# A Solanesol-Derived Scaffold for Multimerization of Bioactive Peptides
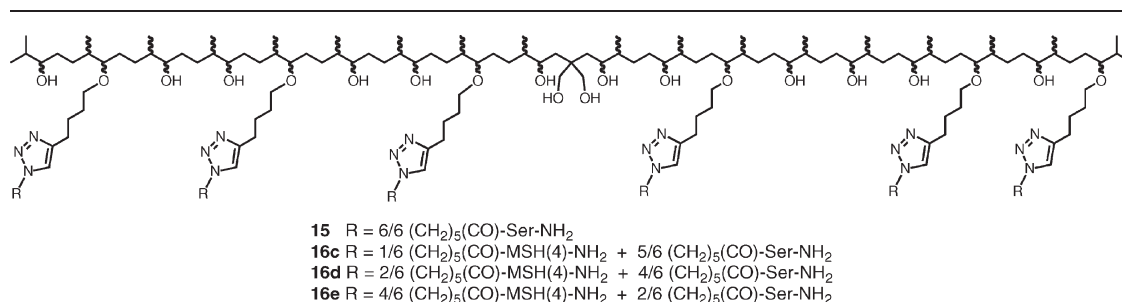
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**15** R = 6/6 $(CH_2)_5(CO)$-Ser-$NH_2$
**16c** R = 1/6 $(CH_2)_5(CO)$-MSH(4)-$NH_2$ + 5/6 $(CH_2)_5(CO)$-Ser-$NH_2$
**16d** R = 2/6 $(CH_2)_5(CO)$-MSH(4)-$NH_2$ + 4/6 $(CH_2)_5(CO)$-Ser-$NH_2$
**16e** R = 4/6 $(CH_2)_5(CO)$-MSH(4)-$NH_2$ + 2/6 $(CH_2)_5(CO)$-Ser-$NH_2$

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 with 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 with 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 with 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 noninvasive 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.

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(1) (a) Weissleder, R.; Mahmood, U. *Radiology* **2001**, *219*, 316–333. (b) Gillies, R. J.; Hruby, V. J. *Expert Opin. Ther. Targets* **2003**, *7*, 137–139. (c) Gillies, R. J.; Hoffman, J. M.; Lam, K. S.; Menkens, A. E.; Piwnica-Worms, D. R.; Sullivan, D. C.; Weissleder, R. *Mol. Imaging* **2005**, *4*, 98–103. (d) Cassidy, P. J.; Radda, G. K. *J. R. Soc., Interface* **2005**, *2*, 133–144.

(2) (a) Handl, H. L.; Vagner, J.; Han, H.; Mash, E.; Hruby, V. J.; Gillies, R. J. *Expert Opin. Ther. Targets* **2004**, *8*, 565–586. (b) Vagner, J.; Handl, H. L.; Monguchi, Y.; Jana, U.; Begay, L. J.; Mash, E. A.; Hruby, V. J.; Gillies, R. J. *Bioconjugate Chem.* **2006**, *17*, 1545–1550. (c) Bowen, M. E.; Monguchi, Y.; Sankaranarayanan, R.; Vagner, J.; Begay, L. J.; Xu, L.; Jagadish, B.; Hruby, V. J.; Gillies, R. J.; Mash, E. A. *J. Org. Chem.* **2007**, *72*, 1675−1680 and 3608. (d) Jagadish, B.; Sankaranarayanan, R.; Xu, L.; Richards, R.; Vagner, J.; Hruby, V. J.; Gillies, R. J.; Mash, E. A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 3310–3313. (e) Handl, H. L.; Sankaranarayanan, R.; Josan, J. S.; Vagner, J.; Mash, E. A.; Gillies, R. J.; Hruby, V. J. *Bioconjugate Chem.* **2007**, *18*, 1101–1109. (f) Vagner, J.; Xu, L.; Handl, H. L.; Josan, J. S.; Morse, D. L.; Mash, E. A.; Gillies, R. J.; Hruby, V. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 1685–1688.

(3) (a) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794. (b) Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. *Angew. Chem., Int. Ed.* **2006**, *45*, 2348–2368. (c) Carlson, C. B.; Mowery, P.; Owen, R. M.; Dykhuizen, E. C.; Kiessling, L. L. *ACS Chem. Biol.* **2007**, *2*, 119–127. (d) Trouche, N.; Wieckowski, S.; Sun, W.; Chaloin, O.; Hoebeke, J.; Fournel, S.; Guichard, G. *J. Am. Chem. Soc.* **2007**, *129*, 13480–13492. (e) Diestler, D. J.; Knapp, E. W. *Phys. Rev. Lett.* **2008**, *100*, 178101/1–178101/4. (f) Choi, S.-K. *Synthetic Multivalent Molecules*; Wiley-Interscience: Hoboken, NJ, 2004.

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 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,d]

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 with cells expressing hMC4R are presented herein.
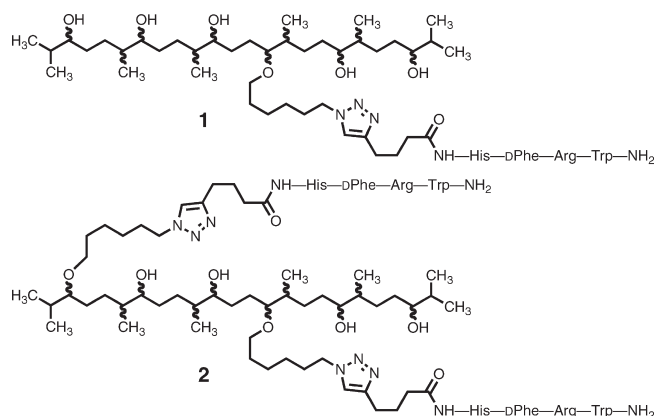


## Results

**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** with 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** with 1.7 equiv of disiamyborane per alkene for a period of 72 h left 4% of the alkene groups intact in the product polyol as determined by mass spectrometry (Figure 1) and titration with bromine (see the Experimental Procedures for details). The principal polyols present in the product

(4) (a) Schwyzer, R.; Kriwaczek, V. M. *Biopolymers* **1981**, *20*, 2011–2020. (b) Wunderlin, R.; Sharma, S. D.; Minakakis, P.; Schwyzer, R. *Helv. Chim. Acta* **1985**, *68*, 12–22. (c) Sharma, S. D.; Granberry, M. E.; Jiang, J.; Leong, S. P. L.; Hadley, M. E.; Hruby, V. J. *Bioconjugate Chem.* **1994**, *5*, 591–601. (d) Sharma, S. D.; Jiang, J.; Hadley, M. E.; Bentley, D. L.; Hruby, V. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13715–13720. (e) Carrithers, M. D.; Lerner, M. R. *Chem. Biol.* **1996**, *3*, 537–542.

(5) (a) Cairo, C. W.; Gestwicki, J. E.; Kanai, M.; Kiessling, L. L. *J. Am. Chem. Soc.* **2002**, *124*, 1615–1619. (b) Griffith, B. R.; Allen, B. L.; Rapraeger, A. C.; Kiessling, L. L. *J. Am. Chem. Soc.* **2004**, *126*, 1608–1609. (c) Line, B. R.; Mitra, A.; Nan, A.; Ghandehari, H. *J. Nucl. Med.* **2005**, *46*, 1552–1560. (d) Li, R. C.; Broyer, R. M.; Maynard, H. D. *J. Polym. Sci., Part A: Polym. Chem.* **2006**, *44*, 5004–5013. (e) Cilliers, R.; Song, Y.; Kohlmeir, E. K.; Larson, A. C.; Omary, R. A.; Meade, T. J. *Magn. Reson. Med.* **2008**, *59*, 898–902.

(6) (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021. (b) Gil, M. V.; Arévalo, M. J.; López, Ó. *Synthesis* **2007**, 1589–1620. (c) Angell, Y. L.; Burgess, K. *Chem. Soc. Rev.* **2007**, *36*, 1674–1689. (d) Holub, J. M.; Garabedian, M. J.; Kirshenbaum, K. *QSAR Comb. Sci.* **2007**, *26*, 1175–1180.

(7) Moore, W. R. A. D.; O'Dowd, M. In *Properties and Applications of Polyvinyl Alcohol*; Finch, C. A., Ed.; Staples Printers Ltd: Kent, England, 1968; Chapter 4, pp 77−87.

(8) (a) Levesque, G.; Pinazzi, C. *Bull. Soc. Chim. Fr.* **1971**, 1008–1010. (b) Yamaguchi, H.; Azuma, K.; Minoura, Y. *Polym. J.* **1972**, *3*, 12–20. (c) Pinazzi, C. P.; Guillaume, P.; Reyx, D. *Eur. Polym. J.* **1976**, *12*, 9–15. (d) Perera, M. C. S.; Elix, J. A.; Bradbury, J. H. *J. Appl. Polym. Sci.* **1987**, *33*, 2731–2742.

(9) The "high-affinity" ligand, NDP-α-MSH, is Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$; see: (a) Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754–5758. (b) Hadley, M. E.; Anderson, B.; Heward, C. B.; Sawyer, T. K.; Hruby, V. J. *Science* **1981**, *213*, 1025–1027.

(10) The "low-affinity" ligand MSH(4), Ac-His-DPhe-Arg-Trp-NH$_2$, was based on the minimal active sequence for full agonist activity of α-MSH; see: (a) Hruby, V. J.; Wilkes, B. C.; Hadley, M. E.; Al-Obeidi, F.; Sawyer, T. K.; Staples, D. J.; de Vaux, A. E.; Dym, O.; de L. Castrucci, A. M.; Hintz, M. F.; Riehm, J. P.; Rao, K. R. *J. Med. Chem.* **1987**, *30*, 2126–2130. (b) Castrucci, A. M. L.; Hadley, M. E.; Sawyer, T. K.; Wilkes, B. C.; Al-Obiedi, F.; Staples, D. J.; de Vaux, A. E.; Dym, O.; Hintz, M. F.; Riehm, J. P.; Rao, K. R.; Hruby, V. J. *Gen. Comp. Endocrinol.* **1989**, *73*, 157–163. (c) Haskell-Luevano, C.; Hendrata, S.; North, C.; Sawyer, T. K.; Hadley, M. E.; Hruby, V. J.; Dickinson, C.; Gantz, I. *J. Med. Chem.* **1997**, *40*, 2133–2139.

(11) Xu, L.; Vagner, J.; Alleti, R.; Rao, V.; Jagadish, B.; Morse, D. L.; Hruby, V. J.; Gillies, R. J.; Mash, E. A. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2489–2492.

(12) Hamamura, K.; Yamatsu, I.; Minami, N.; Yamagishi, Y.; Inai, Y.; Kijima, S.; Nakamura, T. *J. Label. Compd. Radiopharm.* **2002**, *45*, 823–829. Solanesol (**3**) and solanesyl bromide (**4**) are commercially available from NetChem.

(13) This presumably was due to temporary intermolecular and intramolecular cross-linking during hydroboration, leading to sterically inaccessible, unreactive double bonds. Similar behavior was noted in previous work on the hydroboration of polyisoprene; see refs 8a−8d.

(14) Use of triisopinocampheyldiborane as described in ref 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.

**SCHEME 1.** Synthesis of Polyol/Polyalkyne Scaffold 9[a−c]
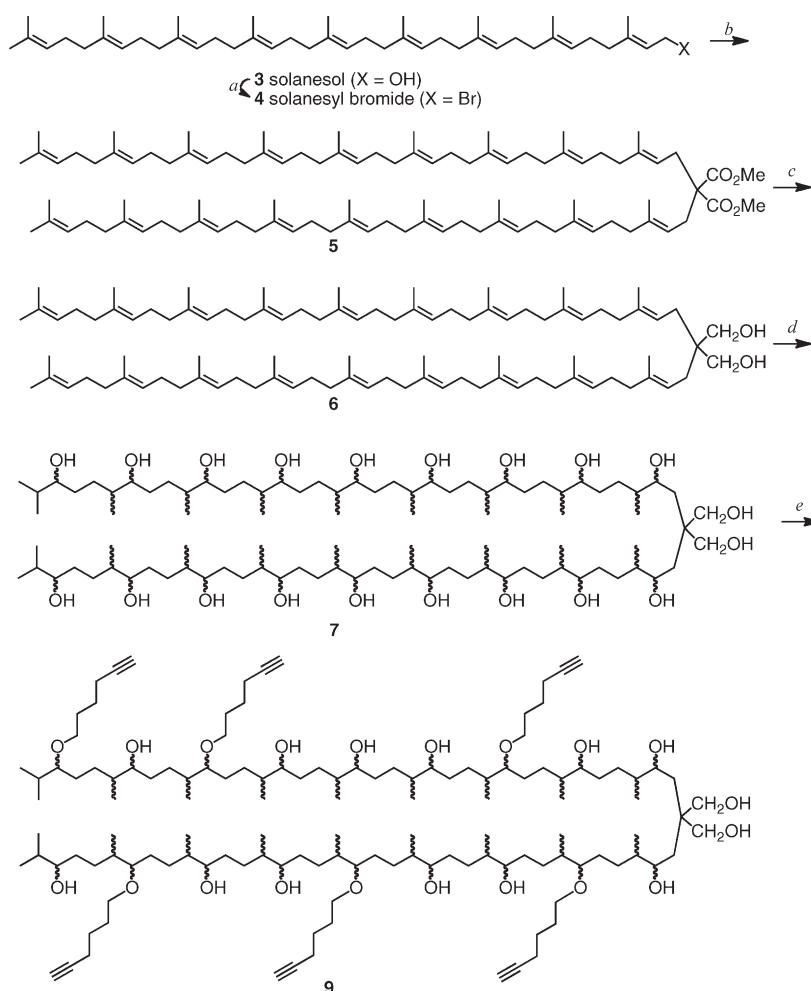


[a]Reagents: (a) PBr$_3$, ether (ref 12); (b) NaH, CH$_2$(CO$_2$Me)$_2$, THF; (c) LiAlH$_4$, ether; (d) disiamylborane, THF; H$_2$O$_2$, NaOH; (e) NaH, Br(CH$_2$)$_4$C≡CH (8). [b]Product 7 resulting from anti-Markovnikov hydration is shown. It is assumed that mixtures of all possible stereoisomers of 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.

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 70% yield based on diol 6. This result was shown to be repeatable in three separate runs. Reaction of 7 with 30 equiv of sodium hydride and 12 equiv 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 69%. 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 *tert*-butanol in the presence of copper sulfate and sodium ascorbate.
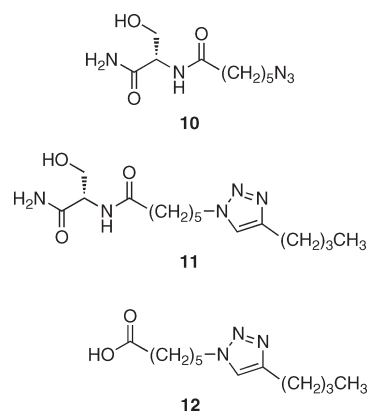


(15) Sharma, S.; Oehlschlager, A. C. *J. Org. Chem.* **1989**, *54*, 5064–5073.
(16) Hidy, P. H.; Hodge, E. B.; Young, V. V.; Harned, R. L.; Brewer, G. A.; Phillips, W. F.; Runge, W. F.; Stavely, H. E.; Pohland, A.; Boaz, H.; Sullivan, H. R. *J. Am. Chem. Soc.* **1955**, *77*, 2345–2346. Serine amide is commercially available from Senn Chemicals USA.
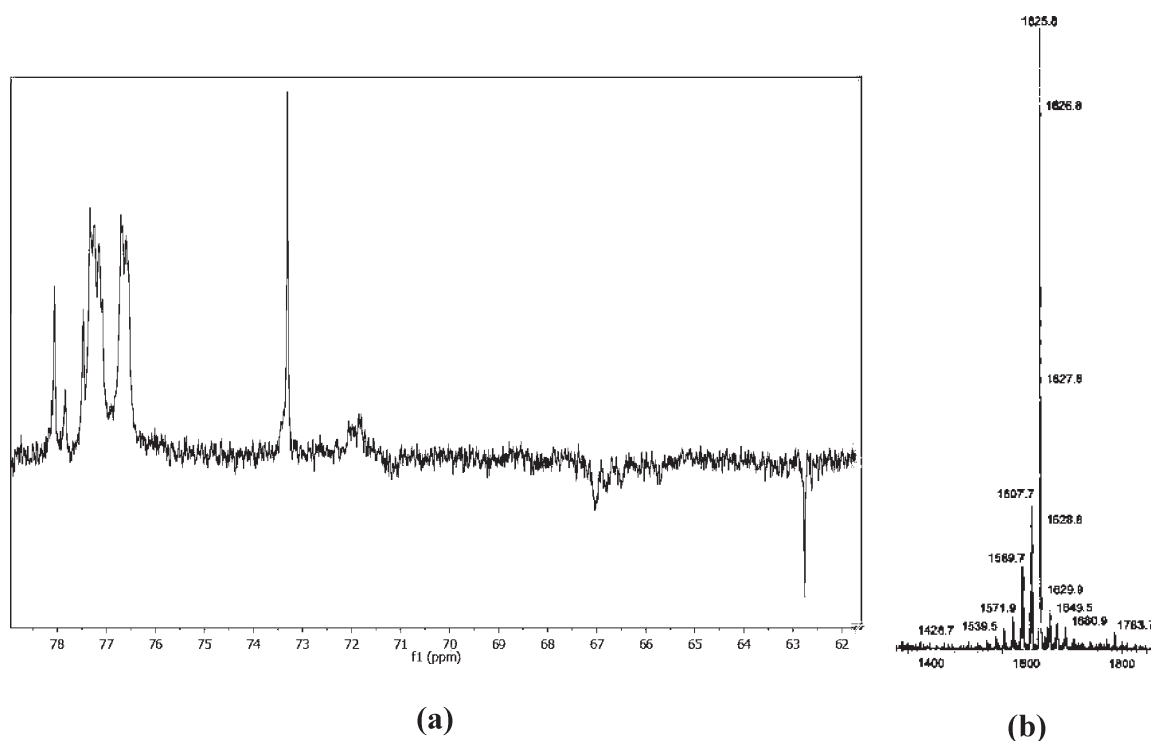
**(a)**



**(b)**

**FIGURE 1.** (a) A portion of the DEPT 135 $^{13}$C NMR spectrum of **7** in CD$_3$OD (see ref 8d). The lines from 76 to 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, octadecaols, nonadecaols, and eicosaols, respectively.
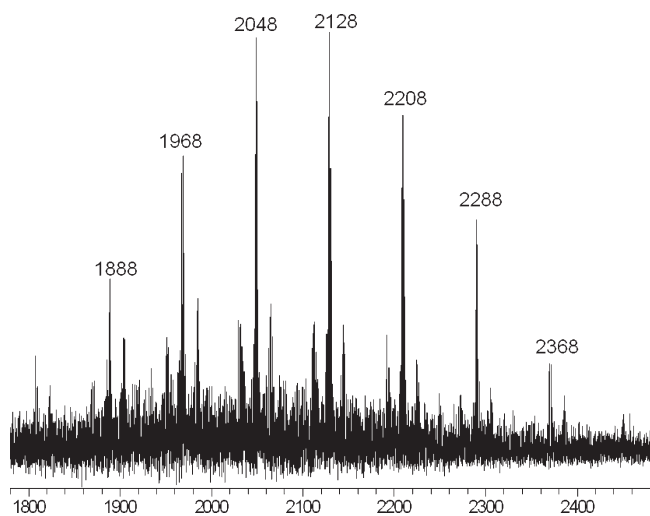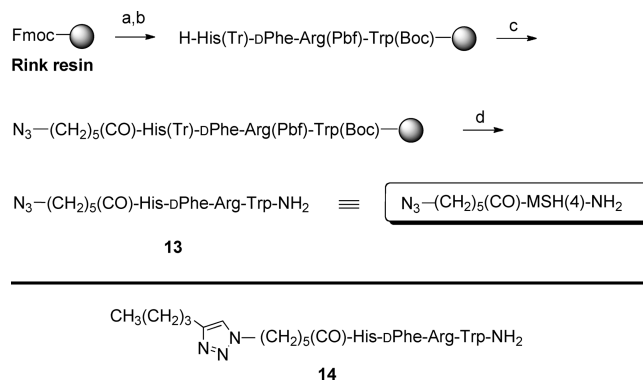


**FIGURE 2.** MS showing the distribution of products in the mixture **9** resulting from alkylation of polyols **7**. Peaks near 1888, 1968, 2048, 2128, 2208, 2288, and 2368 represent incorporation of **3**, **4**, **5**, **6**, **7**, **8**, and **9** alkyne units, respectively, onto the solanesol-derived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

**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

(17) Grandjean, C.; Boutonnier, A.; Guerreiro, C.; Fournier, J.-M.; Mulard, L. A. *J. Org. Chem.* **2005**, *70*, 7123–7132.
(18) Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853–2855.

**SCHEME 2.** **Solid Phase Synthesis**[a]



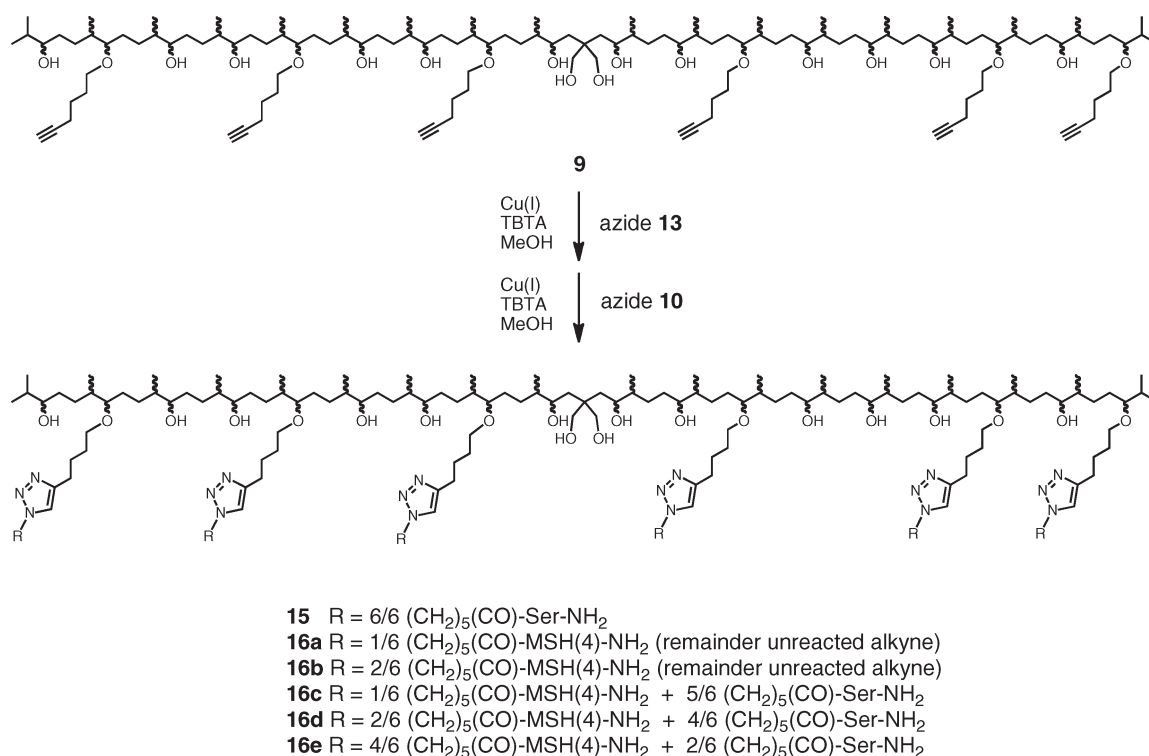$^{a}$Reagents: (a) piperidine; (b) Fmoc/*t*-Bu solid phase synthesis; (c) N$_3$(CH$_2$)$_5$(CO)OH, Cl-HOBt, DIC; (d) TFA/triisopropylsilane/ thioanisole/water (91/3/3/3).

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 by 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$_2$N$_3$)(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.

(19) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154. (b) Hruby, V. J.; Meyer, J.-P. In *Bioorganic Chemistry: Peptides and Proteins*; Hecht, S. M., Ed.; Oxford University Press: New York, 1998; pp 27–64.

**SCHEME 3.** Synthesis of Mixtures of Multivalent Constructs 15 and 16a−e from Solanesol-Derived Molecular Scaffold 9 via CuAAC[a]



15   R = 6/6 (CH₂)₅(CO)-Ser-NH₂
16a  R = 1/6 (CH₂)₅(CO)-MSH(4)-NH₂ (remainder unreacted alkyne)
16b  R = 2/6 (CH₂)₅(CO)-MSH(4)-NH₂ (remainder unreacted alkyne)
16c  R = 1/6 (CH₂)₅(CO)-MSH(4)-NH₂  +  5/6 (CH₂)₅(CO)-Ser-NH₂
16d  R = 2/6 (CH₂)₅(CO)-MSH(4)-NH₂  +  4/6 (CH₂)₅(CO)-Ser-NH₂
16e  R = 4/6 (CH₂)₅(CO)-MSH(4)-NH₂  +  2/6 (CH₂)₅(CO)-Ser-NH₂

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

**TABLE 1. Mass Spectral and HPLC Characterization of Compounds 13 and 14**

| compd | formula [M] | calcd 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% to 90% acetonitrile in water containing 0.1% TFA over 50 min.

Compounds **13** and **14** were purified by reverse-phase C₁₈ 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 CHCl₃.[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 the Supporting Information). The average number of serine amide residues per molecule of **15** was determined to be six.

Reaction of **9** with 1 or 2 equiv of **13** in the presence of copper(I) and TBTA in methanol under microwave irradiation[20]

**TABLE 2. Synthesis and Compositions of 15 and 16a−e**

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

[a]See the Supporting Information.

gave mixtures of MSH(4)-bearing multivalent constructs **16a** and **16b**, respectively (Scheme 3 and Table 2). Reaction of **9** with 1, 2, or 4 equiv 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 CHCl₃.[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 the Supporting Information).

**Binding Studies.** Hek293 cells overexpressing hMC4R were used to assess ligand binding[22] by using two previously

(20) Kappe, C. O.; Dallinger, D.; Murphree, S. S. *Practical Microwave Synthesis for Organic Chemists*; Wiley-VCH: Weinheim, Germany, 2009.

(21) Caplin, S. *Tissue Cult. Assoc. Man.* **1976**, *2*, 439–440. Copper ion could not be removed by dialysis, presumably due to the strong chelatory properties of MSH(4).

(22) The hMC4R vector was originally received from Dr. Ira Gantz; see: Gantz, I.; Miwa, H.; Konda, Y.; Shimoto, Y.; Tashiro, T.; Watson, S. J.; DelValle, J.; Yamada, T. *J. Biol. Chem.* **1993**, *268*, 15174–15179.

**TABLE 3. Assays of Competitive Binding of MSH(4), 11, 14, 15, and 16a−e to hMC4R**

| compd | assay A[a] | | assay B[b] | |
|---|---|---|---|---|
| | $K_i$,[c] $\mu$M | $n$ | $K_i$,[c] $\mu$M | $n$ |
| **11** | nb[d] | 3 | nb[e] | 3 |
| **15** | nb[d] | 3 | nb[e] | 3 |
| MSH(4) | 1.9 ± 0.55 | 5 | 0.76 ± 0.04 | 4 |
| **14** | 2.7 ± 0.21 | 5 | 0.82 ± 0.26 | 3 |
| **16a** | nd[f] | | 1.7 ± 0.39 | 4 |
| **16b** | nd[f] | | 1.2 ± 0.38 | 6 |
| **16c** | nd[f] | | 0.84 ± 0.10 | 4 |
| **16d** | nd[f] | | 0.52 ± 0.02 | 6 |
| **16e** | 2.2 ± 0.17 | 4 | 0.22 ± 0.06 | 4 |

[a]This assay employed probe **17**. [b]This assay employed probe **18**. [c]$K_i$ values were calculated with 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 $\mu$M and $K_D$ = 9.1 $\mu$M. 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.

described europium-based competitive binding assays.[11,23] Assay A[23a] employed Eu-DTPA-NDP-$\alpha$-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) analogue **14**, and for MSH(4)-containing multivalent constructs **16a−e** are given in Table 3.



**17**



**18**
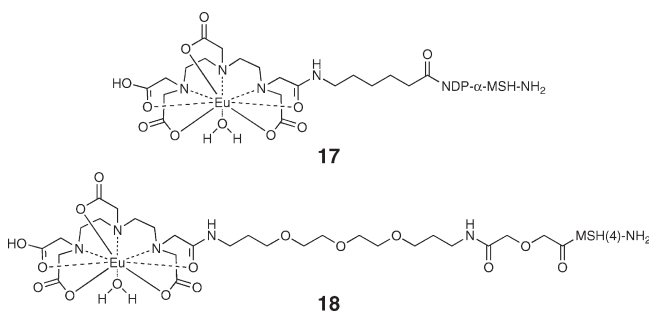
## Discussion

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 by 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 with 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-$\alpha$-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 ($\sim$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−e**, 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−e** (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 side chains 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,

(23) (a) Handl, H. L.; Vagner, J.; Yamamura, H. I.; Hruby, V. J.; Gillies, R. J. *Anal. Biochem.* **2004**, *330*, 242–250. (b) De Silva, C. R.; Vagner, J.; Lynch, R.; Gillies, R. J.; Hruby, V. J. *Anal. Biochem.* **2010**, *398*, 15–23.

(24) Haskell-Luevano, C.; Miwa, H.; Dickinson, C.; Hadley, M. E.; Hruby, V. J.; Yamada, T.; Gantz, I. *J. Med. Chem.* **1996**, *39*, 432–435.

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–e** 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 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 $\mu$L, 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$_4$Cl solution and the mixture was extracted with ether (200 mL). The organic extract was washed with water and 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$_3$) $\delta$ 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$_3$) $\delta$ 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 calcd for C$_{95}$H$_{152}$O$_4$Na (M + Na)$^+$ 1380.1588, obsd 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$_2$SO$_4$ 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$_3$) $\delta$ 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$_3$) $\delta$ 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 Anal. Calcd 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).** [Note: All reagents and solutions were deoxygenated with argon before use.] In a three-necked flask (500 mL) a solution of borane in THF (1 M, 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 (2 M, 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 3 N NaOH (26 mL) and 30% H$_2$O$_2$ (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 20 min of stirring, 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$_4$Cl (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

(25) Vagner, J.; Handl, H. L.; Gillies, R. J.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 211–215.

(26) Shewmake, T. A.; Solis, F. J.; Gillies, R. J.; Caplan, M. R. *Biomacromolecules* **2008**, *9*, 3057–3064.

(27) Xu, L. Unpublished work.

(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 with hexol **A** (see ref 2d for the preparation of **A**) gave a persistent bromine color upon addition of the first drop of bromine solution.
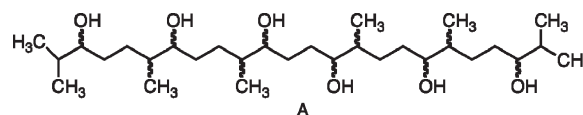
with hexanes ($2 \times 10$ mL) to afford a mixture of polyol/polyalkynes **9** (218 mg). On the basis of mass spectral analysis (see Figure 2) it was found that an average of six alkylations per molecule had occurred. On the basis of this average, the calculated yield is 69%. This reaction was repeated 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 g, 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$_3$-OD) $\delta$ 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$_3$OD) $\delta$ 24.8, 26.1, 28.4, 35.7, 51.0, 54.2, 62.2, 173.9, 174.0; HRMS (ESI) calcd for C$_9$H$_{17}$N$_5$O$_3$ (MH)$^+$ 244.1411, obsd 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 mmol) in methanol (5 mL) were added 1-hexyne (337 mg, 4.11 mmol), 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$_3$OD) $\delta$ 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$_3$OD) $\delta$ 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) calcd for C$_{15}$H$_{28}$N$_5$O$_3$ (MH)$^+$ 326.2187, obsd 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-bottomed flask containing a 1:1 mixture of $t$-BuOH and water (20 mL). 6-Azidohexanoic acid[17] (1.00 g, 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) and washed with 1 N HCl ($2 \times 20$ mL) and brine ($2 \times 20$ mL), then the organic phase was separated, dried over Na$_2$SO$_4$, 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$_3$) $\delta$ 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$_3$) $\delta$ 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) calcd for C$_{12}$H$_{22}$N$_3$O$_2$ (MH)$^+$ 240.1707, obsd 240.1706.

**Solid Phase Synthesis (Scheme 2).** In a syringe (polypropylene reaction tube equipped with a polypropylene frit) Rink amide resin (1 g, 0.68 mmol) was allowed to swell in THF for 1 h. THF was removed and addition of 20% piperidine in DMF (15 mL) for 2 min led to the deprotection of the Fmoc functionality. DMF was removed, 20% piperidine solution in DMF (15 mL) was again added, and the mixture was shaken for 18 min. DMF was removed and the resin washed with DMF ($3 \times 15$ mL),

CH$_2$Cl$_2$ ($3 \times 15$ mL), DMF ($3 \times 15$ mL), 0.5 M HOBt in DMF ($1 \times 15$ mL), 0.5 M HOBt in DMF ($1 \times 15$ mL) plus a drop of bromophenol blue, DMF ($2 \times 15$ mL), and CH$_2$Cl$_2$ ($1 \times 15$ mL), in that order. A solution of Fmoc-Trp(Boc)-OH, the first Fmoc-amino acid (1.05 g, 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 was shaken for 1 h. The resin was then washed with DMF ($3 \times 15$ mL), CH$_2$Cl$_2$ ($3 \times 15$ mL), and DMF ($3 \times 15$ 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 ($3 \times 15$ mL), CH$_2$Cl$_2$ ($3 \times 15$ mL), and DMF ($3 \times 15$ 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-1H-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 by using a 91:3:3:3 mixture of trifluoro-acetic acid, triisopropylsilane, thioanisole, and water (10 mL). The mixture of cleavage cocktail and resin was shaken over-night, the solution was separated from the resin, volatiles were evaporated, the residue was triturated with ether, and the crude product was separated by centrifugation. Purification of the tetrapeptide amides **13** and **14** was accomplished by reverse phase chromatography with a $19 \times 256$ 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 $4.6 \times 75$ 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 $\mu$M. 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 $\mu$mol), azide **10** (10 mg, 50 $\mu$mol), TBTA (3 mg, 5 $\mu$mol), and tetrakis-(acetonitrile)copper(I) hexafluorophosphate (2 mg, 5 $\mu$mol) in dry methanol (1 mL) was irradiated for 4 h in a Biotage micro-wave reactor (100 °C). Additional azide **10** (5 mg, 25 $\mu$mol) 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$_3$ contain-ing dithizone (20 mg/L, $3 \times 30$ mL) to remove copper.[21] The water layer was then washed with CHCl$_3$ ($2 \times 20$ 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 $\mu$mol), MSH(4) azide **13** (variable amount, see Table 2), TBTA (3 mg, 5 $\mu$mol), and tetrakis(acetonitrile)copper(I) hexafluoro-phosphate (2 mg, 5 $\mu$mol) in dry methanol (1 mL/5 $\mu$mol of **9**) were irradiated for 6 h 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 $\mu$mol 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 serina-mide 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 the 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 $\mu$L of the compounds to be tested (dilutions ranging from 2 × 10$^{-4}$ to 1 × 10$^{-11}$ M) and 50 $\mu$L of Eu-labeled ligand (assay A, 10 nM probe **17**; assay B, 0.5 $\mu$M 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 h. 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 $\mu$L/well), and fluorescence was measured on a Wallac VICTOR$^3$ instrument with standard Eu TRF measurement conditions (340 nm excitation, 400 $\mu$s delay, and emission collection for 400 $\mu$s 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 by using the equation $K_i = \text{EC}_{50}/(1 + ([\text{ligand}]/K_D))$, 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 $\mu$M and $K_D$ = 9.1 $\mu$M. 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.

**Supporting Information Available:** A general experimental section, a list of nonstandard abbreviations, copies of the $^1$H 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.