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Development of a time-resolved fluorescence probe for evaluation of competitive binding to the cholecystokinin 2 receptor

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ABSTRACT

The synthesis, characterization, and use of Eu-DTPA-PEGO-Trp-Nle-Asp-Phe-NH_2 (Eu-DTPA-PEGO-CCK4), a luminescent probe targeted to cholecystokinin 2 receptor (CCK2R, aka CCKBR), are described. The probe was prepared by solid phase synthesis. A K_d value of 17 ± 2 nM was determined by means of saturation binding assays using HEK-293 cells that overexpress CCK2R. The probe was then used in competitive binding assays against Ac-CCK4 and three new trivalent CCK4 compounds. Repeatable and reproducible binding assay results were obtained. Given its ease of synthesis, purification, receptor binding properties, and utility in competitive binding assays, Eu-DTPA-PEGO-CCK4 could become a standard tool for high-throughput screening of compounds in development targeted to cholecystokinin receptors.

1. Introduction

The cholecystokinin 2 receptor (CCK2R, aka CCKBR) is a member of the GPCR family expressed in several cancers, including medullary thyroid carcinomas, small-cell lung cancers, gastroenteropancreatic neuroendocrine cancers, stromal ovarian cancers, astrocytomas, and gastrointestinal stromal cancers.[1] In many of these cancers, CCK2R is overexpressed on malignant cell surfaces,[2,3] providing an opportunity for targeted imaging and/or therapy. One strategy for detecting overexpression is to employ simultaneous binding of two or more weak ligands presented on a single scaffold. Such multivalent molecules can selectively bind with high avidity to cells overexpressing the targeted receptors.[4,5]

When developing a new series of compounds, rapid screening for binding efficacy using a high-throughput competitive binding assay is a useful strategy for hit selection. By this method, the binding efficacies of a series of unlabeled molecules may be evaluated against a single labeled probe targeted to the same receptor. While many radiolabelled ligands that bind to CCK receptors have been reported,[6–9] luminescent probes targeted to CCKRs were only recently described. Probes bearing lanthanide chelates have become popular due to their sensitivity and the avoidance of radioactive materials.[10–13] They are of special importance in applications where background signal is a significant problem. During the past decade several europium-diethylenetriaminepentaacetic acid (Eu-DTPA) chelates linked to peptide recognition elements have been reported.[10,13–16] These probes were used to evaluate multivalent molecules targeted to melanocortin or cholecystokinin receptors via dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA)-based ligand binding assays.[17] The functional readout of such assays comes from the time-resolved fluorescence (TRF) generated by the europium ions. While TRF probes **1** and **2** based on a CCK8 recognition element[16] have previously been used to characterize ligand binding to CCK2R in competitive binding assays,[18–20] a redesign of these probes could simplify and improve probe preparation and purification, possibly enhance probe solubility and receptor binding, and provide an additional tool for use in bioassays.

Abbreviations: CCK4, Trp-Nle-Asp-Phe-NH_2; Cl-HOBt, 1-hydroxy-6-chlorobenzotriazole; CuAAC, copper-catalyzed azide–alkyne cyclization; DELFIA, dissociation-enhanced lanthanide fluoroimmunoassay; DIC, diisopropyl carbodiimide; DMEM, Dulbecco's Modified Eagle Medium; DTPA, diethylenetriaminepentaacetic acid; ESI, electrospray ionization; ICR, ion cyclotron resonance; hCCK2R, human cholecystokinin 2 receptor; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; hMC4R, human melanocortin 4 receptor; HOBt, 1-hydroxybenzotriazole; 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; TACP, tetrakis(acetonitrile)copper(I) hexafluorophosphate; TRF, time-resolved fluorescence.

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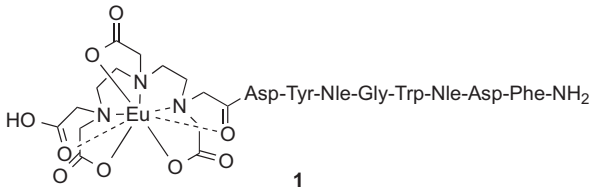
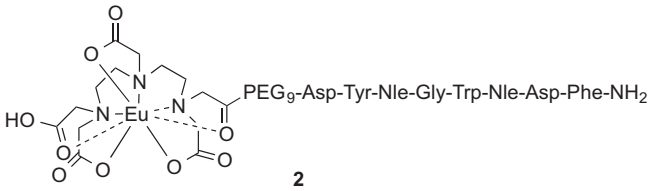




In designing probes for characterization of multivalent binding, we employ the minimum peptide sequence that gives an acceptable level of receptor binding on the theory that a binding competition should involve recognition elements of similar binding affinity. This strategy also minimizes the number of synthetic steps required for probe assembly. In this manner we recently developed a new and useful TRF probe for the evaluation of ligand binding to human melanocortin 4 receptors (hMC4R) and established a robust in vitro binding assay protocol.[15,21] In the present work, we describe a new TRF probe for high-throughput screening of compounds for binding to CCK2R and extend the aforementioned binding assay protocol to bioassays involving this receptor.

2. Materials and methods

2.1. General

Commercial reagents were used as supplied unless otherwise noted. Dichloromethane (DCM) and tetrahydrofuran (THF) were dried by passage through activated alumina. Dimethylsulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF) were dried by contact with activated 4 Å molecular sieves, followed by distillation under reduced pressure. Analytical thin-layer chromatography (TLC) was carried out on pre-coated silica gel 60 F-254 plates with staining by 10% phosphomolybdic acid solution in ethanol or aqueous potassium permanganate solution and heat. Column chromatography was performed using silica gel 60 (200–400 mesh). Melting points were recorded on an Electrothermal® Mel-Temp® apparatus (Model 1001) and are uncorrected. IR spectra were recorded on a Thermo Nicolet iS5 FT-IR Spectrophotometer using NaCl plates. NMR spectra were recorded at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR on a Bruker DRX-500 NMR instrument. Chemical shifts (δ) are expressed in ppm and are internally referenced to chloroform (7.26 ppm and 77.16 ppm for ^{1}H and ^{13}C NMR, respectively). Reactions under microwave irradiation utilized a Biotage Initiator 2.0 microwave reactor. Preparative scale reversed-phase HPLC was performed using a 19 × 250 mm Waters XBridge™ 10 μm OBD™ C_{18} preparative HPLC column. The flow rate was 10 mL/min and a dual channel UV detector was used at 230 and 280 nm. Analytical HPLC was performed on a 3.0 × 150 mm Waters XBridge™ 3.5 μm C_{18} analytical HPLC column. For the analysis of compound **3**, a linear gradient of mobile phase from 10% to 90% MeCN/triethylammonium acetate buffer (0.1% v/v of triethylamine in HPLC grade water adjusted to pH = 6.0 by the addition of acetic acid) was used over 30 min. For all other compounds, a linear gradient of mobile phase from 10% to 90% MeCN/water containing 0.1% TFA was used over 30 min. The flow rate was 0.3 mL/min and a dual channel UV detector was used at 220 and 280 nm. A VWR SympHony™ pH meter (Model SB20) equipped with a Ag/AgCl electrode was used for pH measurements. ESI experiments were performed on a Bruker 9.4 T Apex-Qh hybrid Fourier transform ion-cyclotron resonance (FT-ICR) instrument using standard ESI conditions. Samples were dissolved in MeCN/water (1:1) or MeCN/MeOH (1:1) containing 0.1% formic acid in a concentration range of 1–30 μM. HEK-293 cells engineered[18] to overexpress both hCCK2R and hMC4R were used to measure the affinity of the probe for binding to hCCK2R by means of saturation binding assays. All compounds evaluated in bioassays had purities of ⩾95% as determined by HPLC. Unless otherwise specified, all cell incubations were done in a Fisher Scientific™ Isotemp™ CO_2 incubator (Model 3530) maintained at 37 °C and 5% CO_2 atmosphere. Europium-based TRF competitive binding assays were employed to study the binding of all multivalent constructs and controls. Centrifugations were performed using a VWR Galaxy 7 microcentrifuge or a Fischer Scientific Model 59A microcentrifuge. TRF was measured using a VICTOR™ X4 2030 Multilabel Reader (PerkinElmer) employing the standard Eu TRF measurement settings (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm).

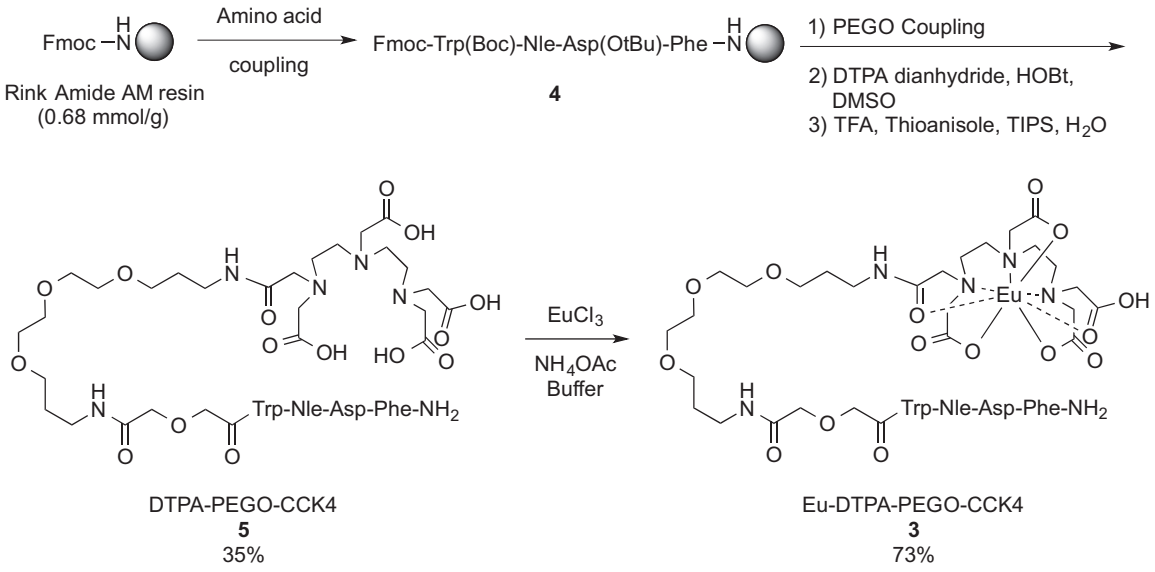
2.2. Solid-phase synthesis

2.2.1. Resin-bound protected CCK4 peptide 4

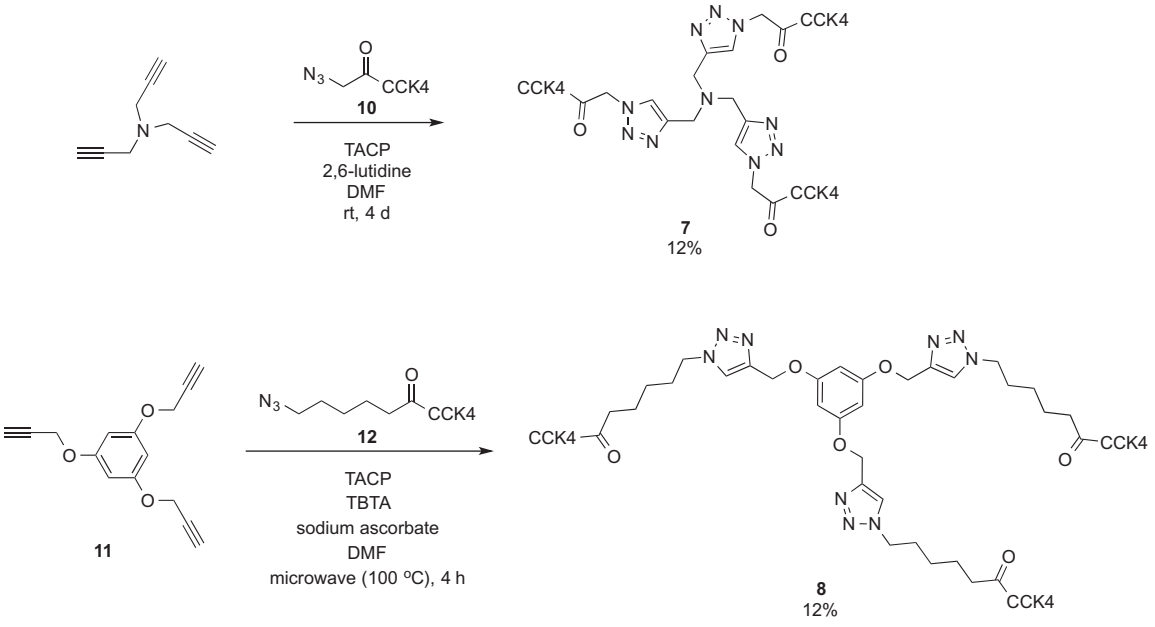
For the synthesis of probe **3** (see Scheme 1) and compounds **6**, **10**, **12**, and **14** (see Schemes 2 and 3), resin-bound protected CCK4 peptide **4** was synthesized manually via an N^{α}-Fmoc solid-phase peptide synthesis strategy on Rink amide AM resin (200–400 mesh, 0.68 mmol/g loading). Resin (1 g) was allowed to swell in THF for 1 h in a polypropylene syringe equipped with a polypropylene frit. THF was removed, a solution of 20% piperidine in DMF (15 mL) was added, and the tube was shaken for 2 min. This solution was removed, 20% piperidine in DMF (15 mL) was again added, and the mixture was shaken for 18 min. After removal of the solution, the resin was washed sequentially with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), 0.5 M HOBt in DMF (15 mL), 0.5 M HOBt in DMF + one drop of 0.01 M bromophenol blue solution in DMF (15 mL), DMF (2 × 15 mL), and DCM (15 mL) in that order. Unless otherwise specified, all resin wash steps in the solid phase peptide synthesis were done by shaking the resin in contact with the wash solvent for 1 min. The amino acid (3 equiv) to be coupled was activated by reaction in a glass vial with 1-hydroxy-6-chlorobenzotriazole (Cl-HOBt, 3 equiv) and diisopropyl carbodiimide (DIC, 6 equiv) in DMF (15 mL) over two min. This solution was then added to the resin and the syringe shaken for 1 h. The coupling solution was removed and the resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). Free amine groups were capped by shaking the resin with acetic anhydride/pyridine (1:1, 6 mL) for 20 min. The resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL), and DMF (3 × 15 mL). The coupling cycle was then repeated for each of the remaining amino acids in the sequence. The Kaiser test[22] was used to determine coupling completion at each attachment step. Following the four coupling cycles, resin-bound **4** was obtained in its protected form.

2.2.2. DTPA-PEGO-CCK4 (5)

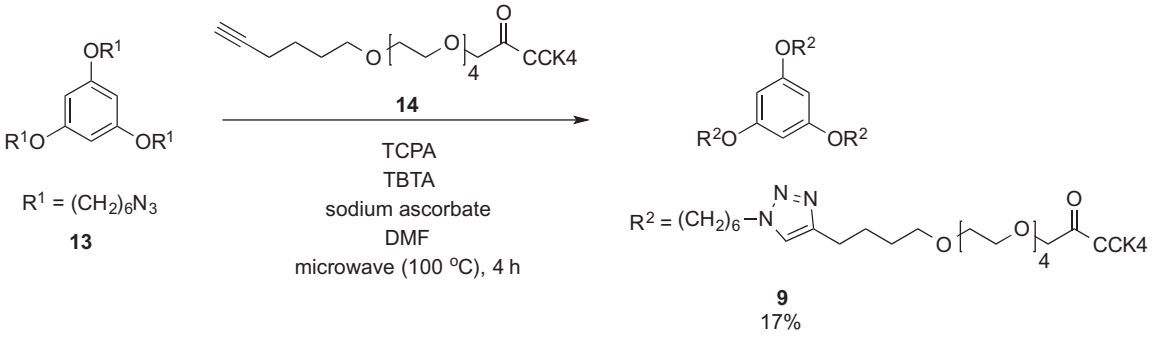
The PEGO linker was attached to a portion (~0.34 mmol) of resin-bound peptide **4** by a conventional coupling cycle following activation of Fmoc-PEGO-OH (Novabiochem 8510310001, 1.02 mmol in 2.04 mL of DCM) with Cl-HOBt (173 mg, 1.02 mmol) and DIC (256 mg, 2.04 mmol) in DMF (5 mL). After 2 h, coupling completion was ascertained by the Kaiser test, and the resin was washed sequentially with DMF (3 × 8 mL), DCM (3 × 8 mL), and DMF (3 × 8 mL).



Scheme 1. Synthesis of the probe Eu-DTPA-PEGO-CCK4 (**3**) using an N^{α}-Fmoc solid-phase peptide synthesis strategy.



Scheme 2. Synthesis of **7** and **8**.



Scheme 3. Synthesis of **9**.

One half of the resin from above (~0.17 mmol) was placed in a syringe reactor and 20% piperidine in DMF (4 mL) was added. The syringe was shaken for 2 min and the solution removed. Additional 20% piperidine in DMF (4 mL) was added and the syringe shaken for 18 min. The solution was removed and the resin washed thoroughly with dry DMSO (9 × 4 mL). DTPA dianhydride (620 mg, 1.7 mmol, 10 equiv) and HOBt monohydrate (520 mg, 3.4 mmol, 20 equiv) were placed in a capped vial with dry DMSO (5 mL). This suspension was heated at 70 °C for 5 min. The suspension cleared, the solution was stirred for 15 min at rt, and was then taken up into the syringe reactor. The mixture was shaken for 1 h and the resin washed with DMSO (2 × 4 mL), THF (2 × 4 mL), 20% aqueous THF (4 mL), 5% diisopropylethylamine in THF (4 mL), THF (3 × 4 mL), and DCM (2 × 4 mL) with five min of shaking for each wash.

A cocktail (3 mL) consisting of TFA, thioanisole, triisopropylsilane, and water (9.1:0.3:0.3:0.3) was injected into the syringe reactor, which was shaken for 4 h at rt. The solution was then collected into a centrifuge tube (15 mL) and the resin washed with further aliquots of TFA (2 × 2 mL × 2 min). The combined TFA solutions were concentrated in the centrifuge tube under a stream of argon and the product precipitated by the addition of cold ether (8 mL). The tube was centrifuged and the supernatant removed. The pellet was washed with cold ether (3 × 6 mL), air dried, dissolved in water/MeCN, and lyophilized. The resultant solid was subjected to reversed-phase HPLC (mobile phase gradient of 0–90% MeCN/water containing 0.1% TFA over 45 min, t_R = 22.9 min), product-containing fractions were combined, and lyophilized to give **5** as a fluffy white solid. Yield 75.0 mg (59 μmol, 35%); HRMS (ESI-ICR) *m*/*z* calculated for $C_{58}H_{84}N_{11}O_{21}$ $[M-H]^-$ 1270.5849, observed 1270.5832.

2.2.3. Ac-Trp-Nle-Asp-Phe-NH_2 (Ac-CCK4, 6)[23]

To resin-bound **4** (0.34 mmol) in a polypropylene syringe equipped with a polypropylene frit was added a solution of 20% piperidine in DMF (8 mL) and the syringe was shaken for two min. The solution was removed, 20% piperidine in DMF (8 mL) was again added, and the mixture was shaken for 18 min. After removal of the solution, the resin was washed sequentially with DMF (3 × 8 mL), DCM (3 × 8 mL), and DMF (3 × 8 mL). A 1:1 mixture of acetic anhydride (960 μL) and pyridine (820 μL) in DMF (3 mL) was taken up into the syringe reactor, which was shaken for 1 h. The resin was washed with DMF (3 × 8 mL), DCM (3 × 8 mL), DMF (3 × 8 mL), THF (3 × 8 mL), and DCM (3 × 8 mL). Cleavage from the resin and isolation according to the procedures described for **5** gave 190 mg of crude product. Following reversed-phase preparative HPLC (mobile phase gradient of 10% at 0 min–50% at 8 min–55% at 20 min, MeCN/water containing 0.1% TFA, t_R = 13.3 min), product-containing fractions were combined and the solution lyophilized to give **6** as a fluffy white solid. Yield 82 mg (0.13 mmol, 39%); mp, decomposes at 232 °C; HRMS (ESI-ICR) *m*/*z* calculated for $C_{32}H_{41}N_6O_7$ $[M+H]^+$ 621.3031, observed 621.3029; analytical HPLC t_R = 17.47 min.

2.2.4. (*S*)-4-(((*S*)-1-Amino-1-oxo-3-phenylpropan-2-yl)amino)-3-((*S*)-2-((*S*)-2-(2-azidoacetamido)-3-(1*H*-indol-3-yl) propanamido)hexanamido)-4-oxobutanoic acid (10)

To a solution of sodium azide (11.5 g, 176 mmol, 2.5 equiv) in water (60 mL) was added bromoacetic acid (10.0 g, 70.5 mmol, 1 equiv) at 0 °C and the resultant solution was stirred at rt for 24 h.[24] The reaction was diluted with aqueous 1 N HCl (150 mL) and extracted with diethyl ether (4 × 50 mL). The organic extracts were combined, dried with anhydrous Na_2SO_4, filtered, and evaporated in vacuo to yield azidoacetic acid as a clear oil. Yield 6.1 g (60.4 mmol, 86%); R_f 0.32 (20% EtOAc/DCM, visualization $KMnO_4$); IR (NaCl plates, cm^{-1}) 3500–2500 (br), 2924, 2113, 1728; ^1H NMR (500 MHz, $CDCl_3$) δ 10.07 (s, 1H), 3.97 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.27, 50.11.

Using the coupling procedures described previously, compound **10** was prepared from azidoacetic acid (0.21 g, 2.04 mmol, 3 equiv) and resin-bound **4** (~0.68 mmol). Cleavage from the resin using of cleavage cocktail (10 mL) and isolation according to the procedures described for **5** gave 355 mg of crude material. Following reversed-phase preparative HPLC (mobile phase gradient of 10% at 0 min–50% at 8 min–55% at 20 min, MeCN/water containing 0.1% TFA, t_R = 14.6 min), product-containing fractions were combined and the solution lyophilized to give **10** as a fluffy white solid. Yield 230.9 mg (0.35 mmol, 51%); HRMS (ESI-ICR) *m*/*z* calculated for $C_{32}H_{40}N_9O_7$ $[M+H]^+$ 662.3045, observed 662.3044.

2.2.5. (*S*)-4-(((*S*)-1-Amino-1-oxo-3-phenylpropan-2-yl)amino)-3-((*S*)-2-((*S*)-2-(6-azidohexanamido)-3-(1*H*-indol-3-yl) propanamido)hexanamido)-4-oxobutanoic acid (12)

Using the coupling procedures described previously, compound **12** was prepared from 6-azidohexanoic acid (0.34 g, 2.04 mmol, 3 equiv) and resin-bound **4** (~0.68 mmol). Cleavage from the resin and isolation according to the procedures described for **5** gave 421 mg of crude material. Following reversed-phase preparative HPLC (mobile phase gradient of 10% at 0 min–50% at 10 min–90% at 25 min, MeCN/water containing 0.1% TFA, t_R = 17.7 min), product-containing fractions were combined and the solution lyophilized to provide **12** as a fluffy white solid. Yield 198 mg (0.28 mmol, 41%); HRMS (ESI-ICR) *m*/*z* calculated for $C_{36}H_{48}N_{10}O_{10}$ $[M+H]^+$ 718.36712, observed 718.36708.

2.2.6. (3*S*,6*S*,9*S*)-9-((1*H*-Indol-3-yl)methyl)-3-(((*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)carbamoyl)-6-butyl-5,8,11-trioxo-13,16,19,22,25-pentaoxa-4,7,10-triazahentriacont-30-ynoic acid (14)

For the synthesis of **14**, procedures modified from those previously described were used to couple 3,6,9,12,15-pentaoxahenicos-20-ynoic acid (0.70 g, 2.10 mmol, 3 equiv, synthesized as described in Supplementary data that accompanies this article) to resin-bound **4** (~0.68 mmol). Specifically, bromophenol blue was not used, a coupling time of 2 h was employed, and post-coupling acylation was not performed. Cleavage from the resin using cleavage cocktail (10 mL) and isolation according to the procedures described for **5** gave a solid which was subjected to reversed-phase preparative HPLC (mobile phase gradient of 10–90% MeCN/water containing 0.1% TFA over 45 min, t_R = 16.9 min). Product-containing fractions were combined and the solution lyophilized to produce **14** as a fluffy white solid. Yield 243 mg (0.27 mmol, 40%); HRMS (ESI-ICR) *m*/*z* calculated for $C_{46}H_{65}N_6O_{12}$ $[M+H]^+$ 893.46550, observed 893.46609.

2.3. Synthesis of the probe Eu-DTPA-PEGO-CCK4 (3)

The metal-free precursor **5** (20.0 mg, 15.7 μmol) was dissolved in 0.1 M ammonium acetate solution at a concentration of 1 mg/mL. The pH was adjusted to 7–8 with aqueous 0.1 M NH_4OH and $EuCl_3 \cdot 6H_2O$ (11.5 mg, 31.4 μmol, 2.0 equiv) was added. The reaction mixture was stirred at rt overnight. Salts were removed using a Sep-Pak® C_{18} reverse-phase (500 mg) column as previously reported.[13,15] Product-containing fractions were concentrated and lyophilized to afford the product Eu-DTPA-PEGO-CCK4 (**3**) as a fluffy white solid (16.2 mg, 11.4 μmol, 73%). The presence of unbound europium ions was confirmed by a xylenol orange assay.[25]

An Empore™ chelating disk (47 mm) was placed on a sintered glass filter holder (47 mm) base fitted to a 1 L filter flask. The solvent reservoir was then clamped on to the base, completing the apparatus. The disk was wetted with 2–5 mL of distilled water, as per the manufacturer's instructions. This resulted in swelling of the disk. Nitric acid (3 M, 20 mL) was added, the disk allowed

to soak in this solution for 1 min, then a vacuum was applied, drawing the acid solution through the filter. The disk was washed on the filter under vacuum with water (2 × 50 mL). The disk was allowed to go dry between each wash. To put the disk in its most active ammonium form, 100 mL of 0.1 M ammonium acetate buffer (pH 5.3, made up in HPLC grade water) was added to the solvent reservoir and the disk allowed to soak for 1 min. The solution was then drawn through the disk by applying a vacuum, and the disk left to dry for 5–20 min before sample loading. During this time, the apparatus was disassembled, cleaned thoroughly with distilled water followed by HPLC grade water, and the apparatus reassembled for use.

A solution of **3** (15.0 mg) in water/MeCN (3 mL/mg, pH 5–7 using the minimum amount of MeCN required to fully dissolve the compound) was passed through the disk at a flow rate of 30–40 mL/min to allow binding interactions. The disk was then washed with MeCN/water (50:50, 30 mL). The combined filtrates were transferred to a round bottom flask and lyophilized to give the purified product **3**. The removal of unchelated Eu ions was confirmed by a xylenol orange assay.[25] Recovery was 14.6 mg (97%); HRMS (ESI-ICR) *m/z* calculated for $C_{58}H_{84}EuN_{11}O_{21}$ $[M+2H]^{2+}$ 711.7527, observed 711.7527; analytical HPLC t_R = 13.82 min.

2.4. Copper-catalyzed azide–alkyne cyclization (CuAAC) reactions

2.4.1. CCK4 Trimer 7

To a microwave vial (0.5–2 mL) purged with argon were added tripropargylamine (571 μL of a 0.035 M solution in DMF, 20 μmol), azide **10** (79.4 mg, 120 μmol, 6 equiv), argon-purged DMF (500 μL), TACP (29 mg, 78 μmol, 3.9 equiv), and 2,6-lutidine (470 μL, 40 μmol, 2 equiv). The vial walls were washed down with 400 μL of argon-purged DMF, the vial sealed, and stirred at rt for 4 days. Volatiles were removed in vacuo and the residue dissolved in water/MeCN (250 mL). The aqueous solution was extracted with a solution of dithizone in chloroform (1.5 mM, 3 × 50 mL) and chloroform (2 × 50 mL), then lyophilized. The residue was subjected to preparative reversed-phase HPLC (mobile phase gradient of 10% at 0 min–55% at 15 min–80% at 25 min, MeCN/water containing 0.1% TFA, t_R = 15.1 min) giving **7** as a white solid after lyophilization. Yield 5.2 mg (2.5 μmol, 12%); HRMS (ESI-ICR) *m/z* calculated for $C_{105}H_{128}N_{28}O_{21}$ $[M+2H]^{2+}$ 1058.48989, observed 1058.49173; analytical HPLC t_R = 21.18 min.

2.4.2. CCK4 Trimer 8

To a microwave vial (0.5–2 mL) purged with argon were added **11** (150 μL of a 0.122 M solution in DMF, 18.3 μmol), azide **12** (80.6 mg, 112.2 μmol, 6 equiv), TBTA (11.9 mg, 22.4 μmol, 1.2 equiv), TACP (8.4 mg, 22.4 μmol, 1.2 equiv), sodium ascorbate (4.9 mg, 24.7 μmol, 1.1 equiv per TACP), and 500 μL of argon-purged DMF. The vial walls were washed down with 400 μL of argon-purged DMF, the vial sealed, and irradiated in a microwave reactor to maintain a temperature of 100 °C for 4 h. Volatiles were removed in vacuo and the residue dissolved in water/MeCN (100 mL). The aqueous solution was extracted with a solution of dithizone in chloroform (0.5 mM, 3 × 40 mL) and chloroform (2 × 40 mL), then lyophilized. The residue was subjected to preparative reversed-phase HPLC (mobile phase gradient of 10% at 0 min–55% at 15 min–80% at 25 min, MeCN/water containing 0.1% TFA, t_R = 16.6 min) affording **8** as a white solid after lyophilization. Yield 5.2 mg (2.2 μmol, 12%); HRMS (ESI-ICR) *m/z* calculated for $C_{123}H_{155}N_{27}O_{24}$ $[M+2H]^{2+}$ 1197.0864, observed 1197.0872; analytical HPLC t_R = 22.42 min.

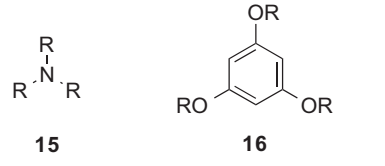
2.4.3. CCK4 Trimer 9

To a microwave vial (0.2–0.5 mL) purged with argon were added **13** (29.0 μL of a 0.518 M solution in DMF, 15 μmol), alkyne **14** (80.4 mg, 90 μmol, 6 equiv), TBTA (9.5 mg, 18 μmol, 1.2 equiv), TACP (6.7 mg, 18 μmol, 1.2 equiv), sodium ascorbate (3.9 mg, 19.8 μmol, 1.1 equiv per TACP), and 150 μL of argon-purged DMF. The vial walls were washed down with 75 μL of argon-purged DMF, the vial sealed, and irradiated in a microwave reactor to maintain a temperature of 100 °C for 4 h. Volatiles were removed in vacuo and the residue dissolved in water (50 mL). The aqueous solution was extracted with a solution of dithizone in chloroform (0.5 mM, 3 × 30 mL) and chloroform (2 × 30 mL). The resulting aqueous solution was lyophilized, giving 30 mg of residue. Preparative reversed-phase HPLC (mobile phase gradient of 10% at 0 min–60% at 13 min–70% at 20 min, MeCN/water containing 0.1% TFA, t_R = 19.8 min) gave **9** as a white solid after lyophilization. Yield 7.9 mg (2.5 μmol, 17%); HRMS (ESI-ICR) *m/z* calculated for $C_{162}H_{234}N_{27}O_{39}$ $[M+3H]^{3+}$ 1060.5714, observed 1060.5729; analytical HPLC t_R = 25.12 min.

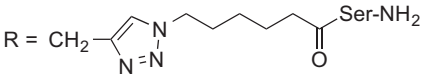
2.5. Biological assays

2.5.1. Preparation of solutions

Stock solutions of the CCK4 trimers **7**–**9**, control compounds **6**, **15**, and **16**, and the probe Eu-DTPA-PEGO-CCK4 (**3**) were made up in DMSO at a nominal concentration of 2.0 mM based on measured weights of solutes. Except for the control compounds **15** and **16**, final concentrations were determined by comparison to a DTrp standard solution (0.50 mM) using analytical HPLC. *Selective growth media* for cell growth was prepared by supplementing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 1% penicillin–streptomycin, 0.1% zeocin, and 0.8% geneticin. *Basic buffer* was prepared by dissolving 5.97 g HEPES and 2 g BSA in 1 L of DMEM. The pH of this solution was adjusted to 7.4 using 2 N NaOH and the solution sterilized by filtration through a 0.22 μm filter (Corning®, 431117, 500 mL bottle-top filter, sterile) under vacuum. *Binding buffer* was prepared by supplementing DMEM (1 L) with HEPES (5.97 g), BSA (2 g), 1,10-phenanthroline (1 mL of a 1 M solution in EtOH), leupeptin (1 mL of a 500 mg/L aqueous solution), and bacitracin (1 mL of a 200 g/L aqueous solution). The pH of this solution was adjusted to 7.4 using 2 N NaOH and the solution sterilized by filtration through a 0.22 μm filter (Corning, 431117, 500 mL bottle-top filter, sterile) under vacuum.



15 **16**



2.5.2. Cell culture

HEK-293 cells expressing both hMC4R and hCCK2R[18] were maintained in selective growth media. For binding assays, cells were plated into six-well plates (Greiner Bio-One, 657160, Cell Culture Multi-well Plates, Polystyrene, six wells) at 240,000 cells per well in a total volume of 3 mL (2 mL of the selective growth media added initially to each well, followed by 1 mL of cell suspension). On the third day after plating, additional selective growth media (1 mL) was carefully added to each well so as not to disturb the cells, which were then left to grow until ~90% confluency was achieved (usually by Day 5) before conducting assays.

2.5.3. Saturation binding assays

Six solutions containing both the probe Eu-DTPA-PEGO-CCK4 (**3**) and Ac-CCK4 (**6**) were made in 1.2 mL of binding buffer in separate micro-centrifuge tubes (one per each well). All six tubes contained 1 μM concentrations of **6**, while the concentration of **3** varied across the six tubes (0.1, 10, 25, 50, 100, and 250 nM). These six solutions were used to assess nonspecific binding. A second set of six solutions (1.2 mL each) contained **3** at the same concentrations without the blocking ligand **6**. These solutions were used to assess total binding.

To commence an assay, cell-containing six-well plates (×2) were removed from the incubator and the selective growth media removed by careful aspiration. The twelve prepared solutions detailed above were carefully transferred (1 mL per well) by pipette down the well walls to minimize disturbance of the cells, which can result in cell loss during media exchanges. The plates were then maintained in a CO_2 incubator at 37 °C for 1 h.

Solutions were then removed by careful aspiration and 600 μL of basic buffer were added to each well. The cells were scraped from each well individually using a Cell Scraper (18 cm, GeneMate) and transferred in suspension to separate 1.7 mL micro-centrifuge tubes. The wells and scraper were rinsed with 600 μL of basic buffer and the rinses combined with the corresponding suspensions. One scraper was used across the wells measuring total binding, and another was used across the wells measuring non-specific binding.

The tubes containing the cell suspensions were centrifuged (3000 rpm for 3 min) in a micro-centrifuge. After removing the supernatant, the cells were re-suspended in basic buffer (1 mL) and incubated for 5 min in a 37 °C water bath during each of three wash cycles. After the final wash, 600 μL of DELFIA Enhancement Solution (Perkin Elmer 1244-104) was added to each cell pellet, the tubes were mixed using a vortex mixer, and incubated for 1 h in a water bath maintained at 37 °C.

Following this incubation, cells and cell fragments were pelleted (5000 rpm for 5 min) and 4 × 100 μL aliquots of supernatant from each tube were transferred to a 96-well plate (Perkin–Elmer, 6005060, tissue culture treated B&W Isoplate-96) for fluorescence measurement using a VICTOR X4 2030 Multilabel Reader.

2.5.4. Competitive binding assays

Immediately before an assay, twelve solutions (1350 μL each) of compounds to be tested were made up in binding buffer in micro-centrifuge tubes at concentrations ranging from 10^{-5} to 10^{-12} M. Each of the tubes also contained the probe Eu-DTPA-PEGO-CCK4 (**3**) at a concentration of 15 nM. Cell-containing six-well plates (×2) were removed from the incubator and the selective growth media removed by careful aspiration. The twelve prepared solutions from above were carefully transferred (1 mL per well) by pipette down the well walls. The plates were then maintained in a CO_2 incubator at 37 °C for 1 h.

After 1 h, solutions were removed by careful aspiration and 600 μL of basic buffer were added to each well. The cells were scraped from each well using a Cell Scraper (18 cm, GeneMate) and transferred in suspension to separate 1.7 mL micro-centrifuge tubes. The wells and scraper were rinsed with 600 μL of basic buffer and the rinses combined with the corresponding suspensions.

The cell suspensions were centrifuged (3000 rpm for 3 min) in a micro-centrifuge. After removing the supernatant, the cells were re-suspended in basic buffer (1 mL) and incubated for 5 min in a 37 °C water bath during each of three wash cycles. After the final wash, 600 μL of DELFIA Enhancement Solution (Perkin Elmer 1244-104) was added to each cell pellet, the tubes were mixed using a vortex mixer, and incubated in a water bath maintained at 37 °C for 1 h.

Following this incubation, cells and cell fragments were pelleted (5000 rpm for 5 min) and 4 × 100 μL aliquots of supernatant from each tube were transferred to a 96-well plate (Perkin–Elmer, 6005060, tissue culture treated B&W Isoplate-96) for fluorescence measurement using a VICTOR X4 2030 Multilabel Reader.

2.6. Data analysis

NMR data were analyzed using MestReNova software (Mestre Lab Research S. L., version 7.1.1). Binding data analysis was performed using GraphPad Prism software (version 5.04). A description of the binding equations used appears in Supplementary data that accompanies this article.

3. Results

3.1. Chemistry

The fully protected resin-bound precursor **4** to probe **3**, Ac-CCK4 (**6**), azides **10** and **12**, and alkyne **14** was prepared by solid phase synthesis on Rink amide AM resin (Scheme 1). For the preparation of probe **3**, Fmoc-PEGO[26] was coupled to the N-terminus of the resin-bound peptide. Following removal of the Fmoc, DTPA, 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 followed by purification by preparative HPLC produced the metal-free precursor DTPA-PEGO-CCK4 (**5**) in 35% yield. Compound **5** was characterized by ESI-ICR MS. Following complexation of Eu^{3+} by **5**, purification and partial desalting by reversed-phase chromatography, final desalting by passage through an Empore chelating disk,[25] and recovery by lyophilization afforded Eu-DTPA-PEGO-CCK4 (**3**) in 73% yield. Compound **3** was characterized by analytical HPLC and ESI-ICR MS. The absence of unchelated Eu^{3+} ion (within a 10 μM limit of detection) was confirmed by means of a xylenol orange spectrophotometric assay.[25]

For preparation of **6**, the resin-bound tetrapeptide **4** was N-terminally acetylated. Side chain deprotection and cleavage of the peptide from the resin, purification by reversed-phase preparative HPLC, and recovery by lyophilization gave **6** in 39% yield. Compound **6** was characterized by analytical HPLC and ESI-ICR MS.

Compounds **7** and **8** were prepared as depicted in Scheme 2. Tripropargylamine is commercially available. The synthesis of phloroglucinol derivative **11** was previously described.[21] Azides **10** and **12** were prepared by coupling 2-azidoacetic acid and 6-azidohexanoic acid,[27] respectively, to the N-terminus of the resin-bound peptide **4** (chemistry not depicted). Side chain deprotection and cleavage of the peptides from the resin, purification by reversed-phase preparative HPLC, and recovery by lyophilization produced **10** and **12** in 51% and 41% yields, respectively. These compounds were characterized by ESI-ICR MS. The copper-catalyzed azide–alkyne cyclization (CuAAC) reaction of tripropargylamine with **10** at room temperature produced **7**. The CuAAC reaction of **11** with **12** under microwave irradiation to maintain a temperature of 100 °C produced **8**. In each case, copper ions were removed from the crude product mixture by complexation with dithizone with removal of the resulting complex by extraction with $CHCl_3$.[28] The water-soluble material was then recovered by lyophilization, subjected to preparative reversed-phase HPLC, and product-containing fractions subjected to lyophilization to give products **7** and **8**, each in 12% yield.

Compound **9** was prepared as depicted in Scheme 3. Phloroglucinol derivative **13** was prepared as described in Supplementary data that accompanies this article. Alkyne **14** was prepared by coupling 3,6,9,12,15-pentaoxahenicos-20-ynoic acid

(prepared as described in Supplementary data) to the N-terminus of the resin-bound peptide **4**. Side chain deprotection and cleavage of the peptide from the resin, purification by reversed-phase preparative HPLC, and recovery by lyophilization produced **14** in 40% yield. This compound was characterized by ESI-ICR MS. The CuAAC reaction of **13** with **14** under microwave irradiation to maintain a temperature of 100 °C produced **9**. Copper ions were removed from the crude product mixture by complexation with dithizone with removal of the resulting complex by extraction with $CHCl_3$.[28] The water-soluble material was then recovered by lyophilization, subjected to preparative reversed-phase HPLC, and product-containing fractions subjected to lyophilization to give **9** in 17% yield.

Compounds **7**-**9** were characterized by analytical HPLC and ESI-ICR MS.

3.2. Bioassays

HEK-293 cells overexpressing both hCCK2R[29] and hMC4R[30,31] were used to measure the affinity of probe **3** for binding to hCCK2R by means of saturation binding assays (Fig. 1).[21] Ac-CCK4 (**6**)[23] was used as the blocking ligand. The K_d value obtained for probe **3** was 17 ± 2 nM.

Competitive binding assays were conducted for compounds **6**–**9**, **15**, and **16** against probe **3**. Sample binding curves appear in Figure 2 and K_i values are listed in Table 1.

4. Discussion

A recent study of TRF probes for melanocortin receptors showed that probes based on ligands with less than the maximal binding affinity offered greater ease of synthesis and, in some cases, excellent performance in competitive binding assays.[15] With this in mind, development and testing of a probe for cholecystokinin receptors based on the minimal active sequence Trp-Met-Asp-Phe-NH_2[32,33] was undertaken.

Eu-DTPA-PEGO-CCK4 (**3**) was synthesized via a solid phase peptide synthesis strategy (Scheme 1). The protected tetrapeptide **4** was assembled on Rink amide AM resin. Methionine was replaced with the isosteric analog norleucine, which is not susceptible to oxidation and is less prone to proteolytic degradation.[34,35] This substitution was expected to afford a probe with increased shelf life[34,36] and high biological activity.[36–38] A PEGO spacer was used to minimize interference of the europium chelate with binding of the ligand to the receptor. Given the hydrophobic nature of CCK4, this was expected to enhance the water solubility of the probe. Following deprotection and cleavage from the resin, purification by reversed-phase HPLC afforded **5** in 35% yield. Treatment of **5** with excess $EuCl_3$ in a pH 7–8 buffer gave the chelate **3**, but passage through a Sep-Pak®C_{18} column left unchelated europium ions as a contaminant as determined by a xylenol orange assay.[25] Complete removal of unchelated europium ions is essential to obtaining reliable bioassay readings. Fortunately, passage of **3** through an Empore chelating disk removed the unchelated europium ions.[25] The overall yield of **3** was 26%, significantly higher than the 16% yield reported for the synthesis of probe **1**.[16]

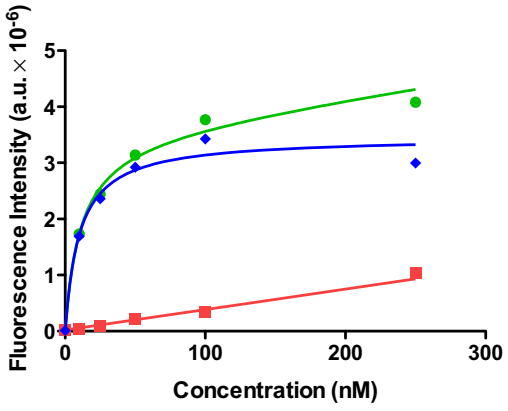


Figure 1. Saturation binding curves for probe **3**. Total binding (●), non-specific binding (■), and specific binding (◆). The calculated K_d = 17 ± 2 nM (n = 5).

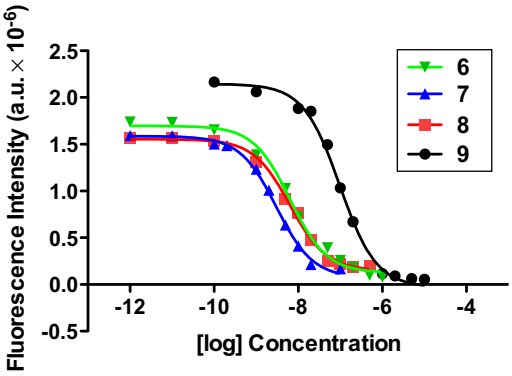


Figure 2. Competitive binding curves for the compounds **6**–**9** against probe **3** (15 nM). Control compounds **15** and **16** were not competitive inhibitors of **3** over the concentration range tested.

Table 1
Results of competitive binding assays[a]

Compound	K_i ± SEM[b] (nM)	Relative binding affinity[c]
Ac-CCK4 (**6**)	6.1 ± 2.1	1
7	1.2 ± 0.4	5
8	3.7 ± 0.4	2
9	30 ± 8	0.2
15	nb[d]	na[e]
16	nb[d]	na[e]

[a] Competition experiments were carried out against probe **3** (K_d = 17 nM, [**3**] = 15 nM) using HEK-293 cells overexpressing hCCK2R and hMC4R.
[b] SEM = standard error of the mean; n = 4 independent determinations.
[c] Ratio of the K_i of Ac-CCK4 (**6**) to the compound K_i value.
[d] nb = no competitive binding observed.
[e] na = not applicable.

Saturation binding assays were conducted to characterize the affinity of probe **3** for CCK2R using HEK-293 cells that overexpress both hCCK2R and hMC4R. Despite having a highly truncated ligand relative to probe **1**, probe **3** bound tightly to CCK2R. The K_d for tetrapeptide **3** was 17 nM, while the reported K_d for octapeptide **1** is 35 nM.[18]

Probe **3** was developed for use in high-throughput screening of unlabeled molecules for binding to CCK receptors. To establish its efficacy for this purpose, trivalent compounds **7**–**9** were synthesized and tested against **3** in competitive binding assays. Three quite different inter-ligand distances were employed in **7**–**9**, since the optimum ligand spacing for multivalent binding to CCK receptors has not been established. All three compounds were prepared by a combination of solid phase synthesis (from **4**) and copper-catalyzed azide–alkyne cyclization as depicted in Schemes 2 and 3. Positive control **6** and negative controls **15**[21] and **16**[21] in which serinamide residues replace the CCK4 ligands were also competed against probe **3**. Results of the competitive binding assays are given in Table 1.

As was expected, compounds **15** and **16** did not interfere with the binding and uptake of probe **3**. Control **6** exhibited a K_i of 6.1 nM, a value consistent with those reported for other

monovalent CCK4 ligands.[20,39] Given the high affinity of CCK4, avidities indicative of multivalent binding were not expected for CCK4 trimers **7**–**9**. Even if the ligand spacing had been optimal, enhanced avidities due to multivalent binding are demonstrable only when weakly binding ligands are employed.[4,5,20] Modest increases in binding affinity were observed for compounds **7** and **8** relative to **6** that can be attributed to statistical and/or proximity effects. Compound **9** exhibited a significantly lower binding affinity compared to CCK4 trimers **7** and **8**. This maybe due to a larger entropy penalty for **9** upon binding to CCK2R or to hydrophobic interactions between the PEG spacer and the CCK4 ligand which hinder binding to the receptor.[40,41] For these reasons, lower affinity ligands are currently being identified for incorporation into multivalent molecules targeting CCK2R.

5. Conclusion

A short and efficient synthesis of a new TRF probe that binds to cholecystokinin-2 receptors has been developed. The combination of this probe with the high expression levels of CCK2R ($\sim 1.1 \times 10^6$ receptors/cell) by the engineered HEK-293 cell line and the use of large cell populations ($\sim 1 \times 10^6$ cells per well at 90% confluence) in the six-well plate assay employed provide binding assay data with very high signal-to-noise ratios. Hence, the Eu-DTPA-PEGO-CCK4 probe **3** could become an important tool for high-throughput screening of binding to cholecystokinin receptors.

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Supplementary data

Supplementary data, including details of the synthesis of **13** and of 3,6,9,12,15-pentaoxahenicos-20-ynoic acid (used in the synthesis of **14**), ^{1}H and ^{13}C NMR spectra of new compounds not containing CCK4, copies of the HPLC chromatograms for compounds **3** and **6**–**9**, and details of the binding assays with binding curves associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.02.028.

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