Linear Scaffolds for Multivalent Targeting of Melanocortin Receptors

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ARTICLE

Linear scaffolds for multivalent targeting of melanocortin receptors†

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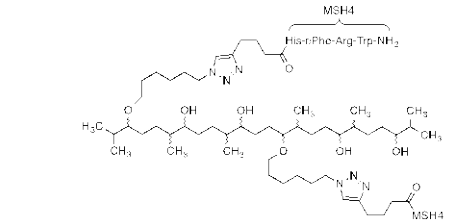
Molecules bearing one, two, three, or four copies of the tetrapeptide His-DPhe-Arg-Trp were attached to scaffolds based on ethylene glycol, glycerol, and D-mannitol by means of the copper-assisted azide-alkyne cyclization. The abilities of these compounds to block binding of a probe at the melanocortin 4 receptor were evaluated using a competitive binding assay. All of the multivalent molecules studied exhibited 30- to 40-fold higher apparent affinites when compared to a monovalent control. These results are consistent with divalent binding to receptor dimers. No evidence for tri- or tetravalent binding was obtained. Differences in the interligand spacing required for divalent binding, as opposed to tri- or tetravalent binding, may be responsible for these results.

Introduction

Recognition of the importance of weak multivalent interactions in physiology has enabled chemists to use this strategy for the production of biologically active synthetic compounds.[1-3] Our research in this area is directed to early detection of melanoma, a serious health problem.[4] Studies have suggested that a majority of melanoma cells express, and in some cases overexpress, melanocortin receptors (MCRs).[5-10] There is also evidence that melanocortin receptors form dimers on cell surfaces.[11-15] Thus, melanocortin receptors present an excellent opportunity for application of a multivalent binding strategy for detection, and potentially treatment, of melanoma.

Previously we reported the synthesis of regioisomeric mixtures of linear multivalent constructs, for example **1**[16] and **2**[17], which were derived from squalene and solanesol, respectively. Since enhanced avidity arising from multivalency is generally more apparent when weakly binding ligands are employed,[18,19] mixtures **1** and **2** incorporated the tetrapeptide His-DPhe-Arg-Trp-NH_2 (MSH4) which exhibits a K_d of ~1 μM for MCRs.[20-22] The closest possible inter-ligand spacing in **1** and **2** is 31 atoms, or approximately 38 Å when the intervening atoms adopt a fully extended conformation.[23] When **1** and **2** were tested in competitive binding assays against time-resolved fluorescence (TRF) probes[16,17,22,24] using HEK 293 cells engineered to overexpress MC4R,[25] the levels of inhibition of probe binding and uptake[26] reflected the number of ligands present per scaffold (statistical effects), but not multivalent binding (simultaneous attachment of two or more ligands).



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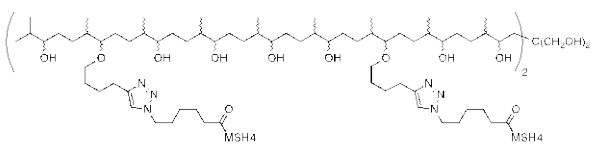

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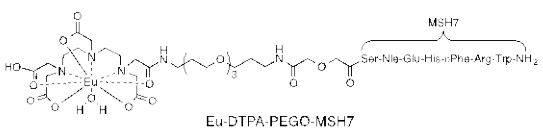
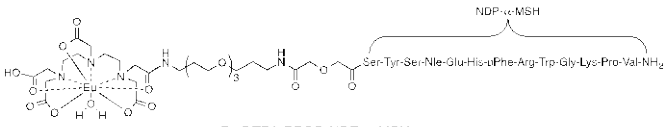


Eu-DTPA-PEGO-MSH7

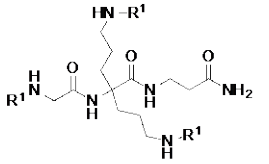
3



Eu-DTPA-PEGO-NDP-α-MSH

4

More recently, multivalent binding to MC4R was demonstrated using compound **5**.[27] The authors of this study suggested that inter-ligand distances of 24±5 Å are necessary for observation of multivalent binding to MC4R. We prepared and tested compounds **6-8** which possess inter-ligand distances in the suggested range and, like **5**, display the three MSH4 ligands in a trigonal fashion. Unlike **5**, compounds **6-8** exhibited K_i values suggestive of bivalent binding, not trivalent binding.[28] To explore further the effects of ligand spacing and orientation on avidity, we designed multivalent MSH4 constructs based on simple linear scaffolds derived from ethylene glycol, glycerol, and D-mannitol. The syntheses of and bioassay results from these compounds are the subject of this article.



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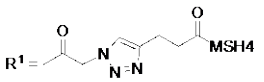
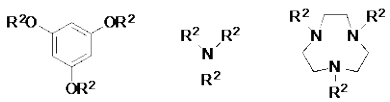
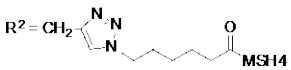




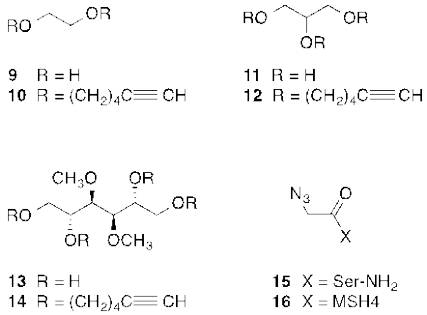
6 **7** **8**



Results

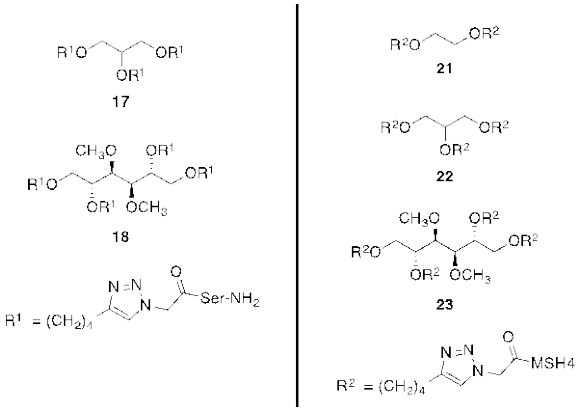
Chemistry

Alkylations of polyols **9**, **11**, and **13**[29] with 6-iodo-1-hexyne produced polyalkynes **10**, **12**, and **14** in 56%, 25%, and 40% yields, respectively. Serinamide azide **15** was prepared in 66% yield by acylation of serinamide with *N*-hydroxysuccinimidyl 2-azidoacetate.[30] MSH4 azide **16** was synthesized in 65% yield by solid phase peptide synthesis as described in the Experimental. Compound **16** was purified by preparative reversed phase HPLC and was characterized by analytical reversed phase HPLC and by high resolution mass spectrometry (see the Electronic Supplementary Information†).

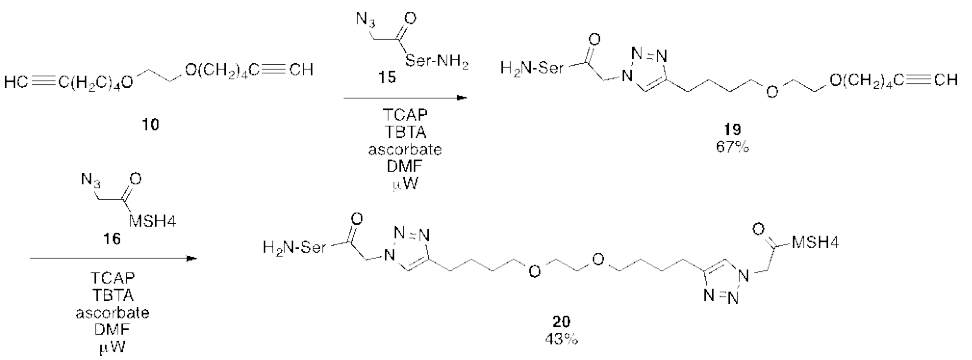


9 R = H
10 R = $(CH_2)_4C{\equiv}CH$
11 R = H
12 R = $(CH_2)_4C{\equiv}CH$
13 R = H
14 R = $(CH_2)_4C{\equiv}CH$
15 X = Ser-NH_2
16 X = MSH4

Copper-catalyzed azide-alkyne cycloaddition (CuAAC)[31] reactions of **15** with compounds **12** and **14** afforded the corresponding serinamide control compounds **17** and **18**, each in 74% yield after purification by gravity column chromatography. The monovalent control **20** was prepared by sequential CuAAC reactions as depicted in Scheme 1. Reaction of **15** with a large excess of **10** gave alkyne **19** in 67% yield after purification by gravity column chromatography. CuAAC reaction of **16** with **19** produced **20** in 43% yield. Compounds **16** and **20** were difficult to separate by preparative HPLC. As a result, the sample of **20** subjected to bioassay contained ~15% of azide **16**. CuAAC reactions of **16** with scaffolds **10**, **12**, and **14** afforded the corresponding di-, tri-, and tetravalent MSH4 compounds **21**, **22**, and **23** in 37%, 83%, and 71% yields, respectively, after purification by preparative HPLC. Samples of compounds **21-23** used in bioassays were ≥95% pure as determined by analytical HPLC analysis and were characterized by high resolution mass spectrometry (see the ESI†).



17
18
21
22
23



10
15
16
19 67%
20 43%

Scheme 1. Synthesis of compound **20**.

Biological Assays. To confirm the competence of the six-well plate protocols,[28] saturation binding assays were performed using the known TRF probe, Eu-DTPA-PEGO-NDP-α-MSH (**4**)[22] and HEK293 cells engineered to overexpress hMC4R (approximately 640,000 copies per cell) and human cholecystokinin 2 receptor (hCCK2R, approximately 1,100,000 copies per cell).[25] Saturation binding curves are depicted in Figure 1. The K_d for **4** calculated from these assays was 12 ± 1 nM, which is reasonably consistent with the value of 4.2 ± 0.5 nM reported from saturation binding assays using a less sensitive high throughput protocol in 96-well plates.[22]

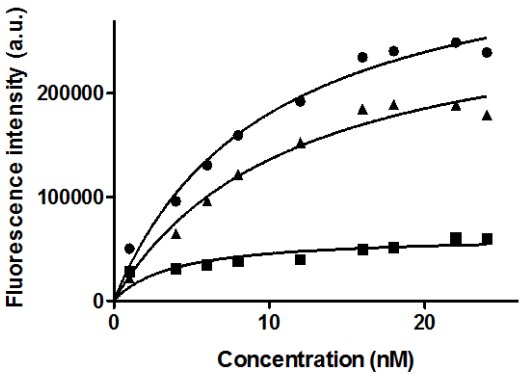


Figure 1. Saturation binding curves for probe **4** generated using the six-well plate assay method. Total binding (●), non-specific binding (▲), and specific binding (■). The calculated K_d = 12 ± 1 nM (n = 4).

Competitive binding assays also employed the six-well plate protocols,[28] HEK293 cells engineered to overexpress hMC4R and hCCK2R,[25] and the TRF probes **3**[22,24] or **4**.[22] Representative binding curves are depicted in Figure 2, and the averaged results for each compound are numerically summarized in Tables 1 and 2. Control compounds **17** and **18** did not inhibit the binding and uptake[26] of probe **3** over the concentration range tested. Mono-, di-, tri-, and tetravalent constructs **20**-**23** exhibited K_i values of 1300, 46, 34, and 39 nM, respectively, against probe **3**. Mono-, di-, and trivalent constructs **20**-**22** were also competed against probe **4** and exhibited K_i values of 2500, 140, and 130 nM, respectively.

Discussion

We previously prepared MSH4-bearing multivalent molecules from linear[16,17,32,33] and spherical[34] scaffolds with inter-ligand distances in the 20-50 Å range suggested by a homology model based on rhodopsin.[35] In competitive binding assays, these constructs generally exhibited the inhibitory potency of MSH4 amplified by the number of ligands present in the construct. Potencies attributable to multivalent binding were not observed. Subsequently, multivalent binding to MC4R was demonstrated using trivalent compound **5**.[22,27] The inter-ligand distances in **5** were estimated to be 24 ± 5 Å, suggesting that the ligand spacing necessary for multivalent binding to MC4R had been overestimated by the homology model. We then prepared compounds **6**-**8** that, like **5**, display three MSH4 ligands in trigonal fashion, but with spacing in the 16–24 Å range. Surprisingly, **6**-**8** displayed inhibitory potencies consistent with divalent binding.[28] We hypothesized that the shorter ligand spacing of **6**-**8** (as compared with **5**) might favor bridging between binding sites on receptor dimers, but not permit binding to a third, more remote binding site in an unsymmetrical receptor trimer. To further explore the effects of ligand spacing and orientation on avidity, we designed MSH4 constructs **20**-**23** based on the simple, inexpensive linear polyols **9**, **11**, and **13**.

Alkylations of **9**, **11**, and **13**[29] with 6-iodo-1-hexyne, followed by CuAAC reactions of the product polyalkynes **10**, **12**, and **14** with serinamide azide **15** and/or MSH4 azide **16** under microwave irradiation gave control compounds **17** and **18** and MSH4 constructs **20**-**23**. The former were purified by silica gel column chromatography, and the latter were purified by reversed-phase preparative HPLC. Compound **20** was ~85% pure as determined by reversed-phase analytical HPLC analysis (see the ESI†). The contaminant was azide **16**, which should have an affinity for MC4R similar to that of **20**.[22] Because of this expectation, impure **20** was used as the monovalent control in the competitive binding assays. Multivalent compounds **21**-**23** were ≥95% pure as determined by reversed-phase analytical HPLC analysis and were characterized by high resolution mass spectrometry (see the ESI†).

To validate the use of the recently described 6-well plate binding assay protocols[28] with TRF probe **4**[22] based on NDP-α-MSH,[36] a saturation binding assay was performed using HEK293 cells engineered to overexpress hMC4R and hCCK2R.[25] The K_d for **4** calculated from this assay was 12 ± 1 nM, while the reported K_d for **4** from use of protocols using 96-well plates was 4.2 ± 0.5 nM.[22] This level of agreement was taken as a validation of the 6-well plate binding assay protocols.

In competitive binding assays, control compounds **17** and **18** did not inhibit the binding and uptake[26] of the TRF probe **3** over the concentration range tested. The monovalent control **20** exhibited a K_i value of 1300 nM, a value consistent with previous results.[22,28] The di-, tri-, and tetravalent constructs **21**-**23** exhibited K_i values of 46, 34, and 39 nM, respectively. These values are consistent with the K_i values observed for trivalent compounds **6**-**8** (compare the data given in Table 1) and are indicative of divalent binding.[27,28]

The possibility that these results might have been due to a technical limit to the dynamic range of the competitive binding assay was excluded by competing NDP-α-MSH[36] against probe **3**. The K_i observed for NDP-α-MSH using the 6-well plate assay was 3.2 ± 0.4 nM, a value consistent with an earlier determination using 96-well plate assays.[22]

Table 1. Results of competitive binding assays using probe **3**.[a]

Compound	K_i ± SEM[b] (nM)	Relative Potency[c]
NDP-α-MSH	3.2 ± 0.4	406
5[d]	4.3 ± 0.4	302
6[e]	45 ± 11	29
7[e]	36 ± 4	36
8[e]	25 ± 4	52
17	NB[f]	NA[g]
18	NB[f]	NA[g]
20	1300 ± 180	1
21	46 ± 4	28
22	34 ± 6	38
23	39 ± 5	33

[a]Competitive binding experiments were carried out against probe **3** (K_d = 21 nM, [**3**] = 20 nM) using HEK293 cells overexpressing hMC4R and CCK2R. [b]SEM = standard error of the mean; n = 4 independent determinations. [c]Relative inhibitory potency compared to monovalent MSH(4) construct **20**. [d]Result taken from reference 22. [e]Result taken from reference 28. [f]NB = no competitive binding observed. [g]NA = not applicable.

Table 2. Results of competitive binding assays using probe **4**.[a]

Compound	K_i ± SEM[b] (nM)	Relative Potency[c]
5[d]	11 ± 1.2	227
20	2500 ± 300	1
21	140 ± 5	18
22	130 ± 4	19

[a]Competitive binding experiments were carried out against probe **4** (K_d = 12 nM, [**4**] = 10 nM) using HEK293 cells overexpressing hMC4R and CCK2R. [b]SEM = standard error of the mean; n = 4 independent determinations. [c]Relative inhibitory potency compared to monovalent MSH(4) construct **20**. [d]Calculated from an EC_{50} value taken from reference 27, wherein a probe similar to **4** was employed.

It is generally held that, like other melanocortin receptors, MC4R forms constitutive dimers on the cell surface.[11-15] The possible modes of divalent binding of **21**-**23** to such dimers are graphically depicted in Figure 3. The maximum inter-ligand distances between the bound ligands, as measured from the *N*-terminal nitrogen atoms of the histidine residues and assuming full extension of all intervening chain segments,[23] are as follows: for **21**-1, 27 Å; for **22**-1, 27 Å; for **22**-2, 28 Å; for **23**-1, 27 Å; for **23**-2, 29 Å; for **23**-3, 30 Å; and for **23**-4, 32 Å. Given the similarity of the K_i values obtained for **21**-**23**, a common binding mode (Mode 1) seems likely. If true, this fixes the maximum distances that can be bridged for binding of a free ligand of **22** or **23** to a third receptor at 27 Å and 30 Å, respectively.

While the MSH4 ligands are positively charged and should be charge-separated in an aqueous solution, full extension of the linear scaffolds and/or linkers seems unlikely, suggesting that shorter inter-ligand distances should predominate in the conformational ensembles. To better address this point, molecular dynamics studies were performed using Molecular Operating Environment (MOE).[37] Representative

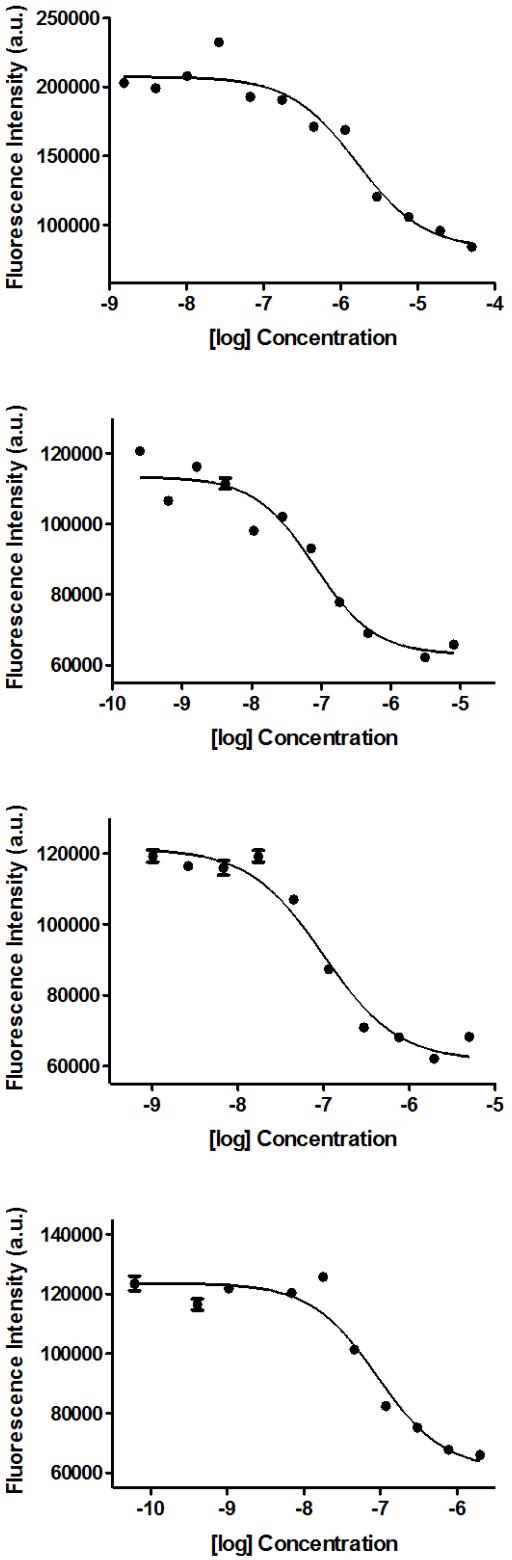


Figure 2. Sample competitive binding curves for compounds **20** (top), **21** (*top middle*), **22** (*bottom middle*), and **23** (*bottom*) against probe **3** (20 nM) generated using the six-well plate assay method. Control compounds **17** and **18** were not competitive inhibitors of **3** over the concentration ranges tested.

conformations of **21-23** from these studies are depicted in Figure 4. The maximum inter-ligand distances observed were, for **21**, 22 Å; for **22**, 23 Å; and for **23**, 28 Å. All are shorter than the distances calculated assuming full extension of the intervening chain segments. The average inter-ligand distances observed during molecular dynamics studies, which are shorter still, are given in the ESI.

Based on previous work with compounds **6-8**, we postulated that an optimum distance for divalent binding to MC4R dimers lies between 17 and 23 Å.[28] Both the observation of inhibitory binding constants for **21-23** that are indicative of divalent binding and the inter-ligand distance analysis for these compounds are consistent with this postulate. We previously suggested that compounds **6-8** had an insufficient "reach" to permit trivalent binding. The same is apparently true for **22** and **23**. Despite having four available ligands, compound **23** exhibited no increase in inhibitory potency, a fact consistent with the observation that dendritic compounds with up to nine MSH4 ligands were not more potent than the structurally related trivalent compound **5**.[38]

Given the adaptability of the synthetic methods described here and elsewhere,[28] we plan to examine the in vitro binding of trivalent molecules that possess an optimized short inter-ligand distance and one longer inter-ligand distance (up to 35 Å). In addition, we assume that a 20- to 40-fold difference in avidity afforded by divalency will be sufficient for distinguishing healthy from abnormal cells through discernment of receptor overexpression. Compounds with two ligands and one imaging or therapeutic agent will therefore be prepared and evaluated in vitro and in vivo.

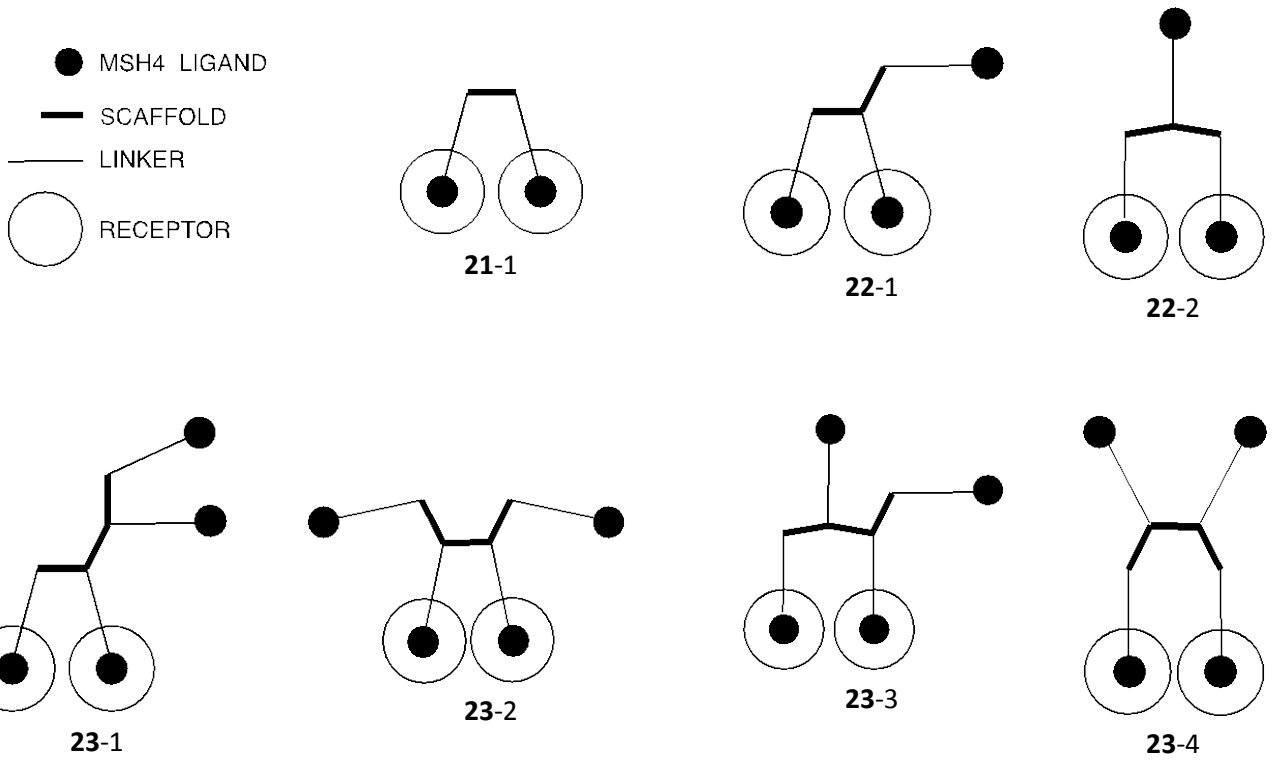
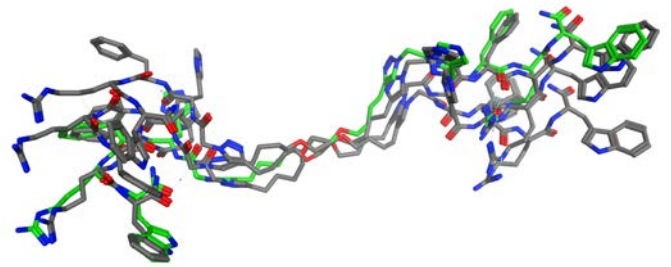


Figure 3. Possible modes of divalent binding of **21-23** to MSH4 dimers.



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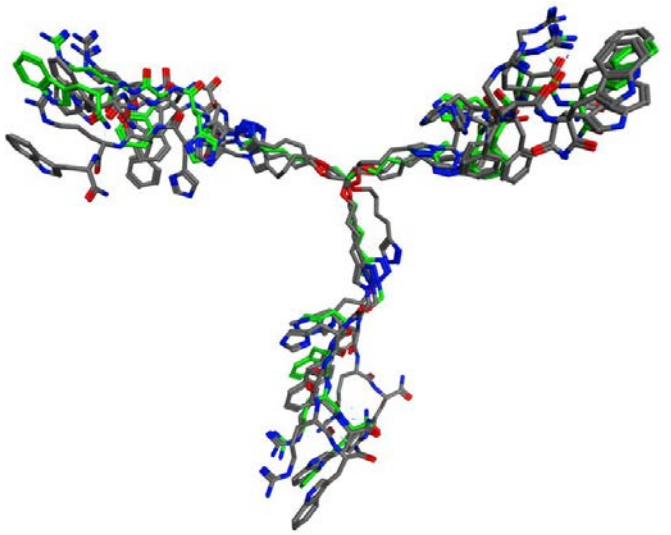

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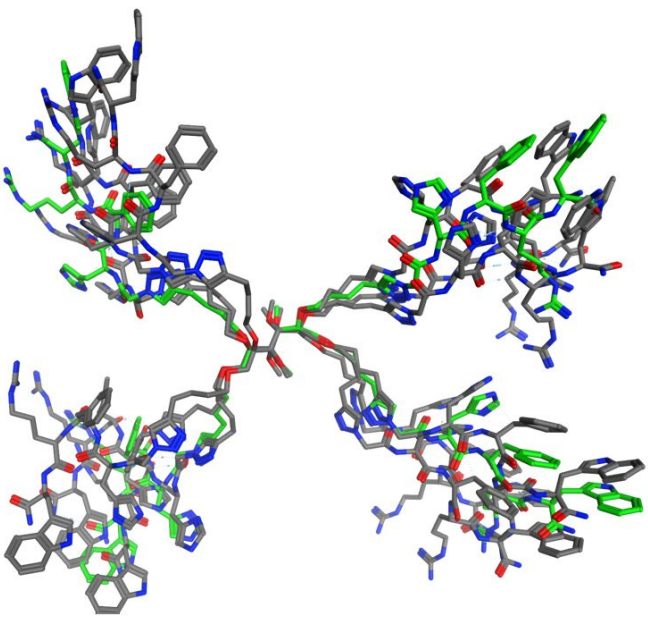

23

Figure 4. Views of representative conformations observed during Molecular Dynamics (MD) studies of compounds **21**, **22**, and **23**. The minimized starting structures appear in green. The lowest energy structures from each of three MD runs are overlaid with the starting structures. The average distances between ligands for the conformational ensembles of **21**, **22**, and **23** are given in the ESI.

Experimental

Chemical Synthesis

General materials and methods. Dichloromethane (DCM), diethyl ether ("ether"), and tetrahydrofuran (THF) were dried by passage through alumina under a positive pressure of argon. Trifluoroacetic acid (TFA), acetic acid, *N*,*N*-dimethylformamide (DMF), acetonitrile (MeCN), ethanol, methanol, and dimethylsulfoxide (DMSO) were used as supplied. $(Boc)_2$(Arg)OH was purchased from Chem-Impex International. Rink Amide AM resin (200–400 mesh) and all other Fmoc amino acids were purchased from Novabiochem. For moisture sensitive reactions, glassware was flame-dried under argon. Solutions were concentrated *in vacuo* using a rotary evaporator. Analytical thin-layer chromatography (TLC) was performed on pre-coated silica gel 60 F-254 glass plates. TLC plates were visualized using UV light and/or staining with iodine vapor, ninhydrin stain (2.25 g ninhydrin, 22.5 mL glacial AcOH, 750 mL *n*-butanol) with heating, or PMA stain (5 g phosphomolybdic acid, 100 mL 95% EtOH) with heating. Gravity column chromatography was performed using silica gel 60 (70-230 mesh). Flash column chromatography was performed using silica gel 60 (230-400 mesh). Preparative HPLC was performed on a 19×250 mm Waters X-Bridge 10 μm OBD C_{18} column. A linear gradient of mobile phase from 10–90% MeCN–water containing 0.1% TFA was used over 45 min. The flow rate was 10 mL min^{-1}, 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 C_{18} column. A linear gradient of mobile phase from 10–90% MeCN–water containing 0.1% TFA was used over 30 min. The flow rate was 0.3 mL min^{-1}, and a dual channel UV detector was used at 220 and 280 nm. Melting points are uncorrected. Infrared spectra were recorded on a Thermo Nicolet iS5 FTIR spectrometer using KBr pellets (solids) or NaCl plates (oils). Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR (Bruker DRX-500). Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) are expressed in Hz. ^{1}H NMR spectra were internally referenced to the residual proton signal in solvent (7.26 ppm for $CDCl_3$ and 3.30 ppm for CD_3OD). ^{13}C NMR spectra were internally referenced to $CDCl_3$ (77.0 ppm) and CD_3OD (49.0 ppm). Characterization by mass spectrometry was performed by the Mass Spectrometry Facility in the Department of Chemistry and Biochemistry at the University of Arizona. ESI experiments were run 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. MALDI-TOF experiments were performed on a Bruker Ultraflex III TOF instrument. Samples in MeOH were dissolved in a saturated solution of sinapinic acid (made up in MeCN–water 3:7 containting 0.1% TFA) in 1:10 v:v ratio. One μL of this mixture was plated on a MALDI plate. Ions were formed by laser desorption with a N_2 laser. Specific rotations were measured on a Rudolph Research Autopol III polarimeter using a 50 mm sample cell (1 mL volume). Cell incubations were done in a Fisher Scientific Isotemp CO_2 incubator (Model 3530) maintained at 37 °C and a 5% CO_2 atmosphere. Centrifugations were performed using a Fischer Scientific Model 59A microcentrifuge. Fluorescence was measured on 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.

Williamson Ether Synthesis

1,2-Bis-*O*-(5-hexynyl)ethylene Glycol (10). A solution of ethylene glycol (**9**, 140 mg, 2.25 mmol) in dry DMF (10 mL) under argon was stirred and cooled in an ice bath. NaH (350 mg, 14.0 mmol) was added, and the suspension allowed to attain room temperature. After 2 h, the reaction mixture was cooled in an ice bath, 6-iodo-1-hexyne[39] (2.8 g, 13.5 mmol) was added dropwise, and the mixture allowed to attain room temperature. After 6 h, second portion of 6-iodo-1-hexyne (1.5 g, 7.2 mmol) was added. After 24 h, the mixture was cooled in an ice bath, the reaction quenched by addition of water (10 mL), and the mixture extracted with ether (4 × 20 mL). The combined ether extracts were washed with water (3 × 20 mL), brine (15 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*, leaving an oily brown residue (1.5 g). Gravity column chromatography on silica gel 60 using 10% EtOAc/hexanes as the eluent afforded 280 mg (1.26 mmol, 56%) of **10** as a pale yellow viscous liquid, R_f 0.43 (10% EtOAc/hexanes, iodine vapor and PMA stains). IR (neat) 3295, 2939, 2116, 1118 cm^{-1}; ^1H NMR (499 MHz, $CDCl_3$) δ 3.57 (s, 4H), 3.49 (t, *J* = 6.4 Hz, 4H), 2.21 (td, *J* = 7.0, 2.6 Hz, 4H), 1.94 (t, *J* = 2.6 Hz, 2H), 1.74 – 1.67 (m, 4H), 1.64 – 1.55 (m, 4H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ 84.4, 70.7, 70.1, 68.3, 28.6, 25.1, 18.2 ppm; HRMS (ICR-ESI) *m/z* calcd for $C_{14}H_{22}O_2Na$ $[M + Na]^+$ 245.1512, found 245.1513.

1,2,3-Tris-*O*-(5-hexynyl)glycerol (12). A solution of glycerol (**11**, 128 mg, 1.38 mmol) in dry DMF (10 mL) under argon was stirred in an ice bath. NaH (298 mg, 12.42 mmol) was added, and the suspension allowed to attain room temperature. After 3 h, the reaction mixture was cooled in an ice bath, 6-iodo-1-hexyne (2.5 g, 12.42 mmol) was added dropwise, and the mixture allowed to attain room temperature. After 48 h, the mixture was cooled in an ice bath, the reaction quenched with NH_4Cl (10 mL), and the mixture extracted with ether (3 × 20 mL). The combined ether extracts were washed with water (3 × 20 mL), brine (15 mL), dried over anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*, leaving an oily brown residue. Gravity column chromatography on silica gel 60 using 10% EtOAc/hexanes as the eluent afforded 114 mg (0.34 mmol, 25%) of **12** as a viscous pale yellow liquid, R_f 0.47 (20% EtOAc/hexanes, iodine vapor and PMA stains). IR (neat) 3296, 2937, 2115, 1115, 624 cm^{-1}; ^1H NMR (499 MHz, $CDCl_3$) δ 3.59 (t, *J* = 6.2 Hz, 2H), 3.57 – 3.52 (m, 1H), 3.52 – 3.43 (m, 8H), 2.26 – 2.17 (m, 6H), 1.96 – 1.91 (m, 3H), 1.73 – 1.55 (m, 12H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ 84.44, 84.35, 77.92, 70.89, 70.83, 69.84, 68.36, 28.67, 25.20, 25.16, 18.22, 18.20 ppm; HRMS (ICR-ESI) *m/z* calcd for $C_{21}H_{32}O_3Na$ $[M + Na]^+$ 355.2244, found 355.2244.

1,2,5,6-*O*-Tetrakis(5-hexynyl)-3,4-*O*-dimethyl-D-mannitol (14). A solution of 3,4-di-*O*-methyl-D-mannitol[29] (**13**, 100 mg, 0.48 mmol) in dry DMF (10 mL) under argon was stirred in an ice bath. NaH (138 mg, 5.78 mmol) was added, and the suspension allowed to attain room temperature. After 3 h, the reaction mixture was cooled in an ice bath, 6-iodo-1-hexyne (1.2 g, 5.78 mmol) was added dropwise, and the mixture allowed to attain room temperature. After 48 h, the mixture was cooled in an ice bath, the reaction quenched with NH_4Cl (10 mL), and the mixture extracted with ether (3 × 20 mL). The combined ether extracts were washed with water (3 × 20 mL), brine (15 mL), dried over anhydrous $MgSO_4$, filtered and concentrated *in vacuo* to leave an oily brown residue. Gravity column chromatography on silica gel 60 using 50% EtOAc/hexanes as the eluent afforded 102 mg (0.19 mmol, 40%) of **14** as a viscous pale yellow liquid, R_f 0.57 (50% EtOAc/hexanes, iodine vapor and PMA stains). IR (neat) 3296, 2932, 2116, 1113, 628 cm^{-1}; ^1H NMR (499 MHz, $CDCl_3$) δ 3.78 (dd, *J* = 10.7, 1.7 Hz, 2H), 3.72 (dt, *J* = 9.1, 6.1 Hz, 2H), 3.61 – 3.41 (m, 18H), 2.27 – 2.21 (m, 8H), 1.98 – 1.95 (m, 4H), 1.78 – 1.56 (m, 16H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) 84.35, 84.33, 79.57, 78.61, 70.77, 69.27, 68.97, 68.37, 60.42, 29.23, 28.75, 25.32, 25.30, 18.23 ppm; HRMS (ICR-ESI) *m/z* calcd for $C_{32}H_{50}O_6Na$ $[M + Na]^+$ 553.3500, found 553.3503.

(*S*)-2-(2-Azidoacetamido)-3-hydroxypropanamide (15). To a suspension of serinamide hydrochloride (400 mg, 2.84 mmol) in dry DMF (10 mL) under argon were added triethylamine (478 µL, 347 mg, 3.4 mmol) and *N*-hydroxysuccinimidyl 2-azidoacetate[30] (1.90 mmol). The mixture was stirred at room temperature overnight. DMF was removed *in vacuo* and the resulting oily residue subjected to gravity column chromatography on silica gel 60 using a gradient of DCM to 10% MeOH/DCM as the eluent, affording 235 mg (1.25 mmol, 66%) of **15** as a white, waxy solid, R_f 0.22 (10% MeOH/DCM, PMA stain). IR (KBr, thin film) 3342 (br), 2957, 2113, 1663 cm^{-1}; ^1H NMR (499 MHz, CD_3OD) δ 4.45 (t, *J* = 5.0 Hz, 1H), 3.99 (d, *J* = 2.3 Hz, 2H), 3.83 (dd, *J* = 11.2, 5.3 Hz, 1H), 3.79 (dd, *J* = 11.2, 4.9 Hz, 1H) ppm; ^{13}C NMR (126 MHz, CD_3OD) δ 174.6, 170.3, 63.0, 56.5, 52.9 ppm; HRMS (ICR-ESI) *m/z* calcd for $C_5H_9N_5O_3Na$ $[M + Na]^+$ 210.0603, found 210.0598.

Solid phase peptide synthesis of MSH4 azide 16. In a syringe (polypropylene reaction tube equipped with a polypropylene frit) rink amide resin (1.0 g, 0.7 mmol, loading capacity 0.71 mmol/g) was allowed to swell in THF for 1 hr. THF was removed and the resin was shaken with 20% piperidine in DMF (15 mL) for 2 min. The solution was removed and the resin shaken for 18 min with a fresh portion of 20% piperidine solution in DMF (15 mL). The resin was then washed with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), 0.5M hydroxybenzotriazole (HOBt) in DMF (15 mL), 0.5 M HOBt in DMF containing a drop of bromophenol blue (15 mL), DMF (2 × 15 mL), and DCM (15 mL). A solution of Fmoc-Trp(Boc)-OH (1.05 g, 2.04 mmol), 6-chloro-1-hydroxybenzotriazole (Cl-HOBt, 345 mg, 2.04 mmol), and diisopropyl carbodiimide (DIC, 512 mg, 4.08 mmol) in DMF (15 mL) was allowed to react for 2 min before being added to the resin, which was shaken with this solution for 2 h. Completion of the coupling was confirmed by the Kaiser test.[40] The same cycle of procedures was repeated for coupling of the other amino acids in the sequence, Fmoc-Arg(pbf)-OH (1.32 g, 2.04 mmol), Fmoc-D-Phe-OH (0.79 g, 2.04 mmol), Fmoc-His(Trt)-OH (2.52 g, 2.04 mmol), and finally for of *N*-terminal attachment of 2-azido acetic acid[27] (0.26 g, 2.04 mmol). Capping of free NH_2 groups was unnecessary as the Kaiser test indicated completion of all the coupling reactions. Cleavage from the resin and side chain deprotection was achieved using a 91:3:3:3 mixture of TFA, triisopropylsilane, thioanisole, and water (10 mL). The cleavage cocktail and resin were shaken overnight, the resulting solution separated, the resin washed with another portion of TFA (5 mL), the TFA phases combined, and volatiles evaporated *in vacuo*. The residue was triturated with cold ether and the crude products separated by centrifugation at 6000 rpm for 3 min. Purifications of the product tetrapeptides were carried out by reverse phase chromatography using a 19×250 mm X-Bridge Preparative C_{18} column. The mobile phase used was 10-90% MeCN and water containing 0.1% TFA within 45 min; the flow rate was 10 mL/min and the UV detector system operated at 230 nm and 280 nm. Lyophilization gave **16** as a white powder; 320 mg (0.44 mmol, 65%); IR (KBr, thin film) 3329 (br), 2929, 2114, 1664, 1202, 1134 cm^{-1}; MS (ESI) calcd. for $C_{34}H_{44}N_{14}O_5$ $(M+2H)^{2+}$ 364.1804, obsd. 364.1808; Analytical HPLC t_R 11.37 min.

CuAAC Reactions

Trivalent serinamide construct 17. A mixture of **12** (10 mg, 0.018 mmol), **15** (25 mg, 0.135 mmol), (tris[(1-benzyl-1*H*-1,2,3-triazol-4-

yl)methyl]amine (TBTA, 19 mg, 0.036 mmol), tetrakis(acetonitrile)copper(I) hexafluorophosphate (TACP, 13 mg, 0.036 mmol), and sodium ascorbate (7 mg, 0.036 mol) in degassed DMF (600 μL) in a microwave reaction tube was purged with argon, sealed, and irradiated in a Biotage microwave reactor to maintain a temperature of 100 °C for 2 h. The mixture was diluted with water (10 mL), washed with DCM (4 × 20 mL), and the resulting aqueous solution lyophilized to give a brownish solid (50 mg). Gravity column chromatography on silica gel 60 using DCM:MeOH:NH_4OH (5:2:0.5) as the eluent gave a light brown sticky oily product. The residue was dissolved in water (10 mL) and lyophilized to afford 20 mg (0.02 mmol, 74%) of **17** as a light brown solid, R_f 0.14 (DCM:MeOH:NH_4OH 5:2:0.5, PMA stain). ^{1}H NMR (499 MHz, D_2O) δ 7.67 (s, 2H), 7.65 (s, 1H), 5.18 – 5.15 (two overlapped s, 6H), 4.35 (t, *J* = 5.0 Hz, 3H), 3.82 – 3.74 (m, 6H), 3.58 – 3.53 (m, 1H), 3.50 (t, *J* = 6.4 Hz, 2H), 3.47 – 3.34 (m, 8H), 3.22 – 3.18 (m, 2H), 2.63 – 2.53 (m, 6H), 1.61 – 1.52 (m, 6H), 1.51 – 1.42 (m, 6H) ppm; HRMS (ICR-ESI) *m/z* calcd for $C_{36}H_{60}N_{15}O_{12}$ $[M + H]^+$ 894.4540, found 894.4568.

Tetravalent serinamide construct 18. A mixture of **14** (10 mg, 0.03 mmol), **15** (21 mg, 0.11 mmol), TBTA (15 mg, 0.028 mmol), TACP (10.7 mg, 0.028 mmol), and sodium ascorbate (5.5 mg, 0.028 mol) in degassed DMF (600 μL) in a microwave reaction tube was purged with argon, sealed, and irradiated in a Biotage microwave reactor to maintain a temperature of 100 °C for 2 h. The mixture was diluted with water (10 mL) and washed with DCM (4 × 20 mL). Brown particles were removed by filtration through a plug of cotton and the resulting aqueous solution lyophilized to give a brownish solid (58 mg). Gravity column chromatography on silica gel 60 using DCM:MeOH:NH4OH (5:2:0.5) as the eluent afforded 17 mg (0.013 mmol, 74%) of **18** as a light brown solid, R_f 0.11 (DCM:MeOH:NH_4OH 5:2:0.5, PMA stain). ^{1}H NMR (499 MHz, D_2O) δ 7.76 (s, 2H), 7.75 (s, 2H), 5.233 (s, 4H), 5.226 (s, 4H), 4.46 – 4.41 (m, 4H), 3.89 – 3.77 (m, 10H), 3.73 – 3.63 (m, 2H), 3.61 – 3.43 (m, 13H), 3.41 (s, 6H), 3.31 – 3.27 (m, 8H), 2.71 – 2.59 (m, 8H), 1.75 – 1.51 (m, 16H) ppm; HRMS (ICR-ESI) *m/z* calcd for $C_{52}H_{87}N_{20}O_{18}$ $[M + H]^+$ 1279.6502, found 1279.6503.

Monoserinamide derivative 19. A mixture of **15** (13.7 mg, 0.073 mmol), **10** (97 mg, 0.438 mmol), TBTA (5.6 mg, 0.029 mmol), TACP (10 mg, 0.029 mmol), and sodium ascorbate (5.7 mg, 0.029 mol) in degassed DMF (600 μL) in a microwave reaction tube was purged with argon, sealed, and irradiated in a Biotage microwave reactor to maintain a temperature of 100 °C for 1 h. The solution turned from pale green to yellow. DMF was removed *in vacuo* and the residue subjected to gravity column chromatography on silica gel 60 eluted with a gradient of 5% MeOH in DCM to 10% MeOH in DCM, affording 20 mg (0.049 mmol, 67%) of **19** as a white waxy solid, R_f 0.18 (MeOH:DCM 1:9; PMA stain). IR (KBr, thin film) 3281, 2935, 2114, 1653, 1119 cm^{-1}; ^{1}H NMR (499 MHz, MeOD) δ 7.80 (s, 1H), 5.21 (s, 2H), 4.44 (t, *J* = 5.1 Hz, 1H), 3.86 – 3.78 (m, 2H), 3.57 (s, 4H), 3.50 (dt, *J* = 10.8, 6.4 Hz, 4H), 2.74 (t, *J* = 7.3 Hz, 2H), 2.22 – 2.17 (m, 3H), 1.81 – 1.52 (m, 8H) ppm; ^{13}C NMR (126 MHz, MeOD) δ 174.5, 168.0, 124.9, 84.9, 71.9, 71.7, 71.2, 69.6, 65.2, 63.0, 62.3, 56.7, 53.0, 30.1, 29.7, 27.1, 26.4, 26.0, 18.8 ppm; HRMS (ICR-ESI) *m/z* calcd for $C_{19}H_{32}N_5O_5$ $[M + H]^+$ 410.2398, found 410.2403.

Monovalent MSH4 derivative 20. A mixture of **19** (10.8 mg, 0.026 mmol), **16** (29 mg, 0.039 mmol), TACP (3.9 mg, 0.01 mmol), TBTA (5.6 mg, 0.01 mmol), and sodium ascorbate (2 mg, 0.01 mol) in degassed DMF (600 μL) in a microwave reaction tube was purged with argon, sealed, and irradiated in a Biotage microwave reactor to maintain a temperature of 100 °C for 2 h. The solution turned from pale green to red. The reaction mixture was diluted with water (20 mL), washed with a solution of dithizone[41] (3 mg/150 mL) in $CHCl_3$ (3 × 20 mL) and $CHCl_3$ (3 × 20 mL), volatiles were removed, and the resulting aqueous solution lyophilized to give a light pink powder (53 mg). Purification was carried out by reverse phase HPLC on a 19×256 mm X-Bridge Preparative C18 column. The mobile phase used was 10-90% MeCN and water containing 0.1% TFA within 45 min; the flow rate was 10 mL/min and the UV detector system operated at 230 nm and 280 nm. Lyophilization gave 13 mg (0.011 mmol, 43%) of **20** as a powder, t_R 13.23 min; HRMS (MALDI) calcd. for $C_{53}H_{74}N_{19}O_{10}$ $(M+H)^+$ 1136.5860, obsd. 1136.5865; Analytical HPLC t_R 12.02 min.

Divalent MSH4 construct 21. A mixture of **10** (4.4 mg, 0.02 mmol), **16** (44 mg, 0.06 mmol), TBTA (8.5 mg, 0.016 mmol), TACP (5.9 mg, 0.016 mmol), and sodium ascorbate (3.1 mg, 0.016 mol) in degassed DMF (600 μL) in a microwave reaction tube was purged with argon, sealed, and irradiated in a Biotage microwave reactor to maintain a temperature of 100 °C for 2 h. The pale solution turned brownish red. The reaction mixture was diluted with water (20 mL), washed with a solution of dithizone[41] (3 mg/150 mL) in $CHCl_3$ (3 × 20 mL) and $CHCl_3$ (3 × 20 mL), volatiles were removed, and the resulting aqueous solution lyophilized to give a light pink powder (58 mg). Purification was carried out by reverse phase HPLC on a 19×256 mm X-Bridge Preparative C18 column. The mobile phase used was 10-90% MeCN and water containing 0.1% TFA within 45 min; the flow rate was 10 mL/min and the UV detector system operated at 230 nm and 280 nm. Lyophilization gave 12.3 mg (0.010 mmol, 37%) of **21** as a powder with a tinge of brown color, t_R 15.58 min; HRMS (MALDI) calcd. for $C_{82}H_{107}N_{28}O_{12}$ $(M+H)^+$ 1675.8617, obsd.1675.8633; Analytical HPLC t_R 12.85 min.

Trivalent MSH4 construct 22. A mixture of **12** (4 mg, 0.012 mmol), **16** (40 mg, 0.054 mmol), TBTA (7.6 mg, 0.014 mmol), TACP (5.3 mg, 0.014 mmol), and sodium ascorbate (2.8 mg, 0.014 mol) in degassed DMF (600 μL) in a microwave reaction tube was purged with argon, sealed, and irradiated in a Biotage microwave reactor to maintain a temperature of 100 °C for 2 h. The pale colored solution turned dark red. The reaction mixture was diluted with water (20 mL), washed with a solution of dithizone[41] (3 mg/150 mL) in $CHCl_3$ (3 × 20 mL) and $CHCl_3$ (3 × 20 mL), volatiles removed and the resulting aqueous solution lyophilized to give a light pink powder (50 mg). Purification was carried out by reverse phase chromatography with a 19×256 mm X-Bridge Preparative C18 column. The mobile phase used was 10-90% MeCN and water containing 0.1% TFA within 45 min; the flow rate was 10 mL/min and the UV detector system operated at 230 nm and 280 nm. Lyophilization gave 25 mg (0.010 mmol, 83%) of **22** as a powder with a tinge of brown color, t_R 16.53 min; HRMS (MALDI) calcd. for $C_{123}H_{159}N_{42}O_{18}$ $(M+H)^+$ 2512.2812, obsd. 2512.2838; Analytical HPLC t_R 12.86 min.

Tetravalent MSH4 construct 23. A mixture of **14** (5 mg, 0.009 mmol), **16** (41 mg, 0.057 mmol), TBTA (7.6 mg, 0.014 mmol), TACP (5.3 mg, 0.014 mmol), and sodium ascorbate (2.8 mg, 0.014 mol) in degassed DMF (600 μL) in a microwave reaction tube was purged with argon, sealed, and irradiated in a Biotage microwave reactor to maintain a temperature of 100 °C for 2 h. The pale colored solution turned dark red. The reaction mixture was diluted with water (20 mL), washed with a solution of dithizone[41] (3 mg/150 mL) in $CHCl_3$ (3 × 20 mL) and $CHCl_3$ (3 × 20 mL), volatiles were removed, and the resulting aqueous solution lyophilized to give a

light pink powder (50 mg). Purification was carried out by reverse phase HPLC on a 19×256 mm X-Bridge Preparative C18 column. The mobile phase used was 10-90% MeCN and water containing 0.1% TFA within 45 min; the flow rate was 10 mL/min and the UV detector system operated at 230 nm and 280 nm. Lyophilization produced 22 mg (0.006 mmol, 71%) of **23** as a powder with tinge of brown color, t_R 16.68 min; HRMS (MALDI) calcd. for $C_{168}H_{219}N_{56}O_{26}$ $(M+H)^+$ 3436.7530, obsd. 3436.7479; Analytical HPLC t_R 12.96 min.

Biological Studies

Preparation of solutions. Unless otherwise noted, solutions were stored at 4 °C. Selective growth media, basic buffer, and binding buffer were prepared as previously described.[28] Compounds **17**, **18**, and **20**-**23** were dissolved in water. Solids were weighed out to make approximately 2 mM solutions (by total MSH4) in 1 mL of water, and 100 μL of each of these solutions was diluted with 900 μL of DMSO. More accurate concentrations were then determined based on the absorbance at 280 nm using the equation y = 4.611 x + 0.045 (generated from a standard curve for an MSH4 construct reported previously)[17] where y is the absorbance and x is the concentration. Solutions of control compounds **17** (1 mM) and **18** (0.75 mM) were prepared in volumetric flasks by dissolving the weighed solid in water and topping up to the mark (1 mL). Dilutions of NDP-α-MSH in water were made from a previously prepared 2 mM stock solution. Stock solutions of probes **3** and **4** were stored at -80 °C. Working solutions of these probes (2 μM in water) were stored at 4 °C. Final concentrations of compounds used in bioassays are given in Table S1 in the Electronic Supplementary Information.†

Cell culture. Details of cell culture were previously described.[28]

Saturation binding assays for probe 4. Binding buffer and basic buffer were warmed in a water bath at 37 °C prior to use. On the day of an experiment, nine solutions of probe **4** ranging in concentration from 2 to 48 nM were made up in binding buffer. For generation of the total binding curve, an aliquot (550 μL) from each concentration of the probe was mixed with binding buffer (550 μL). For generation of the non-specific binding curve, an aliquot (550 μL) from each concentration of the probe was mixed with an aliquot (550 μL) of a 2 μM solution of NDP-α-MSH in binding buffer. Three plates containing cells were removed from the incubator and selective growth media was carefully removed by aspiration using a weak vacuum. The solutions of probe **4** plus binding buffer (1 mL) were added to nine wells and solutions of probe **4** plus NDP-α-MSH in binding buffer (1 mL) were added to another nine wells. The plates were placed in an incubator at 37 °C and 5% CO_2 for 1h. The plates were then removed from the incubator, media was carefully removed by aspiration, and basic buffer (600 μL) was added to each well. Cells were gently scraped from each well of the plate using a cell scraper (18 cm, GeneMate). One scraper was used for the nine wells containing the solutions for determination of total binding, working from low concentration to high concentration of the probe. A new scraper was used for the nine wells containing the solutions for determination of non-specific binding. The cells from each well were transferred to separate Eppendorf tubes using a micropipette. Additional basic buffer (600 μL) was used to rinse each well to collect and transfer the remaining cells. The tubes were centrifuged at 3000 rpm for 3 min in a micro-centrifuge (Fisher Scientific, model 59A). This was the optimum speed to spin down the cells while not rupturing them. The supernatant solution was aspirated, the cells were re-suspended in basic buffer (1.2 mL), and the tubes kept at 37 °C for 5 min in the incubator. Washing was repeated three times. Finally, the supernatant solution was removed by aspiration, enhancement solution (Delfia, PerkinElmer 1244-105, 800 μL/tube) was added to the pelleted cells, the contents mixed using a vortex mixer, and the tubes kept at 37 °C in a water bath for 1 h. Cell debris was spun down at 5000 rpm in the micro-centrifuge. Aliquots (100 μL) of the supernatants were added in quadruplicate to a Perkin-Elmer 96-well plate, the top four rows for total binding and the bottom four rows for non-specific binding at the same probe concentrations. Fluorescence 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). Saturation binding data were analyzed using nonlinear regression analysis and fitted to classic one site total binding and nonspecific binding equations using GraphPad Prism software. A K_d value of 12 ± 1 nM was determined for probe **4** by averaging the results of four determinations.

Competitive binding assays. Binding buffer and basic buffer were warmed in a water bath at 37 °C prior to use. Solutions of probes **3** (40 nM) and **4** (20 nM) were prepared in binding buffer. Serial dilutions of compounds **17**, **18**, and **20**-**23** were prepared in binding buffer (see the ESI†). Aliquots (550 μL) of a diluted probe solution were mixed with aliquots (550 μL) of the solutions from each concentration of a compound to be tested. Two plates containing cells were removed from the incubator and the selective growth media aspirated as described above. The test solutions (1 mL) were added to the 12 wells and the plates incubated for 1 h at 37 °C and 5% CO_2. The plates were removed from the incubator, binding buffer was aspirated, and basic buffer (600 μL) was added to each well. Cells were gently scraped from each well of the plate using a cell scraper. One scraper was used for the 12 wells containing test solutions, working from high concentration to the low concentration of the tested compound. The cells in each well were transferred to separate Eppendorf tubes using a micropipette. Additional basic buffer (600 μL) was used to rinse each well to collect and transfer the remaining cells. The tubes were centrifuged at 3000 rpm for 3 min to spin down the cells without rupturing them. The supernatant solution was aspirated, replaced with basic buffer (1.2 mL), the cells re-suspended, and the tubes kept at 37 °C for 5 min in the incubator. Washing was repeated three times. Finally, the supernatant solution was aspirated, enhancement solution (Delfia, PerkinElmer 1244-105, 800 μL/tube) was added, the contents mixed using a vortex mixer, and the tubes kept at 37 °C for 1 h in a water bath. Cell debris was spun down at 5000 rpm. Aliquots (100 μL) of the supernatants were added in quadruplicate to a Perkin-Elmer 96-well plate, and fluorescence 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). Competitive binding data were analyzed using nonlinear regression analysis using GraphPad Prism software. The K_i values given in Table 1 are the average of four determinations.

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 linear core scaffolds. Pertaining to MC4R, the results support the hypothesis that ligand spacing for multivalent binding to dimeric receptors is on the short side of the previously reported range, 24±5 Å, and that binding as a trimer requires a longer reach.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Copies of NMR spectra of compounds **10**, **12**, **14**, **15**, and **17-19**, analytical HPLC traces from analyses of compounds **16** and **20-23**, a description of the binding equations used in data analysis, and details of the molecular dynamics analysis of compounds **21-23**. See DOI:

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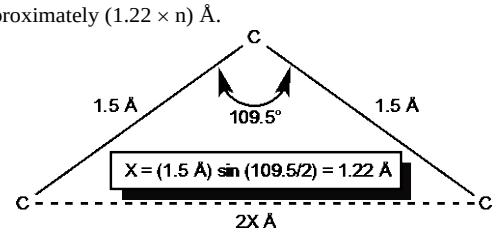

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