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A Solanesol-derived Scaffold for Multimerization of Bioactive Peptides

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Abstract

A flexible molecular scaffold bearing varying numbers of terminal alkyne groups was synthesized in five steps from solanesol. R(CO)-MSH(4)-NH_2 ligands, which have a relatively low affinity for binding at the human melanocortin 4 receptor (hMC4R), were prepared by solid phase synthesis and were N-terminally acylated using 6-azidohexanoic acid. Multiple copies of the azide $N_3(CH_2)_5$(CO)-MSH(4)-NH_2 were attached to the alkyne-bearing, solanesol-derived molecular scaffold via the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. Control studies showed that the binding affinity of the triazole-containing ligand, $CH_3(CH_2)_3(C_2N_3)(CH_2)_5$(CO)-MSH(4)-$NH_2$, was not significantly diminished relative to the corresponding parental ligand, CH_3(CO)-MSH(4)-NH_2. In a competitive binding assay using a Eu-labeled probe based on the superpotent ligand NDP-α-MSH, the monovalent and multivalent constructs appear to bind to hMC4R as monovalent species. In a similar assay using a Eu-labeled probe based on MSH(4), modest increases in binding potency with increased MSH(4) content per scaffold were observed.

Introduction

Early detection and diagnosis of many human cancers would be facilitated by the availability of reagents that could seek out and selectively bind to cancer cells and report their existence and location by non-invasive molecular imaging.[1] One strategy for development of such reagents involves linking imaging agents to molecules that contain multiple copies of individual binding units, or ligands, targeted to cell surface receptors that are displayed by cancer cells.[2] Such multivalent constructs should display enhanced affinity and selectivity for cancer cells based on cooperative binding.[1–3]

The foundation for ligand-guided multivalent attachment of reporter groups to cell surface receptors was laid in part by studies that employed a poly(vinyl alcohol) scaffold (PVA) decorated with fluorescein and R(CO)-NDP-α-MSH-NH_2 ligands.[4] Such constructs bound specifically and irreversibly to mouse and human melanoma cells that expressed melanocortin receptors. The PVA-based multimer system was not extended to other peptide hormone/receptor systems at that time due to problems with the attachment chemistry and solubility of the multimeric constructs. Recent advances in polymer-supported multivalent binding[5] prompted a reexamination of this earlier approach to ligand multimerization with

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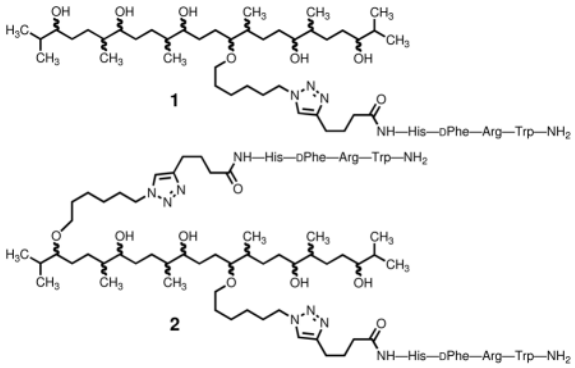
Supporting Information Available: A general experimental section, a list of nonstandard abbreviations, copies of the ^1H and ^{13}C NMR spectra of compounds **5**, **6**, **10**, **11**, and **12**, the APT of **7**, MALDI-TOF spectra of multivalent construct mixtures **15** and **16a–e**, and a description of UV studies. This material is available free of charge via the Internet at http://pubs.acs.org.

the intent of using more efficient attachment chemistry and developing more soluble scaffolds.

With regard to ligand attachment, the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, which generates 1,4-disubstituted triazole products,[6] is a reasonable choice to replace the maleimide electrophile/thiol nucleophile and thiol/disulfide exchange attachment chemistries used previously with PVA.[4c,4d]

With regard to scaffold, PVA derived by incomplete hydrolysis of poly(vinyl acetate) is often more water-soluble at room temperature than is more completely hydrolyzed poly(vinyl acetate).[7] This is presumably due to interruption of hydrogen bonded microcrystalline domains, and suggested that a polymer bearing fewer, stereorandom hydroxyl groups might be less crystalline and more water-soluble. Such a polymer can be prepared from polyisoprene.[8] Since analysis of polymer products is complicated by high molecular weight and polydispersity, we elected to employ more tractable model systems for the establishment of the synthetic methodology necessary to this approach.

Previously we described the preparation from squalene of regiochemically and stereochemically mixed hexol derivatives **1** and **2** which carry one and two copies, respectively, of the linear tetrapeptide amide R(CO)-MSH(4)-NH_2.2d In contrast with CH_3(CO)-NDP-α-MSH-NH_2,[9] CH_3(CO)-MSH(4)-NH_2 ligands have relatively low affinity for binding at the human melanocortin 4 receptor (hMC4R).[10] R(CO)-MSH(4)-NH_2 was selected for this work because synergistic effects are more easily detected for multivalent constructs of low-affinity ligands.[3c] Mixture **2** was found to be about 18 times more potent than mixture **1** in a competitive binding assay against a europium-labeled derivative of MSH(4).[11] The synthetic and biological results with **1** and **2** were encouraging, and work toward a polyisoprene-derived polyol scaffold was begun. However, when solubility problems were encountered during hydroboration and oxidation of polyisoprene, a study of the chemistry of a second model system intermediate in size was deemed appropriate. It was also recognized that study of a larger model system would permit testing of the biological effects of higher levels of ligand multimerization. The synthesis of a scaffold derived from the polyterpene solanesol (**3**), decoration of the scaffold with copies of MSH(4) via CuAAC, and results of competitive binding studies using cells expressing hMC4R are presented herein.



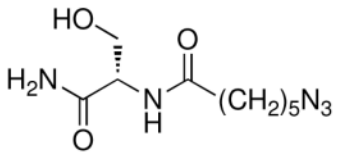
Results

Synthesis

The ready availability of solanesol (**3**) and solanesyl bromide (**4**)[12] made these compounds appealing starting materials for construction of an intermediate-sized polyisoprene scaffold

(Scheme 1). Double alkylation of dimethyl malonate with **4** gave **5** in 72% yield. Reduction of **5** using LAH produced diol **6** in 82% yield. Hydroboration and oxidation of **6** under standard conditions proved to be unsatisfactory since the use of BH_3/THF left an unacceptably high percentage of alkene groups in the product polyol.[13] The use of excess disiamylborane in THF for extended periods gave more satisfactory results.[14] For example, hydroboration of **6** using 1.7 equivalents of disiamyborane per alkene for a period of 72 hours left 4% of the alkene groups intact in the product polyol as determined by mass spectrometry (Figure 1) and titration with bromine (see Experimental Procedures for details). The principal polyols present in the product mixture are formulated as the stereochemically mixed eicosols **7** based on mass spectral and NMR data (Figure 1). While hydroboration of **6** with BH_3 should yield products containing both secondary and tertiary alcohol groups,[8d] disiamylborane was expected to be more regioselective and lead mostly to secondary alcohol groups. The ^{13}C NMR spectrum of **7** (Figure 1) suggests that this expectation was met. Compound 7 was obtained in about 90% yield based on diol **6**. This result was shown to be repeatable in three separate runs. Reaction of **7** with 40 equivalents of sodium hydride and 12 equivalents of 1-bromo-5-hexyne (**8**)[15] in DMF afforded a mixture of polyol/polyalkynes **9**. The product distribution was determined by mass spectrometry (Figure 2). The average number of alkynes was approximately six per molecular scaffold. The product yield based on this level of alkyne incorporation was 70%. This result was shown to be repeatable in three separate runs.

Azide **10** was prepared in 84% yield from serine amide hydrochloride[16] and 6-azidohexanoic acid.[17] Triazole **11** was prepared in 83% yield by reaction of azide **10** with 1-hexyne at room temperature in methanol in the presence of tetrakis(acetonitrile)copper(I) hexafluorophosphate and TBTA.[18] 6-(4-Butyl-1*H*-1,2,3-triazol-1-yl)hexanoic acid (**12**) was prepared in 60% yield by reaction of 6-azidohexanoic acid with 1-hexyne at room temperature in a mixture of water and *t*-butanol in the presence of copper sulfate and sodium ascorbate.



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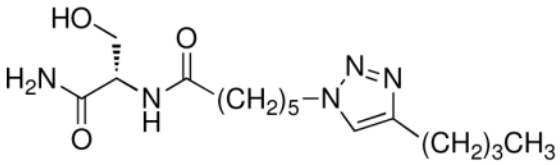

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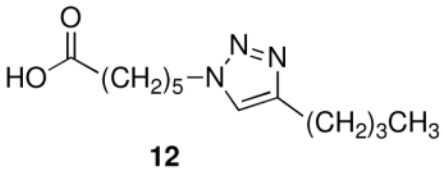

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Solid Phase Synthesis

The low affinity ligand MSH(4)[10] was constructed on Rink amide Tentagel S resin (initial loading 0.62 mmol/g) as depicted in Scheme 2.[19] The product resin retained all side chain protecting groups. 6-Azidohexanoic acid[17] was coupled to the N-terminus of the resin-bound tetrapeptide. Simultaneous side chain deprotection and cleavage of the tetrapeptide from the resin was effected using a mixture of trifluoroacetic acid, 1,2-ethanedithiol, thioanisole, and water (91:3:3:3), producing the desired azide-terminated ligand, $N_3(CH_2)_5$(CO)-MSH(4)-NH_2 (**13**). Triazole-containing ligand $CH_3(CH_2)_3(C_2N_3)(CH_2)_5$(CO)-MSH(4)-$NH_2$ (**14**) was similarly prepared by N-terminal acylation of the resin-bound tetrapeptide with acid **12** in place of 6-azidohexanoic acid. Compounds **13** and **14** were purified by reverse-phase C_{18} preparative HPLC and were characterized by ESI-MS and MALDI-TOF. Details appear in Table 1.

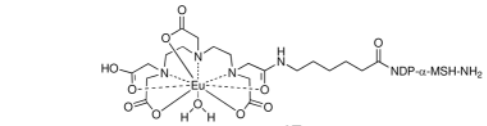
Multimer Assembly

Reaction of the polyol/polyalkyne mixture **9** with an excess of **10** in the presence of copper(I) and TBTA in methanol under microwave irradiation (temperature held constant at 100 °C) gave a mixture of serine amide multivalent constructs **15** (Scheme 3 and Table 2).[20] After dilution with water, copper ions were removed by complexation with dithizone and extraction of the complex with $CHCl_3$.[21] Other organic-soluble molecules (TBTA, excess **10**) were also removed by this extraction. The serine amide-containing product mixture **15** was recovered from the aqueous solution by lyophilization as a white powder and was characterized by MALDI-TOF mass spectrometry (see Supporting Information). The average number of serine amide residues per molecule of **15** was determined to be six.

Reaction of **9** with one or two equivalents of **13** in the presence of copper(I) and TBTA in methanol under microwave irradiation[20] gave mixtures of MSH(4)-bearing multivalent constructs **16a** and **16b**, respectively (Scheme 3 and Table 2). Reaction of **9** with from one, two, or four equivalents of **13**, followed by reaction of the intermediate MSH(4)-containing multivalent constructs with an excess of **10** under these same conditions, gave mixtures of MSH(4)/serine amide multivalent constructs **16c**–**e**. After dilution with water, copper ions were removed by complexation with dithizone and extraction of the complex with $CHCl_3$.21 Other organic-soluble molecules (TBTA, excess **10**) were also removed by this extraction. The resulting aqueous solutions of multivalent constructs were subjected to lyophilization, producing **16a**–**e** as white powders in good to very good yields. These mixtures were characterized by MALDI-TOF and by UV analysis (see Supporting Information).

Binding Studies

Hek293 cells overexpressing hMC4R were used to assess ligand binding[22] using two previously described europium-based competitive binding assays.[11,23] Assay A[23a] employed Eu-DTPA-NDP-α-MSH-NH_2 (**17**) as the labeled probe, and Assay B[11] employed Eu-DTPA-PEGO-MSH(4)-NH_2 (**18**) as the labeled probe. The K_i values for the serine amide-containing control compounds **11** and **15**, for the parental ligand MSH(4), for the triazole-containing MSH(4) analog **14**, and for MSH(4)-containing multivalent constructs **16a**–**e** are given in Table 3.



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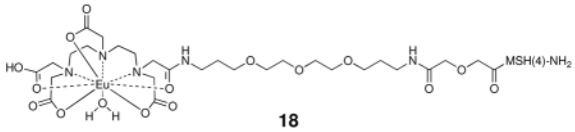

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Discussion

The four-step synthesis of the mixture of polyols **7** starting from solanesol was efficient and highly regioselective. The water-soluble product **7**, which is available in gram quantities, was statistically alkylated using a 12-fold molar excess of 1-bromo-5-hexyne to produce a mixture of polyol/polyalkynes **9** that bore an average of six alkyne residues per scaffold. Presumably, the identity of the alkylating agent and the extent of alkylation might be varied. Microwave-driven CuAAC was used to attach copies of serine amide and MSH(4) derivatives bearing N-terminal azide groups. In principle, this method might be used to attach other ligands, imaging agents, and/or therapeutic agents to scaffold **9**. The constructs so produced were purified from copper and from small molecules, characterized by MALDI-TOF and by UV spectroscopy, and subjected to testing using two previously described competitive binding assays.

In Assay A,[23a] serine amide derivative **11** and the mixture of serine amide multivalent constructs **15** were both ineffective at blocking probe **17** from binding to hMC4R over the range of concentrations tested. The K_i for the monovalent control compound **14** was 1.4 times the value for the parental ligand, indicating that attachment of the triazole-containing "spacer" to the N-terminus of MSH(4) has a modest detrimental effect on ligand binding to hMC4R. In keeping with prior results obtained with the squalene-derived monovalent and divalent MSH(4) constructs **1** and **2**,[11] multimer **16e** was shown to be nearly as potent as MSH(4), but not more so, in Assay A. This result suggests *monovalent binding* of **16e** at *available* hMC4R. Monovalent binding of **16e** would be expected whenever the off-rate of monovalently bound **16e** is faster than the binding of a second "anchoring" ligand arm to hMC4R on the cell surface. The NDP-α-MSH ligand,[9] upon which **17** is based,[23a] is a much stronger binder than MSH(4) and is known to have a slow off-rate (~8 h).[24] Thus, monopolization of neighboring receptors by the superpotent probe **17** would disfavor the binding of a second ligand arm of **16e**. Depletion of receptors at the cell surface by cycling may also have contributed, since internalization results in fewer neighboring surface receptors to which a second ligand arm of **16e** might bind.

Assay B[11] was developed to better balance the off-rates of competed and competing ligands, probe **18** and **16a**–**16e**, respectively. In this assay, serine amide derivative **11** and serine amide multivalent constructs **15** were ineffective at blocking probe **18** from binding to hMC4R over the range of concentrations tested. The K_i for compound **14** was 1.1 times higher than the value for the parental ligand, indicating that attachment of the triazole-containing "spacer" to the N-terminus of MSH(4) had a very modest detrimental effect on ligand binding to hMC4R. The K_i values measured in Assay B differed among the various solanesol-derived constructs **16a**–**16e** (Table 3). Construct **16a** contained, on average, one copy of MSH(4) and five unreacted alkyne groups per scaffold and was 2.2 times *less* potent than the parental ligand, MSH(4). Construct **16b** contained, on average, two copies of MSH(4) and four unreacted alkyne groups per scaffold and was 1.6 times *less* potent than MSH(4). Construct **16c** contained, on average, one copy of MSH(4) and five serinamide

residues per scaffold and was 1.1 times *less* potent than MSH(4). Construct **16d** contained, on average, two copies of MSH(4) and four serinamide residues per scaffold and was 1.5 times *more* potent than MSH(4). Construct **16e** contained, on average, four copies of MSH(4) and two serinamide residues per scaffold and was 3.4 times *more* potent than MSH(4). As demonstrated by comparison of the K_i values for **16a** and **16b** with those for **16c** and **16d**, conversion of the remaining hydrophobic terminal alkyne spacers to the longer, bulkier, but more hydrophilic triazole-serinamide sidechains enhances binding. That this enhancement is not due to specific binding by the serinamide residues is supported by the inactivity of compounds **11** and **15** in Assay B. Enhanced binding for solanesol-derived constructs with higher levels of MSH(4) incorporation was observed, but the small magnitude of the changes in the K_i values was unexpected and inconsistent with prior studies with multivalent MSH(4) constructs.[25] The observed binding enhancements for the series **16c**–**16e** can be entirely attributed to statistics and also suggest *monovalent binding* of **16a**–**e** at *available* hMC4R. It may be that mixtures **16a**–**e** are competent binders as monovalent species, but **16b** and **16d**–**e** are incompetent binders as multivalent species due to improper ligand spacing or presentation.[26] Incompetence could also be due to release of the monovalently-bound multivalent construct from the cell surface before binding of a second ligand arm can occur (*vide supra*). At present, the on rates and off-rates of MSH(4) and related constructs, such as **18** and **16a**–**e**, are unknown. As before, depletion of receptors at the cell surface by cycling should reduce the number of neighboring receptors to which a second ligand arm might bind and limit opportunities for multivalent binding. In support of this point, internalization of probes **17** and **18** contributes significantly to the fluorescence measured in these assays, which may more properly be termed “binding and uptake” assays. In a preliminary study with probe **17** that compared measured fluorescence at 37 °C and 4 °C, as much as 90% of the fluorescence at 37 °C was attributable to internalized probe.[27] Work is presently underway to delineate the details of binding and uptake in the further characterization of the interactions of solanesol-derived multivalent constructs with hMC4R.

Experimental Procedures[28]

Chemical Synthesis (Scheme 1)

Dimethyl 2,2-Bis-[(2E,6E,10E,14E,18E,22E,26E,30E)-3,7,11,15,19,23,27,31,35-nonamethylhexatriaconta-2,6,10,14,18,22,26,30,34-nonaenyl]malonate (5)—To a suspension of NaH (60 mg, 2.5 mmol) in dry THF (10 mL) at 0 °C was added dimethyl malonate (80 μL, 92 mg, 0.7 mmol). The mixture was allowed to warm to RT and was stirred for 3 h. To the mixture was added a solution of solanesyl bromide[12] (**4**, 1.00 g, 1.45 mmol) in dry THF (10 mL) dropwise over 20 min. After 1 h, additional NaH (60 mg, 2.5 mmol) and **4** (200 mg, 0.28 mmol) were added. After 5 h, the reaction was quenched with saturated aqueous NH_4Cl solution and the mixture was extracted with ether (200 mL). The organic extract was washed with water, brine, dried over anhydrous $MgSO_4$, and filtered. Approximately 3 g of $NaHCO_3$ was added to the filtrate to neutralize any HBr formed during evaporation of volatiles using a rotary evaporator. Column chromatography on silica gel 60 eluted with 2% ether/hexanes gave 684 mg (0.50 mmol, 72%) of **5** (R_f 0.45, 5% EtOAc/hexanes) as a viscous pale yellow oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.58 (s, 54H), 1.66 (s, 6H), 1.96–2.09 (m, 64H), 2.59 (d, J = 7.2 Hz, 2H), 3.67 (s, 6H), 4.96 (t, J = 7.2 Hz, 2H), 5.10 (t, J = 6.6 Hz, 16H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 16.0, 17.6, 25.7, 26.7, 30.8, 39.7, 52.2, 57.8, 117.7, 123.9, 124.2, 131.1, 134.8, 135.2, 139.1, 171.8; HRMS calculated for $C_{95}H_{152}O_4Na$ $(M+Na)^+$ 1380.1588, observed 1380.1505.

2,2-Bis-[(2E,6E,10E,14E,18E,22E,26E,30E)-3,7,11,15,19,23,27,31,35-nonamethylhexatriaconta-2,6,10,14,18,22,26,30,34-nonaenyl]propane-1,3-diol (6)—To a suspension of $LiAlH_4$ (12 mg, 0.29 mmol) in dry ether (6 mL) was added diester

5 (200 mg, 0.147 mmol) dropwise at room temperature. The suspension was stirred at RT for 8 h. After completion of the reaction (TLC), the reaction mixture was quenched with saturated aqueous Na_2SO_4 solution and filtered. The filtrate was extracted with diethyl ether and the combined organic extracts were washed with saturated $NaHCO_3$ (15 mL) and brine (15 mL). The ether layer was dried over $MgSO_4$, filtered, and concentrated. Column chromatography on silica gel 60 eluted with 30% ether/hexanes gave 157 mg (0.12 mmol, 82%) of **6** (R_f 0.60, 10% EtOAc/hexanes) as a colorless, viscous oil; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.59 (s, 54H), 1.68 (s, 6H), 1.97–2.07 (m, 68H), 3.56 (s, 4H), 5.10 (t, J = 6 Hz, 18H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 15.9, 17.6, 25.6, 26.6, 29.9, 40.1, 43.4, 68.7, 74.7, 119.4, 124.2, 131.2, 134.8, 137.8. Elemental Analysis. Calculated for $C_{93}H_{152}O_2$ C 85.78, H 11.77; Found C 85.70; H 11.87.

37,37-Bis(hydroxymethyl)-2,6,10,14,18,22,26,30,34,40,44,48,52,56,60,64,68,72-octadecamethyltriheptacontan-3,7,11,15,19,23,27,31,35,39,43,47,51,55,59,63,67,71-octadecaol (7)—[N.B. All reagents and solutions were deoxygenated with argon before use.] In a three-necked flask (500 mL) a solution of borane in THF (1M, 84 mL, 84 mmol) was deoxygenated with argon gas. The flask was immersed in an ice bath, and a solution of 2-methyl-2-butene in THF (2M, 63 mL, 126 mmol), also deoxygenated with argon gas, was added to the borane solution dropwise with stirring at 0 °C. The resulting mixture was maintained at 0 °C for 3 h prior to use. Diol **6** (1.80 g, 1.38 mmol) in THF was then added dropwise at 0 °C. The mixture was stirred at 0 °C for 5 h, kept at 4 °C for 3 days, and then 3N NaOH (26 mL) and 30% H_2O_2 (26 mL) were added. After 36 h at rt, the reaction mixture was diluted with water and extracted with ether. The ether extracts were combined, and the volatile materials, including most of the byproduct 3-methyl-2-butanol, were removed under reduced pressure. The crude product was then washed twice with hexane (20 mL). Alternatively, after dilution with water, the reaction mixture and the aqueous layer were placed in a liquid-liquid extraction apparatus, the aqueous phase saturated with NaCl, and the mixture continuously extracted with ether. After 72 h the ether extracts were separated, volatile components removed under reduced pressure, and the residue washed with hexane. Yield: 1.58 g (0.97 mmol, 70%). The product was characterized by ^{13}C NMR and by mass spectral analysis (see Figure 1). A 10 mM solution of **7** in methanol was titrated with a 10 mM solution of bromine in methanol. It was found that 3.8% of the double bonds remained in the sample of polyol **7** produced in the experiment described above as determined by the amount of bromine decolorized by **7**.[29]

Synthesis of Polyol/Polyalkynes 9—To a suspension of NaH (60% in oil, 183 mg, 4.59 mmol) in dry DMF (10 mL) at room temperature was added polyol **7** (250 mg, 0.153 mmol). After stirring for 20 min, a solution of 1-bromo-5-hexyne[15] (**8**, 298 mg, 1.84 mmol) in dry DMF (2 mL) was added. The mixture was stirred under argon for 3 days, then quenched with aqueous NH_4Cl (20 mL) and extracted with EtOAc (3 × 30 mL). The EtOAc extracts were washed with water (3 × 20 mL) and brine (3 × 15 mL). Volatiles were evaporated and the residue washed with hexanes (2 × 10 mL) to afford a mixture of polyol/polyalkynes **9** (218 mg). Based on mass spectral analysis (see Figure 2) it was found that an average of six alkylations per molecule had occurred. Based on this average, the calculated yield is 69%. This reaction was repeated twice under similar conditions with similar results.

N-(1-Amino-3-hydroxy-1-oxopropan-2-yl)-6-azidohexanamide (10)—To a solution of serine amide hydrochloride[16] (1.10 gm, 7.9 mmol) in DMF (20 mL) at room temperature was added triethylamine (0.80 g, 7.9 mmol). To the resulting white suspension was added 2,5-dioxopyrrolidin-1-yl 6-azidohexanoate[17] (1.88 g, 7.1 mmol) and the reaction mixture was stirred at room temperature. After completion of the reaction (disappearance of 2,5-dioxopyrrolidin-1-yl 6-azidohexanoate as monitored by TLC), volatiles were evaporated

under reduced pressure. Column chromatography on silica gel 60 (50 g) eluted with 10% EtOAc/methanol gave 1.46 g (6.0 mmol, 84%) of **10** (R_f 0.5, 15% methanol/$CHCl_3$) as a white solid, mp 94–96 °C. ^{1}H NMR (500 MHz, $CDCl_3$, CD_3OD) δ 4.36 (t, J = 5 Hz, 1H), 3.79 (dd, 1H), 3.58 (dd, 1H), 3.19 (t, J = 7 Hz, 2H), 2.19 (t, J = 7 Hz, 2H), 1.56 (m, 4H), 1.32 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, CD_3OD) δ 24.8, 26.1, 28.4, 35.7, 51.0, 54.2, 62.2, 173.9, 174.0; HRMS (ESI) calculated for $C_9H_{17}N_5O_3$ $(MH)^+$ 244.1411, observed 244.1412.

N-(1-Amino-3-hydroxy-1-oxopropan-2-yl)-6-(4-butyl-1H-1,2,3-triazol-1-yl)hexanamide (11)—To a solution of azide **10** (100 mg, 0.41 mmole) in methanol (5 mL) were added 1-hexyne (337 mg, 4.11 mmole), TBTA (42 mg, 0.08 mmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (30 mg, 0.08 mmol). The reaction mixture was stirred for 15 h at room temperature. Volatiles were removed under reduced pressure and residue chromatographed on silica gel 60 (10 g) eluted with 10% methanol/$CHCl_3$ to afford **11** (110 mg, 0.34 mmol, 83%) as a solid, mp 124–126 °C. ^{1}H NMR (500 MHz, CD_3OD) δ 0.94 (t, J = 7.5 Hz, 3H), 1.35 (m, 4H), 1.65 (m, 4H), 1.90 (m, 2H), 2.28 (t, J = 7.5 Hz, 2H), 2.67 (t, J = 7.5 Hz, 2H), 3.76 (m, 2H), 4.35 (t, J = 7 Hz, 2H), 4.41 (t, J = 5 Hz, 1H), 7.73(s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 14.1, 23.2, 26.0, 27.0, 30.9, 32.7, 36.5, 51.0, 56.5, 63.1, 123.1, 175.0, 175.8; HRMS (ESI) calculated for $C_{15}H_{28}N_5O_3$ $(MH)^+$ 326.2187, observed 326.2189.

6-(4-Butyl-1H-1,2,3-triazol-1-yl)hexanoic acid (12)—1-Hexyne (5.00 g, 63 mmol), $CuSO_4$ (1.5 g, 6.3 mmol), and sodium ascorbate (2.5 g, 12.6 mmol) were added to a round bottom flask containing a 1:1 mixture of *t*-BuOH and water (20 mL). 6-Azidohexanoic acid[17] (1.00 gm, 6.32 mmol) was added to the solution with stirring. The flask was purged with argon and sealed with a glass stopper to avoid evaporation of 1-hexyne. The reaction mixture was stirred overnight, and then additional 1-hexyne (1.03 g, 12.6 mmol) was added. After 24 h the reaction mixture was diluted with EtOAc (100 mL), washed with 1N HCl (2 × 20 mL) and brine (2 × 20 mL), the organic phase separated, dried over Na_2SO_4, filtered, and concentrated in vacuo. Column chromatography on silica gel 60 (40 g) eluted with 2% methanol/$CHCl_3$ gave the product **12** (910 mg, 3.79 mmol, 60%) as a white solid, mp 41–43 °C. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.87 (t, J = 6.9 Hz, 3H), 1.33 (m, 4H), 1.58 (m, 4H), 1.84 (m, 2H), 2.28 (t, J = 7.2 Hz, 2H) 2.66 (t, J = 7.5 Hz, 2H), 4.27 (t, J = 7.2 Hz, 2H), 7.24 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 13.7, 22.2, 23.9, 25.0, 25.8, 29.9, 31.4, 33.7, 49.9, 120.6, 148.2, 178.0; HRMS (ESI) calculated for $C_{12}H_{22}N_3O_2$ $(MH)^+$ 240.1707, observed 240.1706.

Solid Phase Synthesis (Scheme 2)

In a syringe (polypropylene reaction tube equipped with a polypropylene frit) Rink amide resin (1 gm, 0.68 mmol) was allowed to swell in THF for 1 hr. THF was removed and addition of 20% piperadine in DMF (15 mL) for 2 min led to the deprotection of the Fmoc functionality. DMF was removed, 20% piperadine solution in DMF (15 mL) was again added and the mixture shaken for 18 min. DMF was removed and the resin washed with DMF (3 × 15 mL), CH_2Cl_2 (3 × 15 mL), DMF (3 × 15 mL), 0.5 M HOBt in DMF (1 × 15 mL), 0.5 M HOBt in DMF (1×15 mL) plus a drop of bromophenol blue, DMF (2 × 15 mL), and CH_2Cl_2 (1 × 15 mL), in that order. A solution of the next Fmoc-amino acid (1.05 gm, 2.04 mmol), Cl-HOBt (345 mg, 2.04 mmol), and DIC (512 mg, 4.08 mmol) in DMF (15 mL) was allowed to react for 2 min, then added to the resin and the mixture shaken for 1hr. The resin was then washed with DMF (3 × 15 mL), CH_2Cl_2 (3 × 15 mL), and DMF (3 × 15 mL). Free NH_2 groups were then capped by addition of a 1:1 mixture of acetic anhydride and pyridine (6 mL). After the mixture was shaken for 20 min, the resin was washed with DMF (3 × 15 mL), CH_2Cl_2 (3 × 15 mL), and DMF (3 × 15 mL). The absence of free amine

groups was confirmed by the Kaiser test. The same cycle of procedures was repeated for coupling of the other amino acids in the sequence, and finally for attachment of the N-terminal 6-azidohexanoic acid residue or the N-terminal 6-(4-butyl-1*H*-1,2,3-triazol-1-yl)hexanoic acid residue, thus producing the resin-bound tetrapeptide derivatives related to compounds **13** and **14**, respectively. Cleavage and deprotection were achieved using a 91:3:3:3 mixture of trifluoroacetic acid, triisopropylsilane, thioanisole, and water (10 mL). The mixture of cleavage cocktail and resin was shaken for overnight, the solution was separated from the resin, volatiles were evaporated, the residue triturated with ether, and the crude product separated by centrifugation. Purification of the tetrapeptide amides **13** and **14** was accomplished by reverse phase chromatography using a 19 × 256 mm X-Bridge Preparative C_{18} column. The mobile phase used was 10–90% acetonitrile and water containing 0.1% TFA within 50 min; the flow rate was 15 mL/min and the UV detector system operated at 230 nm. The purity of compounds **13** and **14** was checked by reverse phase HPLC using a 4.6 × 75 mm Symmetry Analytical C_{18} column. The mobile phase was 10–90% acetonitrile and water containing 0.1% TFA within 50 min; the flow rate was 1 mL/min and the UV detector system operated at 230 nm. Compounds **13** and **14** were characterized by MALDI-TOF mass spectrometry. Both the reflectron and linear techniques were used for positive ion detection. The matrix, sinapic acid, and the analyte were dissolved in water:acetonitrile 1:1 containing 0.1% of formic acid and the solutions mixed in a ratio of 100:1. ESI was also used to ionize some of the samples. The samples were dissolved in methanol:water (1:1) at a concentration of ca 50 μM. Standard ESI conditions were applied to detect positively charged ions.

Multimer Assembly (Scheme 3)

Serine Amide Multivalent Constructs 15—A mixture of polyol/polyalkynes **9** (10 mg, 5 mmol), azide **10** (10 mg, 50 mmol), TBTA (3 mg, 5 mmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 5 mmol) in dry methanol (1 mL) was irradiated for 4 hr in a Biotage microwave reactor (100 °C). Additional azide **10** (5 mg, 25 mmol) was then added to the reaction mixture and irradiation was resumed for another 4 h. After the reaction was complete, water (20 mL) was added and the mixture was extracted with $CHCl_3$ containing dithizone (20 mg/L, 3 × 30 mL) to remove copper.[21] The water layer was then washed with $CHCl_3$ (2 × 20 mL) to remove any remaining **10** and TBTA. After lyophilization, 16 mg (88% yield) of **15** was obtained as a white powder. Product **15** was analyzed by MALDI-TOF (see Figure S12 in the Supporting Information).

General Procedure for Reaction of Azide-functionalized MSH(4) Derivative 13 and Azide-functionalized Serine Amide Derivative 10 with Polyol/Polyalkynes 9 to Produce Compounds 16a–e—Mixtures of polyol/polyalkynes **9** (10 or 20 mg, 5 or 10 mmol), MSH(4) azide **13** (variable amount, see Table 2), TBTA (3 mg, 5 mmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 5 mmol) in dry methanol (1 mL/5 mmol of **9**) were irradiated for 6 hr in a Biotage microwave reactor (100 °C). Azide **10** (variable amount, see Table 2) was then added to the reaction mixtures and irradiation was resumed for another 4 h. After the reactions were complete, water (20 mL/5 mmol of **9**) was added and the mixtures were extracted with $CHCl_3$ containing dithizone (20 mg/L, 3 × 30 mL) to remove copper.[21] The water layers were then washed with $CHCl_3$ (2 × 20 mL) to remove any remaining **10** and TBTA. After lyophilization, white powders were obtained (yields are given in Table 2). Products were analyzed by MALDI-TOF and by UV spectroscopy (see Figures S13–17 in the Supporting Information).

Formulation of Solutions

Solutions of multivalent constructs for binding assays were prepared in DMSO (HYBRI-MAX) based on the average incorporation of MSH(4) and/or serinamide as determined by

mass spectrometric analysis. Concentrations of MSH(4) ligand in solution were confirmed by measurement of the UV absorbance at 280 nm using a standard calibration plot (see Supporting Information). In all cases the concentrations calculated from the mass spectral analysis and the weight of the compound used agreed within 5% with the measured UV absorption.

Binding Assays

Quantitative receptor-binding assays were carried out following a previously described method.[11] Hek293 cells overexpressing hMC4R were used to assess ligand binding. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Cells were seeded in a 96-well plate at a density of 15,000 cells per well and were allowed to reach 80–90% confluence. On the day of the experiment, media was aspirated from all wells, and 50 μL of the compounds to be tested (dilutions ranging from 2×10^{-4} to 1×10^{-11} M) and 50 μL of Eu-labeled ligand (Assay A, 10 nM probe **17**, Assay B, 0.5 μM probe **18**) were added to each well. Ligands were diluted in binding media (DMEM, 1 mM 1,10-phenanthroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, 0.3% BSA) and each concentration was tested in quadruplicate. Cells were incubated in the presence of unlabeled and labeled ligands at 37 °C and 5% CO_2 for 1 hour. The cells were washed with wash buffer (50 mM Tris-HCl, 0.2% BSA, 30 mM NaCl), enhancement solution (PerkinElmer 1244-105) was added (100 μl/well), and fluorescence was measured on a Wallac VICTOR^3 instrument using standard Eu TRF measurement conditions (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm). Competitive binding data were analyzed with GraphPad Prism software using nonlinear regression analysis and fitted to a classic one site binding competition equation. Each EC_{50} value was generated from individual competitive binding assays and converted to a K_i value using the equation $K_i = EC_{50}/(1 + ([\text{ligand}]/K_D))$ where [ligand] refers to the concentration of the probe used as the labeled competed ligand. For probe **17**, [ligand] = 10 nM and K_D = 18.8 nM. For probe **18**, [ligand] = 0.5 μM and K_D = 9.1 μM. Results are given in Table 3. The value given represents the average of n independent competition binding experiments, with the error bars indicating standard error of the mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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14. Use of triisopinocampheyldiborane as described in reference [8b] gave mixtures of polyols and isopinocampheol that were difficult to separate. The byproduct from use of disiamylborane, 3-methyl-2-butanol, was more easily removed from the polyol product under vacuum.
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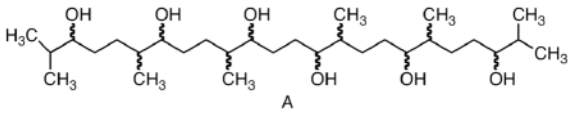
28. The General Experimental section appears in the Supporting Information.

29. This method was validated by titration of 0.10 M solutions of citronellal and geraniol in methanol with a 0.10 M solution of bromine in methanol. A negative control experiment using hexol **A** (see Ref. [2d] for the preparation of **A**) gave a persistent bromine color upon addition of the first drop of bromine solution.



A

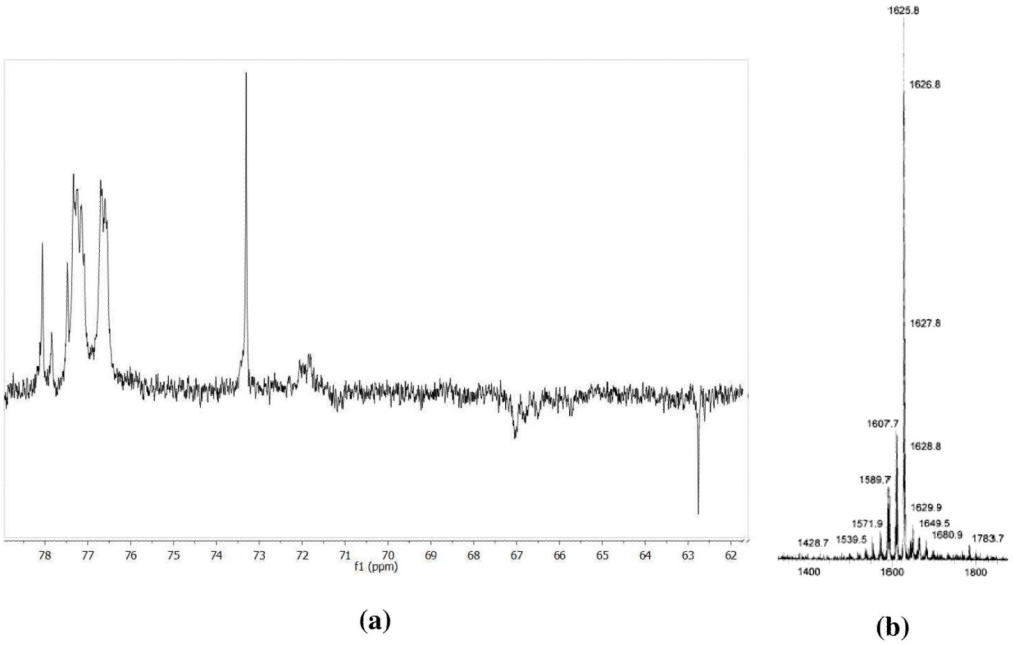


Figure 1.

(a) A portion of the DEPT 135 ^{13}C NMR spectrum of **7** in CD_3OD (see Ref [8d]). The lines from 76–79 ppm are due to methine carbons bearing an OH group. The line at 62.8 ppm is due to the two methylene carbons that bear OH groups. The relatively sharp line at 73.3 ppm is due to residual byproduct 3-methyl-2-butanol in this sample. **(b)** MS showing the distribution of products in the mixture of polyols **7**. Peaks near 1572, 1590, 1608, and 1626 represent solanesol-derived heptadecaols, octadecadols, nonadecaols, and eicosaols, respectively.

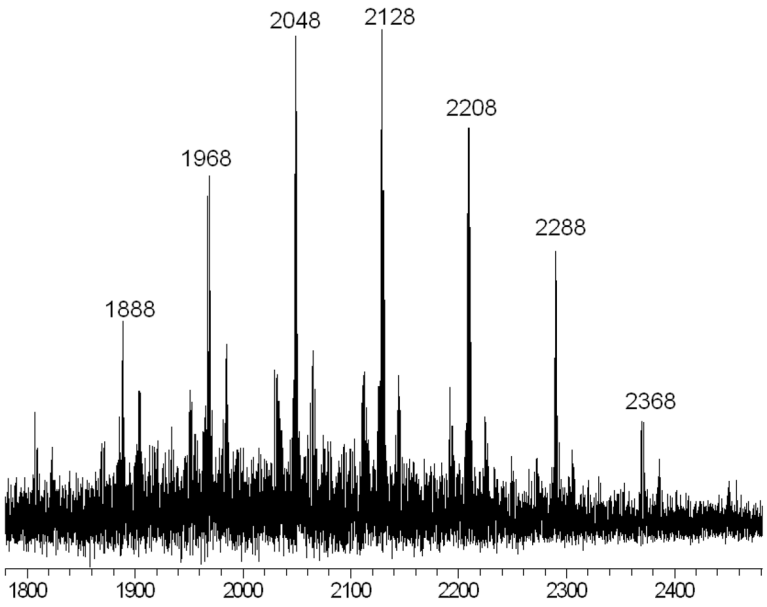
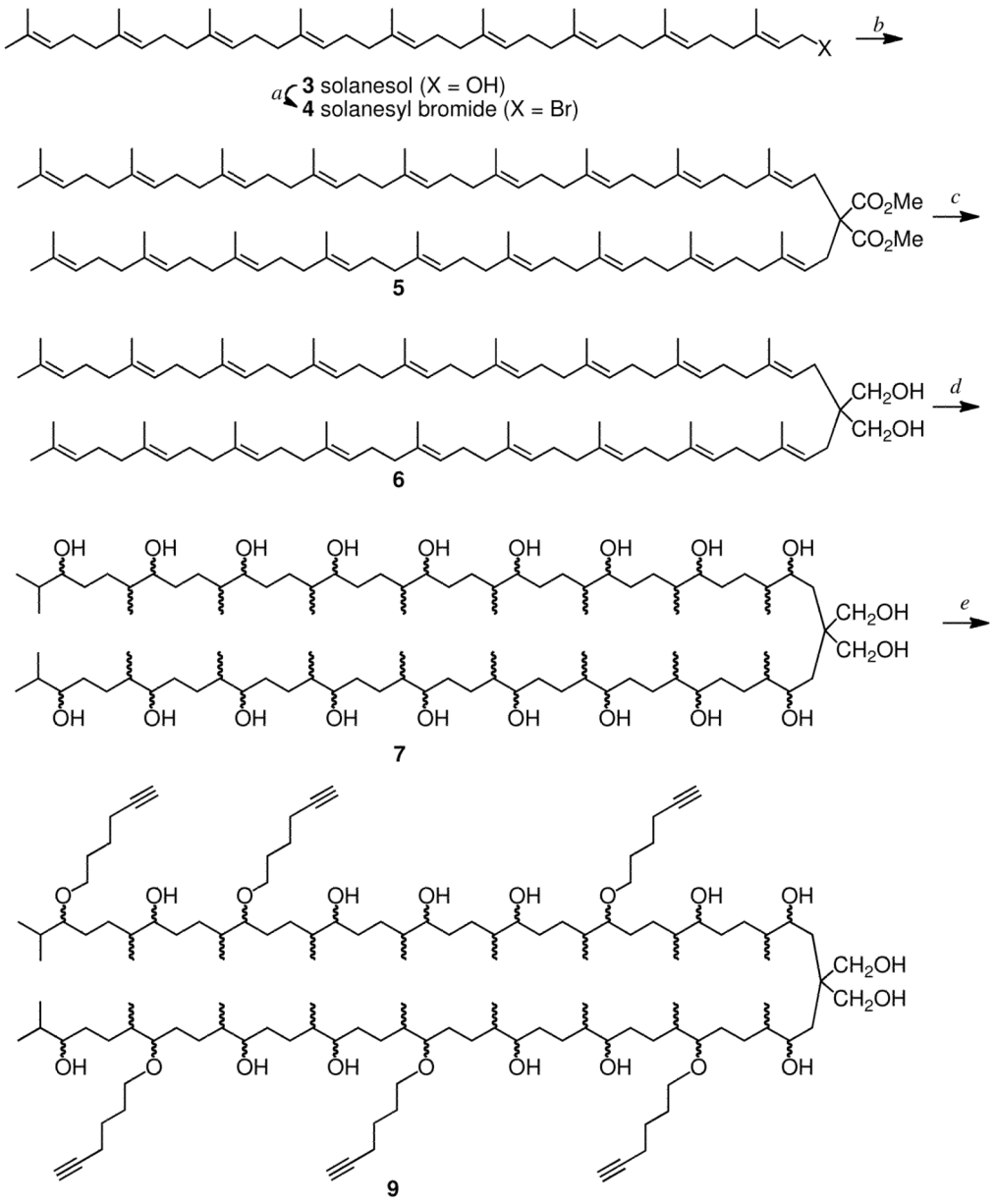
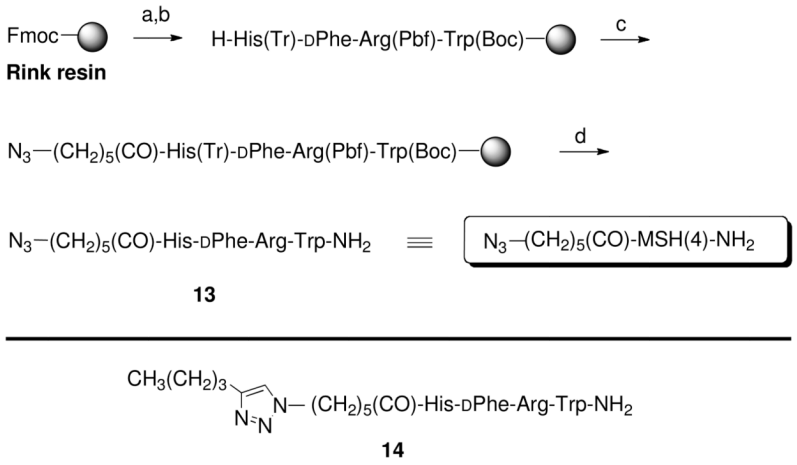


Figure 2.
MS showing the distribution of products in the mixture **9** resulting from alkylation of polyols **7**. Peaks near 1888, 1968, 2048, 2128, 2208, 2288, and 2368 represent incorporation of 3, 4, 5, 6, 7, 8, and 9 alkyne units, respectively, onto the solanesol-derived scaffold.



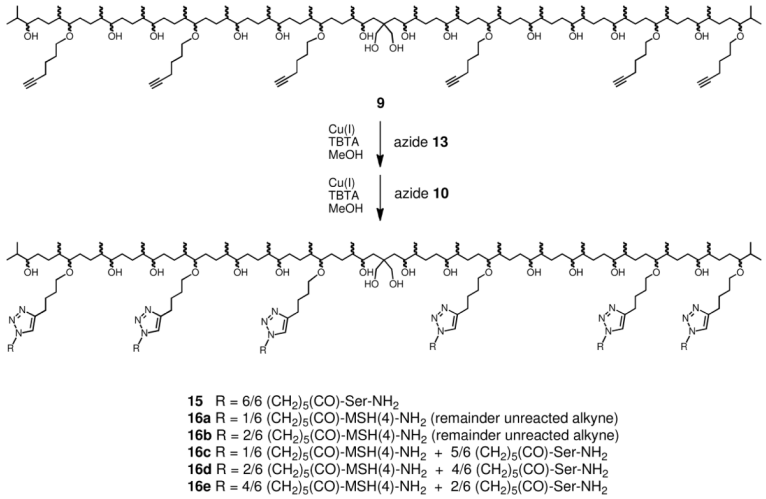
Scheme 1. Synthesis of Polyol/Polyalkyne Scaffold 9^{a-c}

[a]Reagents: (a) PBr_3, ether (Ref. [12]). (b) $NaCH(CO_2Me)_2$, EtOH. (c) $LiAlH_4$, ether. (d) disiamyborane, THF; H_2O_2, NaOH. (e) NaH, $Br(CH_2)_4C{\equiv}CH$ (**8**). [b]Product **7** resulting from anti-Markovnikov hydration is shown. It is assumed that mixtures of all possible stereoisomers of **7** are produced. [c]Structure **9** is a representative hexaalkyne scaffold. The sites of attachment of the 5-hexyn-1-yl groups shown in **9** are arbitrary.



Scheme 2. Solid Phase Synthesis[a]

[a]Reagents: (a) piperidine. (b) Fmoc/*t*Bu solid phase synthesis. (c) $N_3(CH_2)_5(CO)OH$, Cl-HOBt, DIC. (d) TFA/1,2-ethanedithiol/thioanisole/water (91/3/3/3).



Scheme 3. Synthesis of mixtures of multivalent constructs 15 and 16a–16e from solanesol-derived molecular scaffold 9 via CuAAC [a]

[a]Only one set of hexameric products from each mixture is depicted here for illustrative purposes. The sites of sidechain attachment shown are arbitrary.

Table 1

Mass spectral and HPLC characterization of compounds 13 and 14.

Compound	Formula [M]	Calc Mass [Ion]	Mass Found (error)	t_R[a]
13	$C_{38}H_{50}N_{14}O_5$	783.4161 $[M+1]^+$	783.4158 (0.4 ppm)	15.10
14	$C_{44}H_{60}N_{14}O_5$	865.4949 $[M+1]^+$	865.4936 (0.9 ppm)	13.77

[a]Linear gradient of from 10→90% acetonitrile in water containing 0.1% TFA over 50 min.

Table 2

Synthesis and compositions of 15 and 16a–e.

Product	mg 9	mg 13 (equiv)	mg 10 (equiv)	Yield, mg (%)	MALDI-TOF
15	10	0	15 (15)	16 (88)	Figure S12
16a	20	8 (1)	0	22 (76)	Figure S13
16b	20	16 (2)	0	29 (78)	Figure S14
16c	20	8 (1)	15 (7)	31 (76)	Figure S15
16d	20	16 (2)	12 (6)	19 (41)	Figure S16
16e	10	16 (4)	4 (4)	22 (76)	Figure S17

Table 3

Assays of competitive binding of MSH(4), 11, 14, 15, and 16a–e to hMC4R.

	Assay A[a]		Assay B[b]	
Compound	K_i[c]	**n**	K_i[c]	**n**
11	nb[d]	3	nb[e]	3
15	nb[d]	3	nb[e]	3
MSH(4)	1.9 ± 0.55 μM	5	0.76 ± 0.04 μM	4
14	2.7 ± 0.21 μM	5	0.82 ± 0.26 μM	3
16a	nd[f]		1.7 ± 0.39 μM	4
16b	nd[f]		1.2 ± 0.38 μM	6
16c	nd[f]		0.84 ± 0.10 μM	4
16d	nd[f]		0.52 ± 0.02 μM	6
16e	2.2 ± 0.17 μM	4	0.22 ± 0.06 μM	4

[a] This assay employed probe **17**.

[b] This assay employed probe **18**.

[c] K_i values were calculated using the equation $K_i = EC_{50}/(1 + ([\text{ligand}]/K_D))$ where [ligand] refers to the concentration of probe used as the labeled competed ligand. For probe **17**, [ligand] = 10 nM and K_D = 18.8 nM. For probe **18**, [ligand] = 0.5 μM and K_D = 9.1 μM. The value given represents the average of n independent competition binding experiments, each done in quadruplicate.

[d] This compound was unable to prevent probe **17** from binding in the concentration range tested (10^{-5}–10^{-12} M in serine amide).

[e] This compound was unable to prevent probe **18** from binding in the concentration range tested (10^{-5}–10^{-12} M in serine amide).

[f] Not determined.