Modulation of Fluorescence Lifetime with Metal Chelates

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Background

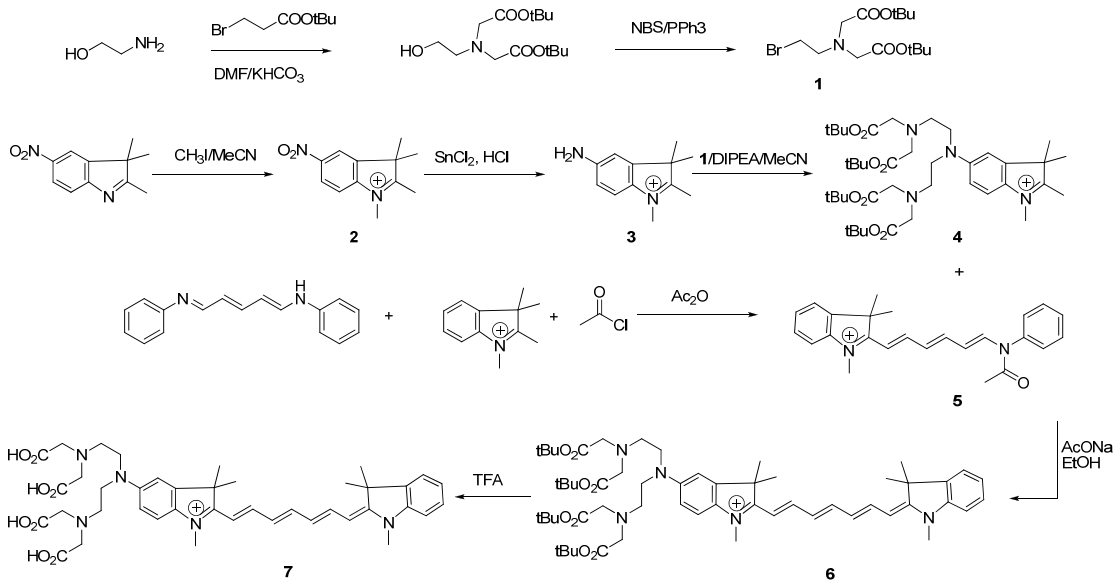
There is strong interest in the fluorescence imaging of biological events using cyanine dyes, which have emissions in or close to the near-infra-red (NIR) region of the spectrum (650-900 nm). At this wavelength light is poorly absorbed and emitted by tissue allowing less interference to and deeper penetration from emitting fluorescent dyes (1). However, a majority of NIR organic dyes have abroad absorption and emission spectra and also very short lifetimes. In order to overcome these limitations, one strategy is to incorporate metal ions as part of the fluorescent probe (1). Metal ions are expected to alter a fluorescent dye's optical properties, particularly its lifetime, and due to differences in electronic configurations, different metals can generate different lifetimes for the same fluorophore probe. Metal ions can be incorporated as part of a probe in their chelated forms, for instance using diethylenetriaminepentaacetic acid (DTPA) which provides a thermodynamically stable coordination site for variety of metals. In addition, different points of attachment of DTPA chelate moieties onto the backbone of cyanine dyes can provide insights into the relationships between structure and optical properties. We synthesized conjugates of DTPA chelates and a cyanine dye in order to study fluorescence lifetime modulation of the dyes.

Chemistry: Two cyanine dye-DTPA analogs were made for these studies. The synthesis of cyanine dye 'A', LS479, **(7)** is shown in Scheme 1. 5-Nitro-2,3,3-trimethylindolenine was first methylated to provide the indole analog **2** in a 60% yield. Reduction of the nitro group, followed by N-alkylation using N,N-bis[(*tert*-butyloxycarbonyl) methyl]-2-bromoethylamine **1** according to a literature method afforded the indole derivative **4** in an overall yield of 64.9%. 1,2,3,3-tetramethyl-indolinium iodide was treated with glutacondialdehyde dianil**,** acetyl chloride and

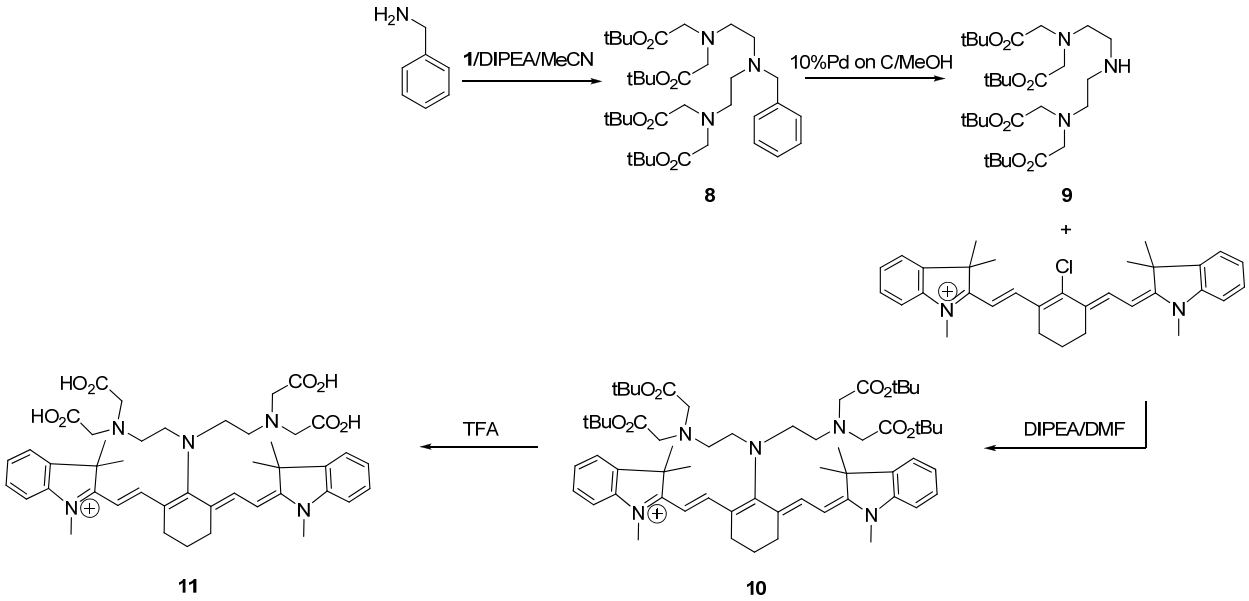
acetic anhydride to obtain hemicyanine intermediate **5** in an 87% yield. Reaction of **5** with the indole derivative **4** under reflux in anhydrous ethanol with anhydrous sodium acetate afforded Boc-protected intermediate **6** which was isolated in good yield (74.5%). The hydrolysis of **6** to afford LS479 was accomplished with TFA and the desired product was purified by preparative HPLC.

Cyanine dye 'B' **(11)** was obtained as outlined in Scheme 2. Benzylamine was N-alkylated with the bromo derivative **1** (Scheme 1) using DIPEA as base in acetonitrile to obtain compound **8**. Intermediate **9** was prepared by removal of the benzyl protective group by hydrogenolysis with hydrogen and 10% palladium on carbon in methanol. N-alkylation of N,N''-tetrakis(t-butyloxycarbonylmethyl)-diethylenetriamine (**9**) with IR 786 perchlorate using DIPEA as base in anhydrous dimethylformamide, according to the published method with modifications as outlined in the experimental section (ref), provided compound **10**. The yield of the reaction is somewhat moderate (10%) which might be attributed to steric hindrance around the secondary amine, as compared to the higher reaction yields seen with a primary amine (76%)[1]. Isolation of compound **10** from the reaction mixture was performed by preparative HPLC. Removal of *t*-butyl protective groups under standard conditions using TFA gave target cyanine dye 'B' (**11**).

Scheme 1.



Scheme 2.



Experimental

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**)[2], 1,2,3,3-Tetramethyl-5-nitro-3H-indolium iodide (**2**)[3], 5-Amino-1,2,3,3-tetramethyl-3H-indolium (**3**)[3] and 1,3,3-Trimethyl-2-(6-(N-phenylacetamido)hexa-1,3,5-trienyl)-3H-indolium (**5**)[4] were obtained according to literature methods. 5-(Bis(2-bis(2-*tert*-butoxy-2-oxoethyl))amino)ethyl)amino)-1,3,3-trimethyl-2-((1*E*,3*E*,5*E*,7*E*)-7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trienyl)-3*H*-indolium **(6)**[5] was synthesized with modifications to the published procedure.

N,N-Bis[(*tert*-butyloxycarbonyl) methyl]-2-bromoethylamine (1). *Tert*-butyl bromoacetate (36.5mL, 225mmol) was dissolved in anhydrous dimethylformamide (500mL), followed by addition of potassium bicarbonate (25.0g, 250mmol). The suspension was cooled in an ice-bath and ethanolamine (6.0mL, 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 (300mL) was added and the mixture was extracted with diethyl ether (3x300mL). The organic fractions were combined, washed with saturated sodium carbonate (2x250mL) 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 (300mL) at room temperature and triphenylphosphine (28.82g, 110mmol) was added with stirring. The reaction was purged with argon and cooled to 0°C in an ice-bath. N-bromosuccinimide (19.6g, 110mmol) 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 a purple oil. The oil was triturated with diethyl ether (3x250mL) with constant manual stirring. The diethyl ether solutions were decanted and the solid was washed with diethyl ether (250mL). The ethereal fractions were combined, the volume reduced to 80 mL, and allowed to stand overnight at 0°C. Diethyl ether (100mL) was added to the cold mixture, mixed, and the solid residue filtered and washed further with diethyl ether (10x4mL). The combined ethereal solutions were percolated through a silica gel column (250g) and eluted with 250mL 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 (29g, 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 ($[M+H]^+$).

1,2,3,3-Tetramethyl-5-nitro-3H-indolium iodide (2). 5-Nitro-2,3,3-trimethylindolenine (8.16g, 40mmol) and methyl iodide (11.44g, 80mmol) were dissolved in anhydrous acetonitrile (200mL). The reaction mixture was refluxed overnight. The color of the reaction mixture turned brown, and it was cooled to room temperature. The final product was collected by filtration as grey-yellowish crystals (8.6g, yield=60%). ^{1}HNMR (400MHz, CD_3OD): δ 8.69 (s, 1H), 8.56 (d, J = 8 Hz, 1H), 8.10 (d, J = 8 Hz, 1H), 4.12 (s, 3H), 1.37 (s, 9H). *m/z* (MS APCI) 220.2 ($[M+H]^+$).

5-Amino-1,2,3,3-tetramethyl-3H-indolium (3). 1,2,3,3-Tetramethyl-5-nitro-3H-indolium iodide **2** (2.2g, 6.35mmol) was added to a solution of stannous chloride (8.43g, 44.45mmol) in hydrochloric acid (32%, 40mL) with stirring. The mixture was refluxed for 16 hours and allowed to cool to room temperature. The reaction mixture was poured onto crushed ice and the solution was rendered alkaline with sodium hydroxide (4M). The mixture was filtered and the solid residue dissolved in *tert*-butyl methyl ether and washed with water (3x30mL). The organic phase was dried over anhydrous sodium sulfate and decolorized with a small amount of activated carbon (Sigma Aldrich). The solvent was removed by rotary evaporation and the title compound was obtained by crystallization from ethyl acetate as grey crystals (1.03g, yield=79%). ^{1}HNMR (400MHz, $CDCl_3$): δ 8.32-8.26 (m, 2H), 7.62 (d, *J* = 8 Hz, 1H), 4.84 (s, 2H), 3.33 (m, 3H), 2.38 (s, 3H), 1.41 (s, 6H). *m/z* (MS APCI) 190.3 ($[M+H]^+$).

5-(Bis(2-(bis(2-*tert*-butoxy-2-oxoethyl)amino)ethyl)amino)-1,2,3,3-tetramethyl-3*H*-indolium (4). 5-Amino-1,2,3,3-tetramethyl-3H-indolium **3** (380mg, 1.7mmol) was added to a solution of N,N-Bis[(*tert*-butyloxycarbonyl) methyl]-2-bromoethylamine **1** (1.94mg, 5.5mmol) and N,N-diisopropylethylamine (6mL) in anhydrous acetonitrile (100mL). The reaction mixture was refluxed under argon for 20 hours and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography (dichloromethane:hexane:methanol=5:1:1). The title compound was obtained as a grey solid (1.13g, yield = 91%). ^{1}HNMR (400MHz, CD_3OD): δ7.53 (d, *J* = 8 Hz, 1H), 7.20 (s, 1H), 6.91-6.89 (dd, 1H), 3.76 (s, 3H), 3.64 (t, J = 12 Hz, 4H), 3.45 (s, 8H), 2.91 (t, *J* = 8 Hz, 4H), 1.57 (s, 6H), 1.50 (s, 3H), 1.35 (s, 36H). *m/z* (MS APCI) 731.7 ($[M]^+$).

1,3,3-Trimethyl-2-(6-(N-phenylacetamido)hexa-1,3,5-trienyl)-3*H*-indolium (**5**). 1,2,3,3-tetramethyl-indolinium iodide (2.0g, 6.7mmol), glutacondialdehyde dianil (1.8g, 6.34mmol), and acetyl chloride (2mL) in acetic anhydride (24mL) were heated at 110°C for 30 minutes. The reaction mixture was evaporated under reduced pressure. The residue was dissolved in dichloromethane (10 mL) and added to a large volume of rapidly stirred diethyl ether. The precipitate was collected and purified by flash chromatography (dichloromethane:hexane:methanol=5:1:1). The title compound was obtained as a purple solid

(2.9g, yield=87%). ^{1}HNMR (400MHz, $CDCl_3$): δ 8.20 (d, *J* = 8 Hz, 1H), 7.91-7.79 (m, 1H), 7.60-7.40 (m, 9H), 7.20-7.16 (m, 2H), 6.90-6.82 (m, 1H), 5.43-5.36 (dd, 1H), 4.19 (s, 3H), 1.96 (s, 3H), 1.70 (s, 6H). *m/z* (MS APCI) 372.3 ($[M+H]^+$).

5-(Bis(2-bis(2-*tert*-butoxy-2-oxoethyl))amino)ethyl)amino)-1,3,3-trimethyl-2-((1*E*,3*E*,5*E*,7*E*)-7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trienyl)-3*H*-indolium (6). 5-(Bis(2-(bis(2-*tert*-butoxy-2-oxoethyl)amino)ethyl)amino)-1,2,3,3-tetramethyl-3*H*-indolium **(4)** (530mg, 0.724mmol), anhydrous sodium acetate (125mg, 1.5mmol) and 1,3,3-trimethyl-2-(6-(N-phenylacetamido)hexa-1,3,5-trienyl)-3H-indolium **5** (300mg, 0.725mmol) were dissolved in anhydrous ethanol (80mL) and the mixture was refluxed for 4 hours under argon. The solvent was removed and the residue was purified by flash chromatography (dichloromethane: methanol=19:1). The title compound was obtained as a blue solid (400mg, yield=74.5%). ^{1}HNMR (400MHz, CD_3OD): δ 7.92-7.87 (m, 1H), 7.68-7.56 (m, 1H), 7.54-7.50 (m, 2H), 7.33-7.24 (m, 3H), 7.16-6.96 (m, 3H), 6.87-6.80 (m, 1H), 6.58-6.36 (m, 2H), 5.86 (d, *J* = 12 Hz, 1H), 3.72 (s, 3H), 3.62-3.54 (m, 4H), 3.45 (s, 8H), 3.35 (s, 3H), 2.93 (t, J = 14 Hz, 4H), 1.73 (s, 6H), 1.62 (s, 6H), 1.48 (s, 36H). *m/z* (MS APCI) 967.3 ($[M+H]^+$).

5-(Bis(2-(bis(carboxymethyl)amino)ethyl)amino)-1,3,3-trimethyl-2-((1*E*,3*E*,5*E*,7*E*)-7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trienyl)-3*H*-indolinium (7). 5-(Bis(2-bis(2-*tert*-butoxy-2-oxoethyl))amino)ethyl)amino)-1,3,3-trimethyl-2-((1*E*,3*E*,5*E*,7*E*)-7-(1,3,3-trimethylindolin-2-ylidene)hepta-1,3,5-trienyl)-3*H*-indolium **(6)** (120mg, 0.124mmol) was dissolved in trifluoroacetic acid (5mL) and stirred at room temperature for 2 hours. The acid was removed under vacuum. The crude product was dissolved in methanol and purified by preparative HPLC on a Zorbax XDB C-18 column (21.2x50mm 5um) at a 20mL/min flow rate. The solvent system consisted of solvent A (0.1%TFA in water) and B (0.1% TFA in acetonitrile) with a gradient of solvent B from 25 to 40% over 12 minutes. The title compound was obtained as a blue solid (65mg, yield=70.6%). ^{1}HNMR (400MHz, D_2O): δ 7.66-7.52 (m, 1H), 7.38-7.19 (m, 2H), 7.19-6.94 (m, 5H), 6.70-6.56 (bs, 1H), 6.42-6.18 (m, 3H), 6.12 (t, *J* = 12 Hz, 1H), 5.40 (s, 1H), 3.72 (s, 3H), 3.28 (s, 3H), 3.16 (s, 8H), 2.86 (bs, 4H), 2.32 (bs, 4H), 1.44 (s, 6H), 1.10 (s, 6H). *m/z* (MALDI-TOF MS) 742.8($[M]^+$).

N'-benzyl-N,N''-tetrakis(*t*-butyloxycarbonylmethyl)-diethylenetriamine (8). A mixture of 2-[*bis*-(t-butyloxycarbonylmethyl)amino]ethyl bromide **2** (6.0g, 17.05mmol), diisopropylethylamine (6mL) and benzylamine (0.91mL, 8.41mmol) in anhydrous acetonitrile (100mL) 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 40mL) and brine (1 x 40mL), 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 (2.48g, 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 (**9**). 10% Palladium on carbon (0.4g) was added to a solution of compound **8** (2.48g, 3.82mmol) in methanol (100mL). 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 (2.12g, 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 (**10**)**.** The secondary amine **9** (0.44g, 0.79mmol), DIPEA (3.95mmol, 0.68mL) and IR 786 perchlorate {2-(2-[2-Chloro-3-([1,3-dihydro-1,3,3-trimethyl-2*H*-indol-2-ylidene]ethylidene)-1-cyclohexen-1-yl]ethenyl)-1,3,3-trimethylindolium perchlorate, Sigma Aldrich} (0.5g, 0.86mmol) 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 (21.2x50mm 5um) with a 20mL/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 (80mg, 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 (**11**). The compound **10** (20mg, 20μmol) was dissolved in triflouroacetic acid (2mL) and stirred at room temperature for 2 hours. The solvent was removed under vacuum to obtain the title compound as a blue solid (15.6mg, 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($[M-2H]^-$).

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