Supporting Information

Solid-Phase Synthetic Strategy & Bioevaluation of a Labeled δ-Opioid Receptor Ligand Dmt-Tic-Lys for *In Vivo* Imaging

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Abbreviations

Dmt	2',6'-dimethyltyrosine
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
Mpr	3-mercaptopropionyl
Ado	8-amino-3,6-dioxaoctanyl
Mtt	4-methyltrityl
Mmt	4-methoxytrityl
DOTA	1,4,7,10-tetraazacyclodecane-N,N',N'',N'''-tetraacetic acid
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HOCt	6-chloro-1-hydroxybenzotriazole
DIC	N,N'-Diisopropylcarbodiimide
TEAA buffer	Triethylamine/Acetic acid buffer
TFA	Trifluoroacetic acid

Materials

The N^{α}-Fmoc protected amino acids and the Wang resin were purchased from SynPep (Dublin, CA) or from Novabiochem (San Diego, CA). The following side chain protections were used for the amino acids: Lys(Mtt); Cys(Mmt). Boc-Dmt-OH was purchased from RSP Amino Acids (Shirley, MA). HOBt, HOCt, DIC, *i*Pr_2NEt and HBTU were purchased from IRIS Biotech (Marktredwitz, Germany). DOTA-NHS ester was purchased from Macrocyclics (Dallas, TX). Cy5-maleimide was purchased from Amersham Biosciences (Piscataway, NJ). Fmoc-8-amino-3,6-dioxaoctanoic acid and S-Tritylsulfanyl-propionic acid were acquired from Bachem (Torrance, CA). Peptide synthesis solvents, dry DMF, dry CH_2Cl_2, and solvents for HPLC were reagent grade, were acquired from VWR (West Chester, PA) or Sigma-Aldrich (Milwaukee, WI), and were used without further purification unless otherwise noted. The polypropylene syringes equipped with a frit and the Domino manual synthesizer were obtained from Torviq (Niles, MI). The C-18 Sep-Pak™ Vac RC cartridges were purchased from Waters (Milford, MA).

HPLC Analysis, Solid Phase Extraction, Size Exclusion Chromatography and Peptide Concentration Determination

The purity of final products was analyzed using Waters high-performance liquid chromatography (HPLC) apparatus and with a Vydac C18 reverse phase column (dia × length: 4.6 mm × 150 mm, pore size: 3 μm). See Table S1 for HPLC methods used for analysis and purification. Peptides were analyzed using a linear gradient of buffer B under various gradient conditions at a flow rate of 0.3 - 1 mL/min and the separations

were monitored at 220 and 280 nm. Purification of compounds was achieved using a Waters 600 HPLC apparatus equipped with a Vydac C18 reverse phase column (22 mm × 250 mm, 5 μm) with similar buffers and 3 - 10 mL/min flow rate. The separations were monitored at 230 and 280 nm.

Table S1. HPLC conditions used for Dmt-Tic compounds.

PHASE A	PHASE B
A1: 0.1% TFA in water	B1: 0.1% TFA in acetonitrile
A2: 0.1% Et_3N/AcOH (TEAA) in water (pH 6.0)	B2: 90% acetonitrile in Phase A2 (pH adjusted to 6.0 with acetic acid)

Method A: 10-40% Phase B1 in Phase A1 in 30 min; **Method B**: 10-60% Phase B1 in Phase A1 in 50 min;
Method C: 10-90% Phase B1 in Phase A1 in 30 min; **Method D**: 20-60% Phase B1 in Phase A1 in 50 min;
Method E: 10-60% Phase B2 in Phase A2 in 50 min; **Method F**: 10-90% Phase B2 in Phase A2 in 40 min

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Solid-Phase Extraction (SPE) was employed where simple isolation of final compound was needed, e.g., europium chelated compound **2** from excess salts and buffers. For this purpose, C-18 Sep-Pak™ cartridges (100 mg or 500 mg) were used and pre-conditioned with 5 column volumes (5 times the volume of packed column bed) each of acetonitrile, methanol and water, in that order. After loading the compound, the column was washed several times with water, and the compound was eluted with acetonitrile/water gradient as needed.

Size exclusion chromatography (SEC) was performed on a borosilicate glass column (2.6 mm × 250 mm, Sigma, St. Louis, MO) filled with medium sized Sephadex G-25 or G-10. The compounds were eluted with an isocratic flow of 1.0 M aqueous acetic acid.

The lyophilized final products were dissolved in DMSO:H_2O (3:2) for characterization by bioassay. The peptide concentrations were determined by monitoring absorbance of peptides against 0.5 mM solution of Tryptophan (D or L) in DMSO at 280

nm. The peptides were initially dissolved in DMSO at approximately 1-5 mM concentration. The peptide ligand and Trp were co-injected into the analytical HPLC using a number of different volumes, and the peptide concentration was calculated from the area under the peaks using a general formula given here (adapted from reference 1):

$$\text{Peptide Conc.} = \frac{[\text{Abs. of Comp.}]}{[\text{Abs. of Trp}]} \times \frac{0.5\ \text{mM}}{\frac{\sum \varepsilon_{280}[\text{Dmt + Cys(or -S-, -SH) + Cy5}]}{\varepsilon_{280}(\text{Trp})}} \times \frac{\text{Vol. of Trp}}{\text{Vol. of Comp.}}$$

… Eq. 1

where individual molar extinction coefficients of chromophores described here are Trp (ε_{280} = 5500), Dmt (ε_{280} = 1490), Cy5 (ε_{280} = 5800), and Cys (ε_{280} = 120 M^{-1} cm^{-1}). Other components in the described compounds do not absorb significantly at this wavelength. Using variable injection volumes (5 or 10 μL), the extinction coefficient of Cy5 was measured in a similar way by comparing the 280 nm absorbance (area under the peak) of a 0.5 mM solution of Cy5 against a 0.5 mM solution of Trp as a standard. The extinction coefficient of Dmt was assumed to be the same as Tyr. The extinction coefficient of SH or -S- group was assumed to be the same as Cys due to its minor contribution relative to other chromophores. See Figure S3 for an example of this assay.

Mass Spectrometry

Mass spectra of positive or negative ions were recorded either with a single stage reflectron MALDI-TOF mass spectrometer (Bruker Rexlex III, Bruker Daltonics, Billerica, MA), using α-cyanocinnamic acid as a matrix, in the reflectron mode; or with a low resolution ESI mass spectrometer (Finnigan, Thermoquest LCQ ion trap instrument, Lake Forrest, CA); and/or with a high resolution Fourier transform mass spectrometer

(FT-ICR MS, Bruker Apex Qh, Bremen, Germany) equipped with an ESI source. For internal calibration, an appropriate mixture of standard peptides was used with an average resolution of *ca.* 10,000 and 60,000 on the Reflex III and the FT-ICR instrument, respectively.

General Method for Solid-Phase Synthesis

Solid-phase syntheses were carried out in polypropylene syringes (5-20 mL) fitted with polyethylene porous disks. Solvents and soluble reagents were removed by vacuum to dry the resin. The Fmoc group was cleaved with piperidine/DMF (1:4) for 2 + 18 min. After deprotection, the resin was washed with DMF (3x), CH_2Cl_2 (3x), 0.2 M HOBt in DMF (2x) and finally with DMF (3x) using 10mL solvent per 1g of resin per treatment. Washings between other synthetic steps were done with DMF (3x) and CH_2Cl_2 (3x). Due to the formation of small amounts of Dmt oligomer on the resin, the resin was treated with 50% piperidine in CH_2Cl_2:MeOH (5:1) for 30 min followed by washings as described above. Finally, the resin was washed with DCM (3x), dried under vacuum, and the compounds were released from the resin using TFA cocktail (10 mL of reagent mixture per 1 g of resin) as follows: 82.5% TFA , 5% water, 5% *i*Pr_3SiH, 5% thioanisole, and 2.5% ethanedithiol.

Synthesis of resin intermediate 5

Lys coupling: In a 50 mL bottle containing 1g of Wang resin (initial loading: 0.93 mmol/g) with a magnetic stir bar, dry CH_2Cl_2 was added and the resin stirred for 1 h to swell it. The solvent was then decanted and the resin washed again. The bottle was closed

with a septum, flushed with nitrogen, and *i*Pr_2NEt (9 equiv, 8.4 mmol, 1.4 mL) in 15 mL of CH_2Cl_2 was added to the resin. The resin slurry was cooled to 0 °C followed by dropwise addition (with a syringe) of mesyl chloride (8 equiv, 7.4 mmol, 0.57 mL) in 2 mL of CH_2Cl_2.[2] The reaction was stirred for 20 min at 0 °C, the ice bath was then removed and the reaction stirred for another 20 min at room temperature. The resin was then transferred to a syringe reactor and washed with dry CH_2Cl_2 (3x) and dry DMF (3x). Fmoc-Lys(Mtt)-OH (2 equiv, 1.86 mmol, 1.2 g) and CsI (2 equiv, 1.86 mmol, 0.5 g) were dissolved in *ca* 10 mL of dry DMF, and *i*Pr_2NEt (2 equiv, 0.32 mL) was added to it. The reaction mixture was added to the resin and the resin stirred overnight at room temperature. The loading efficiency of Lys coupling was determined to be 85% based on the UV determination of Fmoc cleavage (dibenzofulvene release).

Tic coupling: The resin was treated with piperidine/DMF (1:4) for 2 + 18 min for N^{α}-Fmoc deprotection and the resin was washed. Tic was then coupled using Fmoc-Tic-OH (3 equiv, 2.79 mmol, 1.1 g), HOCt (3 equiv, 2.79 mmol, 474 mg) and DIC (6 equiv, 5.58 mmol, 870 μL) in DMF and the resin was stirred for 2 h.

Dmt coupling: The Fmoc group from Tic was cleaved and washed as before. Boc-Dmt-OH (3 equiv, 2.79 mmol, 863 mg), HBTU (3 equiv, 2.79 mmol, 1g) and *i*Pr_2NEt (6 equiv, 0.97 mL) in DMF were added to the resin and the reaction was heated in a household microwave for 3 sec. The reaction was then stirred on a vortex until it cooled to room temperature; the heating/cooling sequence was then repeated for five more times, followed by stirring for another 2 h.

Synthesis of compound 6

300 mg of intermediate resin **5** was treated with 3% TFA, 5% *i*Pr_3SiH in CH_2Cl_2 for 1 min (7x), with CH_2Cl_2 washings (2x) between steps, to cleave the Mtt group from the lysine side chain. After the final step, the resin was washed with CH_2Cl_2 (3x), DMF (3x), 5% *i*Pr_2NEt in CH_2Cl_2 (3x), and finally with DMF (3x). The free amino groups were then coupled with the 3-mercaptopropionyl (Mpr) linker using 3-Tritylsulfanyl-propionic acid (3 equiv, 0.84 mmol, 292 mg), HOCt (3 equiv, 0.84 mmol, 142 mg), and DIC (6 equiv, 1.6 mmol, 261 μL) in DMF (10 mL/g of resin), and the reaction was stirred for 3 h. In order to test the reaction completion, the Kaiser test was performed on a small portion of the resin. The peptide was then cleaved from the resin with 82.5% TFA, 5% *i*Pr_3SiH, 5% H_2O, 5% thioanisole, and 2.5% ethanedithiol. The crude peptide was precipitated with cold ether, centrifuged, and the peptide was washed two more times with cold ether. The crude peptide was then purified by preparative HPLC and characterized by MS to give compound **6** in 52% overall yield (purified yield *ca* 30%).

Synthesis of compound 1

2 mg of purified compound **6** (3.4 μmol) was dissolved in 1 mL of HEPES buffer (pH 7.2) and reacted with Cy5-maleimide dye (1.1 equiv, 3.7 μmol, 3 mg)[*] under argon atmosphere to prevent disulfide formation by air oxidation. The reaction was monitored by analytical HPLC at every hour, and additional aliquots of 0.2 equiv of dye were added until the reaction was complete. The labeled ligand was then loaded onto a pre-conditioned C-18 Sep-Pak™ cartridge and the column washed several times with water

[*] To ease the handling of dyes, the commercially available 5 mg lot was dissolved in 5 mL of water, aliquoted into 1 mL fractions, lyophilized, and stored under argon, until used.

and then with 5% acetonitrile in water. Finally, the compound was eluted out with 50% acetonitrile/water and lyophilized to yield compound **1** as a blue amorphous powder.

Synthesis of compound 2

250 mg of intermediate resin **5** was treated with 3% TFA, 5% *i*Pr_3SiH in CH_2Cl_2 for 1 min (7x), with CH_2Cl_2 washings (2x) between steps, to cleave the Mtt group from the lysine side chain. The resin was washed each time with CH_2Cl_2 (3x). After the final step, the resin was washed with 5 mL each of CH_2Cl_2 (3x), DMF (3x), 5% *i*Pr_2NEt in CH_2Cl_2 (3x), and finally with DMF (3x). The Ado linker was then coupled to the lysine side chain using Fmoc-8-amino-3,6-dioxaoctanoic acid (2 equiv, 0.47 mmol, 181 mg), HOCt (2 equiv, 0.47 mmol, 80 mg), and DIC (4 equiv, 0.94 mmol, 147 μL) in DMF, and the reaction was stirred overnight. Next, the Fmoc protection was cleaved with piperidine/DMF, and the resin washed as described earlier. The orthogonally protected cysteine was coupled using Fmoc-Cys(Mmt)-OH (3 equiv, 0.7 mmol, 430 mg), HOCt (3 equiv, 119 mg), and DIC (6 equiv, 1.4 mmol, 218 μL) in DMF and the resin was stirred for 2 h to give the resin intermediate **7**.

DOTA coupling: The Fmoc protecting group was removed from cysteine, and the free N^{α}-termini were treated with with DOTA-NHS ester (2 equiv, 0.47 mmol, 465 mg)[†] and *i*Pr_2NEt (8 equiv, 320 μL) in DMF. If precipitation occurred during reagent preparation, a few drops of DMSO were added. The reaction was then heated in a microwave for 3 sec, stirred vigorously on a vortex mixer until it cooled to room temperature, and the procedure repeated until reaction completion (about 7 repetitions

[†] Note that the commercially available DOTA-NHS ester (Macrocyclics, TX) contains 3 equiv. of TFA and 1 equiv of HPF^{4-} salt by weight (FW: 989.5 g/mol). Further TFA was neutralized with an excess of *i*Pr_2NEt.

needed in this case), to give the resin intermediate **8**. The peptide was then cleaved from the resin using 82.5% TFA, 5% *i*Pr_3SiH, 5% H_2O, 5% thioanisole, and 2.5% ethanedithiol for 3 h. The crude peptide was isolated from the resin by filtration, the filtrate was reduced to low volume by evaporation using a stream of nitrogen, the peptide precipitated from cold ether, centrifuged and washed two more times with cold ether to give the crude compound. The compound was then purified by preparative HPLC in an acetonitrile/water gradient and characterized by ESI-MS, to give the purified compound intermediate **9**.

Europium chelation: 5 mg of the purified compound **9** (4.4 μmol) was dissolved in 0.1 M ammonium carbonate/ammonium acetate buffer (pH 8), and treated with Europium(III) chloride hexahydrate (3 equiv, 4.8 mg), under argon atmosphere to prevent disulfide formation by air oxidation of this compound. The reaction was stirred for 24 hours, and completion of the complexation reaction was monitored with analytical HPLC under basic buffer conditions (see HPLC *Methods E* and *F* in Table S1 for examples). The reaction mixture was then passed through a pre-conditioned reverse-phase C-18 Sep-Pak™ cartridge to load the compound. The cartridge was repeatedly washed with water to remove excess of metal salts and buffer, then with 5% acetonitrile in water. Finally, the compound was eluted in 50% acetonitrile/water, and lyophilized to yield compound **2**.

Cell Culture & Lanthanide Binding Assays

Chinese hamster ovary (CHO) cells overexpressing the human δ-OR were used to evaluate binding at the δ-OR.[3] The CHO/δ-OR cells were kept under selection with 0.8 mg/mL Hygromycin (10687-010, Invitrogen, Carlsbad, CA, USA) and were grown in

Hams F-12 media supplemented with 10% fetal bovine serum. All cells were maintained under standard conditions (37 °C, 5% CO_2).

Binding assays were performed on CHO/δ-OR cells.[4] Cells were plated in black Costar 96-well plates (cat. no. 3603) at a density of 12,000 cells per well and were allowed to grow for 3 days. On the day of the experiment, media were aspirated from all wells, and then 50 μL of nonlabeled ligand (dilutions ranging from 1e-5 to 1e-11 M) and 50 μL of Eu-DTPA labeled DPLCE[4] (10 nM, K_d = 15.3 nM) were added to each well. Ligands were diluted in binding media (Dulbeccos modified Eagles medium [DMEM], 1 mM 1,10-phenanthroline, 200 mg/L bacitracin, 0.5 mg/L leupeptin, 0.3% BSA) and samples were tested in quadruplicate unless otherwise noted. Cells were incubated in the presence of ligands for 1.5 h at 37 °C and 5% CO_2. Following the incubation, cells were washed four times with wash buffer (50 mM Tris–HCl, 0.2% BSA, 30 mM NaCl) using a Molecular Devices SkanWasher. Enhancement solution (1244-105, PerkinElmer) was added (100 μL/well), and plates were incubated for at least 30 min at 37 °C prior to reading. The plates were read on a Wallac VICTOR[3] instrument using the standard Eu TRF measurement (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm). Competition curves were analyzed with GraphPad Prism software using the sigmoidal dose–response (variable slope) classical equation for nonlinear regression analysis. In the case of saturation binding assays, the one site binding (hyperbola) classical equation for nonlinear regression analysis was used. Nonspecific binding was tested in the presence of 10 μM naloxone.

Small Animal Imaging

All animals were maintained under IACUC-approved protocols. Female SCID mice were obtained from the Arizona Cancer Center shared services (Tucson, AZ, U.S.A). For xenografting, HCT-116 colon cancer cells were obtained from ATCC, Manassas, VA. HCT-116 cells expressing δ-OR were provided by Robert J. Gillies.[5] Imaging was performed using a dark box system built and maintained at The University of Arizona Biomedical Imaging Laboratory. For imaging, animals were anesthetized by i.p. injection of a cocktail containing 72 mg/kg ketamine, 6 mg/kg xylazine and 6 mg/kg acepromazine. Mice were tail-vein injected with 10 or 100 μg dose of ligand **1** and images were acquired at different times post-injection using a VersArray 1300B cooled camera, a filtered fiberoptic light source and a tunable emission filter (CRI, Inc). An excitation wavelength of 640 nm with a 10 nm band pass, emission wavelength of 670-720 nm and 100 ms acquisition time with an F1.2 aperture were used. Saturating intensities were not observed at these parameters. Images were analyzed using Image-Pro® Plus 5 by drawing regions-of-interest (ROIs) over each tumor and non-involved muscle tissue. Histograms were generated for each ROI, and mean fluorescence intensities were determined for each time point.

Table S2. HPLC & MS data of labeled and unlabeled δ-OR ligands

Compound	Mass Calculated[a]	Mass Found	t_R (Purity %) [e]	K'
1 ($C_{69}H_{85}N_8O_{15}S_3$)	1362.53 $(M+1)^{1+}$	1361.859[b] $(M-1)^{1-}$	11.1 (95)	5.3
2 ($C_{52}H_{75}N_{10}O_{16}SEu$)	1281.43 $(M+1)^{1+}$	1279.3 & 1281.2[c] $(M+1)^{+}$	9.6 (96)	4.4
6 ($C_{30}H_{40}N_4O_6S$)	585.27 $(M+1)^{1+}$	585.1[c] $(M+1)^{1+}$	9.9 (96)	4.3

[a] Mass calculated based on the most abundant isotope of the element; [b] MALDI-MS; Due to negative charges on Cy5, -ve ionization mode gave best spectrum; [c] ESI-MS; Eu has two isotopic peaks for 150.9 and 152.9; [e] HPLC Method C (Table 1); t_R is the retention time of compound peak in HPLC; (purity of final product in percentage is given in parenthesis); K' is retention time of compound peak/retention time of solvent peak. Mass calculated from most abundant isotopes of elements.

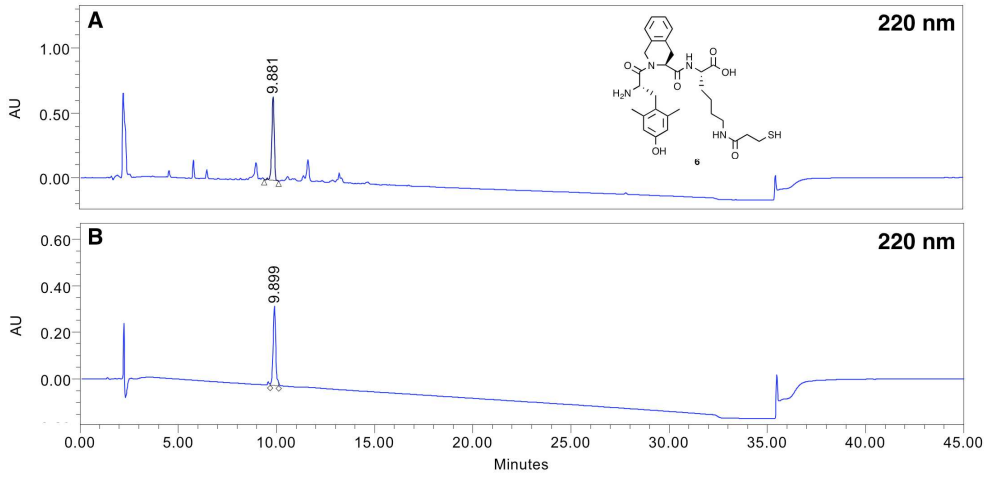

Figure S1. RP-HPLC chromatogram of (a) crude and (b) purified compound **6** run under *Method C*.

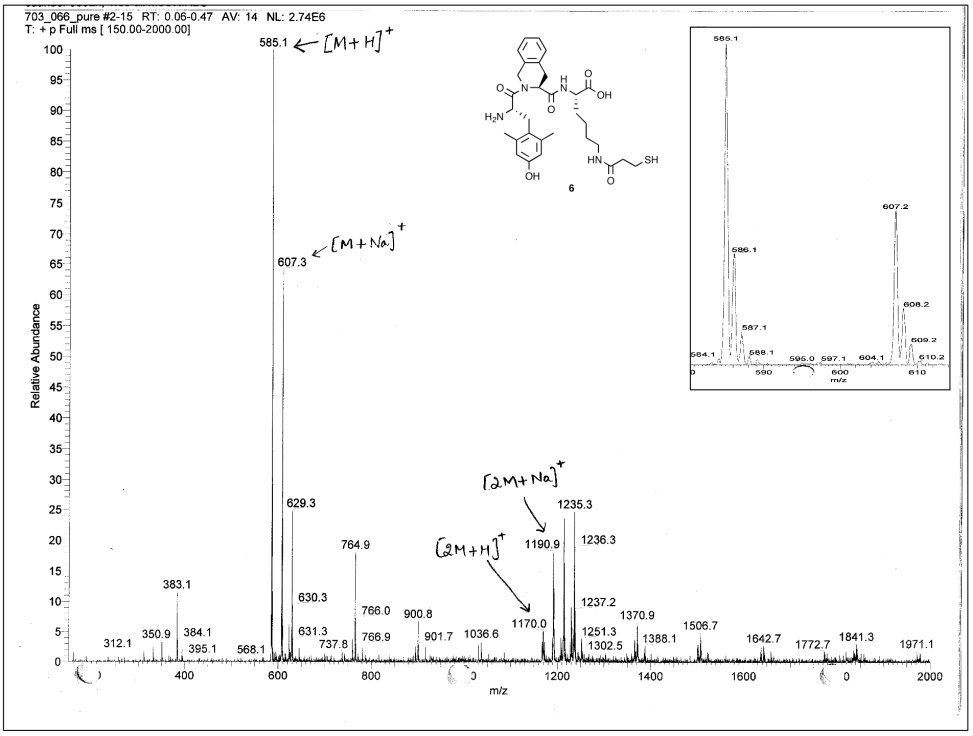

Figure S2. MS of compound **6**.

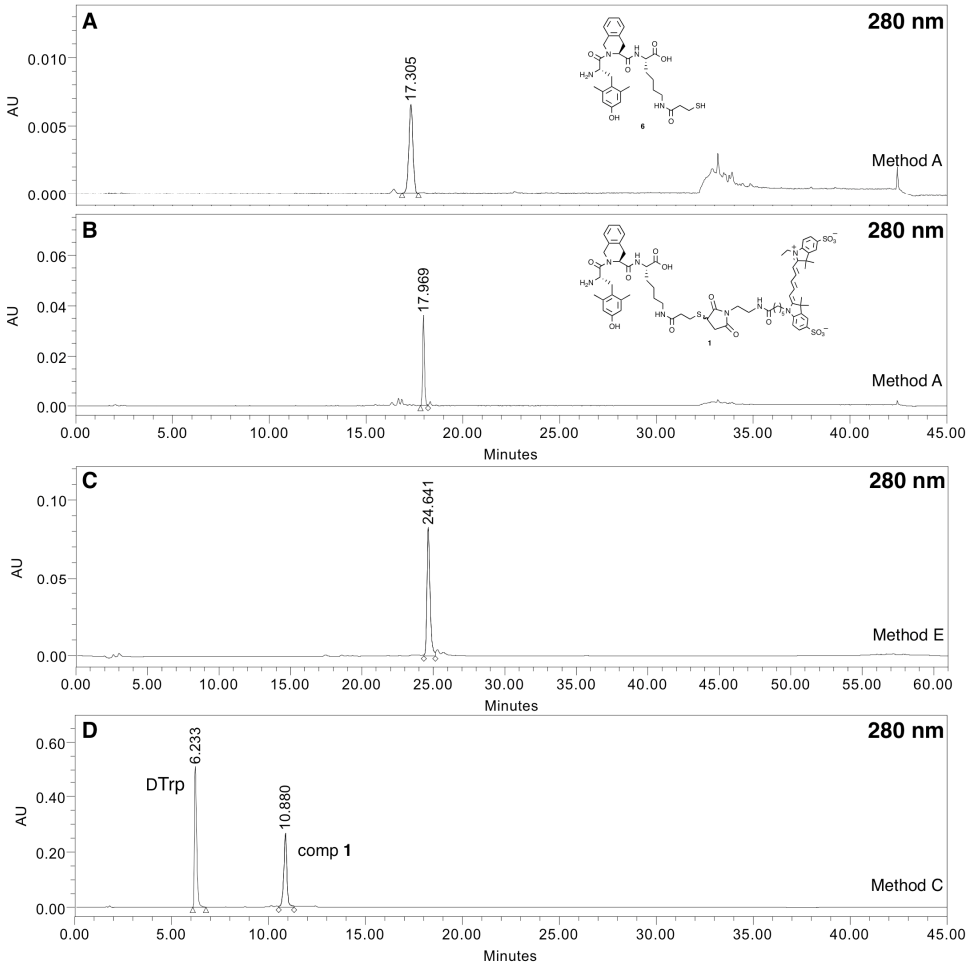

Figure S3. RP-HPLC chromatogram of (a) purified compound **6** in HPLC *Method A* (see Table S1 for methods), (b) Thiol-maleimide reaction of compound **6** with Cy5-maleimide to yield compound **1** (*Method A*), (c) Purified compound **1** run under TEAA buffer condition at pH 6.0 (*Method E*), (d) Concentration measurement of final sample of **1** using 0.5 mM of DTrp as standard (in *Method C*), as follows:

[Abs. of comp **1**] = 2.77×10^6; [Abs. of DTrp] = 4.06×10^6
Vol. of comp. injected = 5 µL; Vol. of DTrp injected = 10 µL
ε_{280} of comp = $(\varepsilon_{280,Dmt} + \varepsilon_{280,Cy5}) / \varepsilon_{280,DTrp} = 1.32$
where $\varepsilon_{280,Dmt} = 1490$, $\varepsilon_{280,Cy5} = 5800$, and $\varepsilon_{280,DTrp} = 5500\ M^{-1}\ cm^{-1}$

Therefore, using equation 1, conc. of compound **1** = 0.51 mM

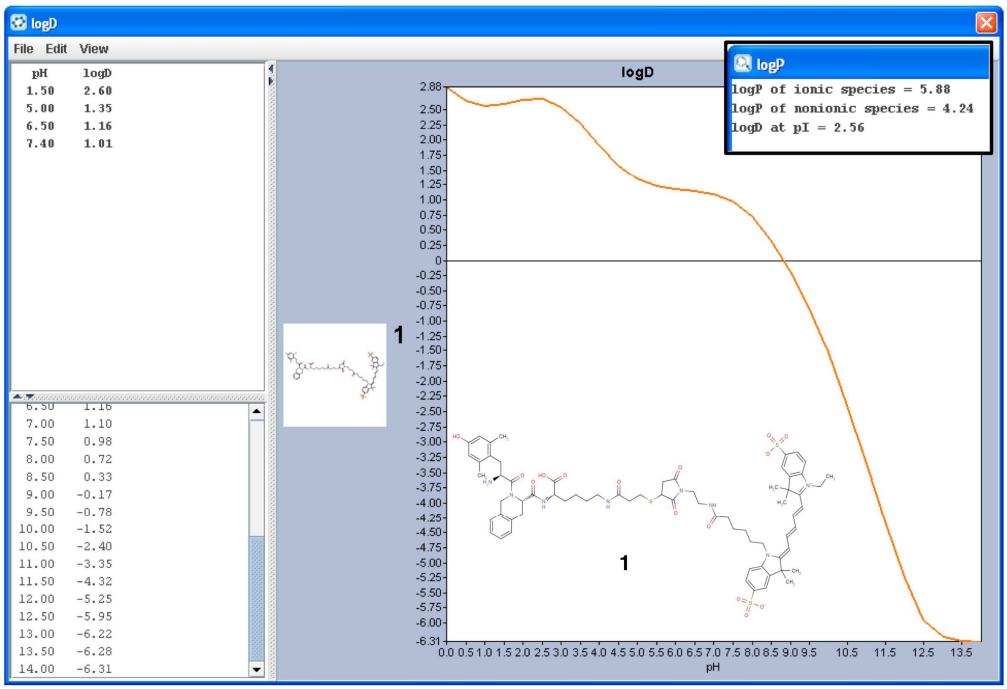

Figure S4. Calculated logD values of compound **1**. The calculations were performed using the partitioning calculator plugin in MarvinSketch 5.1.4 software (ChemAxon, Ltd.). The inset shows clogP values for the same compound (pI = 0.98).

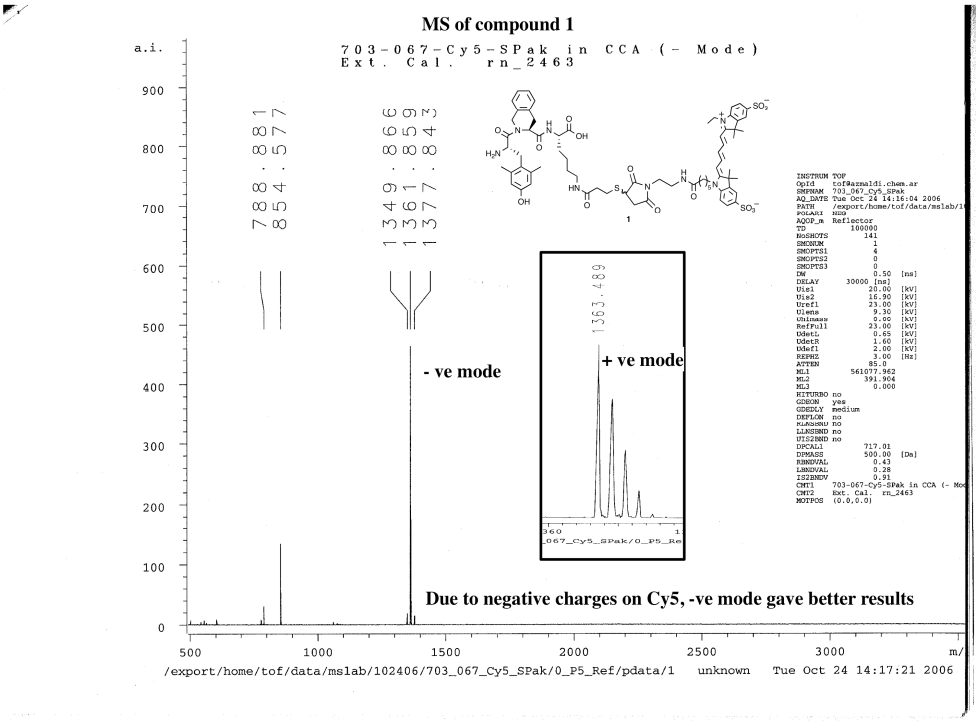

Figure S5. MS of compound **1**.

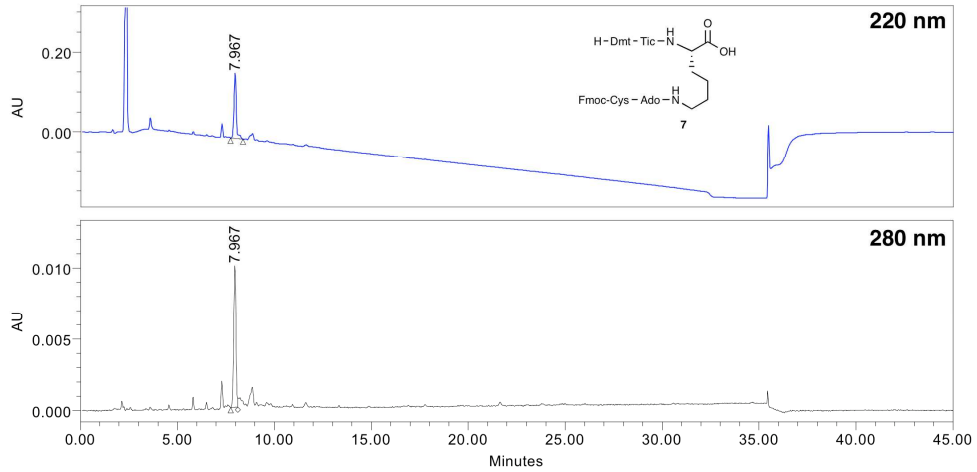

Figure S6. RP-HPLC chromatogram of crude intermediate **7** in *Method C*.

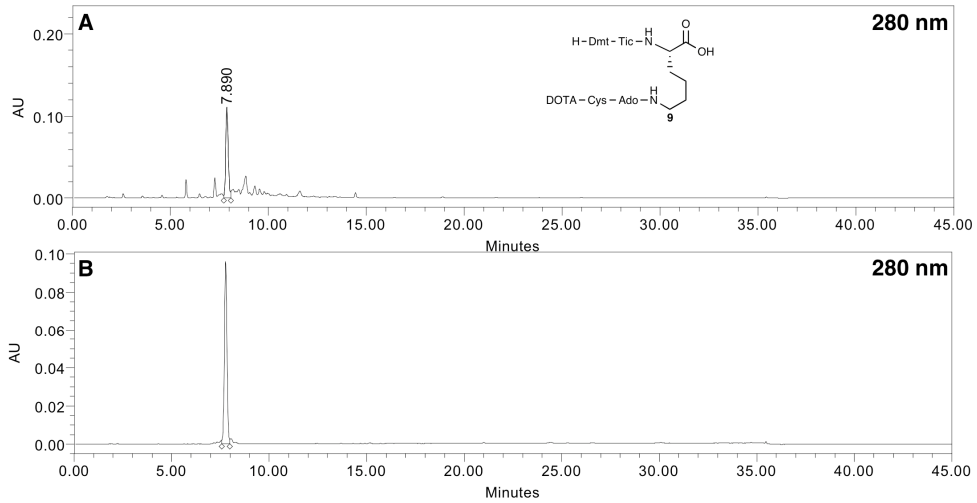

Figure S7. RP-HPLC chromatogram of (a) crude and (b) purified intermediate **9** in *Method C*.

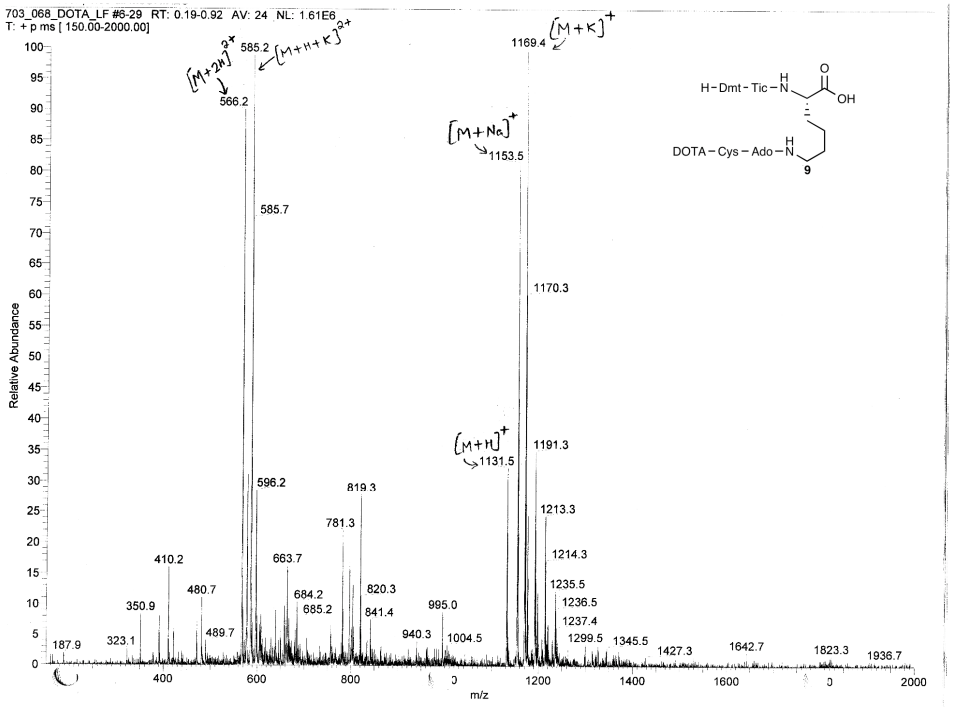

Figure S8. MS of compound **9**.

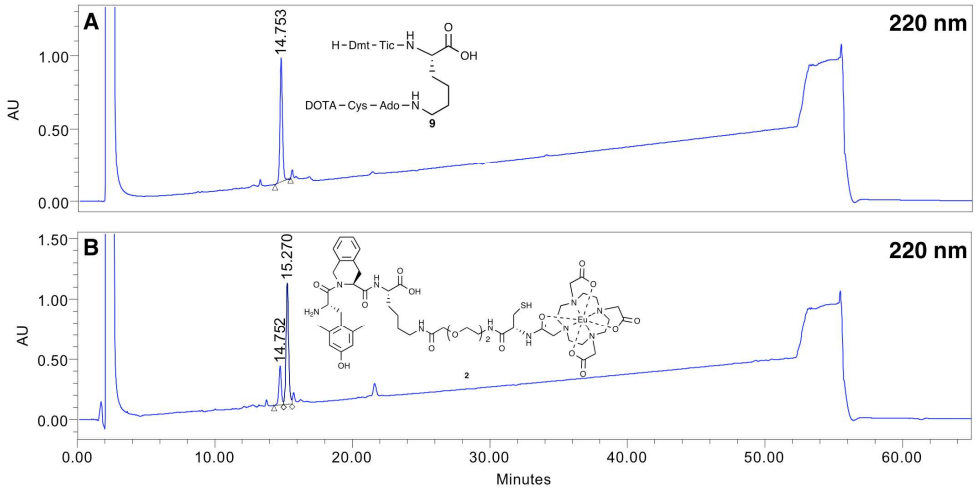

Figure S9. HPLC chromatogram of Europium-DOTA chelation reaction under *Method E*. (a) Purified intermediate **9**, (b) Europium-DOTA complexation to yield product **2**. The complexed product gives a peak with higher retention time. An HPLC column stable at higher pH is necessary to observe chelate peak since the lanthanide-DOTA complex dissociates on the column under TFA conditions (low pH).

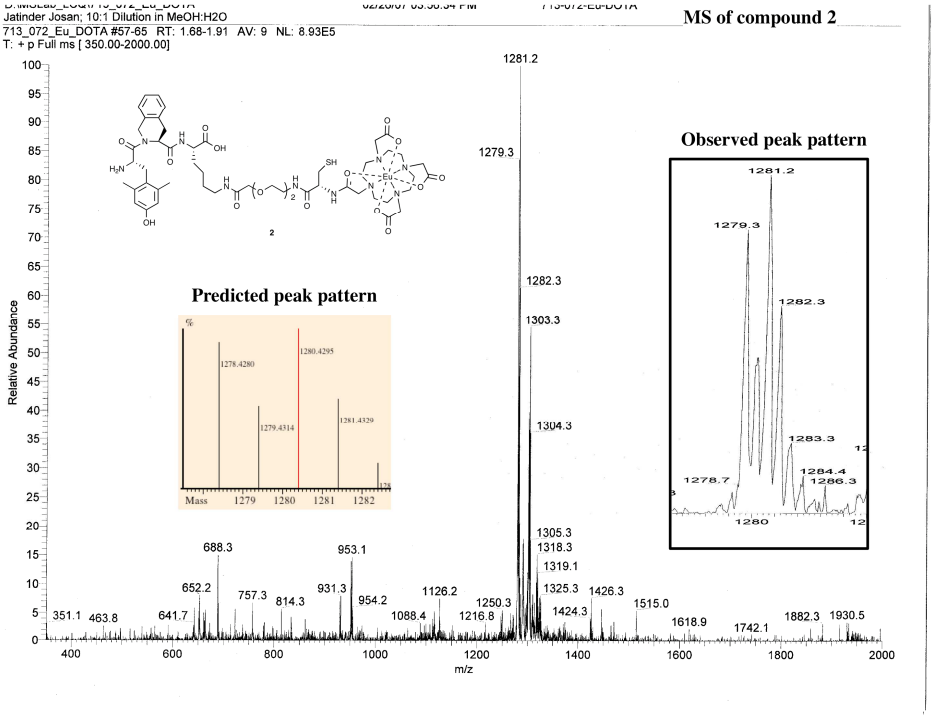

Figure S10. MS of Compound **2**.

REFERENCES:

(1) Moffatt, F.; Senkans, P.; Ricketts, D. *Journal of Chromatography A* **2000**, *891*, 235-242.

(2) Richter, L. S.; Desai, M. C. *Tetrahedron Lett.* **1997**, *38*, 321-322.

(3) Malatynska, E.; Knapp, R. J.; Fang, L.; Li, X.; Wang, Y.; Santoro, G.; Deleon, I.; Waite, S.; Roeske, W. R.; Yamamura, H. I. *Regul. Pept.* **1994**, *54*, 173-174.

(4) Handl, H. L.; Vagner, J.; Yamamura, H. I.; Hruby, V. J.; Gillies, R. J. *Anal. Biochem.* **2005**, *343*, 299-307.

(5) Black, K. C.; Kirkpatrick, N. D.; Troutman, T. S.; Xu, L.; Vagner, J.; Gillies, R. J.; Barton, J. K.; Utzinger, U.; Romanowski, M. *Mol. Imaging* **2008**, *7*, 50-57.