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Design, Synthesis, and Validation of a Branched Flexible Linker for Bioactive Peptides

Martina E. Bowen[†], Yasunari Monguchi[†], Rajesh Sankaranarayanan[†], Josef Vagner[†], Lucinda J. Begay[†], Liping Xu[‡], Bhumasamudram Jagadish[†], Victor J. Hruby^{†,‡}, Robert J. Gillies[‡], and Eugene A. Mash^{*,†}

[†]Department of Chemistry, University of Arizona, Tucson, Arizona 85721-0041

[‡]Department of Biochemistry and Molecular Biophysics, The Arizona Cancer Center, Tucson, Arizona 85724-5024

Abstract

A branched flexible linker that incorporates a fluorescent dansyl moiety was synthesized and used to connect two high affinity NDP-α-MSH ligands or two low affinity MSH(4) ligands. The linker was incorporated into the conjugate by solid-phase synthesis. In vitro biological evaluations showed that potency of binding to the human melanocortin 4 receptor was not diminished for linker–ligand combinations relative to the corresponding ligand alone.

Introduction

Early detection of many human cancers depends on the availability of nontoxic reagents that can seek out and selectively bind to cancer cells and report their existence and location by noninvasive molecular imaging.¹ We are engaged in a program to develop reagents that target human cancers that presently are difficult to detect, such as melanoma and pancreatic cancer. Our strategy involves linking together reporter moieties and two or more ligands that bind to cell surface receptors that are overexpressed in cancer cells.² The resulting multivalent molecules could display enhanced affinity for targeted cells.³

Factors that must be considered in designing linkers for bioactive ligands include the distance that must be spanned, ease of use, biocompatibility, and value-added functionality. The distance that must be spanned between targeted receptors on the cell surface will vary and will often exceed the typical dimensions of small molecules.⁴ In such cases, several

small molecule linkers connected in series will be necessary to span the distance between receptors.

For peptide ligands, linkers should terminate in an amine and a carboxylic acid to facilitate incorporation by solid-phase synthesis. Each linker employed in the assembly of compounds that contain multiple copies of one ligand, or ligands of multiple types, must be validated as noninterfering with the binding of the ligands of interest. Both rigid linkers⁵ and flexible linkers⁶ are in development. The use of water-soluble and biocompatible poly(ethylene oxide) oligomers (PEGs) as linkers is well established.⁷ While several linear PEGs that terminate in amine and carboxylic acid functional groups are commercially available,⁸ to the best of our knowledge, branched PEG oligomers such as **1** that are akin to lysine have not been reported.⁹ Such branching would afford the opportunity to attach, in addition to peptide ligands, fluorescent moieties for imaging or therapeutic moieties for targeted treatment of diseased cells. Linkers such as **1** are modular and can be connected together to bridge larger distances and/or to mix ligand, imaging, and therapeutic moieties on one chain. We report herein a synthesis of a fluorescent derivative of **1**, attachment of “high-affinity” NDP- α -MSH or “low-affinity” MSH(4) variants of α -melanocyte stimulating hormone¹⁰ to produce compounds **2** and **3**, as well as control compounds **4** and **5**, and evaluation of the bioactivities of these peptides.

Results and Discussion

A synthesis of the fluorescent derivative **17** of linker **1** is depicted in Scheme 1. Known amine **611** was allowed to react with acrylonitrile in methanol, producing nitrile **7** in 80% yield. Separately, the sodium salt derived from tri(ethylene glycol) was allowed to react with known iodide **812** in THF, giving alcohol **9** in 65% yield. This alcohol was subjected to Swern oxidation to produce the unstable aldehyde **10**. Reductive coupling of amine **7** and aldehyde **10** using sodium triacetoxyborohydride gave nitrile **11**. This compound, which

possesses the skeleton of linker **1**, was subjected to partial hydrolysis of the ortho ester and subsequently to transesterification using potassium carbonate in dry methanol to give ester **12** in 57% yield from alcohol **9**. Reductive ethylation of the nitrile moiety of **12** was accomplished by the method of Sajiki et al.¹³ Reaction of **13** with dansyl chloride afforded sulfonamide **14** in 47% yield from **12**. Acid-catalyzed removal of the Boc group, saponification of the methyl ester, and protection of the free amine as the trifluoroacetamide (**14** → **15** → **16** → **17**) was effected in 93% yield over the three steps.

Compound **17** incorporates a γ -alkoxy acid terminus instead of a β -alkoxy acid moiety^{7,8} to preclude unwanted β -elimination processes. *N*-Ethylation prior to introduction of the dansyl moiety clears the linker of an acidic proton that could interfere with solid-phase synthesis and/or make the fluorescence properties of the molecule pH sensitive.¹⁴

The solid-phase synthesis of compound **2** consisting of two NDP- α -MSH ligands connected in a head-to-tail fashion by the fluorescent linker is depicted in Scheme 2 and is presented below. Also shown in Scheme 2 and presented is the synthesis of control compound **4** consisting of one NDP- α -MSH ligand connected at the peptide *N*-terminus through the carboxylate of the fluorescent linker.

NDP- α -MSH¹⁰ was constructed on Rink amide Tentagel S resin (**18**, initial loading 0.17 mmol/g).¹⁵ The product resin **19** retained all side-chain protecting groups. Acid **17** was coupled to the *N*-terminus of **19** to give resin **20**. The *N*-trifluoroacetamide protecting group was cleaved by treatment with 15% hydrazine and 15% methanol in THF, and the resulting resin **21** was split into two portions. For the synthesis of **2**, the free amine group of resin **21** was coupled with Fmoc-valine, and solid-phase synthesis continued to completion of the second NDP- α -MSH ligand, giving resin **22**. The *N*-terminus was then capped by *N*-acetylation, producing resin **23**. For the synthesis of control **4**, resin **21** was terminally *N*-acetylated to give resin **24**. Simultaneous side-chain deprotection and cleavage of the peptides from resins **23** and **24** was effected using a mixture of trifluoroacetic acid, 1,2-ethanedithiol, thioanisole, and water (91/3/3/3), producing the desired compounds **2** and **4**. Compounds **3** and **5** incorporating the MSH(4) ligand¹⁰ were similarly prepared.

Compounds **2–5** were purified by reversed-phase C₁₈ preparative HPLC and were characterized by ESI-MS and MALDI-TOF. Mass spectral and HPLC characterization data are given in Table 1. Ligand binding was evaluated using a previously described lanthanide-based binding assay (see the Supporting Information).¹⁶ HEK293 cells overexpressing the human melanocortin 4 receptor (hMC4R) were used to assess ligand binding.¹⁷ Table 2 lists the IC₅₀ values (averaged over *n* experiments) for the ligands NDP- α -MSH and MSH(4), as well as for compounds **2–5**. Compound **2** bound with a 2-fold higher affinity as compared to **4**, possibly a statistical effect as **2** contains two NDP- α -MSH ligands to one for **4**. Synergistic effects are absent because the ligands in **2** are too far apart for enhanced activity from a proximity effect,^{2,5c} but not far enough apart for enhanced binding due to receptor cross-linking. However, this statistical effect disappeared for compounds **3** and **5** that contain the weaker binding MSH(4) ligand. Compounds **2–5** were all slightly more potent binders at the hMC4 receptor when compared with the corresponding parental ligands. Thus, it appears that this linker does not interfere with binding of either the NDP- α -MSH ligand or the MSH(4) ligand at the hMC4 receptor.

Conclusion

This paper describes the synthesis of a branched flexible linker that terminates in amino and carboxylic acid functional groups to facilitate incorporation of the linker into multimeric peptide ligand constructs by solid-phase synthesis. The backbone of the linker is a hybrid

poly(ethylene oxide)/ethylene imine that affords water solubility and biocompatibility to the linker. The branching of the linker permits incorporation of moieties for imaging and/or therapeutic use. In the present work, a fluorescent dansyl group was incorporated. The carboxyl terminus of the linker is based on a γ -alkoxybutanoic acid substructure rather than the common β -alkoxypropionic acid substructure. This prevents unwanted β -elimination reactions from interfering with synthetic manipulations. The utility of this linker was demonstrated by synthesis of compounds containing one or two copies of ligands targeted to the human melanocortin 4 receptor. In vitro binding studies established the noninterference of the linker with ligand binding to this receptor.

Experimental

***tert*-Butyl 1-Cyano-6,9,12-trioxa-3,15-diazaheptadecanoate (7)**

To a solution of **6** (4.34 g, 14.8 mmol) in MeOH (9 mL) was added acrylonitrile (1.56 mL, 1.26 g, 23.7 mmol) dropwise over 12 min. After 19 h, the mixture was concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel (EtOAc/MeOH, 10:1 $\rightarrow$ 2:1) to give **7** (4.07 g, 11.8 mmol, 80%) as a colorless oil: analytical TLC on silica gel, 3:1 EtOAc/MeOH, R_f = 0.31 (ninhydrin); ^1H NMR (CDCl_3) δ 1.39 (9H, s), 2.49 (2H, t, J = 6.8 Hz), 2.78 (2H, t, J = 5.1 Hz), 2.90 (2H, t, J = 6.8 Hz), 3.26 (2H, m), 3.49 (2H, t, J = 5.1 Hz), 3.54–3.82 (10H, m), 5.22 (1H, br s); ^{13}C NMR (CDCl_3) δ 18.5, 28.4, 40.3, 45.0, 48.5, 70.1, 70.2, 70.3, 70.4, 79.0, 118.6, 156.0 (two signals could not be located because of their overlaps with other signals); HRMS (FAB $^+$, NBA) calcd for $\text{C}_{16}\text{H}_{32}\text{N}_3\text{O}_5$ ($\text{M} + \text{H}$) $^+$ 346.2342, found 346.2330.

12-(4-Methyl-2,6,7-trioxabicyclo[2.2.2]octan-1-yl)-3,6,9-trioxatridecan-1-ol (9)

To NaH (499 mg, 20.8 mmol) was added a solution of tri(ethylene glycol) (4.73 g, 4.20 mL, 31.5 mmol) in THF (20 mL) dropwise. After 30 min, a solution of **8** (3.10 g, 10.4 mmol) in THF (10 mL) was added dropwise. The mixture was heated to reflux, and after 2 h, the reaction was quenched with H_2O (10 mL). The mixture was extracted with CH_2Cl_2 (20 mL $\times$ 5), and the combined organic layers were dried (MgSO_4), filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (hexanes/EtOAc/ Et_3N , 29:67:4) to give **9** (2.18 g, 6.80 mmol, 65%) as a pale yellow oil: analytical TLC on silica gel, 12:1 EtOAc/MeOH, R_f = 0.42 (anisaldehyde); ^1H NMR (CDCl_3) δ 0.76 (3H, s), 1.69 (4H, m), 2.57 (1H, t, J = 6.8 Hz), 3.43 (2H, m), 3.51–3.68 (12H, m), 3.84 (6H, s); ^{13}C NMR (CDCl_3) δ 14.5, 23.4, 30.2, 33.2, 61.7, 69.8, 70.4, 70.6, 70.6, 70.9, 72.5, 72.5, 109.0; HRMS (FAB $^+$, NBA) calcd for $\text{C}_{15}\text{H}_{29}\text{O}_7$ ($\text{M} + \text{H}$) $^+$ 321.1913, found 321.1907.

1-*tert*-Butyl 27-Methyl 14-(2-Cyanoethyl)-5,8,11,17,20,23-hexaoxa-2,14-diazaheptacosanedioate (12)

To a solution of oxalyl chloride (856 μL , 9.81 mmol) in CH_2Cl_2 (28 mL) at -78°C was added a solution of DMSO (1.39 mL, 19.6 mmol) in CH_2Cl_2 (7 mL) over 5 min. After 3 min, a solution of **9** (2.86 g, 8.92 mmol) in CH_2Cl_2 (10 mL) was added through a cannula over 7 min. After 15 min, Et_3N (6.22 mL, 44.6 mmol) was added over 3 min, and after 75 min, the cooling bath was removed. After 10 min, H_2O (35 mL) and CH_2Cl_2 (100 mL) were added. The layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (150 mL $\times$ 2). The combined organic layers were dried (MgSO_4), filtered, and concentrated in vacuo. The residue was mixed with **7** (3.35 g, 9.70 mmol), and the mixture was dissolved in 1,2-dichloroethane (100 mL). To the solution at 0°C was added $\text{NaBH}(\text{OAc})_3$ (3.03 g, 14.3 mmol) in one portion. After 3 h, additional $\text{NaBH}(\text{OAc})_3$ (377 mg, 1.78 mmol) was added. After 40 min, H_2O (35 mL) was added, and the layers were separated. The aqueous layer was extracted with CH_2Cl_2 (150 mL $\times$ 3), and the organic layers were combined, dried (MgSO_4), filtered, and concentrated in vacuo. The residue was dissolved in MeOH (80

mL)–H₂O (1 mL). To the solution was added *p*-TsOH·H₂O (899 mg, 4.46 mmol), and after 4.5 h, additional *p*-TsOH·H₂O (170 mg, 0.892 mmol) was added. After 19 h, saturated aqueous NaHCO₃ (5 mL) was added, and the mixture was concentrated in vacuo to ca. 15 mL. EtOAc (300 mL) and brine (20 mL) were added, and the layers were separated. The aqueous layer was extracted with EtOAc (300 mL), and the combined organic layers were dried (MgSO₄), filtered, and concentrated in vacuo. The residue was dissolved in distilled MeOH (100 mL), and K₂CO₃ (2.10 g, 15.2 mmol) was added to the solution at rt in one portion. After 1.5 h, the mixture was cooled to 0 °C, and EtOAc (140 mL) and saturated aqueous NH₄Cl (140 mL) were added. The layers were separated, and the organic layer was washed with brine (20 mL). The combined aqueous layers were extracted with EtOAc (200 mL × 2), and the organic layers were combined, dried (MgSO₄), filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (EtOAc/MeOH, 30:1 → 7:1) to give **12** (2.95 g, 5.11 mmol, 57% from **9**) as a pale yellow oil: analytical TLC on silica gel, 5:1 EtOAc/MeOH, *R*_f = 0.47 (ninhydrin); ¹H NMR (CDCl₃) δ 1.41 (9H, s), 1.89 (2H, m), 2.37 (2H, d, *J* = 7.3 Hz), 2.49 (2H, br m), 2.76 (4H, br, m), 2.95 (2H, br m), 3.28 (2H, m), 3.44–3.88 (27H, m), 5.05 (1H, br s); ¹³C NMR (CDCl₃) δ 16.3, 24.9, 28.4, 30.7, 40.3, 50.9, 51.5, 54.0, 54.0, 68.7, 70.1, 70.2, 70.4, 70.4, 70.5, 70.5, 79.1, 119.3, 156.0, 173.9 (five signals could not be located because of their overlaps with other signals); HRMS (FAB⁺, NBA) calcd for C₂₇H₅₂N₃O₁₀ (M + H)⁺ 578.3653, found 578.3660.

1-*tert*-Butyl 27-Methyl 14-[3-(*N*-Ethyl-*N*-dansyl)aminopropyl]-5,8,11,17,20,23-hexaoxa-2,14-diazaheptacosanedioate (14**)**

To a solution of **12** (338 mg, 0.585 mmol) in MeOH (1.8 mL) was added 5% Rh/C (N. E. Chemcat, Tokyo, Japan, 101 mg), and the system was sealed with a septum. EtNH₂ solution (2 M) in MeOH (2.93 mL, 5.85 mmol) was added, and after 30 min, the air inside was replaced with hydrogen (balloon) by three vacuum/H₂ cycles and additional 2 M EtNH₂ solution in MeOH (1.47 mL, 2.93 mmol) was added. After 21 h, the mixture was passed through a Celite pad, and the pad was washed with MeOH (100 mL). The combined filtrates were concentrated in vacuo to give a 6:4 mixture (331 mg) of monoethylated amine and unethylated amine. The amines mixture was dissolved in MeOH (3.2 mL), and MeCN (143 μL, 2.73 mmol) and 5% Rh/C (97.8 mg) were added. The air inside the flask was replaced with H₂ (balloon) as described above and stirred at rt. After 21 h, the mixture was passed through a Celite pad, and the pad was washed with MeOH (100 mL). The combined filtrates were concentrated in vacuo to give a mixture (328 mg) of monoethylated amine, diethylated amine, and unethylated amine in a ratio of 87:4:9, respectively. The amines mixture was dissolved in MeOH (3.0 mL), and MeCN (134 μL, 2.57 mmol) and 5% Rh/C (93.9 mg) were added. The air inside the flask was replaced with H₂ (balloon) as described above and the mixture stirred at rt. After 22 h, the mixture was passed through a Celite pad, and the pad was washed with MeOH (100 mL). The combined filtrates were concentrated in vacuo to give a mixture of monoethylated amine, diethylated amine, and unethylated amine (306 mg) in a ratio of 87:9:4, respectively. Proton NMR data for the monoethylated amine **13**: ¹H NMR (CDCl₃) δ 1.08 (3H, t, *J* = 6.8 Hz), 1.38 (9H, s), 1.62 (2H, quin, *J* = 6.8 Hz), 1.84 (2H, quin, *J* = 6.8 Hz), 2.34 (2H, t, *J* = 6.8 Hz), 2.54 (2H, t, *J* = 6.8 Hz), 2.60–2.67 (8H, m), 3.25 (4H, m), 3.41–3.61 (23H, m), 5.14 (1H, br s). The mixture of amines was dissolved in a mixture of CH₂Cl₂ (15 mL) and THF (1 mL), and dansyl chloride (170 mg, 0.629 mmol) was added. After 1 h, Et₃N (33.8 μL, 0.242 mmol) was added, and after 14.5 h, additional Et₃N (33.8 μL, 0.242 mmol) was added. After 1 h, EtOAc (150 mL) and H₂O (50 mL) were added, and the layers were separated. The organic layer was washed with brine (10 mL), and the combined aqueous layers were extracted with EtOAc (150 mL). The organic layers were combined, dried (MgSO₄), filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel (CH₂Cl₂/MeOH, 30:1 → 12:1) to give **14** (212 mg, 0.251 mmol, 47% from **12**) as a light green oil. Data for **14**: analytical TLC on silica

gel, 10:1 CH₂Cl₂/MeOH, R_f = 0.30 (longwave UV); ¹H NMR (CDCl₃) δ 1.04 (3H, t, J = 6.8 Hz), 1.40 (9H, s), 1.60 (2H, br quin, J = 6.8 Hz), 1.86 (2H, quin, J = 6.8 Hz), 2.36 (4H, m), 2.57 (4H, m), 2.84 (6H, s), 3.24–3.36 (6H, m), 3.40–3.57 (24H, m), 3.63 (3H, s), 5.02 (1H, br s), 7.13 (1H, d, J = 7.3 Hz), 7.44–7.52 (2H, m), 8.13 (1H, d, J = 7.3 Hz), 8.24 (1H, d, J = 8.5 Hz), 8.48 (1H, d, J = 8.5 Hz); ¹³C NMR (CDCl₃) δ 13.6, 24.8, 26.1, 28.4, 30.7, 40.3, 41.5, 44.5, 45.4, 51.5, 52.5, 53.7, 69.6, 70.0, 70.1, 70.2, 70.3, 70.5, 70.5, 79.1, 115.0, 119.6, 123.0, 127.8, 129.4, 130.0, 130.0, 135.4, 151.6, 155.9, 173.9 (seven signals could not be located because of their overlaps with other signals); HRMS (FAB⁺, MIX) calcd for C₄₁H₇₁N₄O₁₂S (M + H)⁺ 843.4789, found 843.4777.

14-[3-(*N*-Ethyl-*N*-dansyl)aminopropyl]-25-trifluoroacetyl-amino-5,8,11,17,20,23-hexaoxa-14-azapentacosanoic Acid (**17**)

To a solution of **14** (212 mg, 0.251 mmol) in EtOAc (6 mL)–MeOH (2 mL) at rt was added 2 M HCl in Et₂O solution (5.00 mL, 10.0 mmol). After 2 h, additional 2 M HCl in Et₂O solution (7.50 mL, 15.0 mmol) was added, and after an additional 2 h, the mixture was concentrated in vacuo. The residue was coevaporated with MeOH (5 mL $\times$ 3). Analytical TLC on silica gel, 10:1 CH₂Cl₂/MeOH, R_f = 0.11 or 5:1 CH₂Cl₂/MeOH, R_f = 0.32 (longwave UV). To the residue were added MeOH (4 mL), THF (2 mL), and 2 M NaOH (2.51 mL, 5.02 mmol) successively. After 4 h, additional 2 M NaOH (1.26 mL, 2.51 mmol) was added. After 3 h, the mixture was acidified to pH 4 with 2 M HCl, *n*-BuOH (80 mL) and brine (5 mL) were added, and the layers were separated. The organic layer was washed with brine (5 mL), and the combined aqueous layers were extracted with *n*-BuOH (80 mL $\times$ 3). The organic layers were combined, dried (Na₂SO₄), filtered, and concentrated in vacuo. Analytical TLC on silica gel, 5:1 CH₂Cl₂/MeOH, R_f = 0.07 (longwave UV). The residue was dissolved in MeOH (15 mL), and Et₃N (176 μ L, 1.26 mmol) and CF₃CO₂Et (150 μ L, 1.26 mmol) were added. After 18 h, the mixture was concentrated in vacuo. EtOAc (100 mL) and brine (10 mL) were added, and the layers were separated. The aqueous layer was extracted with EtOAc (100 mL $\times$ 3), and the combined organic layers were dried (Na₂SO₄), filtered, and concentrated in vacuo. The residue was separated by PLC (CHCl₃/MeOH, 6:1, developed five times, longwave UV), and the silica gel containing product was collected in a flash column and eluted with CHCl₃/MeOH (4:1 $\rightarrow$ 3:1). The eluent was concentrated in vacuo, EtOAc (100 mL) and 2 M HCl (30 mL) were added to the residue, and the layers were separated. The organic layer was discarded, and the aqueous layer was adjusted to pH 6 with 2 M NaOH and extracted with EtOAc (100 mL). The organic layer was washed with brine (10 mL), and the combined aqueous layer was extracted with EtOAc (300 mL $\times$ 2). The organic layers were dried (Na₂SO₄) and concentrated in vacuo. CHCl₃ (10 mL) was added to the residue, passed through a cotton-packed pasteur pipet, and washed with CHCl₃ (20 mL). The filtrate was concentrated in vacuo to give **17** (192 mg, 0.233 mmol, 93%) as a light green oil: analytical TLC on silica gel, 5:1 CH₂Cl₂/MeOH, R_f = 0.35 (longwave UV); λ_{max} 343 nm (ϵ = 4500, DMSO); ¹H NMR (CDCl₃) δ 0.99 (3H, t, J = 6.8 Hz), 1.85 (2H, quin, J = 6.4 Hz), 2.03 (2H, br m), 2.38 (2H, t, J = 6.4 Hz), 2.85 (6H, s), 3.04 (2H, br m), 3.15 (4H, br m), 3.29–3.38 (4H, m), 3.48–3.61 (22H, m), 3.81 (4H, m), 7.15 (1H, d, J = 7.3 Hz), 7.46–7.55 (2H, m), 7.57 (1H, br s), 8.11 (1H, d, J = 7.3 Hz), 8.21 (1H, d, J = 8.5 Hz), 8.50 (1H, d, J = 8.5 Hz); ¹³C NMR (CDCl₃) δ 13.4, 23.3, 24.9, 31.2, 39.6, 41.8, 44.0, 45.4, 52.3, 52.8, 53.2, 66.2, 66.4, 68.6, 70.1, 70.2, 70.2, 70.3, 70.4, 70.5, 70.7, 115.2, 115.9 (q, J = 288 Hz), 119.3, 123.2, 128.1, 129.4, 130.0, 130.3, 135.0, 151.8, 157.3 (q, J = 37 Hz), 176.2 (three signals could not be located because of their overlaps with other signals); ¹⁹F NMR (CDCl₃) δ –76.6; HRMS (FAB⁺, NBA) calcd for C₃₇H₆₀F₃N₄O₁₁S (M + H)⁺ 825.3931, found 825.3943.

Solid-Phase Synthesis of Compounds 2–5

The Rink resin was washed with DMF, and the N^{α} -Fmoc protecting group was removed with 1:4 piperidine in DMF (1 $\times$ 2 min and 1 $\times$ 20 min). The resin was washed successively with DMF, CH_2Cl_2 , and DMF. The next N^{α} -Fmoc amino acid was coupled by using the HCTU/*i*-Pr₂EtN procedure (3 equiv of N^{α} -Fmoc amino acid, 3 equiv of HCTU, and 3 equiv of *i*-Pr₂EtN; 1 min preactivation) for 1 h. The coupling procedure was repeated if the Kaiser test showed a purple color. If the second coupling did not result in a negative Kaiser test, the resin was washed with DMF and the free amino groups were capped with 50% Ac_2O in pyridine for 10 min.

After coupling Val, Pro, Lys(Boc), Gly, Trp(Boc), Arg(Pbf), *D*-Phe, His(Trt), Glu(O-*t*-Bu), Nle, Ser(*t*-Bu), Tyr(*t*-Bu), and Ser(*t*-Bu) sequentially to the Rink amide resin, the dansylated PEG linker **17** was coupled as above. The trifluoroacetamide group was removed using a solution of hydrazine in MeOH/THF (15/15/70 v/v/v) for 6 h. The resulting resin **21** was divided into two portions, one of which was acetylated to give **24**. Assembly of the second NDP- α -MSH ligand was carried out on the second portion of resin **21** by the procedure described above to give **22**. N-Terminal acetylation of **22** produced **23**. Cleavage of the peptides **2** and **4** from the resins **23** and **24**, respectively, with concomitant side-chain deprotection was effected using a cleavage cocktail (10 mL per 1 g of the resin) consisting of TFA (91%), H_2O (3%), $\text{HSCH}_2\text{-CH}_2\text{SH}$ (3%), and PhSMe (3%) at rt for 3 h. The solution was filtered off, the resin was washed with TFA (2 $\times$ 3 min), the liquid phases were concentrated under a stream of nitrogen, and the product was precipitated using cold Et_2O . The product was washed three times with cold Et_2O , lyophilized, purified, and characterized (see the Supporting Information for methods and Table 1 for compound characterization data). Peptides **3** and **5** were synthesized, purified, and characterized similarly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Scheme 2. Solid-Phase Synthesis of Compounds 2 and 4^a

^a Reagents and conditions: (a) piperidine; (b) Fmoc/*t*-Bu solid-phase synthesis (ref 15); (c) N-protected linker, HCTU, *i*-Pr₂EtN, DMF; (d) THF/MeOH/N₂H₄ (70/15/15); (e) pyridine/Ac₂O (90/10); (f) TFA/HSCH₂CH₂SH/PhSMe/H₂O (91/3/3/3).

TABLE 1

Mass Spectral and HPLC Characterization Data of Compounds 2–5

compd	formula	calcd mass [ion]	mass found	t_R (% purity)	K'
2	$C_{189}H_{275}N_{45}O_{46}S$	789.6135 [(M + 5) ⁵⁺]	789.6113	19 ^a (91)	5.2
3	$C_{101}H_{139}N_{25}O_{18}S$	1012.0304 [(M + 2) ²⁺]	1012.0296	18 ^a (93)	4.8
4	$C_{113}H_{169}N_{25}O_{28}S$	590.3158 [(M + 4) ⁴⁺]	590.3174	18.4 ^a (96)	5.1
5	$C_{69}H_{101}N_{15}O_{14}S$	698.8765 [(M + 2) ²⁺]	698.8730	25.1 ^b (95)	6.6

^aLinear gradient of from 20 to 60% CH₃CN in 0.1% aqueous TFA over 50 min.^bLinear gradient of from 10 to 60% CH₃CN in 0.1% aqueous TFA over 50 min.

TABLE 2

Competitive Binding of NDP- α -MSH, MSH(4), and Compounds 2–5 to hMC4R^a

compd	IC ₅₀	<i>n</i>
NDP- α -MSH	5.86 $\pm$ 1.90 nM	5
2	1.45 $\pm$ 0.44 nM	6
4	3.62 $\pm$ 0.86 nM	6
MSH(4)	1.15 $\pm$ 0.53 μ M	4
3	0.37 $\pm$ 0.12 μ M	6
5	0.42 $\pm$ 0.22 μ M	6

^aThe IC₅₀ value given is the average of *n* independent binding experiments, each done in quadruplicate.