growth media* for cell growth was prepared by supplementing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 1% penicillin-streptomycin, 0.1% zeocin, and 0.8% geneticin. *Basic buffer* was prepared by dissolving 5.97 g of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 2 g BSA in 1 L of DMEM. The pH of this solution was adjusted to 7.4 using 2 N NaOH and the solution sterilized by filtration through a 0.22 μm filter (Corning, 431117, 500 mL bottle-top filter, sterile) under vacuum. *Binding buffer* was prepared by supplementing DMEM (1 L) with HEPES (5.97 g), BSA (2 g), 1,10-phenanthroline (1 mL of a 1 M solution in EtOH), leupeptin (1 mL of a 500 mg/L aqueous solution), and bacitracin (1 mL of a 200 g/L aqueous solution). The pH of this solution was adjusted to 7.4 using 2 N NaOH and the solution sterilized by filtration through a 0.22 μm filter (Corning, 431117, 500 mL bottle-top filter, sterile) under vacuum.

Cell Culture. Dual (MC4R/CCK2R) expressing HEK293 cells⁹ were maintained in selective growth media. For the binding assays, cells were plated into 6 well plates (Greiner Bio-One, 657160, Cell Culture Multi-well Plates, Polystyrene, 6 wells) at 240,000 cells per well in a total volume of 3 mL (2 mL of the selective growth media added initially to each well, followed by 1 mL of cell suspension). On the third day after

plating, additional selective growth media (1 mL) was carefully added to each of the wells so as not to disturb the cells. The cells were left to grow until ~90% confluence was achieved (usually by day 5) before conducting assays.

Saturation Binding Assays. Six solutions containing both the probe Eu-DTPA-PEGO-MSH(7)- NH_2 (**19**) and NDP- α -MSH (**1**) were made in 1.2 mL of binding buffer in separate micro-centrifuge tubes (one per each well). All six tubes contained 1 μM concentrations of NDP- α -MSH (**1**), while the concentration of the probe **19** was varied across the six tubes (1, 10, 25, 50, 100, 250 nM). These six solutions were used to assess nonspecific binding. A second set of six solutions (1.2 mL each) contained the probe **19** at the same concentrations without NDP- α -MSH. These solutions were used to assess total binding.

To commence an assay, cell-containing 6-well plates ($\times 2$) were removed from the incubator and the selective growth media removed by careful aspiration. The twelve prepared solutions detailed above were carefully transferred (1 mL per well) by pipette down the well walls (to minimize disturbance of the cells, which can result in cell loss during media exchanges). The plates were then maintained in a CO_2 incubator at 37 °C for 1 h.

Solutions were then removed by careful aspiration and 600 μL of basic buffer were added to each well. The cells were scraped from each well individually using a Cell Scraper (18 cm, GeneMate) and transferred in suspension to separate 1.7 mL micro-centrifuge tubes. The wells and scraper were rinsed with 600 μL of basic buffer and the rinses combined with the corresponding suspension. One scraper was used across the wells measuring total binding, and another was used across the wells measuring non-specific binding.

The tubes containing the cell suspensions were centrifuged (3000 rpm for 3 min) in a micro-centrifuge. After removing the supernatant, the cells were resuspended in basic buffer (1 mL) and incubated for 5 min in a 37 °C water bath during each of three wash cycles. After the final wash, 600 μL of DELFIA Enhancement Solution (Perkin Elmer 1244-104) was added to each cell pellet, the tubes mixed using a vortex mixer, and incubated for 1 h in a water bath maintained at 37 °C.

Following the incubation, cells and cell fragments were pelleted (5000 rpm for 5 min) and 4 \times 100 μL aliquots of each supernatant were transferred to a 96 well plate (Perkin-Elmer, 6005060, tissue culture treated B&W Isoplate-96) for fluorescence measurement using a VICTOR X4 2030 Multilabel Reader.

Competitive Binding Assays. Immediately before an assay, twelve solutions (1350 μL each) of compounds to be tested were made up in binding buffer in microcentrifuge tubes at concentrations ranging from 10 μM –0.10 nM. Each of the tubes also contained the Eu-DTPA-PEGO-MSH(7)- NH_2 probe **19** at a concentration of 20 nM. Cell-containing 6-well plates ($\times 2$) were removed from the incubator and the selective growth media removed by careful aspiration. The twelve prepared solutions from above were carefully transferred (1 mL per well) by pipette down the well walls. The plates were then maintained in a CO_2 incubator at 37 °C for 1 h.

After 1 h, solutions were removed by careful aspiration and 600 μL of basic buffer were added to each well. The cells were scraped from each well using a Cell Scraper (18 cm, GeneMate) and transferred in suspension to separate 1.7 mL micro-centrifuge tubes. The wells and scraper were rinsed with

600 μL of basic buffer and the rinses combined with the corresponding suspension.

The cell suspensions were centrifuged (3000 rpm for 3 min) in a micro-centrifuge. After removing the supernatant, the cells were resuspended in basic buffer (1 mL) and incubated for 5 min in a 37 °C water bath during each of three wash cycles. After the final wash, 600 μL of DELFIA Enhancement Solution (Perkin Elmer 1244-104) was added to each cell pellet, the tubes mixed using a vortex mixer, and incubated in a water bath maintained at 37 °C for 1 h.

Following the incubation, cells and cell fragments were pelleted (5000 rpm for 5 min) and 4 \times 100 μL aliquots of each supernatant were transferred to a 96 well plate (Perkin-Elmer, 6005060, tissue culture treated B&W Isoplate-96) for fluorescence measurement using a VICTOR X4 2030 Multilabel Reader.

Data Analysis. NMR data were analyzed using MestReNova (Mestre Lab Research S. L., version 7.1.1) software. Biological data analysis was performed using GraphPad Prism software (version 5.04). A description of the binding equations used appears in the Electronic Supplementary Information.

Conclusions

We have demonstrated short and efficient syntheses of multivalent molecules targeted to melanocortin receptors based on three commercially available trigonal core scaffolds. This methodology can be adapted to prepare asymmetric constructs involving two and possibly three different ligands, or combinations of one or two ligands with reporters and/or therapeutic agents. Pertaining to MC4R, we have obtained evidence that the proper ligand spacing for multivalent binding to dimeric receptors is in the short end of the previously reported range, 24 \pm 5 Å.

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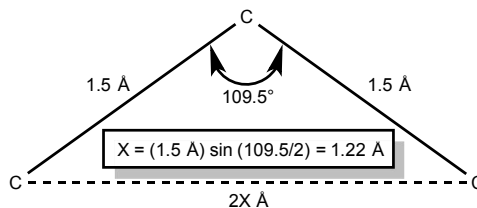
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[†]Electronic Supplementary Information (ESI) available: Copies of NMR spectra of all new compounds, analytical HPLC traces from analyses of compounds **14a-c**, **16a-c**, and **18**, details of the protein assay and normalization of the fluorescence data from competition binding assays with binding curves and the resultant K_i values, and details of the

molecular dynamics analysis of compounds **6**, **14c**, **16c**, and **18**. See DOI: 10.1039/b000000x/

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- (48) *Molecular Operating Environment (MOE)*, 2013.08; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, 2013. Experimental details are given in the Electronic Supplementary Information.
- (49) Given the basicity of the MSH(4) ligand, compounds **14a-c**, **16a-c**, and **18** will all be highly positively charged at pH 7.4. The central tertiary amine moieties of compounds **16a-c** were modeled as ammonium ions, and the tertiary amines of the 1,4,7-triazacyclononane moiety of compound **18** were modeled as the doubly protonated species, consistent with expectations at a physiological pH. Compounds **14a-c** have unprotonated cores, a fact that may influence specific binding with receptors and/or nonspecific binding with negatively charged cell surfaces. While the data generally suggest tighter binding for a more highly charged core, the differences in K_i for comparable constructs are small, and other factors may be responsible.
- (50) This equivalence is not possible for **6**, since the path of attachment of one MSH(4) ligand to the central atom differs from that of the other two ligands.
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