SUPPORTING MATERIAL

Near Infrared Fluorescence Lifetime pH-Sensitive Probes

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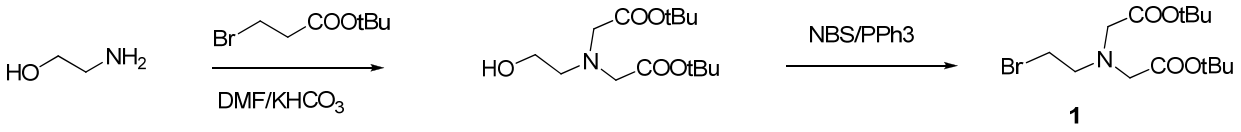
SYNTHESIS

General: All organic precursors and solvents were obtained from commercial sources and used as received. Hydrogenations were performed on a Parr hydrogenation apparatus. Flash chromatography was done on a Biotage SP1 system. Preparative HPLC was performed on an Agilent Technologies 1200 Series equipped with a multi-wavelength detector set to 254 nm and 740 nm. ^{1}H NMR spectra were acquired using a Varian spectrometer operating at 400 MHz. Chemical shifts are reported in parts per million (δ) and referenced to tetramethylsilane (TMS). APCI mass spectrometry (APSI-MS) was performed on an LC/MSD TrapXCl from Agilent Technologies.

N,N-Bis[(*tert*-butyloxycarbonyl) methyl]-2-bromoethylamine (1).



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Tert-butyl bromoacetate (36.5 mL, 225 mmol) was dissolved in anhydrous dimethylformamide (500mL), followed by addition of potassium bicarbonate (25.0 g, 250mmol). The suspension was cooled in an ice-bath and ethanolamine (6.0 mL, 100mmol) was added via syringe over a five-minute period. The reaction was stirred for 30 minutes on ice and then for 20 hours at room temperature. The reaction was evaporated to one fifth its original volume, saturated sodium bicarbonate solution (300 mL) was added and the mixture was extracted with diethyl ether (3x300 mL). The organic fractions were combined, washed with saturated sodium carbonate (2x250 mL) and dried over anhydrous sodium sulfate. The solvents were removed by rotary evaporation to afford the intermediate di-*tert*-butyl ester (**1**) as an oil. It was used in the next step without further purification. The crude oil was dissolved in dichloromethane (300 mL) at room

temperature and triphenylphosphine (28.82 g, 110 mmol) was added with stirring. The reaction was purged with argon and cooled to 0°C in an ice-bath. N-bromosuccinimide (19.6 g, 110 mmol) was added portion-wise over 5 minutes. The reaction was stirred for 1.5 hours at 0°C and the solvents were removed by rotary evaporation to obtain purple oil. The oil was triturated with diethyl ether (3 x 250mL) with constant manual stirring. The diethyl ether solutions were decanted and the solid was washed with diethyl ether (250 mL). The ethereal fractions were combined, the volume reduced to 80 mL, and allowed to stand overnight at 0°C. Diethyl ether (100 mL) was added to the cold mixture, mixed, and the solid residue filtered and washed further with diethyl ether (10 x 4 mL). The combined ethereal solutions were percolated through a silica gel column (250 g) and eluted with 250 mL portions of ether. The fractions containing the product by TLC (hexane:ether=5:1, R_f=0.29) were combined and concentrated by rotary evaporation to obtain an oil residue which was further purified by flash chromatography (hexane:ether from 10:0 to 9:1). The fractions containing the product were combined and the solvents were removed under reduced pressure. The title compound was obtained as a colorless oil (29 g, yield=82.4%). ^{1}HNMR (400MHz, $CDCl_3$): δ 3.48 (s, 4H), 3.44 (t, 2H), 3.13 (t, 2H), 1.46 (s, 18H). m/z (MS APCI) 352.2 ($[M+H]^+$).

N'-benzyl-N,N''-tetrakis(*t*-butyloxycarbonylmethyl)-diethylenetriamine (2). A mixture of 2-[*bis*-(t-butyloxycarbonylmethyl)amino]ethyl bromide **1** (6.0g, 17.05mmol), diisopropylethylamine (6 mL) and benzylamine (0.91 mL, 8.41 mmol) in anhydrous acetonitrile (100 mL) was refluxed for 20 hours under argon. The solvent was removed under reduced pressure and the residue was partitioned between dichloromethane and water. The organic fractions were combined and washed with water (1 x 40 mL) and brine (1 x 40 mL), and dried over anhydrous magnesium sulfate. The solvents were removed and the residue was dissolved in hexane (2-4mL), followed by flash chromatography purification (hexane:diethyl ether = 8:2). The title compound was obtained as a colorless oil (2.48 g, yield=46%). ^{1}HNMR (400MHz, CD_3OD): δ 7.18-7.36 (m, 5H), 3.60 (s, 2H), 3.40 (s, 8H), 2.60 (s, 4H), 2.81-2.85 (m, 4H), 1.44 (s, 36H). APCI MS: m/z: 650.5($[M+H]^+$).

N,N''-tetrakis(*t*-butyloxycarbonylmethyl)-diethylenetriamine (**3**). 10% Palladium on carbon (0.4 g) was added to a solution of compound **2** (2.48 g, 3.82 mmol) in methanol (100 mL). The reaction mixture was placed in a hydrogenation vessel for 6 h with the H_2 pressure set to 50 psi. The catalyst was filtered through Celite®545 (Aldrich) and the solvents were removed under reduced pressure to afford a colorless oil (2.12 g, quantitative). ^{1}HNMR (400MHz, CD_3OD): δ 3.64 (s, 8H), 3.45-3.64 (m, 4H), 3.30-3.32 (m, 4H), 1.50 (s, 36H). m/z (MS APCI) 560.5 ($[M+H]^+$).

2-((*E*)-2-((*E*)-2-(bis(2-(bis(2-*tert*-butoxy-2-oxoethyl)amino)ethyl)amino)-3-((*E*)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-1,3,3-trimethyl-3*H*-indolium (4). The secondary amine **3** (0.44 g, 0.79 mmol), DIPEA (3.95 mmol, 0.68 mL) and IR 786 perchlorate (0.5 g, 0.86 mmol) was dissolved in anhydrous dimethylformamide (15mL). The reaction mixture was purged with argon and heated at 85˚C for 3 hours. The solvent was removed under reduced pressure. The residue was redissolved in methanol and purified by preparative HPLC on a Zorbax XDB C-18 column (21.2 x 5 0 mm 5um) with a 20 mL/min flow rate. The solvent system consisted of solvents A (0.1%TFA in water) and B (0.1% TFA in acetonitrile) with a gradient of solvent B from 85 to 95% over 15 minutes. The title compound

was obtained as a blue solid (80 mg, yield=10%). ^{1}HNMR (400MHz, CD_3CN): δ 7.68 (d, *J* = 13.6Hz, 2H), 7.33-7.28 (m, 4H), 7.10-7.04 (m, 4H), 5.82 (d, *J* = 13.6 Hz, 2H), 4.00 (t, *J* = 6.4 Hz, 4H), 3.40 (s, 6H), 3.33 (s, 8H), 3.06(t, *J* =6.4Hz, 4H), 2.47(t, *J* = 6.8 Hz, 4H), 1.78 (m, 2H), 1.54 (s, 12H), 1.39 (s, 36H). *m/z* (MS APCI) 1007.0 ($[M]^+$).

2-((*E*)-2-((*E*)-2-(bis(2-(bis(carboxymethyl)amino)ethyl)amino)-3-((*E*)-2-(1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-enyl)vinyl)-1,3,3-trimethyl-3*H*-indolium (**LS482**). The compound **4** (20 mg, 20 μmol) was dissolved in triflouroacetic acid (2 mL) and stirred at room temperature for 2 hours. The solvent was removed under vacuum to obtain the title compound as a blue solid (15.6 mg, quantitative). ^{1}HNMR (400MHz, D_2O, NaOH): δ 7.42 (d, *J* = 13.2Hz, 2H), 7.59 (d, *J*= 7.6 Hz, 2H), 7.44 (t, *J* = 7.6 Hz, 2H), 7.27 (t, *J* = 7.6 Hz, 2H), 7.19(d, *J* = 8 Hz, 2H), 5.96 (d, *J* = 13.6 Hz, 2H), 3.79 (t, *J* = 7.6 Hz, 4H), 3.43 (s, 6H), 3.21 (s, 8H), 2.97 (t, *J* = 7.6 Hz, 4H), 2.48 (t, *J* = 6 Hz, 4H), 1.88 (m, 2H), 1.70 (s, 12H). *m/z* (MS APCI) 782.6 ($[M]^+$), 780.5($[M-2H]^-$).

FIGURES

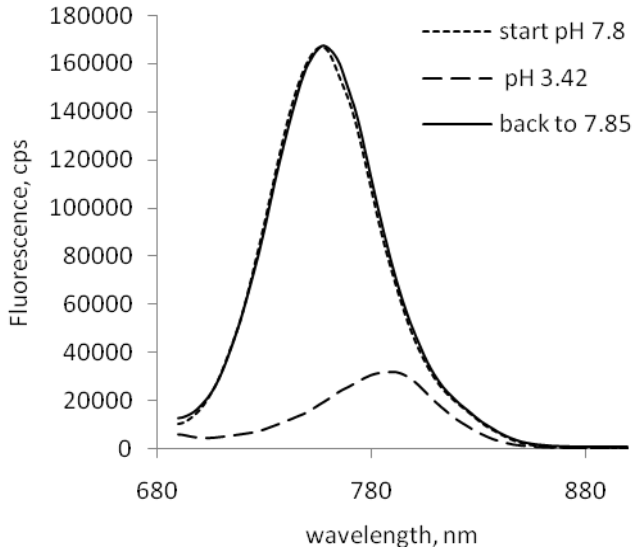

FIGURE S1: The pH dependent fluorescence of LS482. The initial solution of pH 7.8 in water was acidified with HCl (aq) to pH=3.42, then basified back to pH =7.85. Identical fluorescence demonstrates the reversibility of the protonation – deprotonation process. Ex/em. 675/690-900 nm.

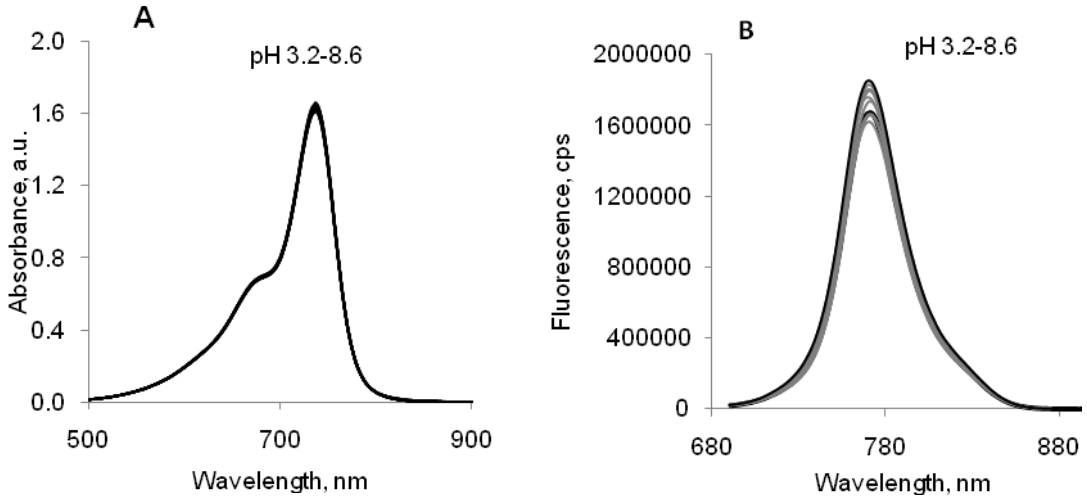

FIGURE S2: (A) Titration absorption spectra of HITC in 0.1 N NaCl aqueous solutions. (B) Titration fluorescence spectra of HITC in aqueous solutions, ex/em. 675/690-900 nm.

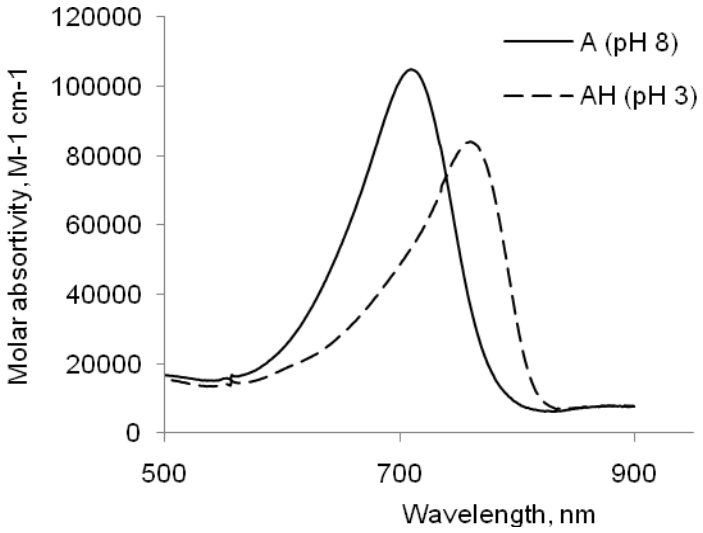

FIGURE S3: Molar absorptivity plots of LS482 in mostly protonated (AH, pH =3) and deprotonated (A, pH=8) forms in Hydrion buffers.

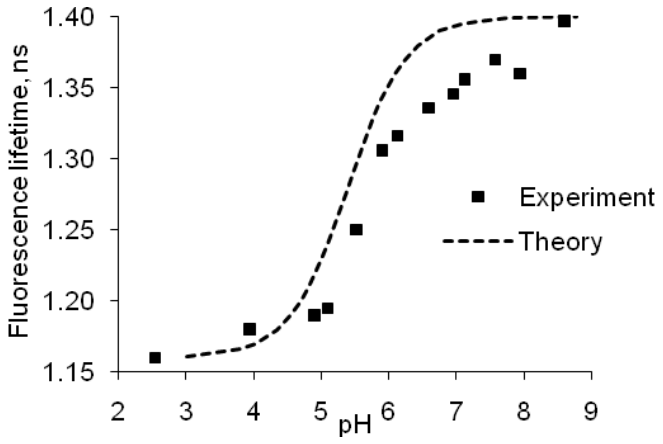

FIGURE S4: Fluorescence lifetime titration curves of LS482 obtained experimentally (DMSO, ex/em 700/780 nm) and from Eq. 11 (see main text)

$pK_{a,\tau}^{*} = 5.39$, $\tau_{AH} = 1.16$ ns, $\tau_{A} = 1.40$.