Electronic Supplementary Information

Spiroguanidine rhodamines as fluorogenic probes for lysophosphatidic acid

Lei Wang,[a] Martha Sibrian-Vazquez,[a] Jorge O. Escobedo,[a] Jialu Wang,[a]
Richard G. Moore[b] and Robert M. Strongin[a,*]

Absorbance and fluorescence measurements

UV Absorbance measurements were performed on a Cary Eclipse™ 50 Bio UV-Vis Spectrophotometer. Fluorescence measurements were performed on a Cary Eclipse™ Fluorescence Spectrophotometer (Agilent Technologies). Fluorescence spectra were recorded at an excitation/emission wavelength of 550 nm/570 nm with excitation and emission bandwidths set at 5 nm.

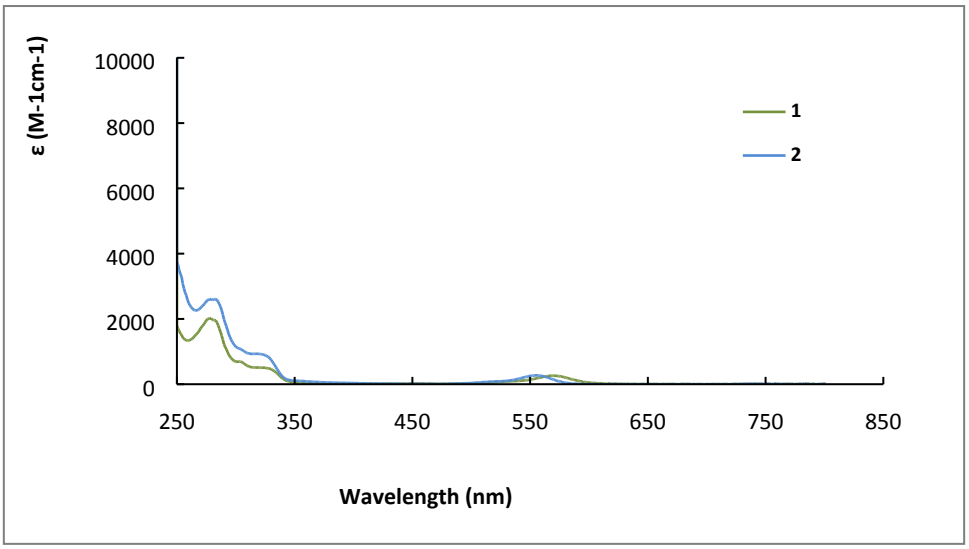


Figure S1. Absorption spectra of 5 μM solutions of **1** and **2** in $CHCl_3$:DMSO 9:1.

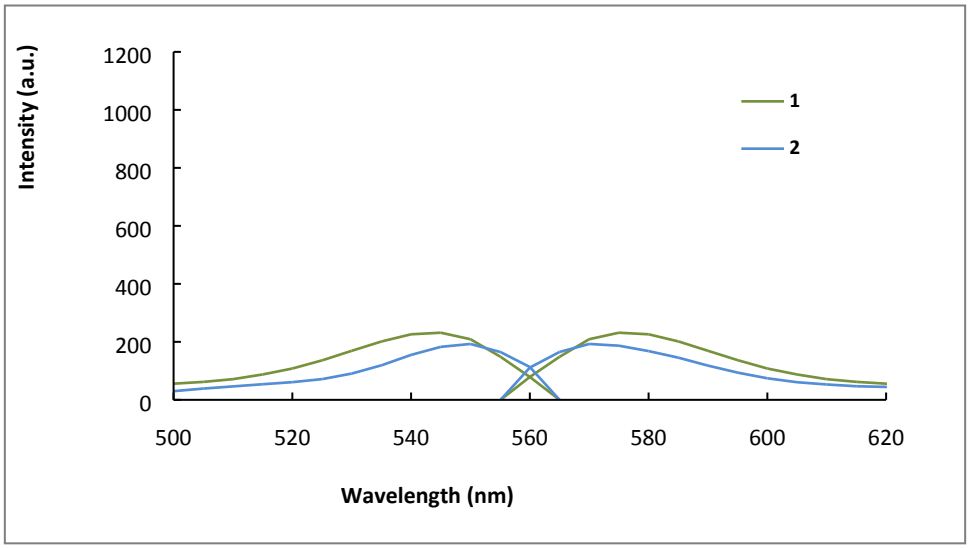


Figure S2. Excitation and emission of 5 μM solutions of **1** and **2**. Ex/Em = 550 nm/570 nm. solvent system: $CHCl_3$: DMSO 9:1.

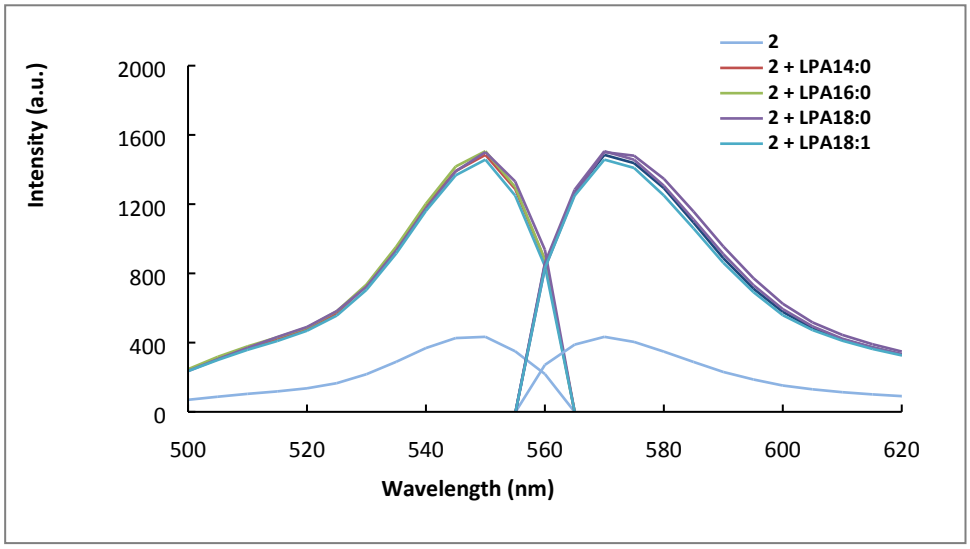


Figure S3. Excitation and emission spectra of **2** and **2** in the presence of LPA 14:0, 16:0, 18:0 and 18:1. Ex/Em = 550 nm/570 nm; final probe concentration: 10 µM; final LPA concentration: 10 µM; solvent system: $CHCl_3$:DMSO 9:1.

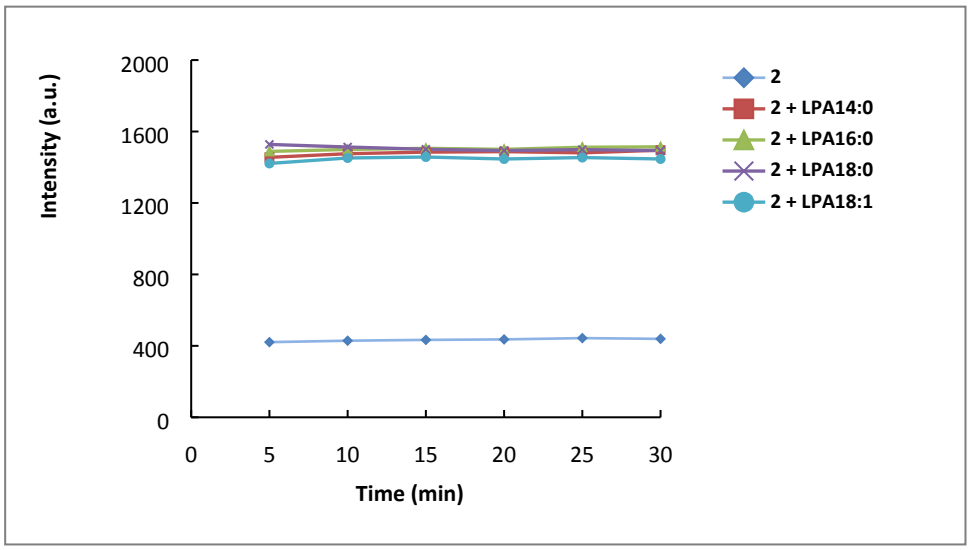


Figure S4. Fluorescence emission intensity of **2** alone and in the presence of LPA 14:0, 16:0, 18:0, 18:1 over time. Ex/Em = 550 nm/570 nm; final probe concentration: 10 µM; final LPA concentration: 10 µM; solvent system: $CHCl_3$:DMSO 9:1.

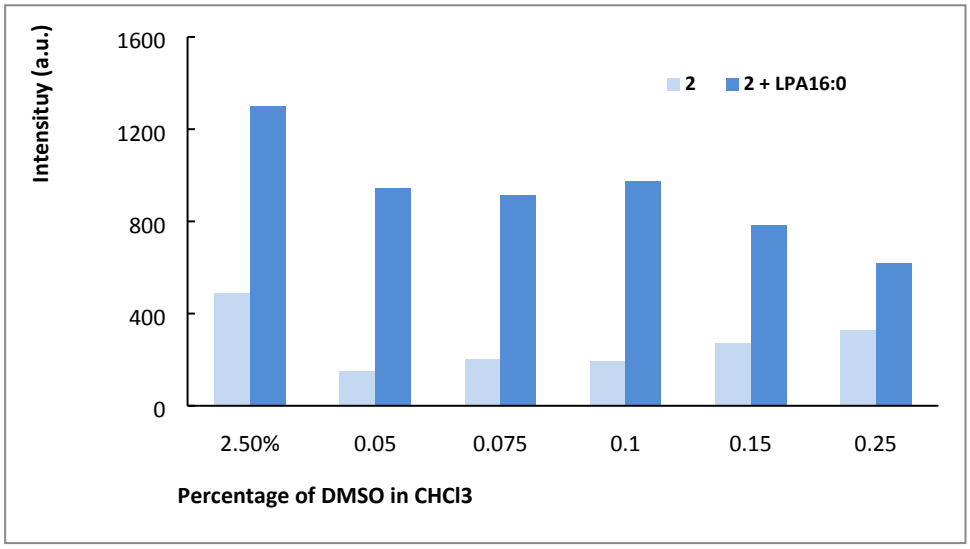


Figure S5. Fluorescence emission intensity of **2** and **2**-LPA in $CHCl_3$ solutions with variable DMSO content. Ex/Em = 550 nm/570 nm; probe concentration: 5 μM; LPA16:0 concentration: 10 μM.

Table S1. Spectroscopic properties of **1** and **2** in EtOH.

	1	**2**
λ_{max} absorbance (nm)	560	560
λ_{max} emission (nm)	570	570
ε ($cm^{-1} \bullet M^{-1}$)	695	1,026
ϕ	0.31	0.39

LC-MS/MS LPA analysis

LPA separation was carried out on a Luna C8 (50 × 2 mm, 3 µm) column at 40 ºC with an injection volume of 10 µL. The mobile phase consisting of a mixture of MeOH:formic acid (10 mM, pH 2.5) 9:1 was delivered at a flow rate of 0.4 mL/min. Ions were created in the negative ion mode setting the sprayer voltage at 3.0 kV and the ion source temperature at 300 ºC.

LPA standard solution preparation

LPA 14:0, 16:0, 18:0, 18:1 and 20:4 in the acid form were obtained by following the SPE procedure developed by Wang *et al.*[1] as follows: 5 mg of the corresponding LPA (sodium or ammonium) salt is dissolved in 3 mL of HPLC grade water. The solution is acidified by addition of 50 µL of 85% H_3PO_4. An SPE cartridge (Waters OASIS™ HLB 3 cc, 400 mg, 30 µm) is preconditioned with 6 mL MeOH, followed by 3 mL H_2O. The acidified LPA solution is loaded onto the cartridge and rinsed with 3 mL H_2O followed by 1 mL $CHCl_3$. The SPE cartridge is dried by applying a stream of N_2, and LPA is eluted with 4 mL of MeOH. The solvent is evaporated and the residue reconstituted in 5 mL MeOH.

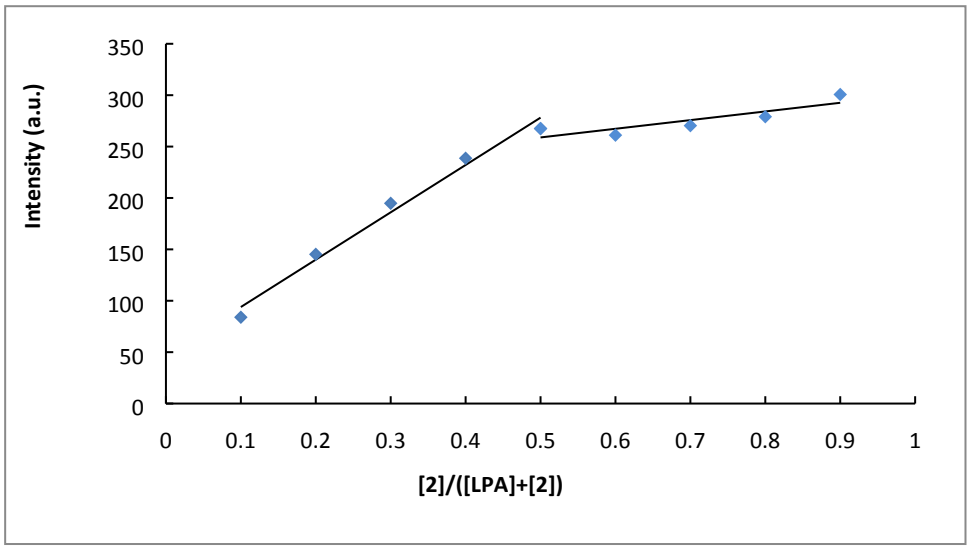


Figure S6. Job's plot for the complex formed between **2** and LPA16:0. [**2**] + [LPA16:0] = 10 µM. Ex/Em = 550 nm/570 nm; solvent system: $CHCl_3$:DMSO 95:5.

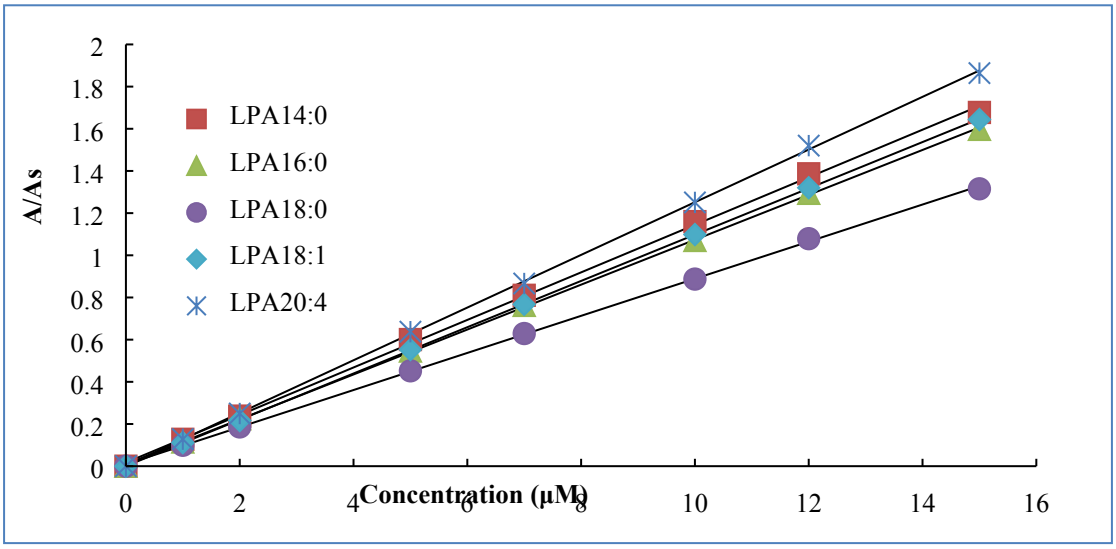


Figure S7. Calibration curves of LPA using the LC-ESI/MS/MS method. The area ratio (A/As) is the peak area of individual LPA divided by the peak area of the internal standard (LPA 17:0). Data points represent the average of 3 runs.

Table S2. Statistical values obtained for the individual LPA species in the LC-ESI/MS/MS method (n=3)

LPA species	Retention time (min)	Linear range (μM)	R^2	LOD (μM)
14:0	3.21	0-15	0.99918	0.02499
16:0	4.09	0-15	0.99987	0.01594
18:0	5.51	0-15	0.99970	0.03968
18:1	4.48	0-15	0.99998	0.01966
20:4	3.71	0-15	0.99977	0.00840

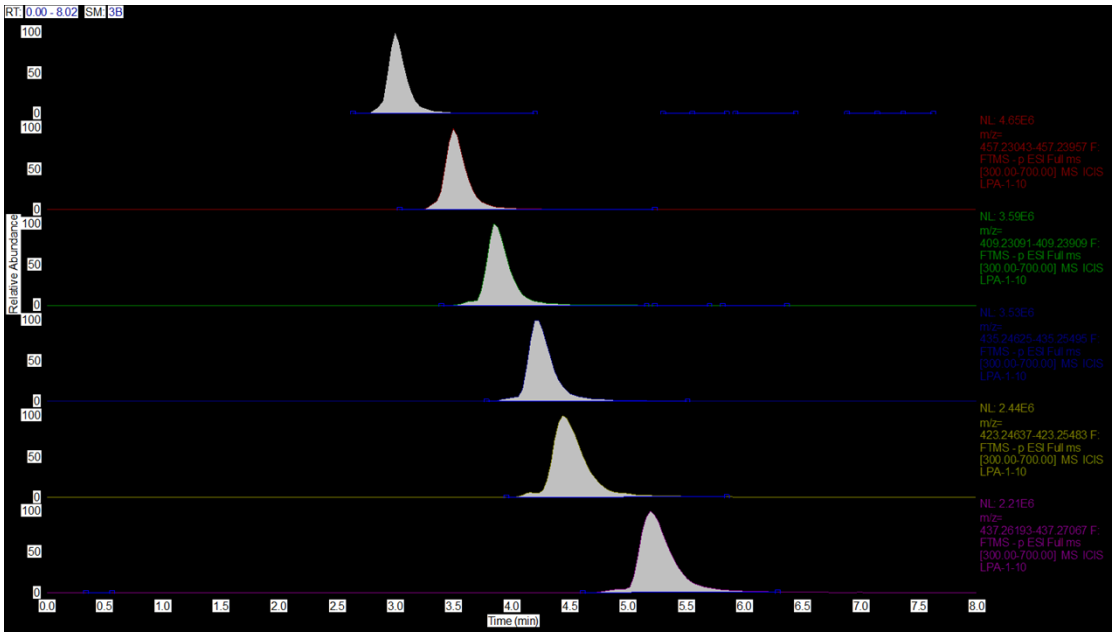


Figure S8. LC-ESI/MS/MS chromatography of a 10 µM standard mixture of LPAs. Column: Luna™ C-8 (50×2 mm, 3 µm) at 40 °C. Injection volume: 10 µL. Mobile phase: MeOH**:**aqueous formic acid (pH 2.5) 9:1 at a flow rate of 0.4 mL/min. Sprayer voltage; 3.0 kV, capillary temperature at 300 °C. Parent and daughter ions were detected in the negative ion mode.

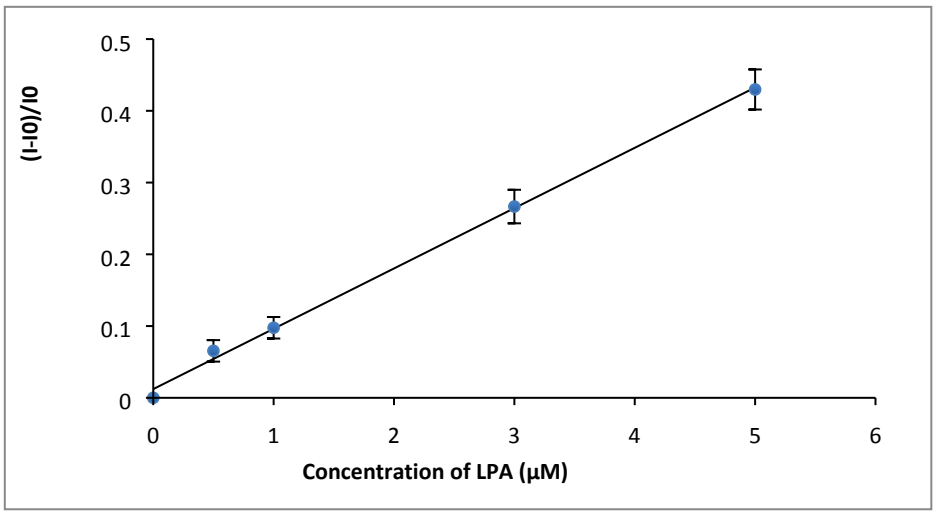
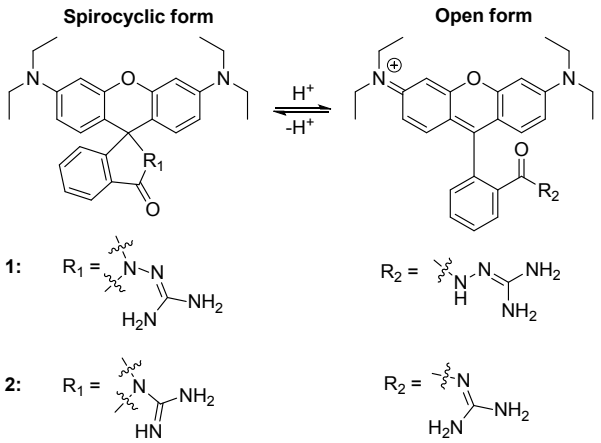


Figure S9. Calibration curve derived from a solution of **2** (10 µM) upon titration with LPA. Ex/Em = 550 nm/570 nm; solvent system: $CHCl_3$:DMSO 95:5.



Scheme S1. Equilibria between spirocyclic and open forms of spiroguanidine rhodamines **1** and **2**.

For a binding ratio of 1:1, equations (1) and (2) were used. A double reciprocal curve was plotted (**Figure S10**), and from the regression equation, $K_a = 4.622 \times 10^5$ M^{-1} ($R^2 = 0.9910$).

$$\frac{1}{I - I_0} = \frac{1}{I_\infty - I_0} + \frac{1}{I_\infty - I_0} \times \frac{1}{K_a} \times \frac{1}{[Probe\ 2]} \quad (1)$$

$$K_a = \frac{\text{Intercept}}{\text{Slope}} \quad (2)$$

I_0, I, I_∞ are the fluorescence intensities of **2** in absence of, in the presence of, and at a concentration corresponding to saturation, respectively, of LPA. K_a is the binding constant.

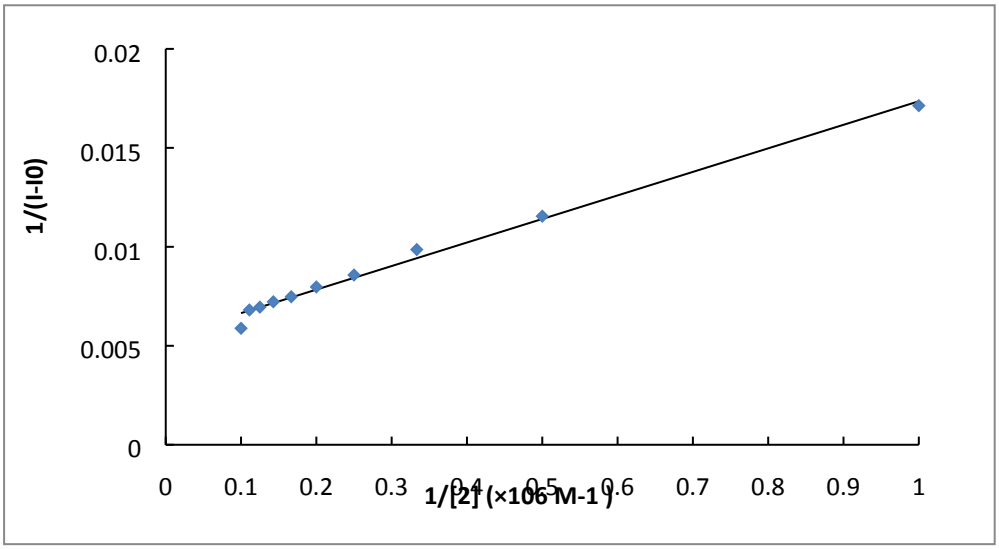


Figure S10. Double reciprocal plot of $1/(I-I_0)$ vs. $1/[\mathbf{2}]$ to calculate the binding constant K_a of **2** to LPA; LPA16:0 concentration: 5 μM.

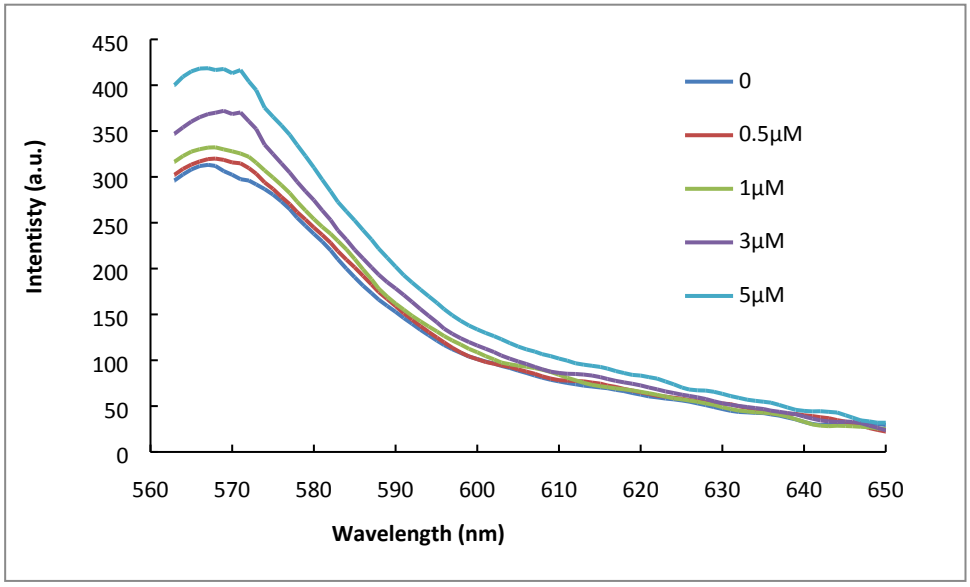


Figure S11. Emission spectra of 10 μM **2** upon titration with LPA. Ex/Em = 550 nm/570 nm; solvent system: $CHCl_3$:DMSO 95:5.

Synthesis of 1 and 2.

Rhodamine B, rhodamine B base, 1,3-*bis*-boc-2-methyl-2-thiopseudourea, sodium methoxide solution (0.5 M in MeOH), and 1,3-*bis*(tert-butoxycarbonyl)guanidine were purchased from Sigma-Aldrich. Anhydrous K_2CO_3 and trifluoroacetic acid were purchased from Fisher Scientific. Mercury(II) chloride was purchased from Acros Organics. Lysophosphatidic acids salts were purchased from Avanti Polar Lipids. Silica gel with a pore diameter of 60Å and particle size of 40-63 μm (230 × 400 mesh) was purchased from Sorbent Technologies. All chemicals were used as received without further purification. NMR spectra were recorded on either a ARX-400 or ARX-600 Advance Bruker spectrometer. MS (HRMS, ESI) spectra were obtained at the PSU Bioanalytical Mass Spectrometry Facility on a Thermo Electron LTQ-Orbitrap Discovery high resolution mass spectrometer.

2-amino-3',6'-bis(diethylamino)spiro[isoindoline-1,9'-xanthen]-3-one (4).[2] To a stirred solution of **3** (Scheme 2, 1 g, 2.1 mmol) in EtOH (60 mL) at rt is added hydrazine monohydrate (98%, 0.6 mL, 12 mmol). The mixture heated at reflux for 12 h. After cooling to rt, the solvent is evaporated under reduced pressure. The solid is dissolved in $CHCl_3$ (50 mL) and the organic phase is washed with H_2O (3 × 50 mL), followed by drying over anhydrous Na_2SO_4. After filtration and removal of the solvent, the crude product is purified by flash chromatography (CH_2Cl_2:MeOH 97.5:2.5) to afford a yellow oil. Yield: 950 mg (95%). The characterization data is consistent with the literature.[2]

(*E*)-tert-butyl(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthene]-2-ylamino)(tert-butoxy-carbonylamino) methylenecarbamate (5). To a stirred solution of **4** (290 mg, 0.64 mmol), $HgCl_2$ (190 mg, 0.70 mmol) and 1,3-*bis*-boc-2-methyl-2-thiopseudourea (188 mg, 0.70 mmol) in anhydrous DMF (10 mL) under Ar is added Et_3N (0.45 mL, 3.18 mmol). The suspension is stirred over an ice bath for 2 h, and then at rt for 12h. The mixture is diluted with $CHCl_3$ and filtered through celite. The filtrate is washed with saturated aqueous $NaHCO_3$ (25 mL) and H_2O (3 × 25 mL). The organic phase is dried over anhydrous Na_2SO_4. After filtration and removal of solvent, the crude product is purified by flash chromatography (EtOAc:hexane 1:2) to

afford a purple solid with a yield of 410 mg (90%). ^{1}H-NMR (600 MHz, $CDCl_3$) δ 1.16 (t, *J* = 6.9 Hz, 12H), 1.37 (s, 9H), 1.38 (s, 9H), 3.33 (m, *J* = 6.8 Hz, 8H), 6.27 (dd, *J* = 32.5, 8.2 Hz, 2H), 6.36 (d, *J* = 14.9 Hz, 2H), 6.77 (s, 1H), 7.10 (t, *J* = 7.3 Hz, 1H), 7.46 (m, 2H), 7.94 (t, *J* = 10.8 Hz, 1H), 9.33 (s, 1H), 11.16 (s, 1H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 12.78, 28.07, 28.35, 44.47, 66.68, 78.80, 83.25, 97.81, 104.55, 108.02, 123.60, 124.19, 128.02, 128.23, 129.03, 133.21, 149.03, 152.35, 153.75, 156.39, 163.56. ESI-MS (*m/z*) for $C_{39}H_{50}N_6O_6$ $[M+H]^+$: calculated 699.3864, observed 699.3932.

1-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthene]-2-yl)guanidine (1). To a stirred solution of **5** (380 mg, 0.55 mmol) in CH_2Cl_2 (2 mL) is slowly added 4 mL of a TFA:CH_2Cl_2 1:1 solution. Stirring at rt is continued until the reaction is complete according to TLC analysis. Solvent and excess TFA are evaporated under reduced pressure to afford the trifluoroacetate salt as a dark purple solid. The solid is dissolved in anhydrous MeOH (3 mL). A 0.5 M NaOMe solution (3 mL) is added and the mixture stirred at rt for 1 h. The solvent is evaporated under reduced pressure. The resulting solid is dissolved in $CHCl_3$ (50 mL) and washed with aqueous saturated $NaHCO_3$ (25 mL) and H_2O (3 × 25 mL). The organic phase is dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, the crude product is purified by flash chromatography (CH_2Cl_2:MeOH 9:1) to afford a purple solid with a yield of 160 mg (60%). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 1.08 (t, *J* = 7.0 Hz, 3H), 3.30 (q, *J* = 7.1 Hz, 8H), 5.51 (d, *J* = 293.9 Hz, 4H), 6.27 (dd, *J* = 8.9, 2.5 Hz, 2H), 6.30 (d, *J* = 2.5 Hz, 2H), 6.56 (d, *J* = 7.3 Hz, 2H), 6.88 (d, *J* = 6.9 Hz, 1H), 7.42 (m, 2H), 7.71 (d, *J* = 6.5 Hz, 1H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 12.49, 43.63, 65.29, 97.15, 105.99, 107.39, 121.78, 123.17, 127.77, 128.72, 130.55, 131.59, 147.93, 152.34, 152.78, 158.53. ESI-MS (*m/z*) for $C_{29}H_{34}N_6O_2$ $[M+H]^+$: calculated 499.2860, observed 499.2846.

***N*-(9-(2-(chlorocarbonyl)phenyl)-6-(diethylamino)-3*H*-xanthen-3-ylidene)-*N*-ethylethanaminium (7)**.[3] To a solution of **6** (500 mg, 1.13 mmol) in anhydrous 1,2-dichloroethane (5 mL), is added a solution of $POCl_3$ (0.26 mL, 2.8 mmol) in 1,2-dichloroethane (5 mL) dropwise over 5 min. The mixture is heated at reflux for 4 h.

After the mixture is cooled to rt, the solvent is evaporated under reduced pressure to yield rhodamine B acyl chloride which is used as is in the next reaction.

(*E*)-tert-butyl-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthene]-2-yl)methanediylidene-dicarbamate (8). 1,3-*bis*(tert-butoxycarbonyl)guanidine (290 mg, 1.13 mmol) and K_2CO_3 (0.62 g, 4.5 mmol) are dissolved in anhydrous MeCN (5 mL) under Ar. The crude rhodamine B acyl chloride is dissolved in anhydrous MeCN (5 mL) and added dropwise to the solution over 3 h. After 12 h, the solvent is evaporated under reduced pressure and the residue washed with aqueous saturated $NaHCO_3$ (25 mL) and H_2O (3 × 25 mL). The combined organic phase is dried over anhydrous Na_2SO_4. After filtration and removal of solvent, the crude product is purified by flash chromatography (EtOAc:hexane 1:2) to afford a yellow solid with a yield of 476 mg (62%). ^{1}H-NMR (600 MHz, $CDCl_3$) δ 1.13 (t, *J* = 7.1 Hz, 12H), 1.35 (s, 9H), 1.41 (s, 9H), 3.30 (m, 8H), 6.18 (dd, *J* = 8.8, 2.6 Hz, 2H), 6.30 (d, *J* = 8.8 Hz, 2H), 6.35 (d, *J* = 2.6 Hz, 2H), 7.16 (d, *J* = 7.7 Hz, 1H), 7.51 (m, 1H), 7.58 (td, *J* = 7.5, 1.1 Hz, 1H), 7.94 (d, *J* = 7.6 Hz, 1H), 10.75 (s, 1H). ^{13}C-NMR (151 MHz, $CDCl_3$) δ 12.78, 28.15, 28.18, 44.40, 68.30, 78.74, 81.78, 97.72, 106.68, 107.23, 123.59, 125.09, 127.65, 128.75, 129.20, 135.16, 139.08, 148.97, 150.16, 153.42, 154.20, 156.14, 171.05. ESI-MS (*m/z*) for $C_{39}H_{49}N_5O_6$ $[M+H]^+$: calculated 684.3756, observed 684.3794.

3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthene]-2-carboximidamide (2). To a stirred solution of **8** (300 mg, 0.44 mmol) in CH_2Cl_2 (2 mL) is added slowly 4 mL of a TFA:CH_2Cl_2 1:1 solution. Stirring at rt is continued until the reaction is complete based on TLC analysis. Solvent and excess TFA are evaporated under reduced pressure to afford the trifluoroacetate salt as a dark purple solid, which is dissolved in anhydrous MeOH (3 mL). A 0.5 M NaOMe solution (3 mL) is added, and the mixture stirred at rt for 1h. The solvent is evaporated under reduced pressure and the resulting solid is dissolved in $CHCl_3$ (50 mL), washed with aqueous saturated $NaHCO_3$ (25 mL) and H_2O (3 × 25mL). The combined organic phases are dried over anhydrous Na_2SO_4. After filtration and removal of solvent, the crude product is purified by flash chromatography (CH_2Cl_2:MeOH 9:1) to afford a light purple solid

with a yield of 110 mg (52%). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 1.09 (t, J = 7.0 Hz, 12H), 3.34 (q, J = 7.1 Hz, 8H), 6.42 (dd, J = 9.0, 2.6 Hz, 2H), 6.45 (d, J = 2.5 Hz, 2H), 6.72 (d, J = 8.9 Hz, 2H), 7.01 (d, J = 7.8 Hz, 1H), 7.58 (m, 1H), 7.68(m, J = 1.1 Hz, 1H), 7.69 (bs, 3H), 7.99 (d, J = 7.7 Hz, 1H). ^{13}C-NMR (151 MHz, DMSO-d_6) δ 12.38, 43.69, 79.18, 97.67, 103.08, 108.69, 123.65, 124.39, 124.53, 127.22, 129.43, 136.51, 149.17, 151.72, 153.52, 153.64, 169.01. ESI-MS (*m/z*) for $C_{29}H_{33}N_5O_2$ $[M+H]^+$: calculated 484.2707, observed 484.2751.

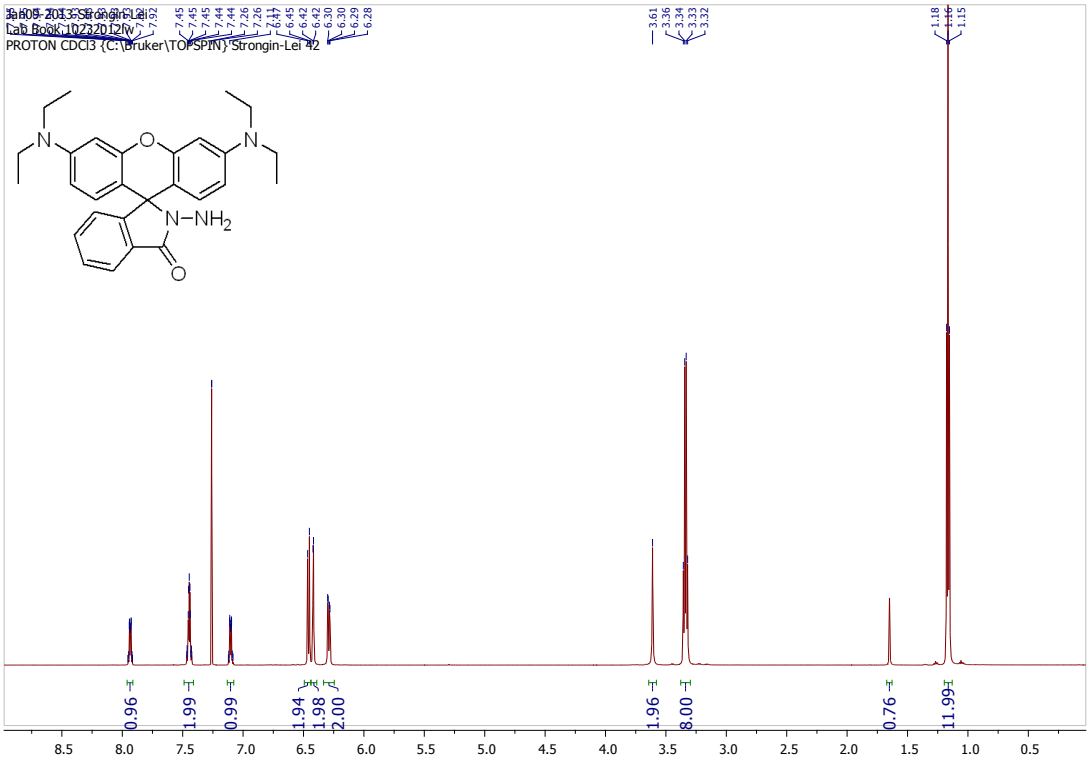


Figure S12. ^{1}H-NMR spectrum of **4** in $CDCl_3$.

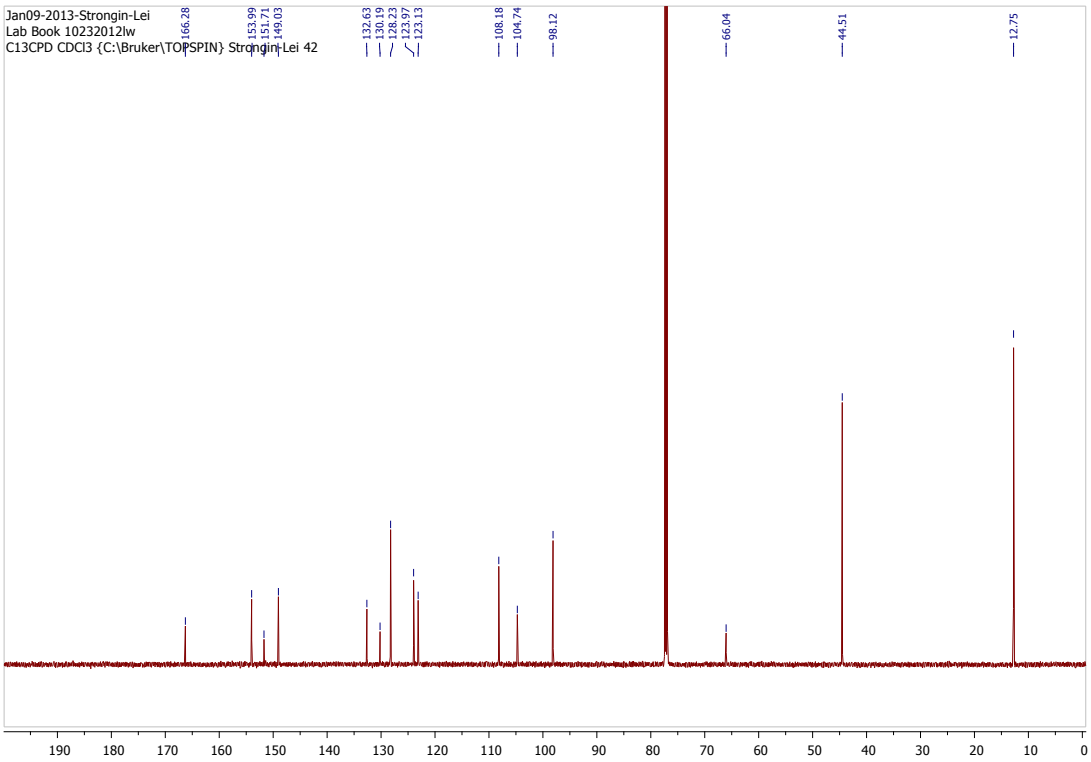


Figure S13. ^{13}C-NMR spectrum of **4** in $CDCl_3$.

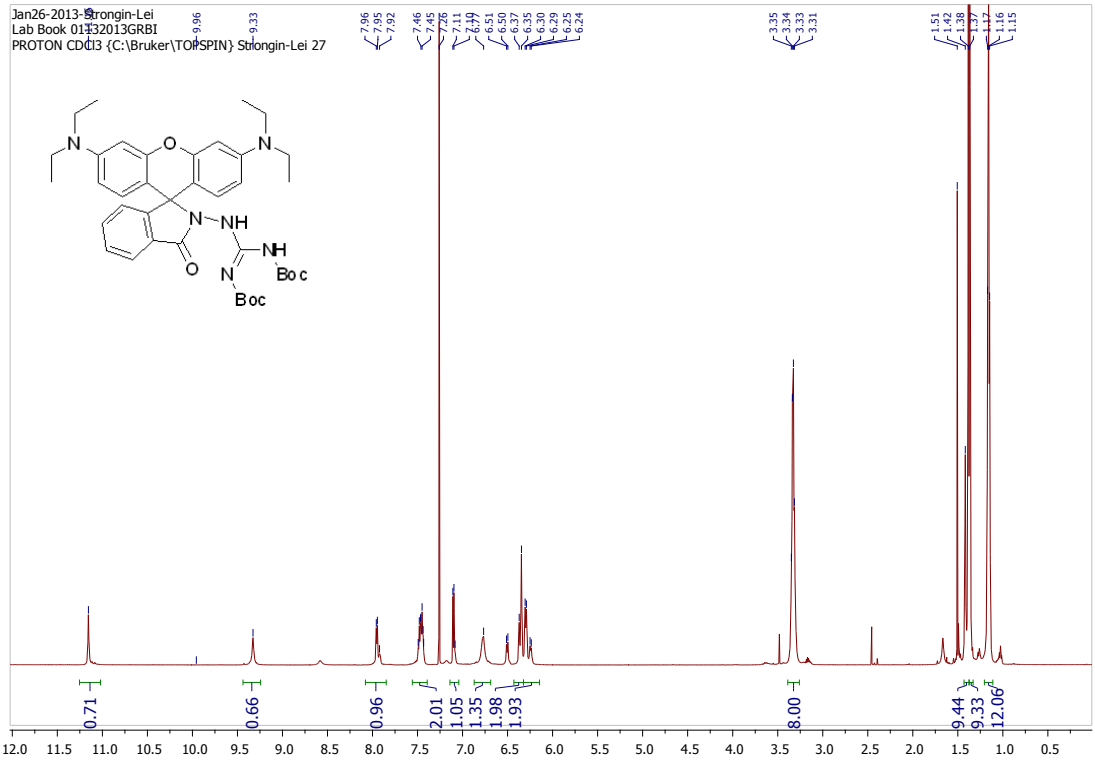


Figure S14. ^{1}H-NMR spectrum of **5** in $CDCl_3$.

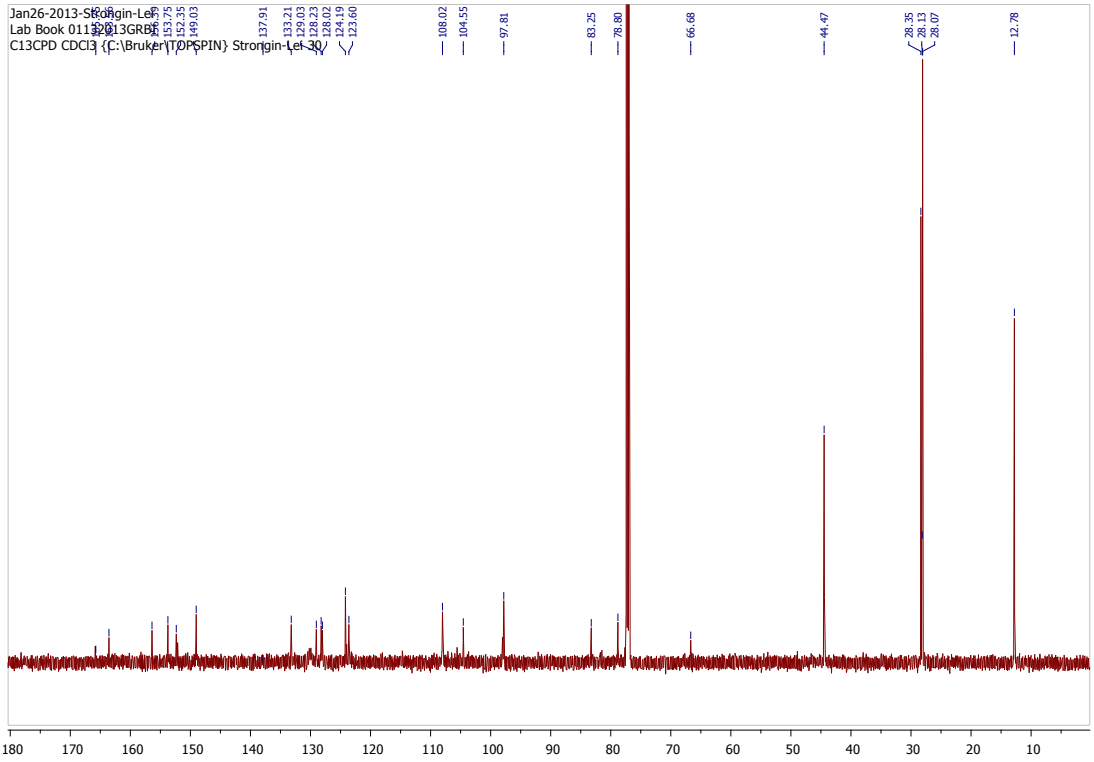


Figure S15. ^{13}C-NMR spectrum of **5** in $CDCl_3$.

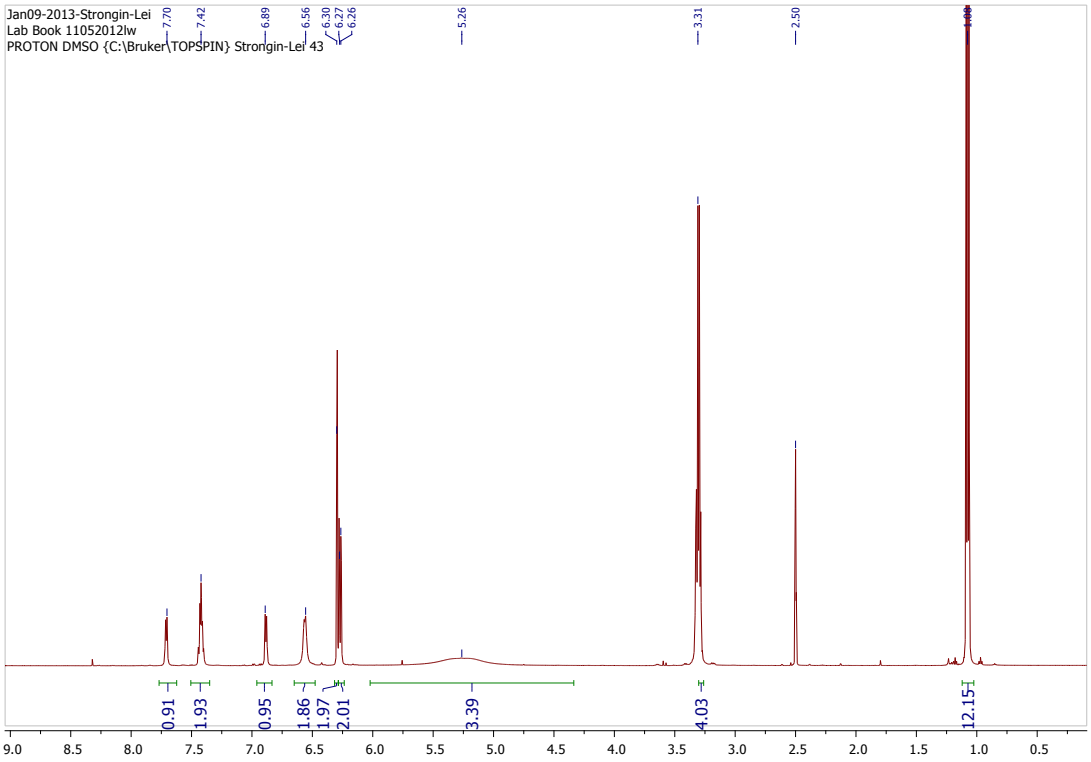


Figure S16. ^{1}H-NMR spectrum of **1** in DMSO-d_6.

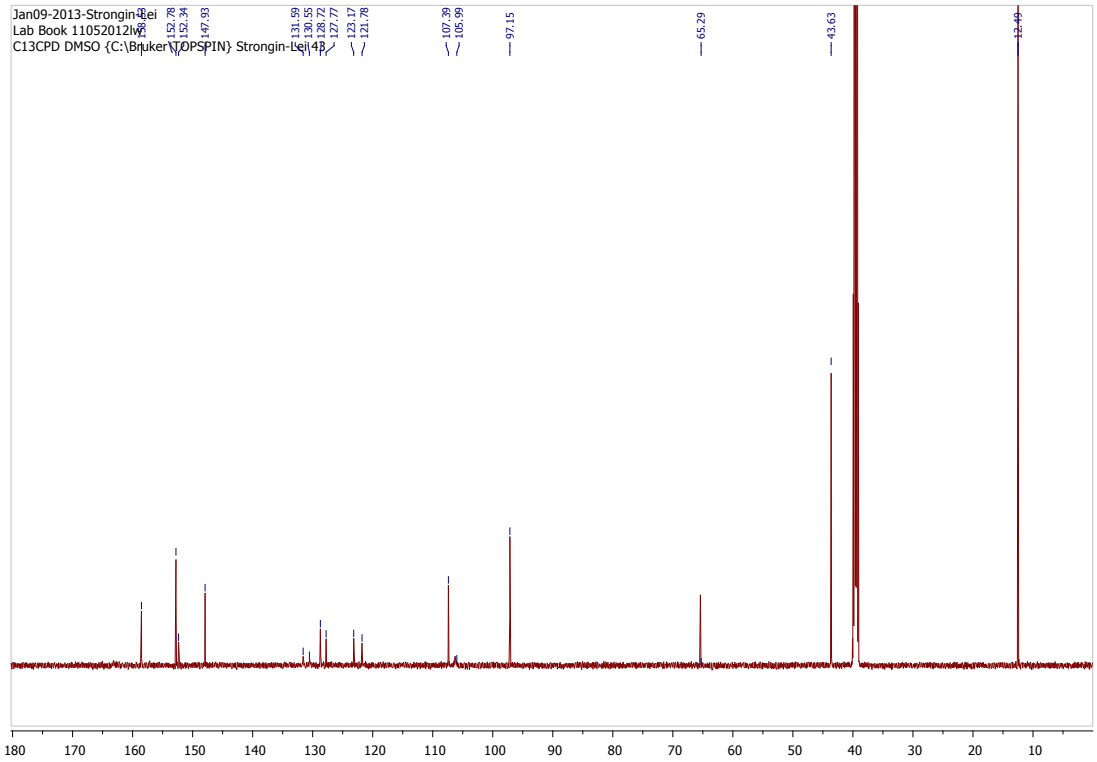


Figure S17. ^{13}C-NMR spectrum of **1** in DMSO-d_6.

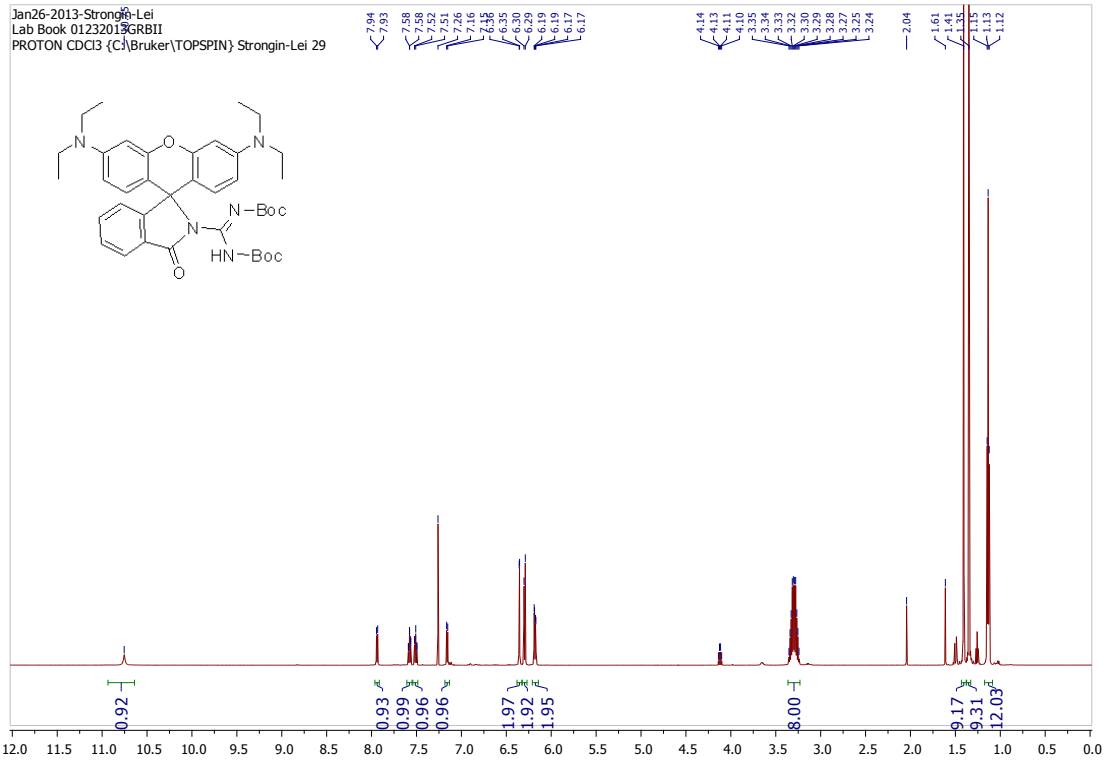


Figure S18. ^{1}H-NMR spectrum of **8** in $CDCl_3$.

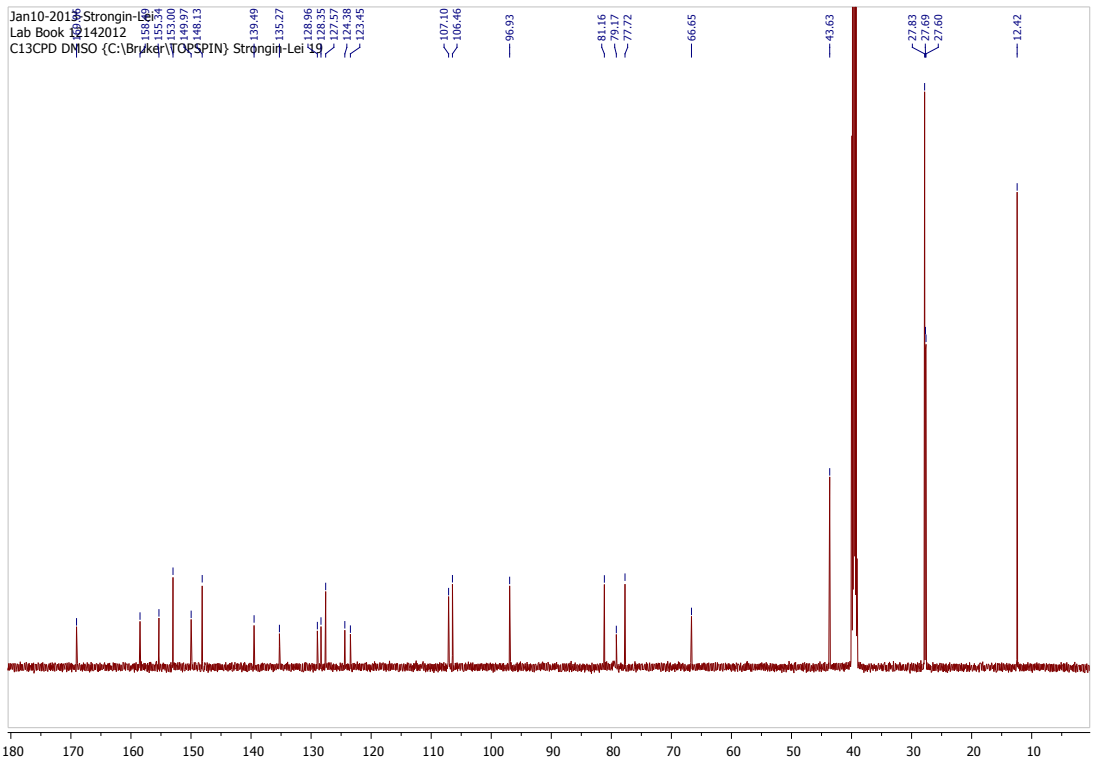


Figure S19. ^{13}C-NMR spectrum of **8** in $CDCl_3$.

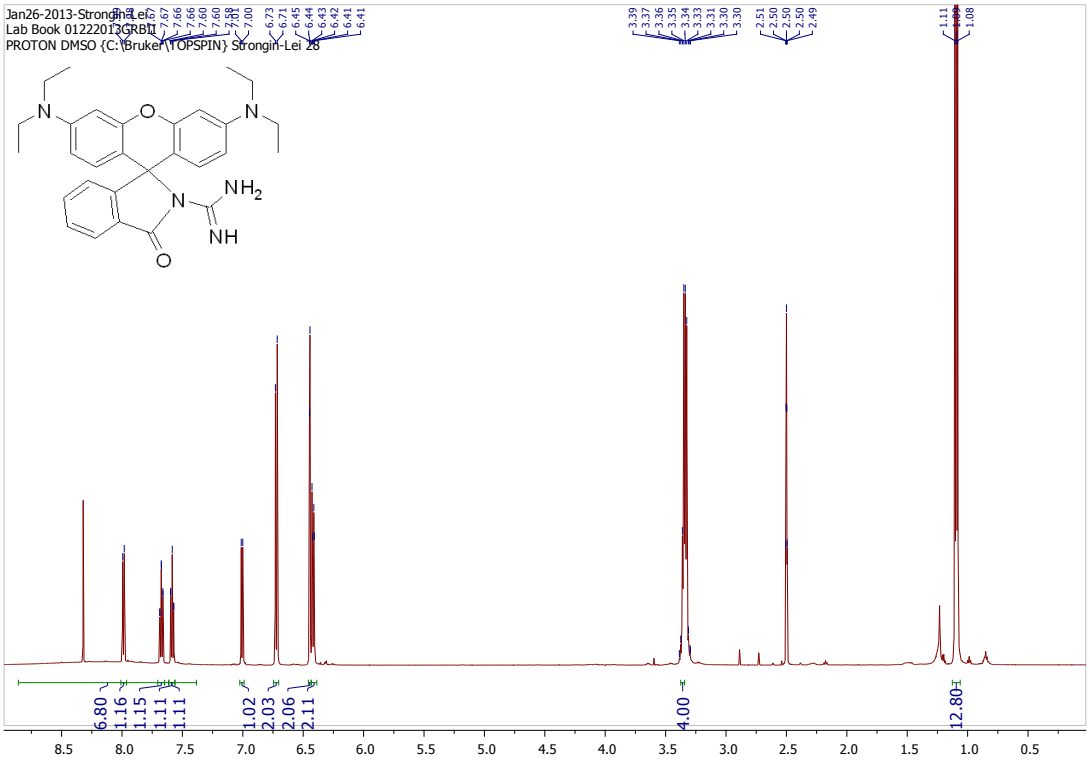


Figure S20. ^{1}H-NMR spectrum of **2** in DMSO-d_6.

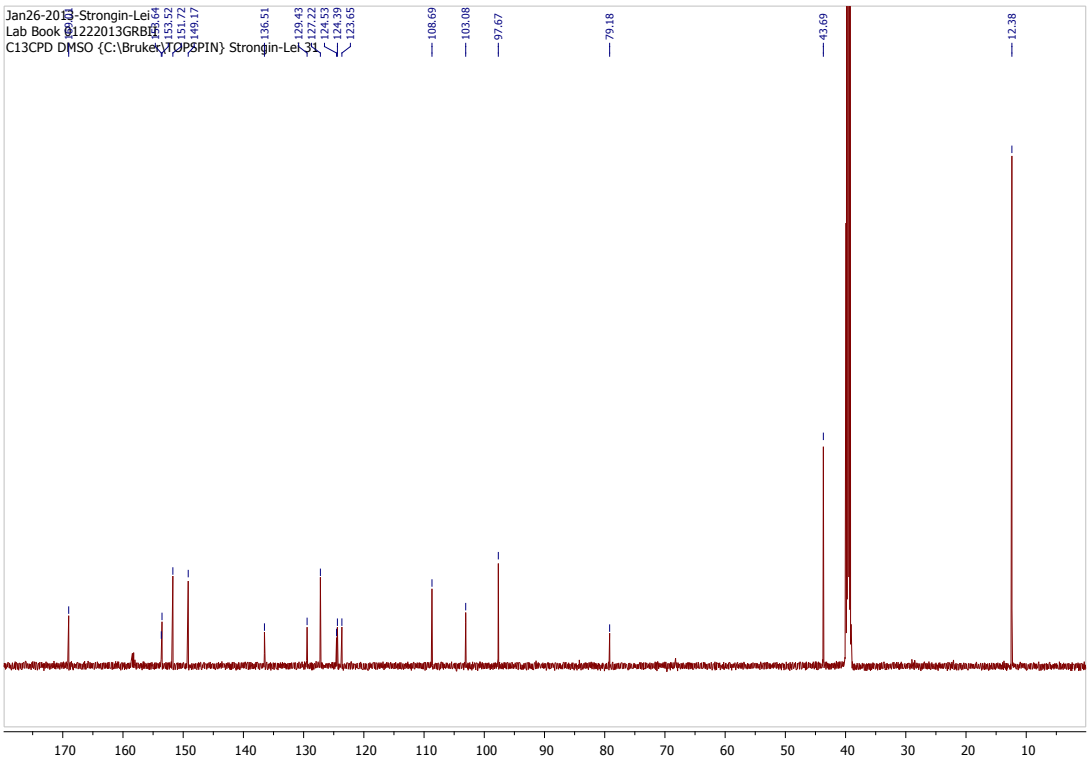


Figure S21. ^{13}C-NMR spectrum of **2** in DMSO-d_6.

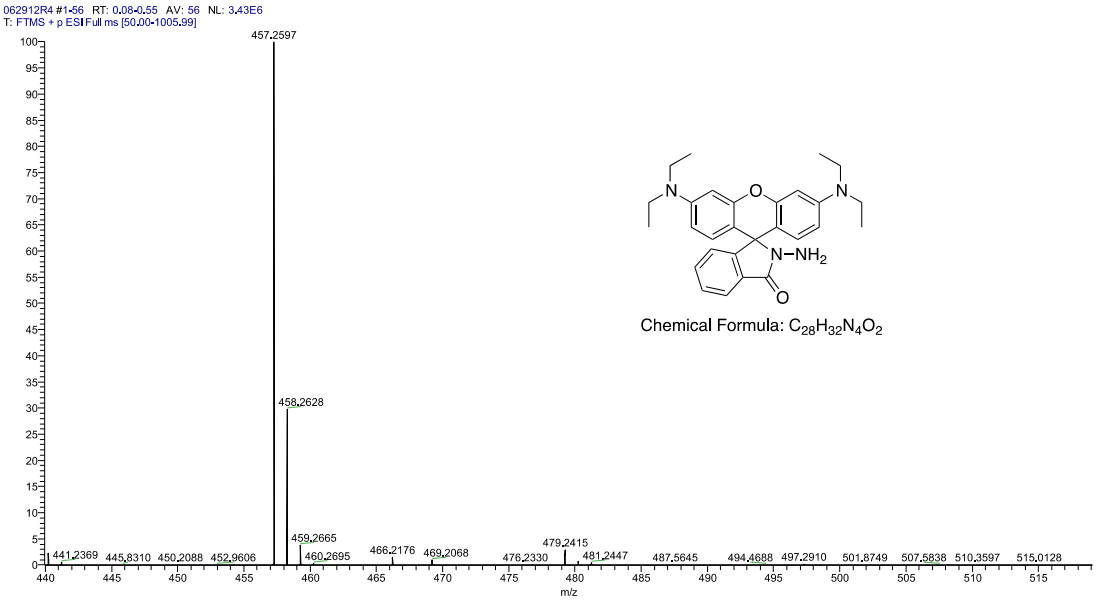


Figure S22. ESI-MS of **4**.

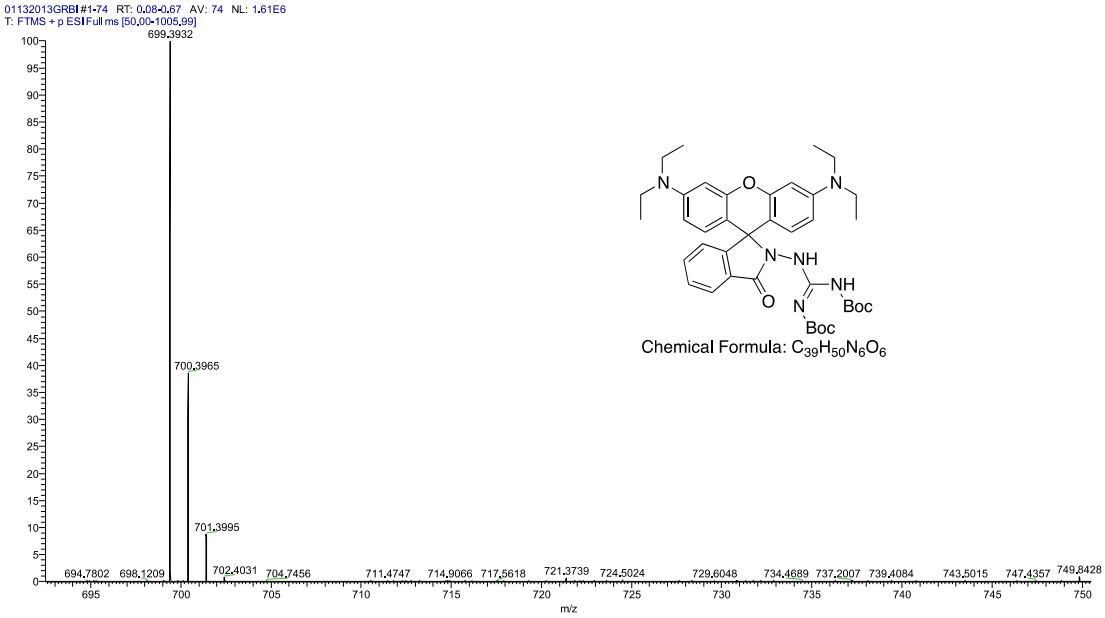


Figure S23. ESI-MS of **5**.

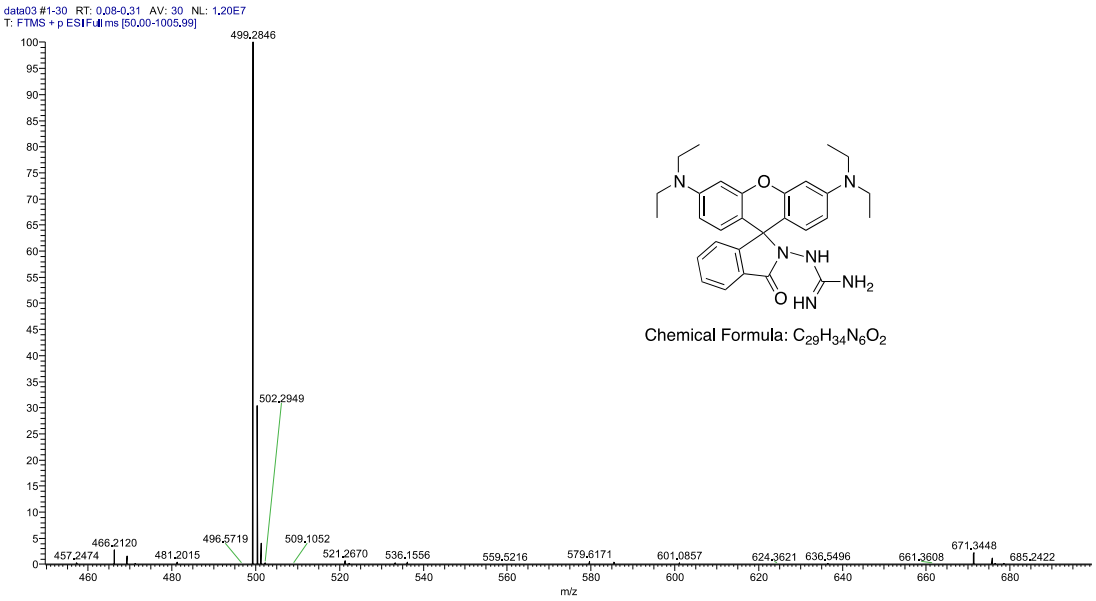


Figure S24. ESI-MS of **1**.

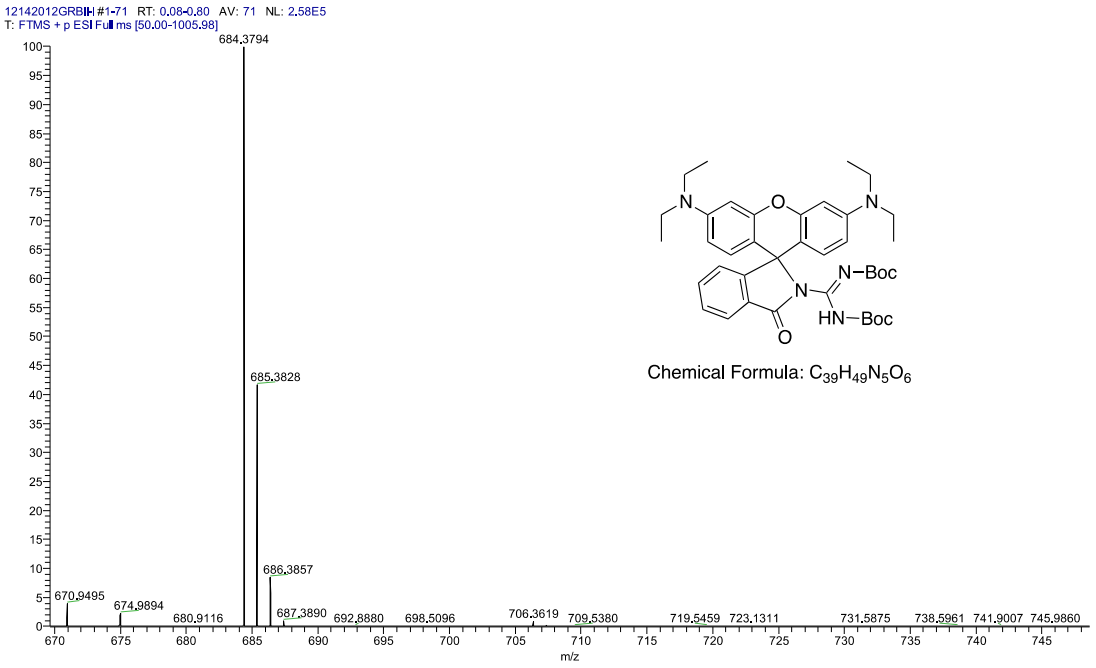


Figure S25. ESI-MS of **8**.

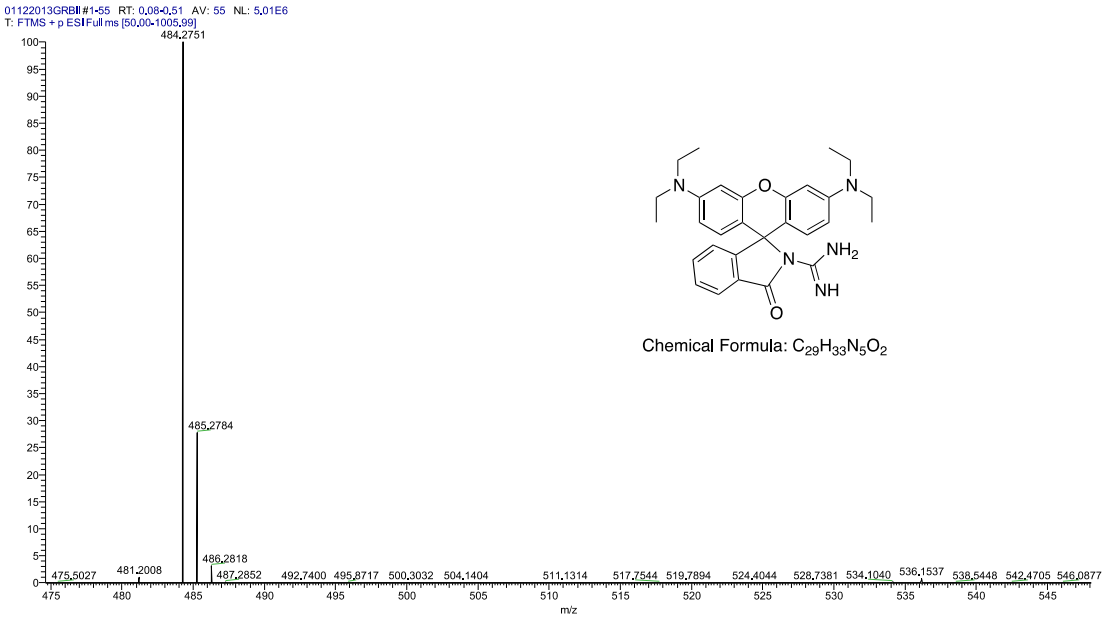


Figure S26. ESI-MS of **2**.

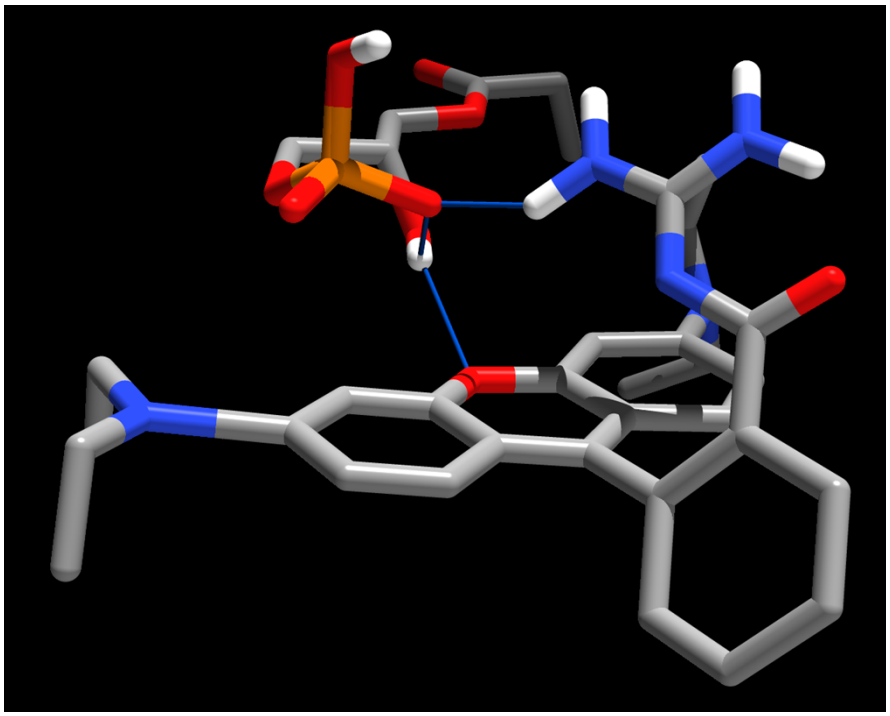


Figure S27. Energy-Minimized structure of **2** in the presence of LPA.

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[a]Department of Chemistry, Portland State University, Portland, OR 97201, USA. E-mail: *strongin@pdx.edu*; Tel: +1-503-725-9724.

[b]Women and Infants Hospital, Brown University, 101 Dudley Street, Providence, RI 02905, USA. E-mail: *RMoore@wihri.org*; Tel: +1-401-453-7520.