Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc



A sucrose-derived scaffold for multimerization of bioactive peptides

Venkataramanarao Rao^a, Ramesh Alleti^a, Liping Xu^b, Narges K. Tafreshi^b, David L. Morse^b, Robert J. Gillies^b, Eugene A. Mash^{a,*}

^a Department of Chemistry and Biochemistry, The University of Arizona, Tucson, AZ 85721-0041, USA

^b Department of Functional and Molecular Imaging, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612, USA

ARTICLE INFO

Article history: Received 23 June 2011 Revised 18 August 2011 Accepted 24 August 2011 Available online 27 August 2011

Keywords: Multimeric ligands Melanocortin 4 receptor ligands Cholecystokinin 2 receptor ligands

ABSTRACT

A spherical molecular scaffold bearing eight terminal alkyne groups was synthesized in one step from sucrose. One or more copies of a tetrapeptide azide, either $N_3(CH_2)_5(C=0)$ -His-DPhe-Arg-Trp-NH₂ (MSH4) or $N_3(CH_2)_5(C=O)$ -Trp-Met-Asp-Phe-NH₂ (CCK4), were attached to the scaffold via the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. Competitive binding assays using Eu-labeled probes based on the superpotent ligands Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂ (NDP-α-MSH) and Asp-Tyr-Met-Gly-Trp-Met-Asp-Phe-NH₂ (CCK8) were used to study the interactions of monovalent and multivalent MSH4 and CCK4 constructs with Hek293 cells engineered to overexpress MC4R and CCK2R. All of the monovalent and multivalent MSH4 constructs exhibited binding comparable to that of the parental ligand, suggesting that either the ligand spacing was inappropriate for multivalent binding, or MSH4 is too weak a binder for a second 'anchoring' binding event to occur before the monovalently-bound construct is released from the cell surface. In contrast with this behavior, monovalent CCK4 constructs were significantly less potent than the parental ligand, while multivalent CCK4 constructs were as or more potent than the parental ligand. These results are suggestive of multivalent binding, which may be due to increased residence times for monovalently bound CCK4 constructs on the cell surface relative to MSH4 constructs, the greater residence time being necessary for the establishment of multivalent binding.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Early detection and diagnosis of many human cancers could be aided by reagents that seek out and selectively bind to cancer cells and report their existence and location by non-invasive molecular imaging.^{1–4} One strategy for development of such reagents involves attaching imaging agents to molecular scaffolds that bear multiple copies of ligands to receptors present on the surface of cancer cells.^{5–11} Such multivalent constructs could display

enhanced affinity and selectivity for cancer cells based on cooperative binding. $^{\rm 12-16}$

Previously we described the preparation and testing of multivalent constructs derived from squalene¹⁷ and solanesol.¹⁸ These constructs bore sidechains based on the ligand Ac-His-DPhe-Arg-Trp-NH₂ (MSH4)¹⁹⁻²¹ which has a low micromolar affinity for binding to the melanocortin 4 receptor (MC4R).²² In our prior work, the azide N₃(CH₂)₅(C=O)-His-DPhe-Arg-Trp-NH₂ (1, Scheme 1) was attached to a linear scaffold (e.g., 2) bearing terminal alkyne groups using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC),²³⁻²⁶ producing triazole-containing multivalent constructs (e.g., **3**).¹⁸ The abilities of **3** and of similar monovalent and multivalent constructs based on MSH4 to bind to MC4R were tested in competitive binding assays against probes $\mathbf{4}^{11}$ and $\mathbf{5}^{27}$ using Hek293 cells engineered to overexpress this receptor. Probe **4** is based on the superpotent ligand Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂ (NDP- α -MSH),^{28,29} while the probe 5 is based on MSH4. Interestingly, all of the solanesol-derived monovalent and multivalent constructs bearing MSH4 bound monovalently to MC4R when competed against either probe.¹⁸ To determine whether or not the solanesol-derived linear scaffold was responsible for this unexpected binding behavior, we have prepared and tested monovalent and multivalent constructs using



Abbreviations: Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; CCK2R, cholecystokinin 2 receptor; CCK4, Trp-Met-Asp-Phe-NH₂; Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; CuAAC, copper(I)-catalyzed azide-alkyne cycloaddition; DCM, dichloromethane; DIC, diisopropyl carbodiimide; DMEM, Dulbecco's Modified Eagle Medium; DMF, *N*,*N*-dimethylformamide; DTPA, diethylenetriaminepentaace-tic acid; EC₅₀, effective concentration, 50%; ESI, electrospray ionization; Fmoc, 9-fluorenylmethyoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HRMS, high resolution mass spectroscopy; MALDI-TOF, matrix-assisted desorption/ionization time-of-flight; MC4R, melanocortin 4 receptor; MEM, minimum essential medium; MSH4, Ac-His-DPhe-Arg-Trp-NH₂; TBTA, tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine; *t*-Bu, *tert*-butyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; TRF, time-resolved fluorescence.

^{*} Corresponding author. Tel.: +1 520 621 6321; fax: +1 520 621 8407. *E-mail address:* emash@u.arizona.edu (E.A. Mash).



Scheme 1. Synthesis of multivalent constructs 3.



Scheme 2. Synthesis of sucrose derivative 9.

azide **1** and a spherical scaffold derived from sucrose (vide infra). To determine whether or not this binding behavior was ligand and/or receptor dependent, we have also prepared and tested monovalent and multivalent constructs derived from this spherical scaffold and an azide that incorporates the ligand Trp-Met-Asp-Phe-NH₂ (CCK4) which has a low nanomolar affinity for binding to the cholecystokinin 2 receptor (CCK2R).³⁰

While searching for alternatives to linear scaffolds for ligand display, our attention was drawn by Olestra, a non-digestible fat substitute (e.g., **6**, Fig. 1).^{31,32} We reasoned that sucrose-derived molecules might make useful scaffolds for multimerization if ligands and other moieties of interest, such as imaging and therapeutic agents, could be attached to the termini of the fatty acid chains. Additionally, we determined that the 1,4-disubstituted-





Figure 1. A space-filling representation of one component of Olestra (6). This representation © 1997 by Daniel J. Berger and may be copied without limit if its use is for non-profit educational purposes.



Figure 2. Superposition of *cis*-3-pentene (**7**) and 1,4-dimethyl-1*H*-1,2,3-triazole (**8**). The methyl carbon-to-methyl carbon distances are 6.04 Å and 5.00 Å for **7** and **8**, respectively. The conformers of **7** and **8** used in this comparison were generated using Spartan '02 v1.0.5 for Macintosh.

1*H*-1,2,3-triazole groups that are produced via CuAAC map reasonably well onto the *cis*-1,2-disubstituted alkene groups of unsaturated fatty acid components of Olestra (compare **7** and **8**, Fig. 2). These considerations led to the synthesis and biological testing of sucrose-derived multivalent constructs that display one or more copies of MSH4 or CCK4 as described herein.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. General experimental

Dichloromethane (DCM), diethyl ether, and tetrahydrofuran (THF) were dried by passage through activated alumina. Other solvents and commercial reagents were used as supplied. For moisture sensitive reactions, glassware was flame-dried under argon. Analytical thin-layer chromatography (TLC) was carried out on pre-coated Silica Gel 60 F-254 plates with visualization by UV

exposure, by exposure to I₂ vapor, or by staining with 10% phosphomolybdic acid solution in ethanol or with 5% H₂SO₄ in ethanol and heat. Gravity-driven column chromatography was accomplished using Silica Gel 60 (63–210 $\mu m).$ 1H NMR and ^{13}C NMR spectra were recorded at 300 MHz or 500 MHz for ¹H NMR and at 75 MHz or 125 MHz for ¹³C NMR. Chemical shifts (δ) are expressed in ppm and are internally referenced (7.24 ppm for CDCl₃ and 3.31 ppm for CD₃OD for ¹H NMR and 77.0 ppm for CDCl₃ and 49.15 ppm for CD₃OD for ¹³C NMR). Analytical HPLC was performed on a 4.6 \times 75 mm Waters Symmetry[®] C₁₈ column and preparative HPLC was performed on a 19×256 mm Waters X-Bridge Preparative C₁₈ column. The mobile phase was 10–90% acetonitrile and water containing 0.1% trifluoroacetic acid (TFA) within 50 min. The flow rates were 1 mL/min and 15 mL/min for analytical and preparative runs, respectively. The dual UV detector system operated at 230 and 280 nm. Matrix-assisted desorption/ionization time-of-flight (MALDI-TOF) experiments were carried out on a Bruker Ultraflex III MALDI TOF-TOF instrument. 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% formic acid and the solutions mixed in a ratio of 100:1. ESI was also used to ionize some of the samples. These experiments were performed on an ESI Bruker Apex Qh 9.4 T FT-ICR instrument using standard ESI conditions. The samples were dissolved in water/acetonitrile 1:1 containing 0.1% formic acid in a concentration range of 1-30 µM.

2.1.2. Scaffold synthesis (Scheme 2)

2.1.2.1. Octa-O-(5-hexyn-1-yl)-β-D-fructofuranosyl-α-D-glucopy-To a suspension of NaH (330 mg, 13.7 mmol) in ranoside (9). dry dimethylformamide (DMF, 10 mL) under argon were added sucrose (200 mg, 0.58 mmol), 6-bromo-1-hexyne³³ (1.13 g, 6.97 mmol), and tetrabutylammonium bromide (50 mg, 0.15 mmol). The mixture was stirred at room temperature for 48 h. The reaction was guenched with satd NH₄Cl and the mixture extracted with ethyl acetate $(3 \times 15 \text{ mL})$. The combined organic extracts were washed with water, brine, dried over Na₂SO₄, and filtered. Removal of volatiles in vacuo afforded a residue that was subjected to silica gel chromatography (63–210 µm) using ethyl acetate/hexanes (2:8) as elutant. This afforded 250 mg (0.25 mmol, 43%) of 9 as a colorless oil, $R_{\rm f}$ 0.6 (ethyl acetate/hexanes, 3:7), $[\alpha]_{\rm D}^{25}$ 17.0 (c 0.5, CHCl₃); IR (cm⁻¹) 3308, 2928, 2854, 1213, 1152, 1094; ¹H NMR (500 MHz, CDCl₃) δ 1.57-1.71 (m, 32H), 1.95 (m, 8H), 2.18-2.23 (m, 16H), 3.17 (dd, / = 9.5 Hz, / = 3.4 Hz, 1H), 3.28 (t, / = 9.5 Hz, 1H), 3.35–3.71 (m, 20H), 3.77–3.92 (m, 5H), 4.07 (d, J = 7.2 Hz, 1H), 5.50 (d, J = 3.8 Hz, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 18.3, 24.7, 25.3, 28.6, 28.7, 29.1, 29.2, 29.6, 31.7, 62.3, 68.4, 68.5, 69.5, 70.2, 70.5, 70.6, 70.8, 71.0, 71.1, 71.7, 72.3, 72.4, 72.7, 79.5, 80.5, 81.6, 82.9, 84.0, 84.2, 84.3, 89.9, 104.3; HRMS (MALDI-TOF) calcd for C₆₀H₈₆NaO₁₁ [M+Na]⁺ 1005.6068, observed 1005.6068.

2.1.3. Solid phase synthesis (Scheme 3)

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 20% piperidine in DMF (15 mL) was added for 2 min to cleave the 9-fluorenylmethyoxycarbonyl (Fmoc) group. The liquid phase was removed, and 20% piperidine solution in DMF (15 mL) was again added and the mixture shaken for 18 min. The liquid phase was removed, and the resin was washed with DMF (3×15 mL), DCM (3×15 mL), DMF (3×15 mL), 0.5 M 1-hydroxybenzotriazole (HOBt) in DMF (15 mL), 0.5 M HOBt in DMF (15 mL) plus a drop of bromophenol blue, DMF (2×15 mL), and DCM (15 mL), in that order. A solution of the first Fmoc-amino acid, Fmoc-Phe (1.05 g, 2.04 mmol), and diisopropylcarbodiimide (DIC, 512 mg, 4.08 mmol) in DMF

Table 1Mass spectral and HPLC characterization of compounds 11 and 12

Compound	Formula [M]	Calcd mass [lon]	Mass found (error)	$t_{\rm R}^{\rm a}$
11	$\begin{array}{c} C_{36}H_{47}N_9O_7\\ C_{44}H_{60}N_{14}O_5 \end{array}$	718.3671 [M+1] ⁺	718.3669 (0.3 ppm)	23.52
12		800.4454 [M+1] ⁺	800.4450 (0.5 ppm)	24.16

 $^{\rm a}$ Linear gradient of from 10–>90% acetonitrile in water containing 0.1% TFA over 50 min.

(15 mL) was allowed to react for 2 min, then added to the resin and the mixture shaken for 1 h, at which time the blue color had disappeared. The resin was then washed with DMF (3×15 mL), DCM $(3 \times 15 \text{ mL})$, and DMF $(3 \times 15 \text{ mL})$. Free NH₂ groups were 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), DCM (3×15 mL), and DMF $(3 \times 15 \text{ mL})$. The absence of free amine groups was confirmed by the Kaiser test. The above cycle of procedures was repeated for coupling of each of the amino acids in the sequence, and finally for attachment of the N-terminal 6-azidohexanoic acid³⁴ residue or the N-terminal 6-(4-butyl-1H-1,2,3-triazol-1-yl)hexanoic acid¹⁸ residue, thus producing the resin-bound peptide derivatives related to compounds 11 and 12. 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 diethyl ether, and the crude product collected by centrifugation. Purification of the tetrapeptide products 11 and 12 was accomplished by preparative reversed phase HPLC. Yields ranged from 39% to 46% over several batches. The purity of compounds 11 and 12 was checked by analytical reversed phase HPLC. Compounds 11 and 12 were recovered from solution by lyophilization and were further characterized by ESI mass spectrometry (see Table 1).

2.1.4. Multimer synthesis (Scheme 4)

2.1.4.1. Serine amide multimer 13. A mixture of 9 (10 mg. 10 µmol), azide **10¹⁸** (39 mg, 162 µmol), trisl(1-benzyl-1**H**-1.2.3triazol-4-yl)methyl]amine (TBTA, 4.3 mg, 8 µmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (3 mg, 8 µmol) in dry methanol (1.5 mL) was irradiated for 4 h in a Biotage microwave reactor (100 °C). After the reaction was complete, water (25 mL) was added and the mixture was extracted with CHCl₃ containing dithizone (20 mg/L, 3×15 mL) to remove copper.³⁵ The water layer was then washed with DCM (2×15 mL) to remove any remaining **10** and TBTA. After lyophilization, fractionation of the residue by preparative HPLC (10 \rightarrow 90% acetonitrile in water containing 0.1% TFA within 50 min, $t_{\rm R}$ 11.1 min) and recovery by lyophilization afforded 4.5 mg (1.5 μ mol, 15% yield) of 13 as a white solid, mp 72–73 °C, $[\alpha]_D^{25}$ 6.5 (**c** 0.55, CHCl₃); IR (cm⁻¹) 3283, 2926, 2852, 1635, 1212, 1152; ¹H NMR (500 MHz, CD₃OD) δ 1.31– 1.36 (m, 16H), 1.57-1.70 (m, 48H), 1.88-191 (m, 16H), 2.27 (t, J = 7.0 Hz, 16H), 2.68–2.72 (m, 16H), 3.06–3.09 (m, 1H), 3.15–3.20 (m, 1H), 3.33–3.95 (m, 43H), 4.05 (d, **J** = 7.5 Hz, 1H), 4.33–4.42 (m, 23H), 5.50 (d, **J** = 3.5 Hz, 1H), 7.81 (br s, 8H); ¹³C NMR (125 MHz, CD₃OD) & 24.6, 25.6, 25.7, 25.8, 28.8, 29.2, 29.5, 35.1, 49.9, 55.1, 61.8, 66.2, 68.0, 69.6, 70.0, 70.2, 70.4, 70.6, 70.9, 71.0, 71.7, 71.9, 72.2, 72.5, 77.9, 79.2, 80.3, 81.3, 82.0, 83.7, 89.3, 92.5, 104.1, 109.4, 111.5, 113.4, 115.8, 122.1, 122.2, 147.3, 173.7, 174.5; HRMS (MALDI-TOF) calcd for C₁₃₂H₂₂₂N₄₀NaO₃₅ [M+Na]⁺ 2950.6719, observed 2950.6423.

2.1.4.2. Procedure for reaction of azide-functionalized MSH4 derivative 1 and 1-azidohexane with 9 to produce multivalent constructs 14a. A mixture of 9 (56 mg, 56 μmol), azide 1¹⁸ (15 mg, 19 μmol), TBTA (2.0 mg, 3.8 μmol), and tetrakis(acetoni-

trile)copper(I) hexafluorophosphate (1.4 mg, 3.8 µmol) in dry methanol (1.5 mL) was irradiated for 2 h in a Biotage microwave reactor (100 °C). After the reaction was complete, water (25 mL) was added and the mixture extracted with CHCl₃ containing dithizone (20 mg/ L, 3×15 mL) and with DCM (3×15 mL) to remove copper,³⁵ TBTA, and excess 9. After lyophilization, the residue (30 mg) was taken up in dry methanol (1.5 mL), 1-azidohexane (97 mg, 763 µmol), TBTA (6.3 mg, 11.8 µmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (4.4 mg, 11.8 µmol) were added, and the reaction mixture was irradiated for 4 h in a Biotage microwave reactor (100 °C). Following workup as above and lyophilization, the residue was fractionated by preparative HPLC ($10 \rightarrow 90\%$ acetonitrile in water containing 0.1% TFA within 50 min, t_R 29–33 min) to afford **14a** as an oil; yield 13 mg (4.9 µmol, 26%). Product 14a was analyzed by MAL-DI-TOF (see Fig. S2 in the Supplementary data) and by UV spectroscopy.18

2.1.4.3. Procedure for reaction of azide-functionalized MSH4 derivative 1 and azide-functionalized serine amide derivative 10 with 9 to produce multivalent constructs 14b. A mixture of **9** (56 mg, 56 µmol), azide **1**¹⁸ (15 mg, 19 µmol), TBTA (2.0 mg, 3.8 μ mol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (1.4 mg, 3.8 µmol) in dry methanol (1.5 mL) was irradiated for 2 h in a Biotage microwave reactor (100 °C). After the reaction was complete, water (25 mL) was added and the mixture extracted with CHCl₃ containing dithizone (20 mg/L, 3×15 mL) and with DCM $(3 \times 15 \text{ mL})$ to remove copper,³⁵ TBTA, and excess **9**. After lyophilization, the residue (29 mg) was taken up in dry methanol (1.5 mL), azide 10^{18} (54 mg, 224 μmol), TBTA (6.9 mg, 3.8 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (4.8 mg, 3.8 µmol) were added, and the reaction mixture was irradiated for 4 h in a Biotage microwave reactor (100 °C). Following workup as above and lyophilization, the residue was fractionated by preparative HPLC ($10 \rightarrow 90\%$ acetonitrile in water containing 0.1% TFA within 50 min, $t_{\rm R}$ 12.5–16.5 min) to afford **14b** as a white powder; yield 3.0 mg (0.87 µmol, 5%). Product 14b was analyzed by MALDI-TOF (see Fig. S3 in the Supplementary data) and by UV spectroscopy.¹⁸

2.1.4.4. General procedure for reaction of azide-functionalized MSH4 derivative 1 and azide-functionalized serine amide derivative 10 with 9 to produce multivalent constructs Mixtures of 9 (variable amount, see Table 2), azide 14с-е. 1^{18} (variable amount, see Table 2), TBTA (0.43 mg/µmol of **9**), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (0.3 mg/µmol of 9) in dry methanol (100 μ L/ μ mol of 9) were irradiated for 2-4 h in a Biotage microwave reactor (100 °C). Azide **10**¹⁸ (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 (2.5 mL/µmol of 9) was added and the mixtures were extracted with CHCl₃ containing dithizone (20 mg/L, 3×15 mL) and with DCM (3×15 mL) to remove copper,³⁵ TBTA, and excess 10. After lyophilization, the residues were fractionated by preparative HPLC $(10\rightarrow 90\%)$ acetonitrile in water containing 0.1% TFA within 50 min, t_R 12.5–16.5 min) to afford products

Table 2				
Synthesis	of	13	and	14a-

Product	mg 9	mg 1 (equiv)	mg 10 (equiv)	Yield, mg (%)	MALDI-TOF
13	10	0	39 (16)	4.5 (15)	Figure S1
14a	56	15 (0.34)	97 (45) ^a	13 (26)	Figure S2
14b	56	15 (0.34)	63 (16)	3.0 (5)	Figure S3
14c	18	29 (2)	44 (10)	44 (61)	Figure S4
14d	7.0	17 (3)	17 (10)	14 (42)	Figure S5
14e	8.0	31 (5)	20 (10)	22 (49)	Figure S6

^a 1-Azidohexane used in place of 10.



Figure 3. MALDI-TOF mass spectrum of 14e showing the distribution of products. The numbers indicate the number of attached MSH4 ligands. The insert shows the anticipated statistical product distribution assuming attachment of 4.1 ligands per scaffold. For peak identification and for spectra of 13, 14a–14e, and 15a see the Supplementary Data.

14c–14e as white powders; yields are given in Table 2. Products were analyzed by MALDI-TOF (see Fig. 3 and Figs. S4–S6 in the Supplementary data) and by UV spectroscopy.¹⁸

2.1.4.5. Procedure for reaction of azide-functionalized CCK4 derivative 11 and azide-functionalized serine amide derivative 10 with 9 to produce multivalent constructs 15a. A mixture of **9** (205 mg, 209 µmol), azide **11** (30 mg, 41 µmol), TBTA (4.3 mg, 8.2 µmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (3 mg, 8.2 µmol) in dry DMF (1.5 mL) was irradiated for 2 h in a Biotage microwave reactor (100 °C). After the reaction was complete, the DMF was evaporated under reduced pressure to afford a residue that was subjected to silica gel chromatography (63-210 µm) using DCM/methanol (9:1). After evaporation of the volatile materials, the residue (40 mg) was taken up in dry DMF (1.5 mL), azide **10¹⁸** (57 mg, 230 µmol), TBTA (8 mg, 16 µmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (6 mg. 16 µmol) were added, and the reaction mixture was irradiated for 6 h in a Biotage microwave reactor (100 °C). Following workup as described above for compounds 14c-e and lyophilization, the residue was fractionated by preparative HPLC ($10 \rightarrow 90\%$ acetonitrile in water containing 0.1% TFA within 50 min, $t_{\rm R}$ 29 min) to afford 15a as white solid; yield 14 mg (4.1 µmol, 17%). Product 15a was analyzed by MALDI-TOF (see Fig. S7 in the Supplementary data) and by UV spectroscopy.¹⁸

Table 3		
Synthesis	of	15a–d

Product	mg 9	mg 11 (equiv)	mg 10 (equiv)	Yield, mg (%)	MALDI-TOF
15a	205	30 (0.2)	57 (10) ^a	14 (17) ^a	Figure S7
15b	17	25 (2)	50 (12)	15 (23)	nr ^b
15c	19	41 (3)	47 (10)	11 (13)	nr ^b
15d	11	40 (5)	27 (10)	15 (26)	nr ^b

 a Equivalents and yield based on 40 mg (23 $\mu mol)$ of purified mono-CCK4 intermediate; see Experimental Section for details.

^b MALDI-TOF spectra showed no ions that could be identified as belonging to product. Yield determined by sample weight and UV spectroscopy.

2.1.4.6. Procedure for reaction of azide-functionalized CCK4 derivative 11 and azide-functionalized serine amide derivative 10 with 9 to produce multivalent constructs 15b-d. A mixture of 9 (variable amounts, see Table 3), azide 11 (variable amounts, see Table 3), TBTA (0.43 mg/µmol of 9), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (0.3 mg/µmol of 9) in dry DMF (1.5 mL) was irradiated for 2 h in a Biotage microwave reactor (100 °C). Azide **10¹⁸** (variable amounts, see Table 3) was then added to the reaction mixtures and irradiation was resumed for another 4 h. After the reactions were complete, water (2.5 mL/ umol of **9**) was added and the mixtures were extracted with CHCl₂ containing dithizone (20 mg/L, 2×15 mL) to remove copper.³⁵ The water layers were then washed with DCM (2×15 mL) to remove any remaining **10** and TBTA. After lyophilization, the residues were fractionated by preparative HPLC ($10 \rightarrow 90\%$ acetonitrile in water containing 0.1% TFA within 50 min, $t_{\rm R}$ 22–25 min) to afford products 15b-d as white solids; yields are given in Table 3. Products were analyzed by UV spectroscopy.¹⁸

2.2. Biological studies

2.2.1. Formulation of solutions

Solutions of MSH4 and CCK4 constructs for binding assays were made up in water and dimethylsulfoxide (HYBRI-MAX), respectively, based on the expected incorporations of MSH4 or CCK4. Concentrations of ligand in solution were then determined by measurement of the UV absorbance at 280 nm using a standard calibration plot.¹⁸

2.2.2. Binding assays

Quantitative receptor-binding assays were carried out following a previously described method.¹¹ Hek293 cells engineered to express both CCK2R and MC4R were used to assess ligand binding. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. For MC4R assays, cells were seeded in PerkinElmer, tissue culture treated, black-frame with white well, 96-well plates (part # 6005060) at a density of 1.5×10^4 cells per well and were allowed to reach 80–90% confluence. For CCK2R assays, 2.5×10^5 cells were plated in each well of

Table 4 Competitive binding of MSH4, CCK4, 12, 13, 14a–14e, 15a–15d, and 17 to MC4R or CCK2R

Compound	MC4R		CCK2R	
	K_i^a (μ M)	n ^b	K_i^a (nM)	n ^b
MSH4	1.3 ± 0.38	5	nd ^c	
17	1.9 ± 0.14	5	nd	
14a	7.3 ± 1.1	4	nd	
14b	1.6 ± 0.16	4	nd	
14c	0.54 ± 0.04	4	nd	
14d	0.23 ± 0.02	4	nd	
14e	0.17 ± 0.02	4	nd	
CCK4	nd		3.1 ^d	
12	nd		18 ± 5.7	3
15a	nd		67 ± 9.4	3
15b	nd		1.5 ± 0.7	3
15c	nd		2.0 ± 0.3	3
15d	nd		0.80 ± 0.2	3
13	nb ^e	3	nb ^e	2

^a K_i values were calculated using the equation $K_i = \text{EC}_{50}/(1 + ([ligand]/K_D))$ where [ligand] = 10 nM and K_D = 8.3 nM for probe **4** and [ligand] = 2 nM and K_D = 34.6 nM for probe **16**.

^b The value given represents the average of n independent competition binding experiments, each done in quadruplicate.

^c Not determined.

d Taken from Ref.30.

^e Compound **13** was unable to inhibit the binding of probe **4** or probe **16** in the concentration range tested $(10^{-5}-10^{-12} \text{ M in serine amide})$.

SigmaScreen poly-o-lysine coated 96-well, black/clear bottom plates (catalog # M5307-SEA) and incubated for 48 h at 37 °C. On the day of the experiment, media was removed from all wells. Ligands were diluted in binding buffer (MEM, 25 mM Hepes [pH 7.4], 0.3% BSA, 1 mM 1,10-phenanthroline, 0.5 mg/L leupeptin, and 200 mg/L bacitracin). Test compound solutions (50 μ L), in a range of dilution concentrations, were added to 50 µL of a 10 nM solution of probe 4 (MC4R assay) or a 2 nM solution of probe 16 (CCK2R assay). Each concentration was tested in quadruplicate. Cells were incubated in the presence of unlabeled and labeled ligands at 37 °C and 5% CO2 for 1 h. Following incubation, media was removed and the wells washed $3 \times$ with wash buffer (50 mM tris(hydroxymethyl)aminomethane hydrochloride, 0.2% BSA, 30 mM NaCl). Enhancement solution (PerkinElmer 1244-105) was added (100 μ L/well) and plates were incubated for 30 min at 37 °C before measuring fluorescence using a VICTOR™ X4 2030 Multilabel Reader (PerkinElmer) instrument and the standard Eu time-resolved fluorescence (TRF) measurement settings (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 $K_i = EC_{50}/(1 + ([ligand]/K_D))$ where [ligand] refers to the concentration of the probe used as the labeled competed ligand. For probe **4**, [ligand] = 10 nM and $K_D = 8.3$ nM. For probe **16**, [ligand] = 2 nM and $K_D = 34.6$ nM. Results are given in Table 4. The value given represents the average of n independent competition binding experiments.

3. Results

3.1. Chemistry

Reaction of sucrose with 24 equiv of sodium hydride in DMF, followed by addition of 12 equiv of 6-bromo-1-hexyne³³ afforded octaalkyne **9** in 43% yield after chromatography (Scheme 2). Azides **1** and **10** were prepared as previously described.¹⁸ Azide **11** was

prepared by solid phase synthesis^{36,37} on Rink amide Tentagel S resin as depicted in Scheme 3. 6-Azidohexanoic acid³⁴ 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, triisopropylsilane, thioanisole, and water (91:3:3:3), producing the desired azide-terminated ligand, N₃(CH₂)₅(C=O)-Trp-Met-Asp-Phe-NH₂ (**11**). The triazole-containing ligand CH₃(CH₂)₃(C₂N₃)(CH₂)₅(C=O)-Trp-Met-Asp-Phe-NH₂ (**12**) was prepared by N-terminal acylation of the resin-bound tetrapeptide with 6-(4-butyl-1*H*-1,2,3-triazol-1-yl)hexanoic acid¹⁸ in place of 6-azidohexanoic acid. Compounds **11** and **12** were purified by reversed phase C₁₈ preparative HPLC (yields ranged from 39% to 46%) and were characterized by analytical HPLC and ESI-MS. Details appear in Table 1.

Reaction of **9** with an excess of **10**¹⁸ in the presence of tetrakis(acetonitrile)copper(I) hexafluorophosphate and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in methanol in a Biotage microwave reactor at 100 °C gave the corresponding serine amide octamer **13** (Scheme 4). Copper ions were removed from this mixture by complexation with dithizone and removal of the complex by extraction with CHCl₃.³⁵ Other small organic-soluble molecules (TBTA, excess **10**) were also removed during this extraction. The water-soluble product **13** was purified by preparative reversed phase HPLC, recovered by lyophilization, and characterized by MALDI-TOF mass spectrometry (see Fig. S1 in the Supplementary data).

Reaction of **9** with varying amounts of **1** in the presence of copper(I) and TBTA in methanol in a Biotage microwave reactor at 100 °C, followed by reaction either with an excess of 1-azidohexane or with the serinamide-derived azide **10** under the same conditions, gave the corresponding multimeric mixtures **14a–14e**. Copper ions and small organic molecules were removed as previously described. The crude product mixtures were further purified by preparative reversed phase HPLC and were characterized by MALDI-TOF mass spectrometry (see Fig. 3 and Figs. S2–S6 in the Supplementary data). The average numbers of R(C=O)-MSH4-NH₂ ligands per scaffold were determined to be 1.0, 1.0, 1.3, 2.6, and 4.1 for the multimeric mixtures **14a–14e**, respectively, by UV spectroscopy (see Section 4 and the Supplementary Data for details).¹⁸

Similarly, reaction of **9** with varying amounts of azide **11** in the presence of copper(I) and TBTA in DMF in a Biotage microwave reactor at 100 °C, followed by reaction with an excess of azide **10** under the same conditions, gave the corresponding multimeric



$$C_4H_9$$

N-(CH₂)₅-C(=O)Trp-Met-Asp-Phe-NH₂
N²N

12

Scheme 3. Solid phase synthesis. Reagents: (a) piperidine; (b) Fmoc/tBu solid phase synthesis; (c) $N_3(CH_2)_5CO_2H$, Cl-HOBt, DIC; (d) trifluoroacetic acid/triisopropylsilane/thioanisole/water (91/3/3/3).



Scheme 4. Synthesis of multivalent compounds 13, 14a-14e, and 15a-d via CuAAC. The positions of ligand attachment are presumed to be random.

mixtures **15a–15d**. Copper ions and small organic molecules were removed as previously described. The crude product mixtures were further purified by preparative reversed phase HPLC and were characterized, in the case of **15a**, by MALDI-TOF mass spectrometry (see Fig. S7 in the Supplementary Data). Satisfactory mass spectra of **15b–15d** could not be obtained. The average numbers of R(C=O)-CCK4-NH₂ ligands per scaffold were determined to be 1.0, 1.3, 2.2, and 3.6 for the multimeric mixtures **15a–15d**, respectively, by UV spectroscopy.¹⁸

3.2. Bioassays

Hek293 cells overexpressing both MC4R^{22,38} and CCK2R^{30,39} were used to assess ligand binding using previously described europium-based competitive binding assays that employed Eu-DTPA-NDP- α -MSH-NH₂ (**4**) or Eu-DTPA-CCK8-NH₂ (**16**) as the labeled probe.¹¹ The K_i values for the parental ligands CH₃C(C=O)-His-DPhe-Arg-Trp-NH₂ (listed as MSH4) and Trp-Met-Asp-Phe-NH₂ (listed as CCK4), for the corresponding triazole-containing control compounds **12** and **17**¹⁸, and for the constructs **13**, **14a–14e**, and **15a–15d** are listed in Table 4.



4. Discussion

The one-step synthesis of octaalkyne **9** starting from sucrose was reasonably efficient and can provide access to multigram quantities of this product. Presumably, the identities of the sugar starting material and the alkylating agent can be varied to produce many such scaffolds. Microwave-driven CuAAC was used to attach zero, one, or an average of 1.3, 2.6, or 4.1 copies of the MSH4 azide 1 to scaffold 9. The remaining alkyne residues of the scaffold were then reacted with 1-azidohexane to produce 14a, or with serinamide-derived azide 10 to produce 13 and 14b-e. The constructs so produced were purified from copper and from small molecules by extraction, further purified by preparative reversed phase HPLC, and characterized by MALDI-TOF mass spectrometry and by UV spectroscopy. While MALDI-TOF analysis was reasonably consistent with statistical attachment of ligands in the case of 14c, the mass spectra for 14d and 14e suggested that ligand attachment was not statistical (see Fig. 3 and Figs. S4–S6 in the Supplementary data). MALDI-TOF analysis of a 1:1 mixture of monovalent and divalent MSH4 constructs on a squalene scaffold^{17,27} demonstrated a highly attenuated sensitivity for detection of the divalent construct. Thus, MALDI-TOF analysis of multimers 14c-14e is qualitative, with actual distributions of MSH4 ligands somewhere between the MALDI-TOF limit of analysis and the expected statistical distributions. For this reason, concentrations of multivalent species for bioassays were determined by UV analysis (see calculations in the Supplementary data).

Constructs **15a–15d** bearing one or an average of 1.3, 2.2, or 3.6 CCK4 ligands were similarly prepared from scaffold **9**, CCK4 azide **11**, and azide **10**. In principle, other ligands, imaging agents, and/ or therapeutic agents might be attached to scaffold **9**.

6481

Constructs 13, 14a-e, and 15a-d were subjected to biological testing using previously described competitive binding assays.¹¹ Serinamide derivative 13 was ineffective at blocking probes 4 and 16 from binding to cells displaying MC4R and CCK2R over the range of concentrations tested (Table 4). The K_i for the monovalent MSH4 control compound 17 was 1.5 times the value for the parental ligand, indicating that attachment of the triazole-containing 'spacer' to the N-terminus of MSH4 has a modest detrimental effect on ligand binding to MC4R.¹⁸ The K_i values differed among the various sucrose-derived constructs 14a-e. Comparison of the K_i values for **14a** and **14b** suggests that termination of the non-MSH4-bearing sidechains with the more hydrophilic serinamide residues, as opposed to hydrophobic alkyl groups, enhances construct binding. That this enhancement is not due to specific binding by the serinamide residues is supported by the inactivity of compound **13**. While the *K*_i values decrease with higher levels of MSH4 incorporation (compare **14b-e**), the observed changes can be attributed to statistics and proximity effects and suggest effective, but monovalent binding of 14b-e at available MC4R. These results are consistent with results from solanesol-derived multivalent MSH4 constructs¹⁸ and divalent MSH4 constructs de-rived from a flexible linker.⁸ One might suppose that **14b–e** are competent binders as monovalent species, but are incompetent binders as multivalent species due to improper ligand spacing and/or presentation for binding.⁴⁰ However, as to ligand spacing, estimates of the distance between ligand binding sites for adjacent receptors range from 20 to 50 Å.⁹ If a spherical distribution of ligands is assumed for these sucrose-derived multivalent constructs, the maximum distance between ligands is approximately 40 Å.⁴¹ If a linear array of ligands is assumed for the solanesol-derived multivalent constructs, the distances between ligands are variable with a maximum separation of approximately 90 Å.⁴² As to ligand presentation, it seems unlikely that all of the MSH4 multivalent and bivalent constructs studied would improperly present a second ligand for binding, given the wide range of permitted N-terminal and C-terminal modifications of this ligand⁴³ and the fact that the observed binding affinities for the first ligand are comparable to the parental ligand.

$$CH_3(CH_2)_3$$

 $N = (CH_2)_5(CO)$ -His-DPhe-Arg-Trp-NH₂
 $N = N$

Monovalent binding of multivalent MSH4 constructs would be expected if the off rates of monovalently bound constructs are faster than the binding of a second ligand arm to MC4R on the cell surface. The NDP- α -MSH ligand, upon which **4** is based, is known to have a slow off-rate ($t_{1/2} \sim 8$ h).⁴⁴ The off rates of MSH4 and related constructs, such as **14a–b** and **17**, are unknown, but are expected to be much larger than the rates for NDP- α -MSH and presumably for **4**. For rigid linkers that are too short to bridge between adjacent receptors, small increases in avidity have been attributed to statistically- and proximity-enhanced rebinding.⁷ In these cases, the shorter the linker the greater the avidity.^{6,7} These observations are consistent with fast off-rates for monovalently bound MSH4 constructs.

Another factor that may inhibit multivalent binding of **14c–e** is reduction in the number of MC4R receptors at the cell surface due to receptor cycling. In support of this point, internalization of probe **4** contributes significantly to the fluorescence measured in these assays, which may more properly be termed 'binding and uptake' assays. In a preliminary study 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.⁴⁵

Results from assays with CCK4 constructs 15a-d stand in contrast with results from assays with MSH4 constructs 14b-e. The K_i for the monovalent CCK4 control compound **12** was six times the value for the parental ligand, indicating that attachment of the triazole-containing 'spacer' to the N-terminus of CCK4 has a significant detrimental effect on ligand binding to CCK2R. The K_i values differed among the various sucrose-derived constructs 15a–d. The K_i value for 15a which bears one copy of the CCK ligand was 22 times the value for the parental ligand and 3.7 times the value for the control compound **12**. Apparently, attachment of the CCK4 ligand to the sucrose-derived scaffold has a much greater negative effect on binding than was observed for MSH4. This observation is in keeping with the more restrictive limits on N-terminal substitution for this ligand.⁴⁶ The K_i values decrease with higher levels of CCK4 incorporation, with 15b-d of comparable potency and each 30–80 times as potent as **15a**. suggestive of multivalent binding. Since the affinity of the parental CCK4 ligand for its receptor is 420 times that of the parental MSH4 ligand for its receptor, it seems reasonable to assume that the off-rates of **15a-d** are lower than the off-rates for **14b-e**, perhaps providing sufficient time for a second binding event to occur before dissociation of the monovalently bound construct. The fact that the greater multivalency of 15d does not more considerably enhance the avidity observed for **15c** or **15b** may be a consequence of the spatial distribution of the CCK4 ligands attached to this spherical scaffold, which necessitates that on average half of the ligands must be oriented away from the cell surface. This fact limits the number of simultaneous attachments that can be made through ligand binding. For a fully loaded sucrose-derived scaffold bearing eight copies of ligand, there can be at most four simultaneous attachments. For steric and/or electrostatic reasons, it is unlikely that full scaffold loading with ligand could commonly be achieved. Assuming half loading (four ligands per scaffold) or less, as is the case for 14c-e and 15b-d, the analysis above suggests that bivalent attachment is a more realistic expectation.

Acknowledgments

This work was supported by grants R33 CA 95944, RO1 CA 97360, RO1 CA 123547, and P30 CA 23074 from the National Cancer Institute.

Supplementary data

Supplementary data (mass spectra of multivalent constructs **13**, **14a–e**, and **15a**, analysis of ligand distribution for **14c–e** and **15b–d**, and ¹H and ¹³C NMR spectra of compounds **9** and **13**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.08.053.

References and notes

- 1. Weissleder, R.; Mahmood, U. Radiology **2001**, 219, 316.
- 2. Gillies, R. J.; Hruby, V. J. Expert Opin. Ther. Targets 2003, 7, 137.
- 3. Cassidy, P. J.; Radda, G. K. J. R. Soc. Interface 2005, 2, 133.
- 4. Morse, D. L.; Gillies, R. J. Biochem. Pharmacol. 2010, 80, 731.
- Handl, H. L.; Vagner, J.; Han, H.; Mash, E.; Hruby, V. J.; Gillies, R. J. Expert Opin. Ther. Targets 2004, 8, 565.
- Vagner, J.; Handl, H. L.; Gillies, R. J.; Hruby, V. J. Bioorg. Med. Chem. Lett. 2004, 14, 211.
- 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.
- 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.
- Handl, H. L.; Sankaranarayanan, R.; Josan, J. S.; Vagner, J.; Mash, E. A.; Gillies, R. J.; Hruby, V. J. *Bioconjugate Chem.* 2007, 18, 1101.
- 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.

- Xu, L.; Vagner, J.; Josan, J. S.; Lynch, R. M.; Morse, D. L.; Baggett, B.; Han, H.; Mash, E. A.; Hruby, V. J.; Gillies, R. J. Mol. Cancer Ther. 2009, 8, 2356.
- 12. Mammen, M.; Choi, S.-K.; Whitesides, G. M. Angew. Chem., Int. Ed. 1998, 37, 2754.
- Kiessling, L. L.; Gestwicki, J. E.; Strong, L. E. Angew. Chem., Int. Ed. 2006, 45, 2348.
- Krishnamurthy, V. M.; Estroff, L. A.; Whitesides, G. M. In *Fragment-based Approaches in Drug Discovery*; Jahnke, W., Erlanson, D. A., Eds.; Wiley-VCH: Weinheim, 2006; pp 11–53.
- Carlson, C. B.; Mowery, P.; Owen, R. M.; Dykhuizen, E. C.; Kiessling, L. L. ACS Chem. Biol. 2007, 2, 119.
- Choi, S.-K. Synthetic Multivalent Molecules; Wiley-Interscience: Hoboken, NJ, 2004.
- 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.
- Alleti, R.; Rao, V.; Xu, L.; Gillies, R. J.; Mash, E. A. J. Org. Chem. 2010, 75, 5895.
 The 'low-affinity' ligand MSH4, Ac-His-DPhe-Arg-Trp-NH₂, was based on the
- minimal active sequence for full agonist activity of α-MSH; see: 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 Lauro Castrucci, A. M.; Hintz, M. F.; Riehm, J. P.; Rao, K. R. J. Med. Chem. **1987**, 30, 2126.
- 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.
- 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.
- 22. The MC4R 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.
- 23. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. Angew. Chem., Int. Ed. 2001, 40, 2004.
- 24. Gil, M. V.; Arévalo, M. J.; López, Ó. Synthesis 2007, 1589.
- 25. Angell, Y. L.; Burgess, K. Chem. Soc. Rev. 2007, 36, 1674.
- Holub, J. M.; Garabedian, M. J.; Kirshenbaum, K. QSAR Comb. Sci. 2007, 26, 1175.

- 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.
- The 'high-affinity' ligand, NDP-α-MSH, is Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂; see: 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.
- Hadley, M. E.; Anderson, B.; Heward, C. B.; Sawyer, T. K.; Hruby, V. J. Science 1981, 213, 1025.
- 30. Horwell, D. C. Neuropeptides 1991, 19, 57.
- 31. Akoh, C. C. Food Sci. Technol. 2002, 117, 695.
- 32. Bimal, C.; Guonong, Z. Food Rev. Int. 2006, 22, 245.
- 33. Sharma, S.; Oehlschlager, A. C. J. Org. Chem. 1989, 54, 5064.
- Grandjean, C.; Boutonnier, A.; Guerreiro, C.; Fournier, J.-M.; Mulard, L. A. J. Org. Chem. 2005, 70, 7123.
- 35. Caplin, S. Tissue Cult. Assoc. Man. 1976, 2, 439.
- 36. Merrifield, R. B. J. Am. Chem. Soc. 1963, 85, 2149.
- 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.
- Yang, X.; Wang, Z.; Dong, W.; Ling, L.; Yang, H.; Chen, R. J. Prot. Chem. 2003, 22, 335.
- Noble, F.; Wank, S. A.; Crawley, J. N.; Bradwejn, J.; Seroogy, K. B.; Hamon, M.; Roques, B. P. *Pharmacol. Rev.* **1999**, *51*, 745.
- Shewmake, T. A.; Solis, F. J.; Gillies, R. J.; Caplan, M. R. Biomacromolecules 2008, 9, 3057.
- 41. This estimate is based on molecular dynamics studies of compound 13 in a water environment. Studies of components present in 15a–d are in progress.
- 42. This estimate is based on examination of Dreiding molecular models in which the chain is fully extended.
- 43. Holder, J. R.; Haskell-Luevano, C. Med. Res. Rev. 2004, 24, 325.
- Haskell-Luevano, C.; Miwa, H.; Dickinson, C.; Hadley, M. E.; Hruby, V. J.; Yamada, T.; Gantz, I. J. Med. Chem. 1996, 39, 432.
- 45. Xu, L. Unpublished work.
- 46. de Tullio, P.; Delarge, J.; Pirotte, B. *Curr. Med. Chem.* **1999**, 6, 433. and references cited therein.