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Synthesis and Characterization of Time-resolved Fluorescence Probes for Evaluation of Competitive Binding to Melanocortin Receptors

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Abstract

Probes for use in time-resolved fluorescence competitive binding assays at melanocortin receptors based on the parental ligands MSH(4), MSH(7), and NDP-α-MSH were prepared by solid phase synthesis methods, purified, and characterized. The saturation binding of these probes was studied using HEK-293 cells engineered to overexpress the human melanocortin 4 receptor (hMC4R) as well as the human cholecystokinin 2 receptor (hCCK2R). The ratios of non-specific binding to total binding approached unity at high concentrations for each probe. At low probe concentrations, receptor-mediated binding and uptake was discernable, and so probe concentrations were kept as low as possible in determining K_d values. The Eu-DTPA-PEGO-MSH(4) probe exhibited low specific binding relative to non-specific binding, even at low nanomolar concentrations, and was deemed unsuitable for use in competition binding assays. The Eu-DTPA-PEGO probes based on MSH(7) and NDP-α-MSH exhibited K_d values of 27±3.9 nM and 4.2±0.48 nM, respectively, for binding with hMC4R. These probes were employed in competitive binding assays to characterize the interactions of hMC4R with monovalent and divalent MSH(4), MSH(7), and NDP-α-MSH constructs derived from squalene. Results from assays with both probes reflected only statistical enhancements, suggesting improper ligand spacing on the squalene scaffold for the divalent constructs. The K_i values from competitive binding assays that employed the MSH(7)-based probe were generally lower than the K_i values obtained when the probe based on NDP-α-MSH was employed, which is consistent with the greater potency of the latter probe. The probe based on MSH(7) was also competed with monovalent, divalent, and trivalent MSH(4) constructs that

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Supplementary Data

Supplementary data (copies of the HPLC chromatograms for compounds **2–7**, **13a–15a**, and **13b–15b**, HRMS data for compounds **2–4**, and the ^{1}H and ^{13}C NMR spectra of compounds **8–10**, **12a**, and **12b**) associated with this article can be found, in the online version, at doi:

previously demonstrated multivalent binding in competitive binding assays against a variant of the probe based on NDP-α-MSH. Results from these assays confirm multivalent binding, but suggest a more modest increase in avidity for these MSH(4) constructs than was previously reported.

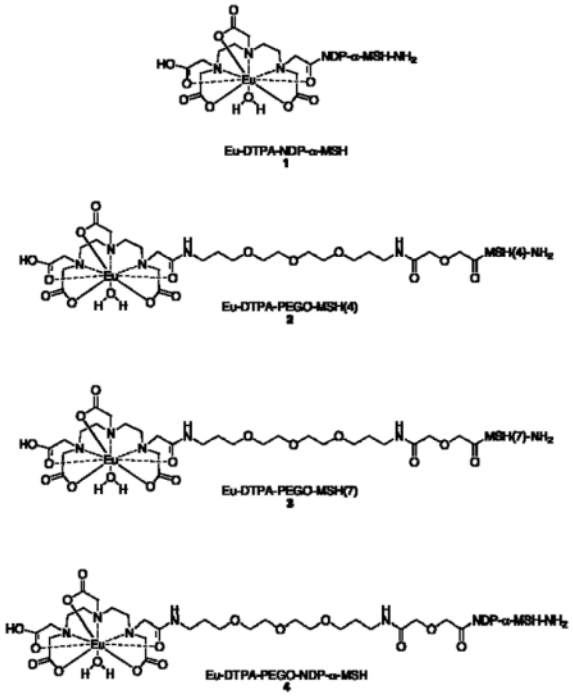
Keywords

competition binding assays; fluorescent probes; melanocortin 4 receptor; saturation binding assays; time-resolved fluorescence

1. Introduction

The affinity of a molecule for binding to a receptor is often quantified by a competitive binding assay against a labeled ligand of known potency. For example, labeled forms of Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 [NDP-α-MSH], a superpotent ligand that binds to melanocortin receptors,[1,2] have been used for this purpose.[3,4] In such assays, one often assumes thermodynamic control, i.e., that the respective on-rates and off-rates of both the competing and competed ligands are similar so that all bound and unbound states are in equilibrium. If this is not the case, details of how the assay is carried out (order and timing of reagent addition, timing of measurements taken) can affect the outcome. Determination of on-rates and off-rates for binding of molecules to living cells is difficult since ligands and labeled probes may be taken up by the cells and because receptors may cycle to and from the cell surface. In the absence of knowledge of ligand and probe on-rates and off-rates, a close match between the affinities of the competed probe and the competing ligand in a competitive binding assay would seem prudent–but is it necessary? This issue was examined experimentally as reported herein.

We have investigated the binding of multivalent molecules, including several derived from the weak ligand Ac-His-DPhe-Arg-Trp-NH_2 [MSH(4)][5–7], to human melanocortin 4 receptors (hMC4R).[8] MSH(4) was selected because synergistic effects are generally more easily detected for multivalent constructs of low-affinity ligands.[9–13] Probe **1** is based on NDP-α-MSH and was used to determine the values for many of K_i our multivalent constructs. However, we became concerned that competitions between superpotent probes such as **1** and multivalent constructs based on much weaker ligands such as MSH(4) were inherently unbalanced, and that perhaps the measured avidity of a competing multivalent construct depended on the affinity of the competed fluorescent probe. In a preliminary study, we prepared the Eu-DTPA-PEGO-MSH(4) probe **2** and tested it in saturation and competitive binding assays.[14] The K_d of **2**, determined by saturation binding to HEK-293 cells overexpressing hMC4R, was 9.1 μM, compared with a reported K_d for **1** of 8.3 nM.[15] We report herein syntheses of the structurally related probes Eu-DTPA-PEGO-MSH(7) **3** and Eu-DTPA-PEGO-NDP-α-MSH **4**, studies of the saturation binding of **2–4** with hMC4R, and the use of **3** and **4** in competitive binding assays involving monovalent and divalent MSH(4), MSH(7), and NDP-α-MSH constructs derived from squalene[16] and monovalent, divalent, and trivalent MSH(4) constructs that previously exhibited multivalent binding in competitive binding assays against a variant of probe **4**.[17]



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 I_2 vapor, or by staining with 10% phosphomolybdic acid solution in ethanol or with 5% H_2SO_4 in ethanol and heat. Gravity column chromatography was accomplished using silica gel 60 (63–210 μm). NMR spectra were recorded at 300 MHz or 500 MHz for ^{1}H NMR and at 75 MHz or 125 MHz for ^{13}C NMR. Chemical shifts (δ) are expressed in ppm and are internally referenced (7.24 ppm for $CDCl_3$ and 3.31 ppm for CD_3OD for ^{1}H NMR and 77.0 ppm for $CDCl_3$ and 49.15 ppm for CD_3OD for ^{13}C NMR). Preparative HPLC was performed on a 19 × 256 mm Waters X-Bridge Preparative C_{18} column. The mobile phase was 10–90% acetonitrile and water containing 0.1% trifluoroacetic acid (TFA) within 50 min. The flow rate was 15 mL/min. The dual UV detector system operated at 230 and 280 nm. ESI experiments were performed on an ESI Bruker Apex Qh 9.4 T FT-ICR instrument using standard ESI conditions. The samples were dissolved in acetonitrile/water 1:1 containing 0.1% formic acid in a concentration range of 1–30 μM.

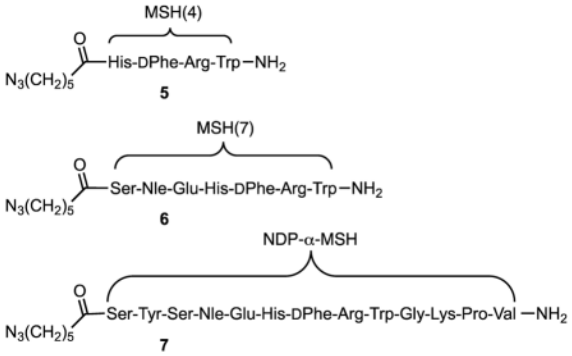
2.1.2. Solid phase synthesis—For the synthesis of probes **2–4**, resin-bound MSH(4), MSH(7), and NDP-α-MSH were synthesized manually using an N -Fmoc/*t*-Bu solid-phase peptide synthesis strategy and standard DIC/HOBt activation on Rink amide Tentagel S resin (approximately 0.24 mmol of active sites per gram). For the synthesis of ligands **5–7**, **16**, and **17**, resin-bound MSH(4), MSH(7), and NDP-α-MSH were synthesized manually using an N -Fmoc/*t*-Bu solid-phase peptide synthesis strategy and standard DIC/Cl-HOBt activation on Rink amide resin (approximately 0.68 mmol of active sites per gram) as follows. Resin (1.0 g) in a syringe (polypropylene reaction tube equipped with a

polypropylene frit) was allowed to swell in THF for 1 h. THF was removed, and 20% piperidine in DMF (15 mL) was added to deprotect the Fmoc functionality. After 2 min, the DMF/piperidine solution was removed, 20% piperidine in DMF (15 mL) was again added, and the mixture was shaken for 18 min. The DMF/piperidine solution was removed and the resin washed with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), 0.5 M HOBt in DMF (1 × 15 mL), 0.5 M HOBt in DMF (1 × 15 mL) plus a drop of 0.01 M bromophenol blue in DMF, DMF (2 × 15 mL), and DCM (1 × 15 mL), in that order. For Rink amide resin, a mixture of the appropriate Fmoc-amino acid (3 equivalents), Cl-HOBt (3 equivalents), and DIC (6 equivalents) in DMF (15 mL) was allowed to react for 2 min, was then added to the resin, and the mixture shaken for 1 h, during which time the blue color disappeared. The resin was then washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). Free NH_2 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 × 15 mL). The absence of free amine groups was confirmed by the Kaiser test. The above cycle of procedures was repeated for coupling of the other amino acids in the sequence, producing the resin-bound peptide derivatives. Further details for production of probes **2–4** and azides **5–7** are given in sections **2.1.2.1.** and **2.1.2.2.**

2.1.2.1. Synthesis and characterization of probes 2–4: Attachment of the PEGO linker was performed using DIC/HOBt activation (3 equiv Fmoc-PEGO[18], 3 equiv of HOBt, and 3 equiv of DIC). Next, the DTPA chelator was attached to the N-terminus of the resin–bound construct as follows. After Fmoc removal, the resin was washed with DMSO. DTPA dianhydride[19] (10 equiv) and HOBt (30 equiv) were dissolved in dry DMSO (1 mL) at 50 °C and then stirred for 20 min at rt. This mixture was injected into the syringe reactor which was shaken overnight, then the resin washed with DMSO, THF, 20% aqueous THF, THF, 5% DIEA in THF (5 min), THF, DMF, THF, and DCM. A cleavage mixture consisting of trifluoroacetic acid, water, 1,2-ethanedithiol, and thioanisole (91:3:3:3, 10 mL/g of resin) was injected into the syringe reactor containing the resin and the mixture shaken for 4 h at rt. The solution was then filtered off and the resin washed twice with TFA. Filtrates were collected, concentrated under a stream of nitrogen, and the product was precipitated by addition of cold ether to the residue. The peptide pellet was washed three times with cold ether, dried, dissolved in 1.0 M acetic acid, and lyophilized. The lyophilized DTPA-PEGO-MSH(4), DTPA-PEGO-MSH(7), and DTPA-PEGO-NDP-α-MSH constructs were purified by preparative HPLC and characterized by FT-ICR MS.

The metal-free precursors were dissolved in 0.1 M ammonium acetate, the pH was adjusted to 8 with aqueous 0.1 M NH_4OH, and 3 equiv of $EuCl_3 \bullet 6H_2O$ in water were added. The reaction mixture was stirred at rt overnight. The excess $EuCl_3$ and ammonium salts were removed using a Sep-Pak® C_{18} reverse-phase column with repetitive washing (20 mL of HPLC grade water). The final products **2–4** were eluted using 50% aqueous acetonitrile (4 mL), concentrated, lyophilized, and characterized by analytical HPLC and FT-ICR MS. Data appear in Table 1.

2.1.2.2. Synthesis and characterization of azides 5–7: Azide **5** was prepared from the corresponding resin-bound tetrapeptide and 6-azidohexanoic acid as previously described.[20] Azides **6** and **7** were prepared in a manner similar to **5**. Cleavage from the resin and deprotection were achieved using a cleavage mixture of trifluoroacetic acid, triisopropylsilane, thioanisole, and water (91:3:3:3). The mixture of cleavage cocktail and resin was shaken overnight, the solution was separated from the resin, volatiles were evaporated, the residue triturated with ether, and the crude product separated by centrifugation. Following purification by preparative HPLC, compounds **5–7** were characterized by ESI MS. Data appear in Table 1.



2.1.3. Scaffold synthesis and characterization (Scheme 1)

2.1.3.1. Synthesis and characterization of 2,6,10,15,19,23-hexamethyltetracosane-3,7,11,14,18,22-hexaol (8): In a three-necked flask a solution of borane-tetrahydrofuran complex in THF (1 M, 132 mL, 132 mmol) was deoxygenated with argon gas. The flask was immersed in an ice bath, and 2-methyl-2-butene (2 M, 132 mL, 264 mmol) also deoxygenated with argon gas was added to the borane solution dropwise with stirring at 0 °C. The resulting disiamyl borane was maintained at 0 °C for 3 h prior to use. A solution of squalene (3.0 gm, 7.3 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 d, then 3 N NaOH (80 mL) and 30% H_2O_2 (80 mL) were added. The reaction mixture was stirred at rt for 12 h and was then diluted with EtOAc (500 mL), washed with water (2 × 200 mL), brine (2 × 100 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo* to leave a viscous oil. The oil was subjected to gravity column chromatography on silica gel 60 eluted with chloroform-methanol (95:5), giving 1.94 g (51%) of **8** as a white, foamy solid, mp 42–46 °C, R_f 0.3 (silica gel 60, 1:9 MeOH:$CHCl_3$). IR (neat) cm^{-1} 3350 (br), 2956, 1464, 1376. NMR spectra are complex since the product is a mixture of regioisomers and stereoisomers. The ^1H NMR and ^{13}C NMR spectra appear in the Supplementary Data that accompanies this paper. Significant lines from the ^1H NMR spectrum are listed here. ^1H NMR (500 MHz, $CDCl_3$) δ 0.85–0.97 (approximately 24H, overlapping methyl doublets), 1.10–1.21 (approximately 6H, methyl singlets), 1.20–1.80 (approximately 26H, m), 3.30–3.50 (approximately 6H, m); HRMS (ESI) calcd for $C_{30}H_{63}O_6$ $(M+H)^+$ 519.4619, found 519.4619.

2.1.3.2. Synthesis and characterization of alkynes 9 and 10: To a suspension of NaH (113 mg, 4.74 mmol) in DMF (10 mL) was added a solution of **8** (821 mg, 1.58 mmol) in DMF (5 mL) and the mixture was stirred at rt for 15 min. Tetrabutylammonium iodide (294 mg, 0.8 mmol) and a solution of 1-bromo-5-hexyne[21] (1.27 g, 7.90 mmol) in DMF (3 mL) were added to the reaction mixture and stirring was continued for 24 h. The mixture was then diluted with ether (150 mL), washed with water (3 × 50 mL), brine (20 mL), dried (Na_2SO_4), filtered, and concentrated *in vacuo* to give a pale yellow oil. The oil was subjected to gravity column chromatography on silica gel 60 eluted with 2% MeOH/$CHCl_3$, giving 220 mg (21%) of bisalkyne **10** as a viscous gum, R_f 0.7 (silica gel 60, 1:9 MeOH/$CHCl_3$). Further elution of the column with 5% MeOH/$CHCl_3$ gave 410 mg (43%) of monoalkyne **9**, also as a viscous gum, R_f 0.4 (silica gel 60, 1:9 MeOH/$CHCl_3$). NMR spectra are complex since the products are mixtures of regioisomers and stereoisomers. The ^1H NMR and ^{13}C NMR spectra appear in the Supplementary Data that accompanies this paper. Significant lines from the ^1H NMR spectra are listed here. Spectral data for **9**: IR (neat) cm^{-1} 3350 (br), 2955, 2117, 1462, 1378; ^1H NMR (500 MHz, $CDCl_3$) δ 0.80–1.00 (approximately 24H, overlapping methyl doublets), 1.10–1.22 (approximately 6H, methyl singlets), 1.10–1.80 (approximately 29H, m), 1.96 (1H, t, J = 2.5 Hz), 2.22 (2H, td, J = 7.0 Hz, 2.5 Hz), 3.30–3.50 (approximately 8H, m); HRMS (ESI) calcd for $C_{36}H_{71}O_6$ $(M+H)^+$

599.5245, found 599.5241. Spectral data for **10**: IR (neat) cm^{-1} 3350 (br), 2955, 2117, 1462, 1376; ^1H NMR (500 MHz, $CDCl_3$) δ 0.80–1.00 (approximately 24H, overlapping methyl doublets), 1.10–1.22 (approximately 6H, methyl singlets), 1.10–1.90 (approximately 32H, m), 1.92–1.95 (2H, m), 2.16–2.22 (4H, m), 2.90–3.60 (approximately 10H, m); HRMS (ESI) calcd for $C_{42}H_{79}O_6$ $(M+H)^+$ 679.5871, found 679.5862.

2.1.4. Multimer synthesis and characterization (Scheme 1)

2.1.4.1. Synthesis and characterization of 12a: To a mixture of alkyne **9** (30 mg, 50 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added serinamide azide **11**[20] (18 mg, 75 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) and the reaction monitored by TLC (20% MeOH/$CHCl_3$). After 4 h, volatiles were evaporated and the residue loaded onto a silica gel 60 column. Elution with 5% MeOH/$CHCl_3$ afforded 22 mg (26 μmol, 52%) of **12a** as an gummy solid, R_f 0.3 (silica gel 60, 1:9 MeOH/$CHCl_3$). IR (neat) cm^{-1} 3350 (br), 2933, 1677, 1546, 1461, 1377. NMR spectra are complex since the product is a mixture of regioisomers and stereoisomers. The ^1H NMR and ^{13}C NMR spectra appear in the Supplementary Data that accompanies this paper. Significant lines from the ^1H and ^{13}C NMR spectra are listed here. ^1H NMR (500 MHz, CD_3OD) δ 0.85–0.94 (approximately 24H, overlapping methyl doublets), 1.12–1.19 (approximately 6H, methyl singlets), 1.20–1.80 (approximately 29H, m), 1.92 (2H, pentet, J = 7 Hz), 2.29 (2H, t, J = 7 Hz), 2.72 (2H, t, J = 7 Hz), 2.90–3.50 (approximately 7H, m), 3.73–3.81 (2H, m), 4.36 (2H, t, J = 7 Hz), 4.39–4.44 (1H, m), 7.74 (1H, s); ^{13}C NMR (125 MHz, CD_3OD) δ 15.9, 17.8, 18.1, 19.5, 19.6, 19.7, 26.1, 26.2, 26.3, 27.1, 27.5, 27.6, 29.4, 29.7, 30.9, 31.1, 32.8, 34.9, 36.6, 36.8, 39.4, 40.3, 51.2, 52.4, 56.5, 63.2, 73.4, 77.7, 78.0, 78.3, 79.1, 79.4, 79.6, 123.3, 175.2. 176.0; HRMS (ESI) calcd for $C_{45}H_{88}N_5O_9$ $(M+H)^+$ 842.6577, obsd 842.6582.

2.1.4.2. Synthesis and characterization of 12b: To a mixture of alkyne **10** (25 mg, 36 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added serinamide azide **11**[20] (22 mg, 90 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) and the reaction monitored by TLC (20% MeOH/$CHCl_3$). After 4 h, volatiles were evaporated and the residue loaded onto a silica gel 60 column. Elution with 10% MeOH/$CHCl_3$ afforded 38 mg (32 μmol, 90%) of **12b** as a gummy solid, R_f 0.5 (silica gel 60, 2:8 MeOH/$CHCl_3$). IR (neat) cm^{-1} 3335 (br), 2936, 1658, 1546, 1461, 1374. NMR spectra are complex since the product is a mixture of regioisomers and stereoisomers. The ^1H NMR and ^{13}C NMR spectra appear in the Supplementary Data that accompanies this paper. Significant lines from the ^1H and ^{13}C NMR spectra are listed here. ^1H NMR (500 MHz, CD_3OD) δ 0.85–0.94 (approximately 24H, overlapping methyl doublets), 1.12–1.19 (approximately 6H, methyl singlets), 1.20–1.80 (approximately 35H, m), 1.91 (4H, pentet, J = 7 Hz), 2.29 (4H, t, J = 7 Hz), 2.72 (4H, t, J = 7 Hz), 2.80–3.50 (approximately 10H, m), 3.73–3.80 (4H, m), 4.36 (4H, t, J = 7 Hz), 4.41 (2H, t J = 5 Hz), 7.75 (2H, s); ^{13}C NMR (125 MHz, CD_3OD) δ 16.0, 18.1, 18.8, 19.0, 19.6, 26.1, 26.3, 27.2, 27.6, 30.9, 31.1, 36.6, 51.2, 56.6, 63.3, 73.4, 77.7, 123.3, 175.2, 176.0; HRMS (ESI) calcd for $C_{60}H_{114}N_{10}O_{12}$ $(M+2H)^{2+}$ 583.4303, obsd 583.4305.

2.1.4.3. Synthesis and characterization of 13a: To a mixture of alkyne **9** (20 mg, 33 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added MSH(4) azide **5** (29 mg, 36 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) for 4 h. The reaction mixture was diluted with water (25 mL) and extracted (3 × 30 mL) with a solution of dithizone (3 mg) in $CHCl_3$ (150 mL). The aqueous phase was washed with $CHCl_3$ (2 × 20 mL) and lyophilized to give a white solid. Purification by HPLC (stationary

phase 19 × 256 mm X-Bridge Preparative C_{18} column, mobile phase 10–90% acetonitrile and water containing 0.1% TFA within 50 min, flow rate 15 mL/min, UV detection at 230 nm) yielded 8 mg (5 μmol, 18%) of **13a**, t_R 21.6–24.9 min; analytical HPLC (stationary phase 3 × 150 mm 3.5 Å Waters C_{18} X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–90% B in A over 45 min, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile, detection at 220 and 280 nm), t_R 20.2 min (broad); HRMS (ESI) calcd for $C_{74}H_{122}N_{14}O_{11}$ $(M+2H)^{2+}$ 691.4703, obsd 691.4699.

2.1.4.4. Synthesis and characterization of 13b: To a mixture of alkyne **10** (15 mg, 22 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added MSH(4) azide **5** (36 mg, 46 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) for 4 h. The reaction mixture was diluted with water (25 mL) and extracted (3 × 30 mL) with a solution of dithizone (3 mg) in $CHCl_3$ (150 mL). The aqueous phase was washed with $CHCl_3$ (2 × 20 mL) and lyophilized to give a white solid. Purification by HPLC (stationary phase 19 × 256 mm X-Bridge Preparative C_{18} column, mobile phase 10–90% acetonitrile and water containing 0.1% TFA within 50 min, flow rate 15 mL/min, UV detection at 230 nm) yielded 26 mg (11 μmol, 53%) of **13b**, t_R 18.2 - 22.6 min; analytical HPLC (stationary phase 3 × 150 mm 3.5 Å Waters C_{18} X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–90% B in A over 45 min, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile, detection at 220 and 280 nm), t_R 18.6 min (broad); HRMS (ESI) calcd for $C_{118}H_{181}N_{28}O_{16}$ $(M+3H)^{3+}$ 748.8064, obsd 748.8063.

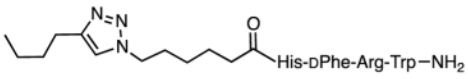
2.1.4.5. Synthesis and characterization of 14a: To a mixture of alkyne **9** (10 mg, 17 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added MSH(7) azide **6** (20 mg, 18 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) for 4 h. The reaction mixture was diluted with water (25 mL) and extracted (3 × 30 mL) with a solution of dithizone (3 mg) in $CHCl_3$ (150 mL). The aqueous phase was washed with $CHCl_3$ (2 × 20 mL) and lyophilized to give a white solid. Purification by HPLC (stationary phase 19 × 256 mm X-Bridge Preparative C_{18} column, mobile phase 10–90% acetonitrile and water containing 0.1% TFA within 50 min, flow rate 15 mL/min, UV detection at 230 nm) yielded 7 mg (4 μmol, 24%) of **14a**, t_R 25.2–27.9 min; analytical HPLC (stationary phase 3 × 150 mm 3.5 Å Waters C_{18} X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–90% B in A over 45 min, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile, detection at 220 and 280 nm), t_R 20.2 min (broad); HRMS (ESI) calcd for $C_{88}H_{146}N_{17}O_{17}$ $(M+3H)^{3+}$ 571.0355, obsd 571.0365.

2.1.4.6. Synthesis and characterization of 14b: To a mixture of alkyne **10** (7.5 mg, 11 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added MSH(7) azide **6** (26 mg, 23 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) for 4 h. The reaction mixture was diluted with water (25 mL) and extracted (3 × 30 mL) with a solution of dithizone (3 mg) in $CHCl_3$ (150 mL). The aqueous phase was then washed with $CHCl_3$ (2 × 20 mL) and lyophilized to give a white solid. Purification by HPLC (stationary phase 19 × 256 mm X-Bridge Preparative C_{18} column, mobile phase 10–90% acetonitrile and water containing 0.1% TFA within 50 min, flow rate 15 mL/min, UV detection at 230 nm) yielded 13 mg (5 μmol, 40%) of **14b**, t_R 23.1–25.9 min; analytical HPLC (stationary phase 3 ± 150 mm 3.5 Å Waters C_{18} X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–90% B in A over 45 min, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile, detection at 220 and 280 nm), t_R 19.4 min (broad); HRMS (ESI) calcd for $C_{146}H_{228}N_{34}O_{28}$ $(M+4H)^{4+}$ 726.4360, obsd 726.4353.

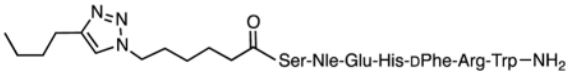
2.1.4.7. Synthesis and characterization of 15a: To a mixture of alkyne **9** (5 mg, 8 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added NDP- -MSH azide **7** (16 mg, 9 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) for 4 h. The reaction mixture was diluted with water (25 mL) and extracted (3 × 30 mL) with a solution of dithizone (3 mg) in $CHCl_3$ (150 mL). The aqueous phase was then washed with $CHCl_3$ (2 × 20 mL) and lyophilized to give a white solid. Purification by HPLC (stationary phase 19 × 256 mm X-Bridge Preparative C_{18} column, mobile phase 10–90% acetonitrile and water containing 0.1% TFA within 50 min, flow rate 15 mL/min, UV detection at 230 nm) yielded 11 mg (5 μmol, 58%) of **15a**, t_R 23.7–26.2 min; analytical HPLC (stationary phase 3 × 150 mm 3.5 Å Waters C_{18}X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–90% B in A over 45 min, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile, detection at 220 and 280 nm), t_R 19.5 min (broad); HRMS (ESI) calcd for $C_{118}H_{191}N_{24}O_{25}$ $(M+3H)^{3+}$ 781.4799, obsd 781.4789.

2.1.4.8. Synthesis and characterization of 15b: To a mixture of alkyne **10** (3.7 mg, 5 μmol), TBTA (3 mg, 6 μmol), and tetrakis(acetonitrile)copper(I) hexafluorophosphate (2 mg, 6 μmol) in dry methanol (1 mL) was added NDP- -MSH azide **7** (20 mg, 11 μmol). The reaction mixture was subjected to microwave irradiation (Biotage Initiator, 100 °C) for 4 h. The reaction mixture was diluted with water (25 mL) and extracted (3 × 30 mL) with a solution of dithizone (3 mg) in $CHCl_3$ (150 mL). The aqueous phase was then washed with $CHCl_3$ (2 × 20 mL) and lyophilized to give a white solid. Purification by HPLC (stationary phase 19 × 256 mm X-Bridge Preparative C_{18} column, mobile phase 10–90% acetonitrile and water containing 0.1% TFA within 50 min, flow rate 15 mL/min, UV detection at 230 nm) yielded 13 mg (3 μmol, 62%) of **15b**, t_R 22.1–24.9 min; analytical HPLC (stationary phase 3 × 150 mm 3.5 Å Waters C_{18} X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–90% B in A over 45 min, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile, detection at 220 and 280 nm), t_R 17.9 min (broad); HRMS (ESI) calcd for $C_{206}H_{317}N_{48}O_{44}$ $(M+3H)^{3+}$ 1389.1342, obsd 1389.1344.

2.1.4.9. Synthesis and characterization of 16 and 17: Triazole **16** was prepared from the corresponding resin-bound tetrapeptide as previously described.[20] Triazole **17** was prepared in a similar manner from the corresponding resin-bound heptapeptide. Following attachment of the N-terminal 6-(4-butyl-1*H*-1,2,3-triazol-1-yl)hexanoic acid residue, 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 overnight, the solution was separated from the resin, volatiles were evaporated, the residue triturated with ether, and the crude product separated by centrifugation. Purification of **17** was accomplished by reversed phase chromatography using 19 × 256 mm X-Bridge Preparative C_{18} column. The mobile phase 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. HRMS (ESI) calcd for $C_{58}H_{85}N_{17}O_{11}$ $(M + 2H)^{2+}$ 597.8301, obsd 597.8295.

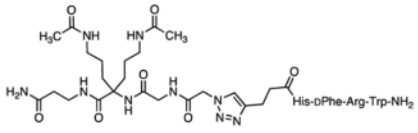


16



17

2.1.4.10. Synthesis and characterization of 18–20: The preparation and characterization of triazoles **18–20** was previously described.[17]



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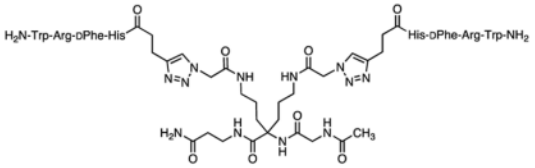
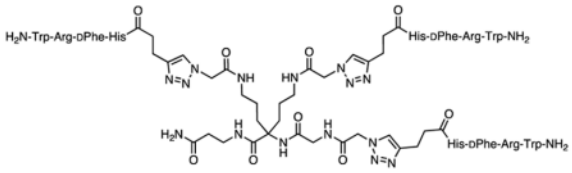


19



20

2.2. Biological studies

2.2.1. Formulation of Solutions—Stock solutions of probes **2–4**, squalene-derived constructs **12a–15a** and **12b–15b**, and control compounds **16**,[20] **17**, and **7** were made up in water. Concentrations were initially based on measured weights of solutes and were confirmed by UV analysis.

2.2.2. Binding Assays

2.2.2.1. Saturation Binding Assays: Quantitative receptor-binding assays were carried out following previously described methods.[3,4,14,15] HEK-293 cells engineered to express both hMC4R and hCCK2R were used to assess ligand binding.[15] Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Cells were seeded in Costar, tissue culture treated, black frame with transparent well, 96-well plates (part #3603) at a density of 2×10^4 cells per well and were incubated at 37 °C to reach 80–90% confluence. Ligands were diluted in binding buffer (1L DMEM, 5.97 g Hepes, 0.2% BSA, 1 mM 1,10-phenanthroline, 0.5 mg/L leupeptin, 200 mg/L bacitracin, pH adjusted to 7.4 using 2 N NaOH). On the day of an experiment, serial dilutions of Eu-labeled probes **2–**

4 (100 μL total volume) were made in different well plates in preparation for addition to plates containing cells. Plates containing cells were removed from the incubator and media was removed. Binding buffer (50 μL/well) was added to the first four rows of a plate and 25 μM NDP-α-MSH in binding buffer (50 μL/well) was added to the next four rows to determine non-specific binding. Fifty μL of the solutions of pre-diluted probes **2–4** were then added to the cell-containing plates and the plates were incubated at 37 °C. After 1 h the media was removed and the plates were washed (3 × 175 μL/well) using wash buffer (1L DMEM, 1 g BSA, 20 μM EDTA, 0.01% Tween20). Enhancement solution (PerkinElmer 1244–105) was added (105 μL/well) and the plates were incubated for 30 min at 37 °C before fluorescence was measured using a VICTOR™ X4 2030 Multilabel Reader (PerkinElmer) employing the standard Eu time-resolved fluorescence (TRF) measurement settings (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm).

2.2.2.2. Competition Binding Assays: Serial dilutions of compounds to be tested were made in different well plates in binding buffer (1L DMEM, 5.97 g Hepes, 0.2% BSA, 1 mM 1,10-phenanthroline, 0.5 mg/L leupeptin, 200 mg/L bacitracin, pH adjusted to 7.4 using 2 N NaOH, 50 μL total volume) before adding to plates containing cells. Stock solutions of Eu-labeled probes were diluted with binding buffer to a concentration of 40 nM for **3** and 10 nM for **4**. Plates containing cells were removed from the incubator, media was removed and replaced with binding buffer containing the Eu-labeled probe (50 μL/well), the solutions of compounds to be tested (concentration range generally 10^{-4}–10^{-11} M) were added, and the plates incubated at 37 °C. After 1 h the media was removed and the plates were washed (3 × 175 μL/well) using wash buffer (1L DMEM, 1 g BSA, 20 μM EDTA, 0.01% Tween20). Enhancement solution (PerkinElmer 1244–105) was added (105 μL/well) and the plates were incubated for 30 min at 37 °C before fluorescence was measured using using a VICTOR™ X4 2030 Multilabel Reader (PerkinElmer) employing the standard Eu time-resolved fluorescence (TRF) measurement settings (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm).

2.2.2.3. Data Analysis: Binding data were analyzed using GraphPad Prism software. Saturation binding data were analyzed using nonlinear regression analysis and fitted to classic one site total binding and nonspecific binding equations. Results from saturation binding experiments are depicted in Figure 2 and given in Table 2. The values of K_d given represent averages of four independent saturation binding experiments, each consisting of four data points per concentration tested. Competitive binding data were analyzed using nonlinear regression analysis and fitted to a classic one site binding competition equation. Each IC_{50} value was generated from individual competitive binding assays and converted to a K_i value using the equation $K_i = IC_{50}/(1 + ([\text{ligand}]/K_d))$ where [ligand] refers to the concentration of the probe used as the labeled competed ligand. For probe **3**, [ligand] = 20 nM and K_d = 27 nM. For probe **4**, [ligand] = 5 nM and K_d = 4.2 nM. Results from competition binding experiments are given in Table 3. The values of K_i given represent averages of four independent competition binding experiments, each consisting of four data points per concentration tested.

3. Results

3.1. Chemistry

Fully protected resin-bound precursors to probes **2–4** and azides **5–7** were prepared by solid phase synthesis on Rink amide Tentagel S resin (for probe synthesis) or on Rink amide resin (for ligand synthesis). For the preparation of probes **2–4**, Fmoc-PEG[18] was coupled to the N-terminus of the resin-bound peptides. Following removal of the Fmoc, DTPA,[19] activated as the HOBt ester, was attached to the N-terminus of the PEGO spacer. Simultaneous side

chain deprotection and cleavage of the peptide from the resin produced the metal-free precursors to **2–4** which were purified by preparative HPLC and characterized by FT-ICR MS. Following complexation of Eu^{3+}, the Eu-DTPA-PEGO probes **2–4** were purified by reversed phase chromatography and characterized by analytical HPLC and FT-ICR MS (Table 1).

For the preparation of azides **5–7**, 6-azidohexanoic acid[22] was coupled to the N-terminus of the resin-bound peptides. Simultaneous side chain deprotection and cleavage of the peptide from the resin produced compounds **5–7** which were purified by reversed phase preparative HPLC and characterized by ESI MS (Table 1). Yields ranged from 39–60%.

Reaction of squalene with excess disiamylborane, followed by oxidation, produced hexaol **8** in 51% yield as a white foam (Scheme 1). Analysis by ^{13}C NMR (Figure 1) confirmed that hydroboration had been regioselective and had yielded a mixture of diastereomeric secondary alcohols as depicted in **8**.[16,20] Treatment of **8** with three equivalents of sodium hydride and five equivalents of 6-bromo-1-hexyne[21] in DMF gave a mixture of alkylation products from which monoalkynes **9** and dialkynes **10** were isolated in 21% and 43% yields, respectively, by silica gel column chromatography. It is assumed that **9** and **10** are mixtures of regioisomers due to random alkylation at oxygen atoms along the carbon backbone.

Copper(1)-catalyzed azide-alkyne cycloaddition (CuAAC) reactions[23] of **9** and **10** with excesses of the serine amide-derived azide **11**[20] gave the corresponding triazole-containing products **12a** and **12b** in 52% and 90% yields, respectively, following chromatographic purification (Scheme 1). These compounds were characterized by NMR and ESI MS. CuAAC reactions of **9** and **10** with excesses of azide **5** gave the corresponding triazoles **13a** and **13b**. Copper ions were removed from the crude product mixtures by complexation with dithizone and removal of the complex by extraction with $CHCl_3$.[24] The water-soluble products **13a** and **13b** were purified by preparative reversed phase HPLC, recovered by lyophilization in 18% and 53% yields, respectively, and characterized by ESI MS. In a similar manner, CuAAC reactions of **9** and **10** with excesses of azides **6** and **7** gave the corresponding triazole-containing products **14a**, **14b**, **15a**, and **15b** in 24%, 40%, 58%, and 62% yields, respectively, after workup and purification (Scheme 1). These compounds were characterized by ESI MS. The synthesis and characterization of compound **16** was previously reported.[20] Compound **17** was prepared in a similar manner by N-terminal acylation of the resin-bound heptapeptide with 6-(4-butyl-1*H*-1,2,3-triazol-1-yl)hexanoic acid.[20] The synthesis and characterization of compounds **18–20** were previously reported.[17]

3.2. Bioassays

HEK-293 cells overexpressing both hMC4R[8,25] and hCCK2R[26] were used to measure the affinities of probes **2–4** for binding to hMC4R by means of saturation binding assays (Figure 2).[15] K_d values for probes **2–4** are given in Table 2. Europium-based time-resolved fluorescence competitive binding assays using Eu-DTPA-PEGO-MSH(7) (**3**) and Eu-DTPA-PEGO-NDP-α-MSH-NH_2 (**4**) as the competed probes were employed to study the binding of monovalent and bivalent serinamide, MSH(4), MSH(7), and NDP-α-MSH constructs **12a–15a** and **12b–15b**. Competitive binding assays using probe **3** as the competed probe were employed to study the binding of monovalent, divalent, and trivalent MSH(4) constructs **18–20**.[17] K_i values for these compounds and for the control compounds **16**, **17**, and **7** are given in Table 3.

4. Discussion

Previously we described the preparation and testing of scaffolds derived from squalene,[14,16] solanesol,[20] and sucrose[27] with one or more sidechains bearing MSH(4), a ligand with a low

micromolar affinity for binding to hMC4R. MSH(4) was chosen because synergistic effects are generally more easily detected for multivalent constructs of low-affinity ligands.[9–13] All of the monovalent and multivalent MSH(4) constructs derived from squalene, solanesol, and sucrose exhibited competitive binding to hMC4R that was comparable to the parental ligand. The competed probe used in these studies was Eu-DTPA-NDP-α-MSH (**1**), which is based on the superpotent ligand NDP-α-MSH. The off-rate of NDP-α-MSH is approximately eight hours.[28] In addition, evidence suggests that receptor-bound **1** is taken up by the cells. [29] For these reasons, it was clear that competition binding assays involving **1** and hMC4R on living cells do not take place under thermodynamic control. This fact raised the possibility that the competition for binding to hMC4R between **1** and any variant of MSH(4) was biased in favor of **1**, to the detriment of multivalent binding.

To determine what effects might result from the use of probes with a lesser affinity for hMC4R, the set of Eu-DTPA-PEGO probes **2–4** was prepared by standard solid phase methods. Saturation binding assays for **2–4** were carried out using HEK-293 cells that stably overexpress both hMC4R (6×10^5 receptors per cell) and hCCK2R (1×10^6 receptors per cell).[15] These cells were employed so that results obtained here will be comparable with results from studies with multimeric constructs that bear ligands targeted to hCCK2R and with multimeric constructs that bear mixtures of ligands targeted to both receptors.[30] In all cases, non-specific binding was determined by blocking binding to hMC4R with a high concentration of NDP-α-MSH. Although probe **2** exhibited 20% as much non-specific binding as did probes **3** and **4** (see Figure 2), the difference between total binding and non-specific binding for **2** was small, even at very low concentrations of the probe. Thus, the measured K_d has a large associated error (93 ± 100 nM), and plans to use probe **2** in competition binding assays were abandoned.[31]

As with probe **2**, graphs of total and non-specific binding for probes **3** and **4** appear to be linear and parallel at high concentrations (see Figure 2), consistent with some form of receptor-independent binding and/or uptake as opposed to receptor saturation. However, at sufficiently low concentrations, the differences between total and non-specific binding for probes **3** and **4** were significant, and permitted the calculation of reasonable K_d values (see Table 2) so that use of these probes in competition binding assays was deemed possible.

For competition assays, a range of ligand potencies attached to monovalent, bivalent, and trivalent constructs was desired. Azides **5–7** were prepared by standard solid phase methods. Scaffolds **9** and **10** were prepared from squalene via hexaol **8** as depicted in Scheme 1.[16] Attachment of azides **5–7** to **9** and **10** by means of microwave-assisted copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC)[23] produced monovalent and bivalent constructs **13a** and **13b** incorporating MSH(4), **14a** and **14b** incorporating MSH(7), and **15a** and **15b** incorporating NDP-α-MSH. Control compounds **12a** and **12b** were prepared by CuAAC reaction of serine amide-derived azide **11**[20] with **9** and **10**. In addition, MSH(4)-containing compounds **18–20**, which were recently shown to exhibit enhanced avidity with increased ligand number when competed against an NDP-α-MSH-based probe,[17] were here competed against MSH(7)-based probe **3**. Compounds **16**,[20] **17**, and **7** were used as reference compounds in place of the parental ligands.

Results of the competition binding assays are given in Table 3. The serine amide-containing control compounds **12a** and **12b** were both ineffective at blocking probes **3** and **4** from binding to hMC4R over the range of concentrations tested. For the squalene-derived monovalent and divalent constructs, the trends in K_i values are consistent for all three ligands tested, MSH(4) (compare **16**, **13a**, and **13b**), MSH(7) (compare **17**, **14a**, and **14b**), and NDP-α-MSH (compare **7**, **15a**, and **15b**). Squalene-derived monovalent compounds exhibit a higher K_i value than the control, often a factor of two, presumably due to

compromised ligand binding, while the corresponding divalent compound exhibits a lower K_i value, often a factor of two that can be ascribed to statistical doubling of the number of ligands. In all but one case, the K_i value obtained using probe **4** was higher than the corresponding K_i value obtained using probe **3**, a result that is consistent with the stronger binding of **4** versus **3** to hMC4R. In view of past results with monovalent and multivalent compounds derived from squalene,[14,16] solanesol,[20] sucrose,[27] and compounds **18–20**,[17] the results herein suggest that the MSH(4) ligands in compounds **13b–15b** are spaced too far apart for manifestation of multivalent binding.

Enhanced avidity interpretable as multivalent binding was observed when compounds **18–20** were competed against probe **3**, although the lowering of the K_i values with each additional ligand (approximately nine-fold at each step) was attenuated when compared with K_i values calculated from published EC_{50} data for competition binding assays that employed a Eu-DTPA-PEGO-NDP-α-MSH probe similar to **4** (approximately 16-fold and 22-fold, see Table 3).[17]

Although different sets of data were produced, similar conclusions were reached from competition binding experiments that employed probes **3** and **4**, regardless of the potency of the ligand being competed. Probe **3** requires less time and effort to prepare, and so may find favor over probe **4** in future competition binding studies. However, confirmation of observed binding behaviors by the use of both probes would seem a prudent practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

BSA	bovine serum albumin
Cl-HOBt	6-chloro-1-hydroxybenzotriazole
CuAAC	copper(I)-catalyzed azide-alkyne cycloaddition
DCM	dichloromethane
DIC	diisopropyl carbodiimide
DIEA	diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DTPA	diethylenetriaminepentaacetic acid
IC_{50}	half maximal inhibitory concentration
ESI MS	electrospray ionization mass spectrometry
Fmoc	9-fluorenylmethyoxycarbonyl

Fmoc-PEGO	1-(9*H*-fluoren-9-yl)-3,19-dioxo-2,8,11,14,21-pentaoxa-4,18-diazatricosan-23-oic acid
FT-ICR MS	Fourier transform ion cyclotron resonance mass spectrometry
hMC4R	human melanocortin 4 receptor
HOBt	1-hydroxybenzotriazole
HRMS	high resolution mass spectroscopy
MEM	Minimum Essential Medium
MSH(4)	His-DPhe-Arg-Trp
MSH(7)	Ser-Nle-Glu-His-DPhe-Arg-Trp
NDP-α-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
PEGO	19-amino-5-oxo-3,10,13,16-tetraoxa-6-azanonadecan-1-oic acid
TBTA	tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
TEAA	triethylammonium acetate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
TRF	time-resolved fluorescence

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31. In a prior study (see reference 14), a K_d of 9.1 ± 1.4 μM was measured for probe **2**, and much less non-specific binding relative to total binding was observed. The study employed HEK-293 cells that transiently overexpressed only hMC4R (6×10^5 receptors per cell), a large excess of MSH(4) instead of NDP-α-MSH as the blocking agent in the determination of non-specific binding, and a different brand of 96-well plates. It is unclear which factors, if any, account for the different outcome for probe **2** in the present study.

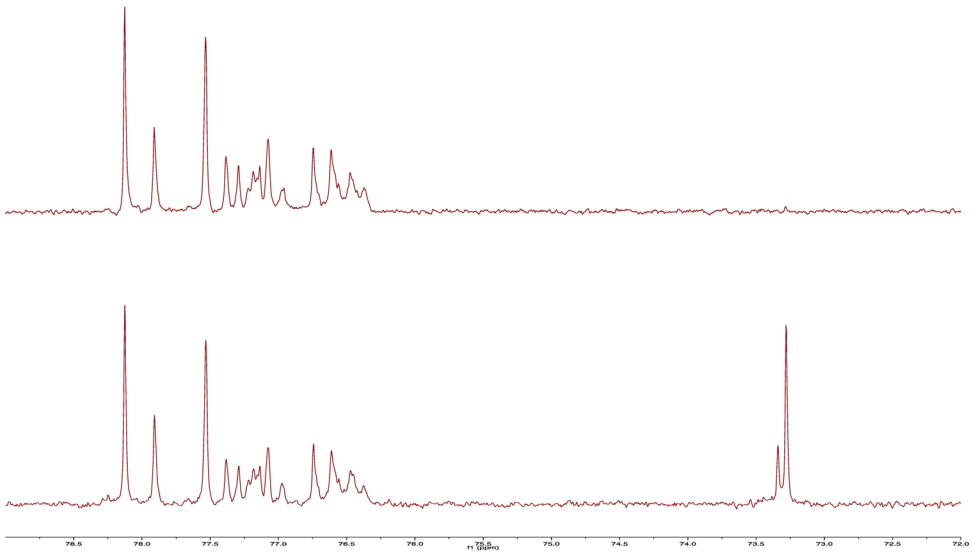


Figure 1.

Top: A portion of the DEPT 135 ^{13}C NMR spectrum of **8** in CD_3OD. Bottom: A portion of the ^{13}C NMR spectrum of **8** in CD_3OD. The lines near 73 ppm are due to tertiary carbons bearing OH groups and the lines between 76–79 ppm are due to secondary carbons bearing OH groups. The spectral complexity occurs because **8** is a mixture of regioisomers and stereoisomers.

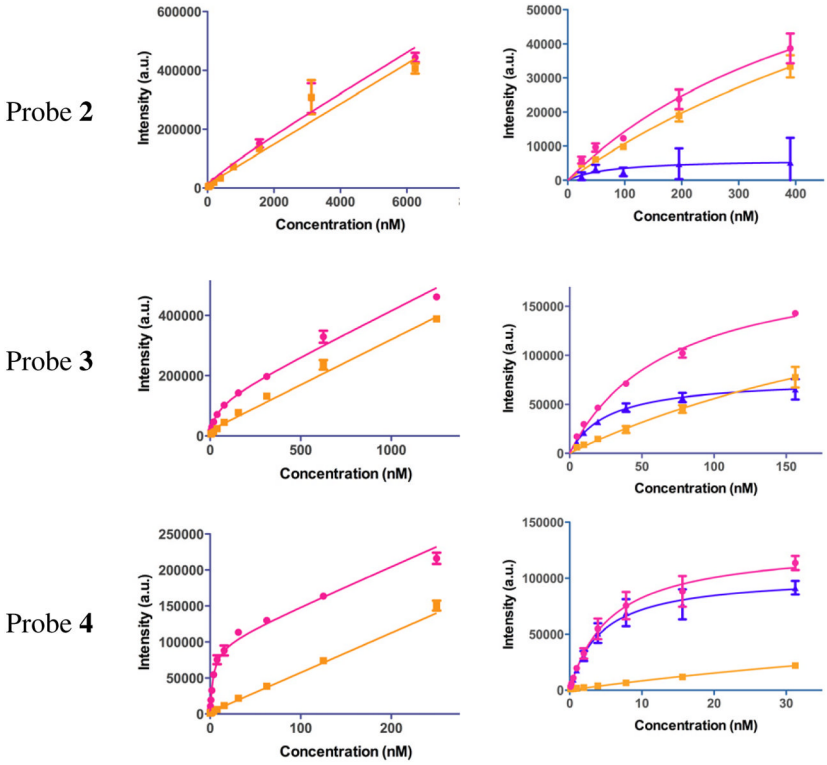
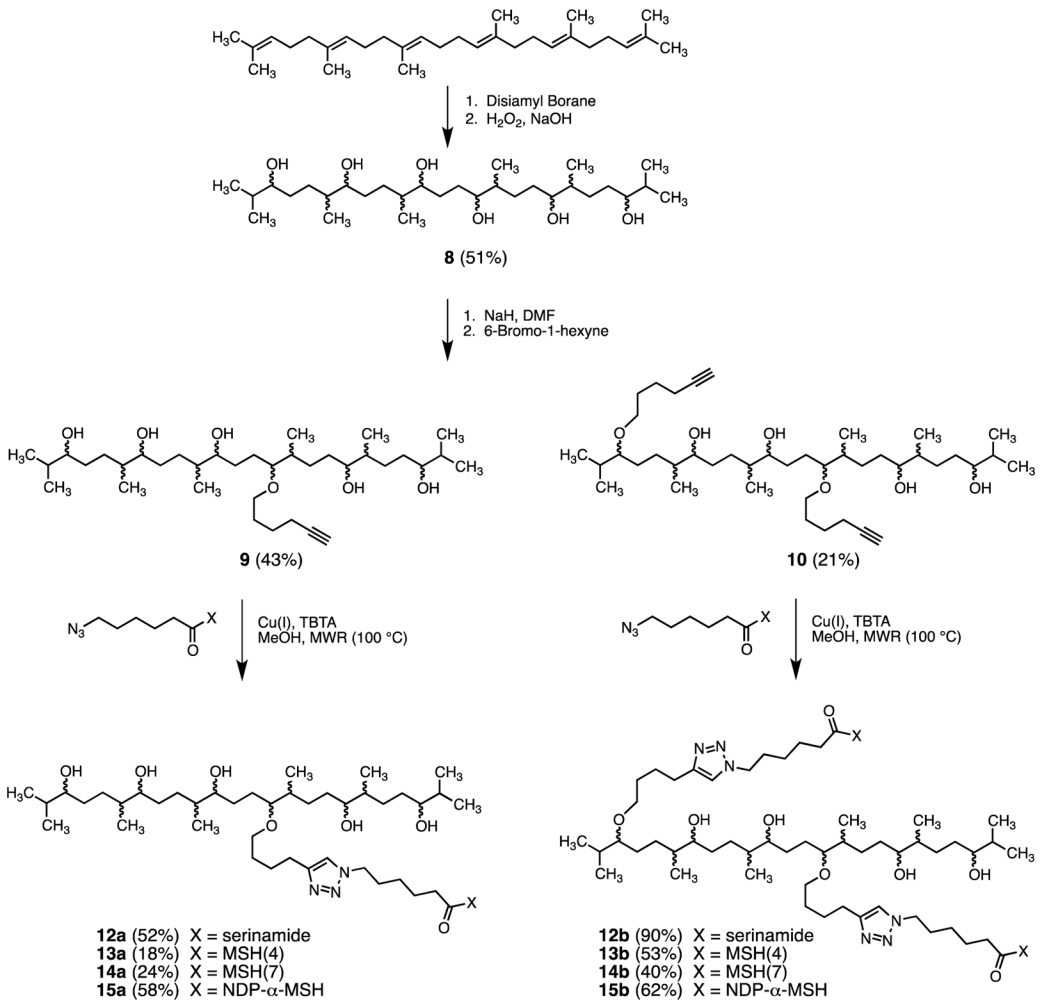


Figure 2.

Saturation binding curves for Eu-DTPA-PEGO probes **2–4**. Total binding (●). Non-specific binding (■). Specific binding (▲). The graphs in column 3 are truncated expansions of the graphs in column 2 and include a specific binding curve.



Scheme 1.

Synthesis of monovalent constructs **12a–15a** and divalent constructs **12b–15b**.[a]

[a]Compounds **8**, **9**, and **10** were obtained as complex mixtures of stereoisomers and regioisomers. Compounds **12a–15a** and **12b–15b**, derived from **9** and **10**, respectively, are likewise assumed to be the related mixtures of stereoisomers and regioisomers.

Table 1

MS and HPLC characterization of compounds **2–7**.

Compound	Formula [M]	Calculated Masses [Ion]	Masses Found (error)	t_R (min)	Yields (%)
2[a]	$C_{60}H_{85}N_{16}O_{19}{}^{151}Eu$	1486.5532 $[M+1]^+$	1486.5526 (0.4 ppm)	10.5	20
	$C_{60}H_{85}N_{16}O_{19}{}^{153}Eu$	1488.5546 $[M+1]^+$	1488.5549 (0.2 ppm)		
3[a]	$C_{74}H_{108}N_{19}O_{25}{}^{151}Eu$	1815.7119 $[M+1]^+$	1815.7113 (0.3 ppm)	12.9	9
	$C_{74}H_{108}N_{19}O_{25}{}^{153}Eu$	1817.7133 $[M+1]^+$	1817.7139 (0.3 ppm)		
4[a]	$C_{104}H_{155}N_{26}O_{33}{}^{151}Eu$	817.0254 $[M+3]^{3+}$	817.0248 (0.7 ppm)	12.6	11
	$C_{104}H_{155}N_{26}O_{33}{}^{153}Eu$	817.6925 $[M+3]^{3+}$	817.6926 (0.1 ppm)		
5[b]	$C_{38}H_{50}N_{14}O_5$	783.4161 $[M+1]^+$	783.4158 (0.4 ppm)	13.6	39–45
6[b]	$C_{52}H_{73}N_{17}O_{11}$	556.7910 $[M+2]^{2+}$	556.7909 (0.3 ppm)	15.4	42–50
7[b]	$C_{82}H_{118}N_{24}O_{19}$	872.4575 $[M+2]^{2+}$	872.4567 (0.9 ppm)	14.6	50–60

[a]Analyzed on a 3 × 150 mm 3.5 Å Waters C_{18} X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–60% B in A over 45 min, where A is 0.1% TEAA in water (pH 6) and B is 90% acetonitrile and 10% A, detection at 220 and 280 nm. Purity >95%. Characterized by FT-ICR MS.

[b]Analyzed on a 3 × 150 mm 3.5 Å Waters C_{18} X-Bridge column, flow rate 0.3 mL/min, linear gradient from 10–90% B in A over 45 min, where A is 0.1% TFA in water and B is 0.1% TFA in acetonitrile, detection at 220 and 280 nm. Purity >95%. Characterized by ESI MS.

Table 2

Binding constants for Eu-DTPA-PEGO probes **2–4** with hMC4R.

Probe	K_d[a]
2	93±100 nM[b]
3	27±3.9 nM[c]
4	4.2±0.48 nM[d]

[a]The value given is the average of four independent binding experiments, each done in quadruplicate.

[b]Includes data up to [**2**] = 400 nM.

[c]Includes data up to [**3**] = 150 nM.

[d]Includes data up to [**4**] = 30 nM.

Table 3

Competitive binding of Eu-DTPA-PEGO probes **3** and **4** against monovalent constructs **12a–15a,** bivalent constructs **12b–15b**, monovalent, divalent, and trivalent compounds **18–20**, and control compounds **16**, **17**, and **7** with hMC4R.

Compounds	Probe 3 $K_i^{a,b}$ (nM)	Probe 4 $K_i^{a,c}$ (nM)
12a	ncb[d]	ncb[d]
12b	ncb[d]	ncb[d]
16	350±28	990±40
13a	600±16	2200±150
13b	150±38	810±56
17	1.8±0.2	18±1.0
14a	2.4±0.8	33±0.6
14b	1.7±0.2	15±1.5
7	3.1±0.4	0.14±0.01
15a	6.0±0.2	15±1.8
15b	1.5±0.9	3.4±1.4
18	330±52	3900±600[e]
19	37±10	250±58[e]
20	4.3±0.4	11±1.2[e]

[a]The K_i was calculated using the equation $K_i = IC_{50}/(1+([\text{ligand}]/K_d))$, where [ligand] refers to the concentration of the probe used as the labeled competed ligand.

[b]Here [ligand] = 20 nM, the concentration of probe **3**.

[c]Here [ligand] = 5 nM, the concentration of probe **4**.

[d]ncb = no competitive binding.

[e]Calculated from EC_{50} values taken from reference 17, wherein a probe similar to **4** was employed.