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Biological perspectives of a FRET based pH-probe exhibiting molecular logic gate operation with altering pH

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Materials and Methods: Reagents and solvents were purchased from Alfa Aesar and TCI and were used without any further purification. ^{1}H and ^{13}C NMR spectra (chemical shifts in δ ppm) were recorded on a JEOL AL FT–NMR (400 MHz) spectrometer, using TMS as internal standard. FT-IR spectra in KBr were recorded on a perkin elmer FT-IR spectrometer. The UV-Vis absorption spectra were recorded on Perkin Elmer 1700 spectrophotometer using a quartz cuvette (path length = 1.0 cm). Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Agilent). Stock solution of probe **1** ($c = 1x10^{-3}$ M) was prepared in ethanol-water (1:9, v/v). For each absorption and emission experiment, 30 µL of stock solution or probe was taken and diluted to make the concentrations 10 µM in a 3 mL probe solution. For interaction studies, 0.1 M solutions of different metal ions were used. The pH experiment in the range of pH = 2-10 were performed in Britton-Robinson (B-R) aqueous universal buffer solutions by mixing appropriate volumes of acids and basic buffer components. The binding constant of probe **1** for DNA were calculated by reported methods.[S1]

Photo-activated DNA cleavage studies

The extent of photoactivated DNA cleavage of supercoiled pUC19 DNA (30 µM, 0.2 µg) in 10 mM phosphate buffer (5% EtOH; pH 7.0) at RT was performed through agarose gel electrophoresis by photoirradiation of the probe **1** with ultraviolet light of 312 nm (8 W). The different concentration of the probe **1** in DMF was prepared and used after dilution to the final volume of 20 µL using the buffer medium. Probe was irradiated with ultraviolet light for 90 mins in a dark chamber and kept for incubation at 37 °C for an hour which was further analysed using gel electrophoresis. Mechanistic study of the probe was performed using different additives (KI, 0.2 mM; NaN_3, 0.2 mM; DMSO, 2 µL; L-His., 0.2 mM) at 312 nm for 90 mins prior to the addition of the probe. In case of D_2O experiment, D_2O itself was used for dilution to the volume of 20 µL. After incubation at 37 °C, 3 µl of loading dye (0.25% bromophenol blue, 30% glycerol (3.0 µL) and 0.25% xylene cyanol) was added to the probe and was finally loaded on agarose gel (1%) consisting of 1 µg ml^{-1} ethidium bromide. Gel electrophoresis was run for 2.0 h in Tris–acetate EDTA (TAE) buffer at 60 V. NC-DNA bands were visible in UV light and its image was clicked. The cleavage extent of SC-DNA to NC-DNA was calculated from the intensities of the bands and the quantification of cleaved SC-DNA was conducted using UVITEC Fire Reader V4 gel documentation system and UVI band software. The observed error was 4–6% in defining the intensity of the gel bands.

pKa calculations

The pKa value of probe **3** have been calculated using equation (1)

$$\boldsymbol{Log\left[\frac{Amax - A}{A - Amin}\right] = pH - pKa} \qquad (\mathbf{1}\boldsymbol{A})$$

$$\boldsymbol{Log[\frac{Imax - I}{I - Imin}] = pH - pKa} \qquad (\mathbf{1}\boldsymbol{B})$$

Where A and I is the absorbance and emission intensity with variation in pH and A_{max}, A_{min} and I_{max}, I_{min} is the maximum and minimum absorbance and emission intensity with variation of pH.[S2]

Quantum yield estimation

The quantum yield of probe **3** and its ring open form were calculated utilising equation (2)

$$\emptyset sample = \emptyset ref \left(\frac{Isample}{Iref}\right)\left(\frac{ODref}{ODsample}\right)\left(\frac{n2sample}{n2ref}\right) \quad \textbf{Eq (2)}$$

Where Φ represents the quantum yield, I, represent the integrated emission area and OD, the optical density of sample and reference (ref) fluorophore. The integrated emission area of probe **1** and open ring form (**3**) were calculated at λ_{ex}= 510 nm.

Energy Transfer Efficiency (E_T)

The energy transfer efficiency (E_T) in probe **1** have been estimated by employing equation (3).

$$E_T = 1 - F_{DA} / F_D \quad \textbf{Eq (3)}$$

Where, E_T is energy transfer efficiency, F_{DA} and F_D fluorescence intensity in the presence of acceptor and absence of acceptor.

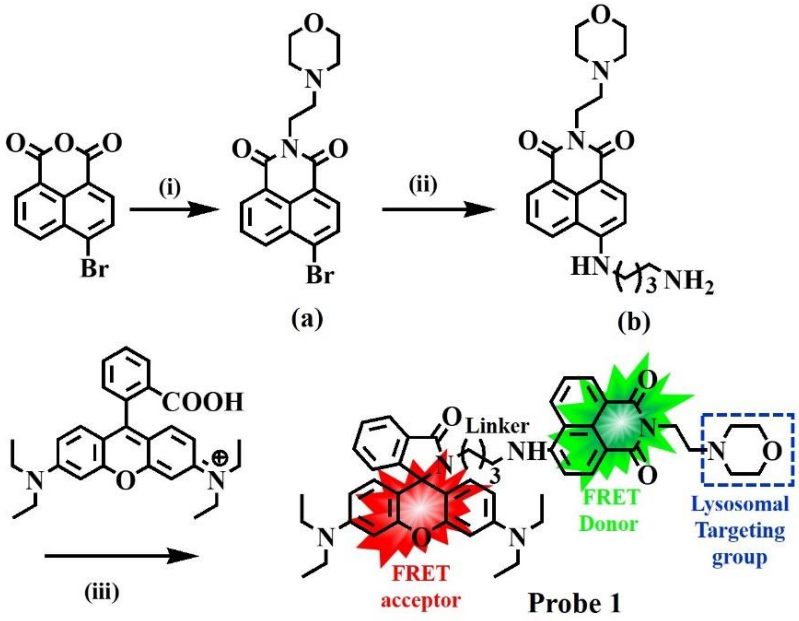
Synthesis:

Compound (a): The suspension of 4-bromo-1,8-naphthalic anhydride (0.554 g, 2 mmol) was taken in anhydrous ethanol (10 ml) and 4-(2-ethyl amine) morpholine (325 µl, 2.5 mmol) was added. The reaction mixture was stirred at 50°C for 5 h. After complete reaction (monitored on TLC), cold water (25 ml) was added to the reaction mixture and filtered. The precipitate was washed with 10% aqueous Na_2CO_3 solution followed by water and dried to obtain a light yellow colour crystalline solid. Yield: 80% (0.62 g, 1.70 mmol). m.p. 155-161°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.64-8.63 (d, 1H, *J* = 6 Hz), 8.57-8.53 (d, 1H, *J* = 6.8 Hz), 8.40-8.38 (d, 1H, *J* = 7.6 Hz), 8.04-8.02 (d, 1H, *J* = 8.4 Hz), 7.86-7.82 (m, 1H, *J1* = 8.4 Hz; J_2 = 7.6 Hz) 4.33-4.30 (m, 2H, *J* = 6.8 Hz), 3.66-3.64 (m, 4H), 2.70-2.66 (m, 2H, J_1J_2=6.8Hz*)* 2.58-2.56 (m, 4H). Anal. Calc. For $C_{18}H_{17}N_2O_3Br$: C, 55.54; H, 4.40; N, 7.20%. Found: C, 55.25; H, 4.27; N, 7.15%.

Compound (b): The solution of compound a (0.5 g, 1.28 mmol) was taken in anhydrous pyridine (10 ml), 1,4-diamino butane (3 ml, 30 mmol) and triethylamine (200 µl) were added and reaction mixture was refluxed for overnight. After the completion of reaction (as monitored on TLC), the solvent was evaporated under reduced pressure and cold water was added to the reaction mixture to precipitate out the desired product, which was filtered and dried in air. Pure compound was obtained by column chromatography (elution with methanol) to afford an orange colour compound. Yield: 81% (0.41 g, 1.03 mmol); m.p. 190-195°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.54-8.52 (m, 1H, *J* = 6.4 Hz), 8.44-8.41 (d, 1H, *J* = 8.4 Hz), 8.20-8.18 (d, 1H, *J*= 7.2 Hz), 7.58-7.54 (m, 1H, J_1 = 7.6 Hz, J_2 = 8.4Hz), 6.66-6.64 (d, 1H, *J* = 8.4Hz), 6.61 (s, -NH), 4.32-4.29 (m, 2H, J_1 = 7.2 Hz, J_2 = 8.4 Hz), 3.69-3.67 (m, 4H), 3.38-3.37, 2.86-2.83, 2.69-2.66, 2.59, 1.93-1.88, 1.71-1.59. Anal. Calc. For $C_{22}H_{28}N_4O_3$: C, 66.64; H, 7.12; N, 14.13%. Found: C, 66.57; H, 7.01; N, 14.02%.

Probe 1: The solution of compound **2** (0.35g, 0.88 mmol) and Rhodamine B (0.42g, 0.88 mmol) were taken in ethanol and refluxed for 24 h. After completion of reaction monitored on TLC, the reaction mixture was evaporated. The crude was suspended in water and extracted with DCM and washed with water (three times) and kept over $MgSO_4$. The solvent was evaporated under vacuum and crude product obtained was purified column chromatography using DCM and elution with methanol. Yield: 35% (0.250 g, 0.31 mmol); m.p. 216-220°C ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm): 8.59-8.57 (d, 1H, *J* = 8 Hz), 8.38-8.36(d, 1H, *J* = 6.4 Hz), 8.17-8.15(d, 1H, *J* = 8.4 Hz), 7.74-7.28 (H14) , 7.62-7.57 (H16, H6), 7.47-7.45 (H15, H8), 7.00-6.98 (H17), 6.54-6.62 (H18), 6.30 (H24), 6.27 (H19-23), 4.11-4.08 (t, -NH, J_1 = 6.8 Hz, J_2 = 7,2 Hz), 3.48 (H1), 3.29 (H4), 3.23-3.18 (q, H26, J_1 = 6.8 Hz, J_2 = 7,2 Hz), 3.06-3.00 (H13, H10) , 2.46- 2.45 (t, H2, J_1 = 6.8 Hz, J_2 = 7,2 Hz), 2.40 (H3), 1.68-1.65 (m, H12), 1.58- 1.55 (m, H11), 1.00-0.967 (t, H25, J_1 = 6.8 Hz, J_2 = 7,2 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 167.4, 157.1, 153.2, 148.8, 134.7, 133.1, 131.2, 129.1, 128.8, 124.7, 124.1, 122.7, 108.5, 105.7, 66.7, 56.3, 53.9, 48.0, 44.1, 33.8, 25.8, 24.9 and 12.8; Anal. Calc. For $C_{50}H_{56}N_6O_5$:

C, 73.15; H, 6.87; N, 10.24%. Found: C, 73.07; H, 6.67; N, 10.12%. HRMS m/z: calcd for $C_{50}H_{56}N_6O_5$ $[M + H]^+$: 821.4346, found: 821.4343.



Scheme 1. Synthesis of probe **1**, (i) 4-(2-ethyl amine) morpholine/ Ethanol /reflux/overnight (ii) 1,4 –diamino butane /TEA/ pyridine/ reflux (iii) Rhodamine-B/ Ethanol / reflux.

Table S1: Quantum yield of probe **1** and ring-opened derivative (**2**).

Quantum yield (ϕ)	Compared to rhodamine -B	Compared to quinine sulfate
Probe **1**	0.018	0.00159
Probe **1**+H^+ (**2**)	0.453	0.04

References:

S1 (a) R. B. P. Elmes, M. Erby, S. M. Cloonan, S. J. Quinn, D. C. Williams, T. Gunnlaugsson, *Chem. Commun*., 2011, **47**, 686–688. (b) M. T. Carter, M. Rodriguez, A. J. Bard, *J. Am. Chem. Soc*. 1989, **111**, 8901-8911. (c) J. D. McGhee, P. H. V. Hippel, *J. Mol. Biol*. 1974, **86**, 469-489. (d) D. L. Boger, B. E. Fink, S. R. Brunette, W. C. Tse, M. P. Hedrick, *J. Am. Chem. Soc.* 2001, **123**, 5878-5891. **S2.** M. H. Lee, N. Park, C. Yi, J. H. Han, J. H. Hong, K. P. Kim, D. H. Kang, J. L. Sessler, C. Kang, J. S. Kim, *J. Am. Chem. Soc*. 2014, **136**, 14136−14142.

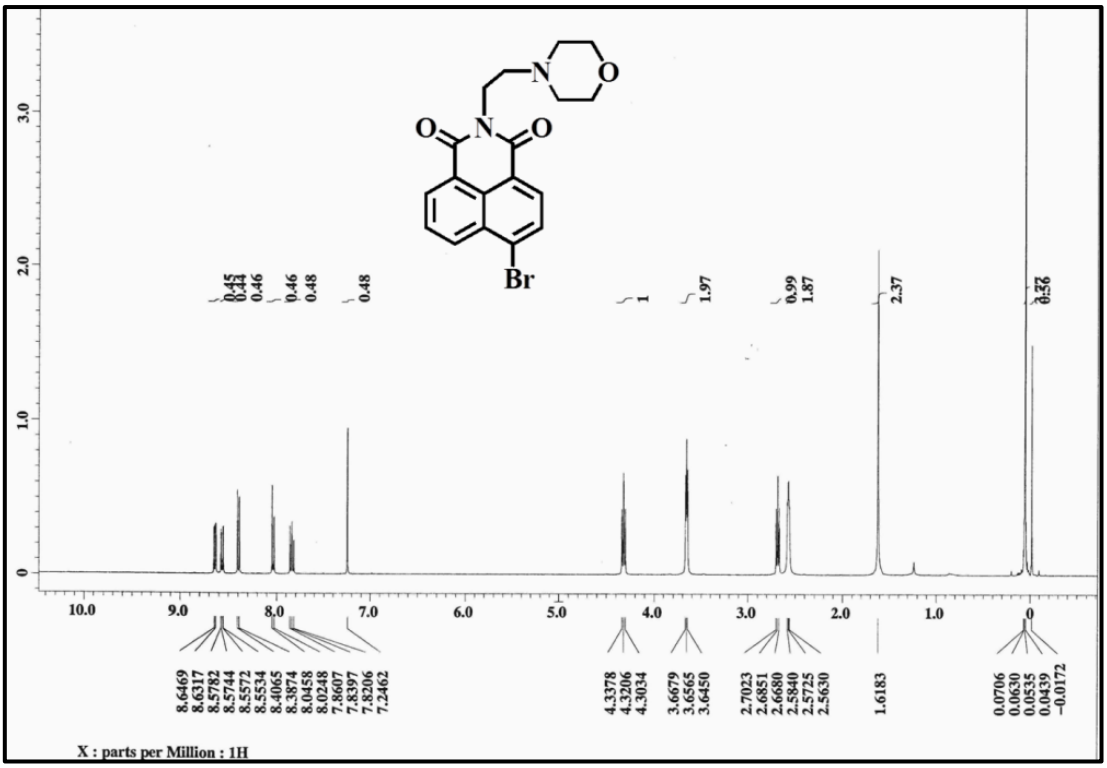


Figure S1. ^{1}H NMR spectrum of compound (**a**) in $CDCl_3$.

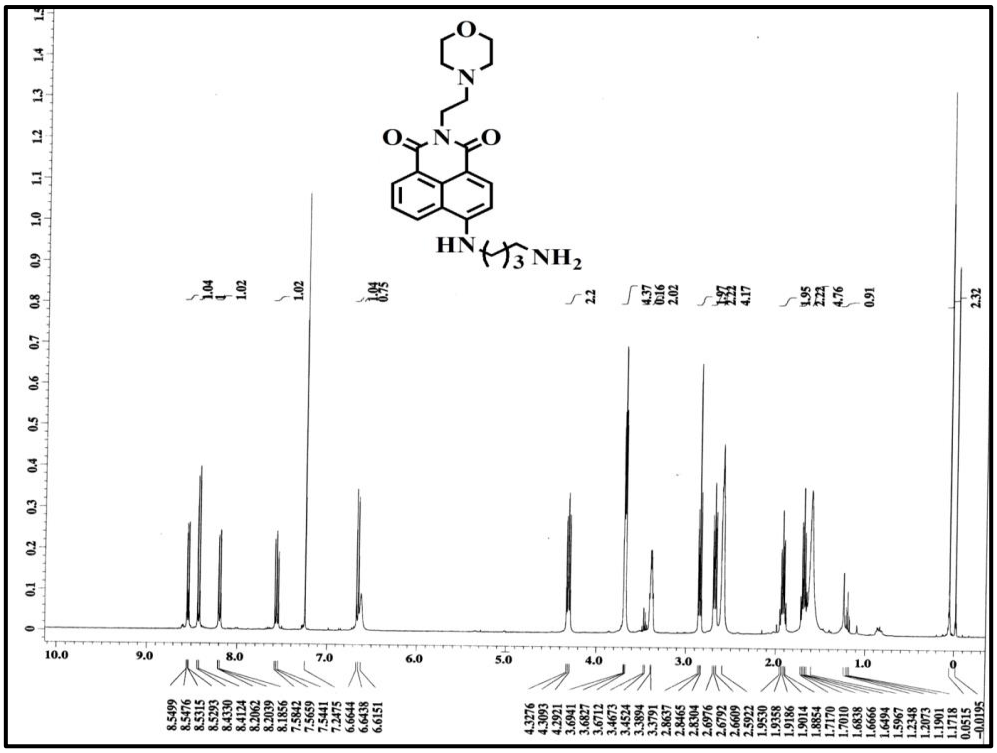


Figure S2. ^{1}H NMR spectrum of compound (**b**) in $CDCl_3$.

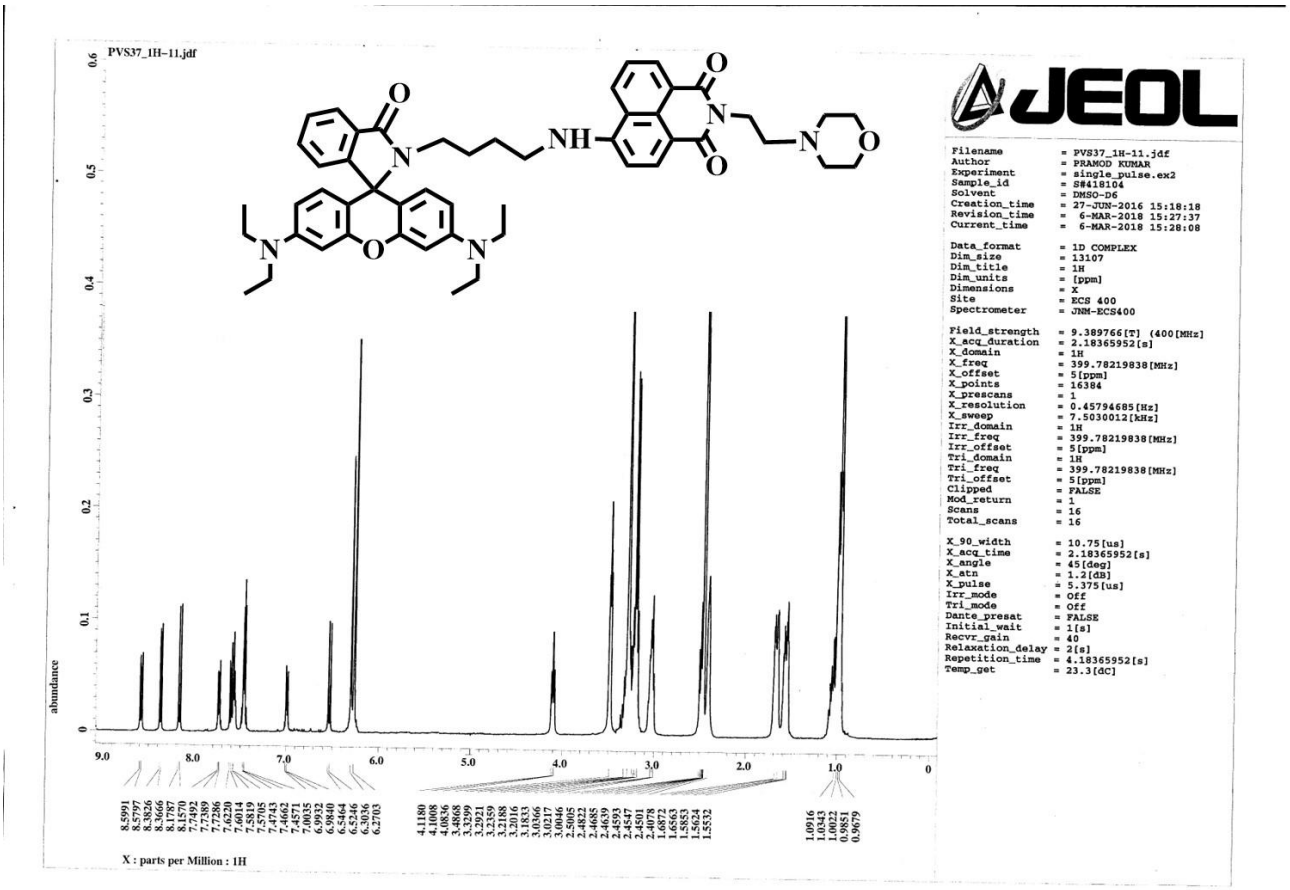


Figure S3. ^{1}H NMR spectrum of probe **1** in DMSO-D_6.

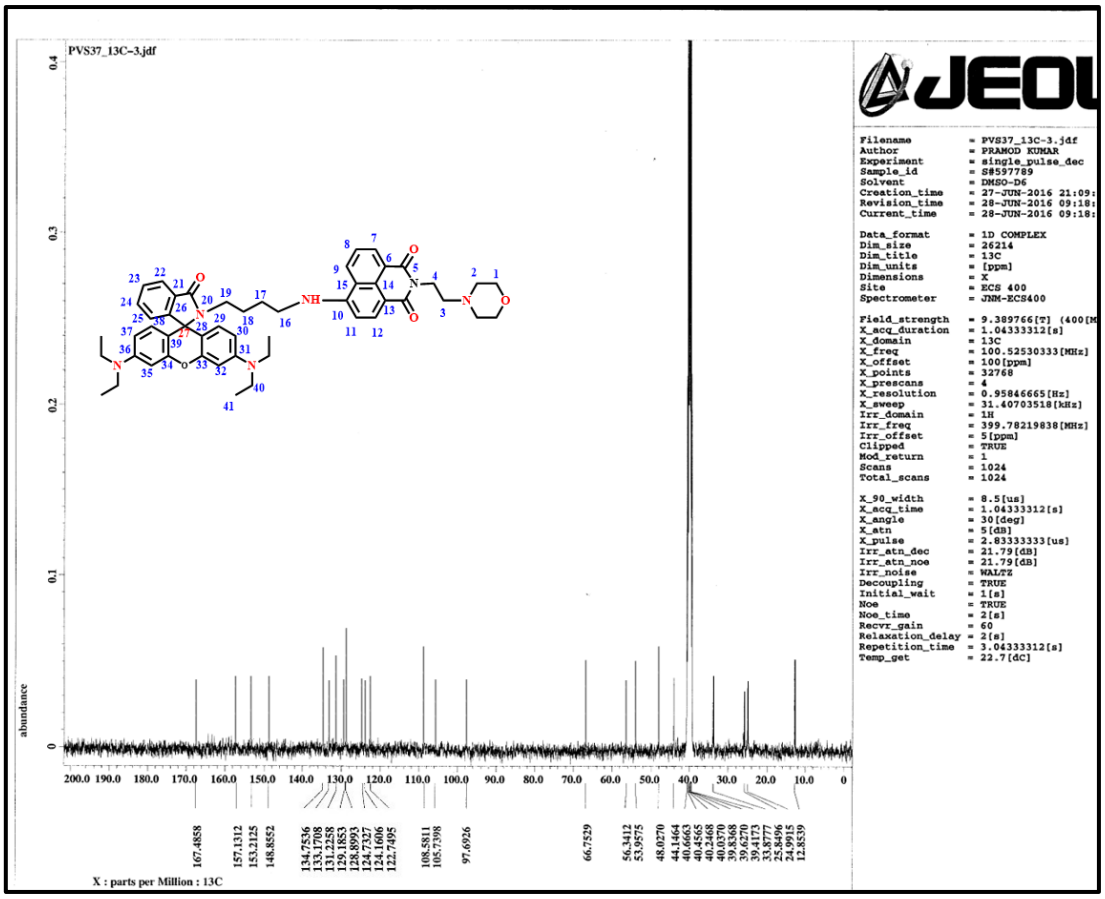


Figure S4. ^{13}C NMR spectrum of probe **1** in DMSO-D_6.

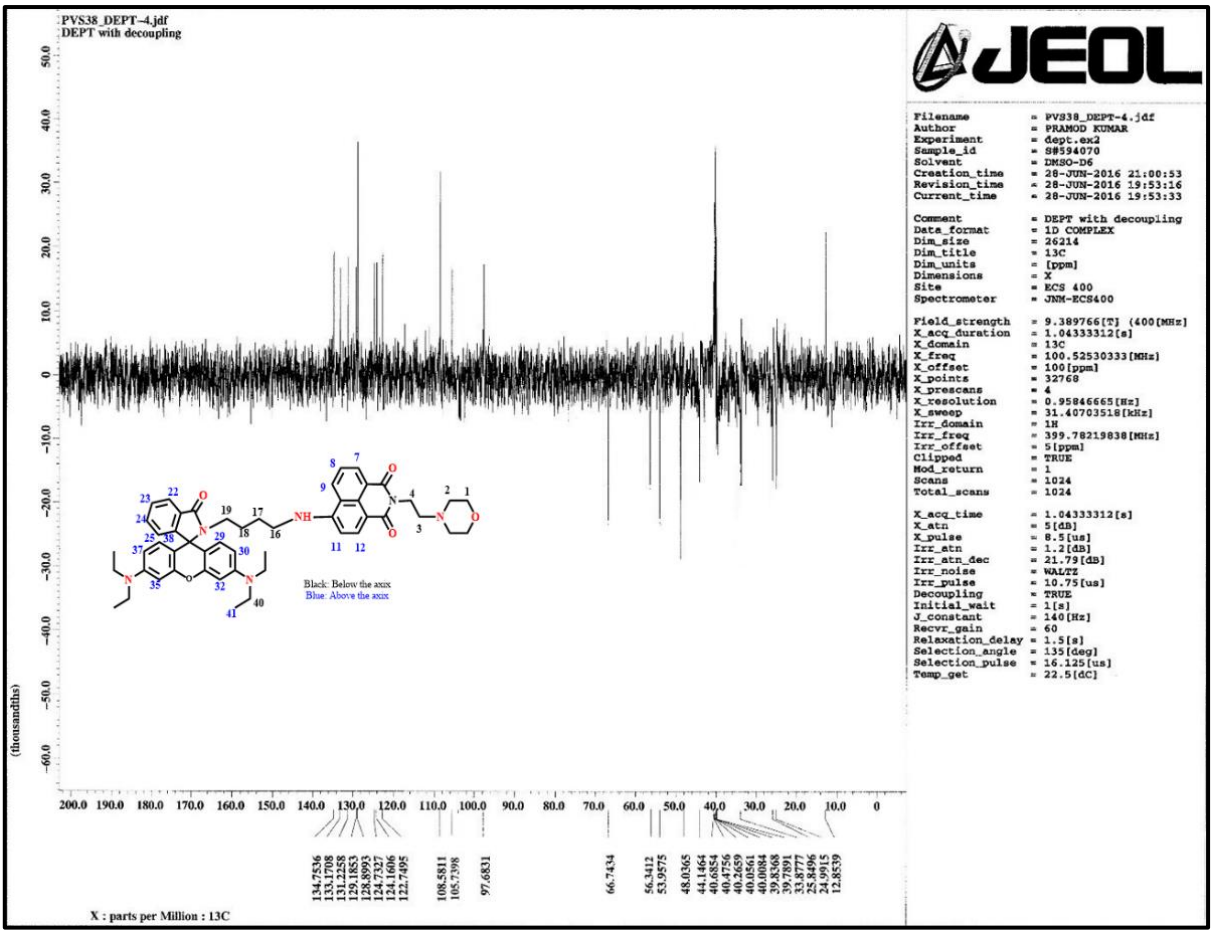


Figure S5. ^{13}C DEPT NMR spectrum of probe **1** in DMSO-D_6**.**

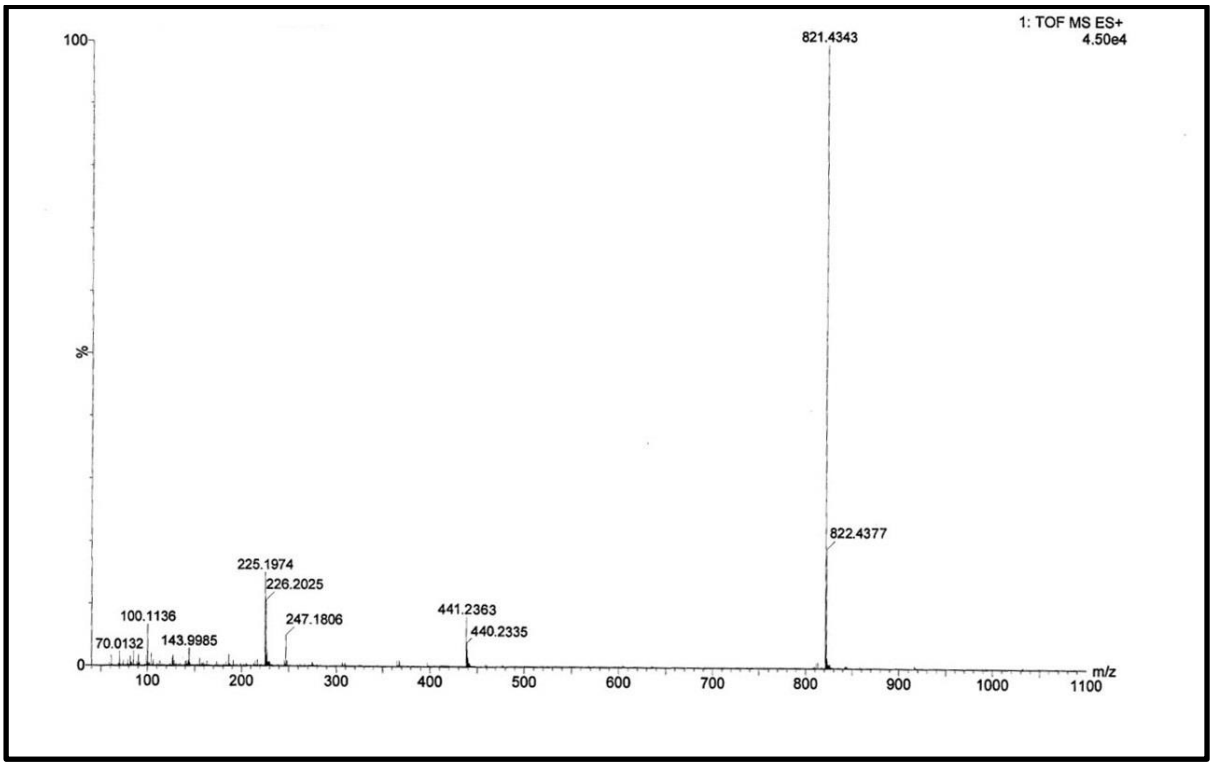


Figure S6. ESI-MS spectrum of probe **1**.

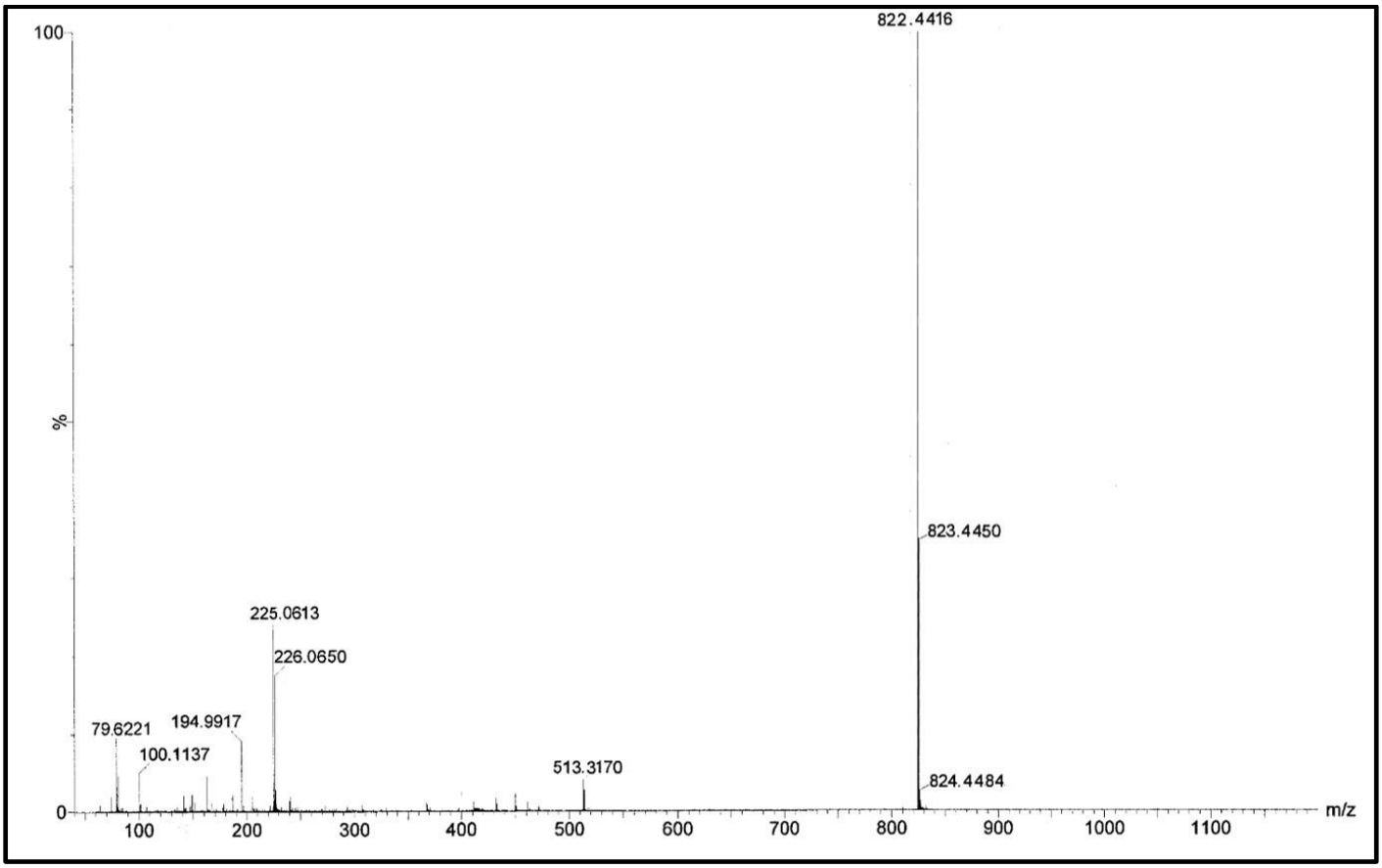


Figure S7. ESI-MS spectrum of probe **1**+H^+ (**2**).

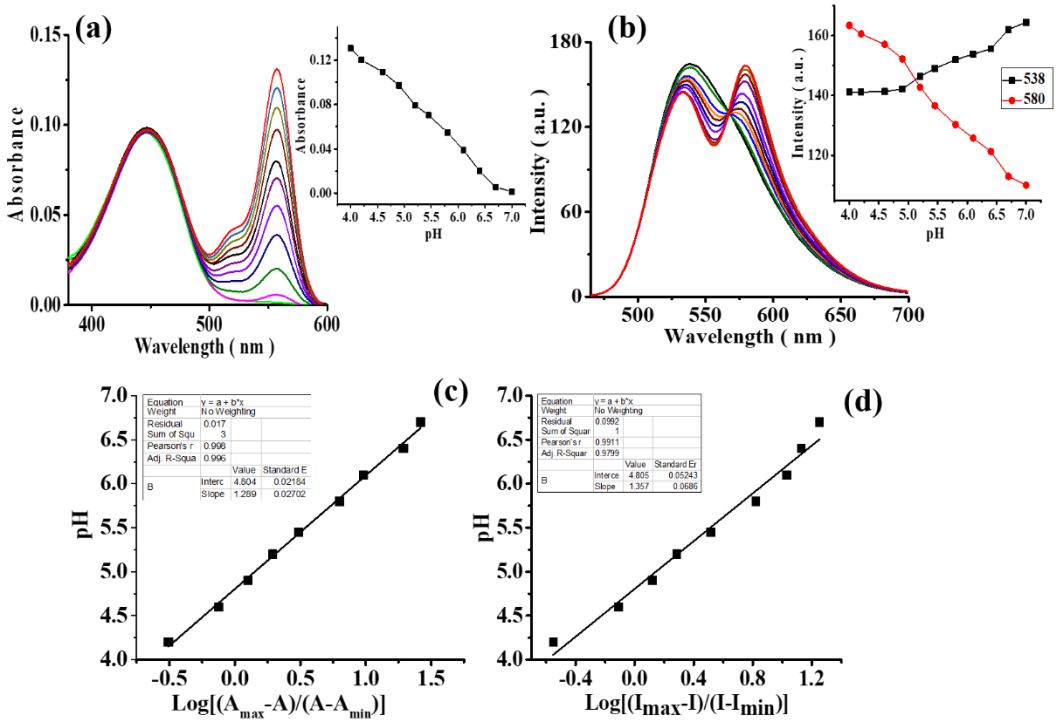


Figure S8. (a) Absorption spectra and (b) emission spectra exhibited by probe **1** (10 μM) with variation in pH 4.0 – pH 7.0 in Britton−Robinson (B−R) buffers (5% EtOH) at RT. Inset plot of (a) absorption spectral changes at λ_{max} = 560 nm (rhodamine band); (b) emission spectra changes at λ_{max} = 580 nm (rhodamine band) and 542 nm (napthalimide) with λ_{ex} =445 nm. Plot of pH vs. (c) log[(A_{max}-A)(A-A_{min})] (d) log[(I_{max}-I)(I-I_{min})], where A is the absorbance with variation in pH and I is the observed ratio of fluorescence intensity of probe **1** at 542 and 580 nm upon excitation at 445 nm[S2].

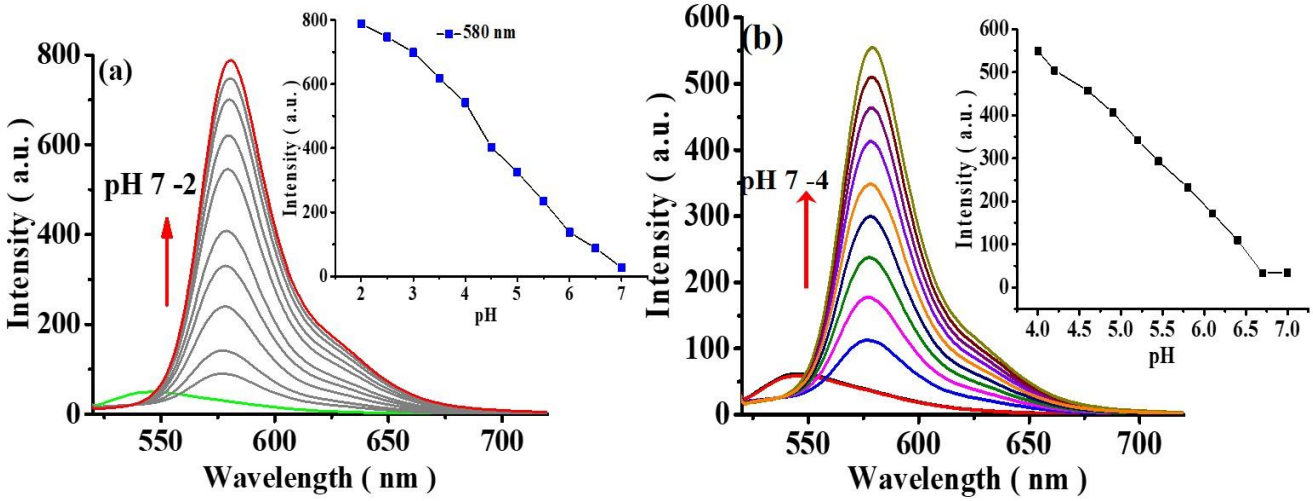


Figure S9. Emission spectra of probe **1** (a) pH 7 - 2 (b) pH 7 – 4. Inset: plot of corresponding changes in emission intensity at λ_{em}= 580 nm (at Rhodamine band) by probe **1** due to spirolactum ring cleavage with changing pH 7 to 2 in B−R buffer (5% EtOH) and λ_{ex}= 510 nm (slit width = 5 nm, 298 K).

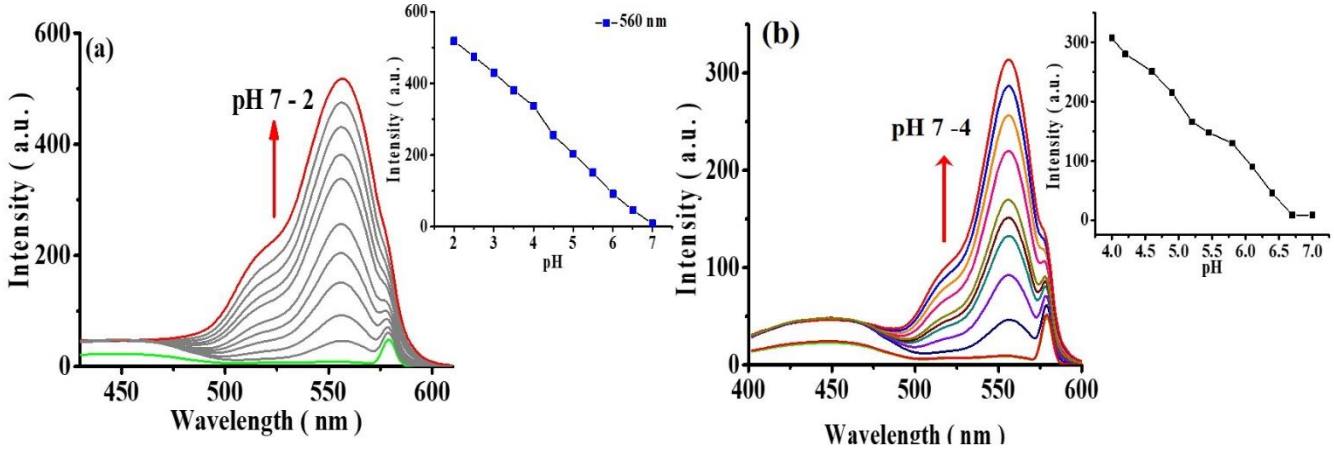


Figure S10. Excitation spectra of probe **1** with (a) pH 7 – 2 (b) pH 7.0 – 4.0. Inset: plot of emission spectral changes exhibited by probe **1** with changing pH in B−R buffers (5% EtOH) at RT and λ_{ex}= 580 nm (slit width 5 nm, 298 K).

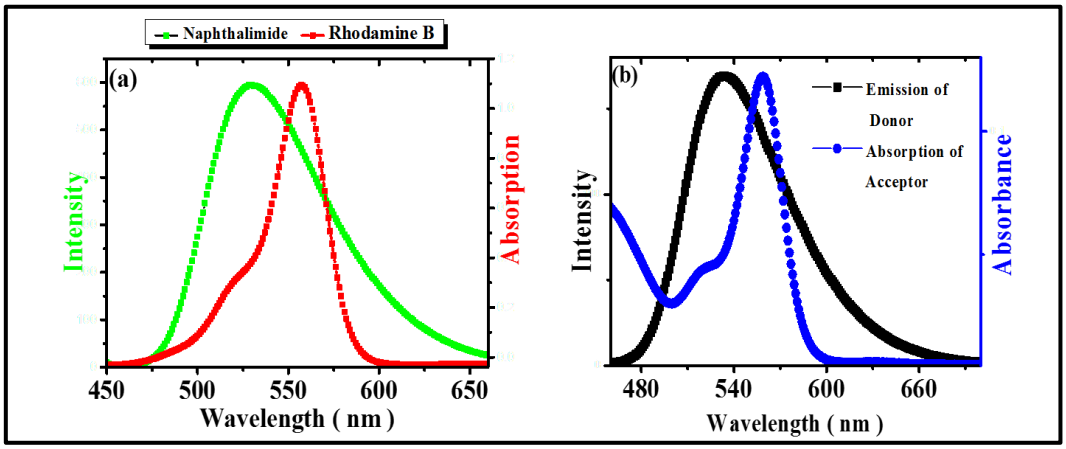


Figure S11. Normalized absorption and emission spectra of (a) Rhodamine-B and comp **b** and (b) probe **1** at pH 4.0 (acceptor) and at pH 7.0 (donor) respectively in B−R buffer (5% EtOH) at RT (slit width = 5 nm).

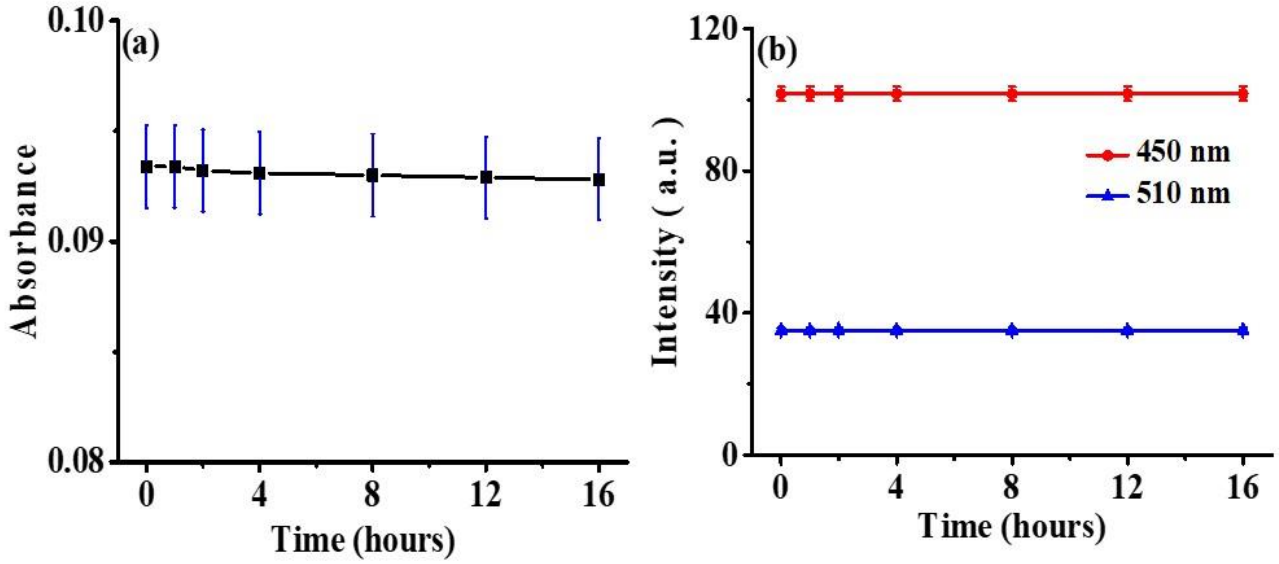


Figure S12. The photo stability experiment of probe **1** up to 16 hours (a) absorption (b) emission spectral changes in B−R buffer (5% EtOH, pH 7.0) at RT.

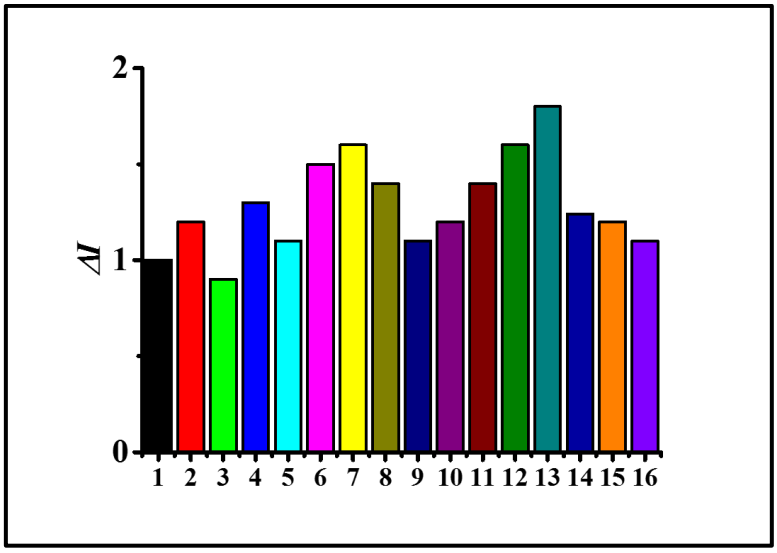


Figure S13. Interaction study of probe **1** upon addition of glutathione (1), homocysteine (2), cysteine (3), nitric oxide (4) (NO), hydrogen peroxide (5) (H_2O_2), glucose (6), glycine (7), valine (8), glutamic acid (9), threonine (10), serine (11), aspartic acid (12), tryptophan (13), lysine (14), arginine (15) and ATP (16) in B-R buffer, pH 7.0 prepared in 5% EtOH at RT (λ_{ex}= 450 nm, slit width 5 nm).

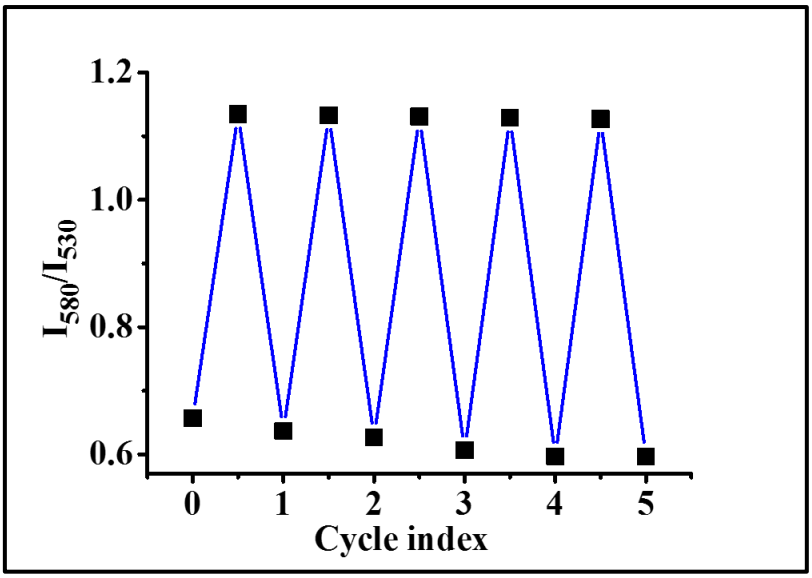


Figure S14. Reversibility experiment of probe **1** with changing pH between 7.0 and 4.0 up to 5 times.

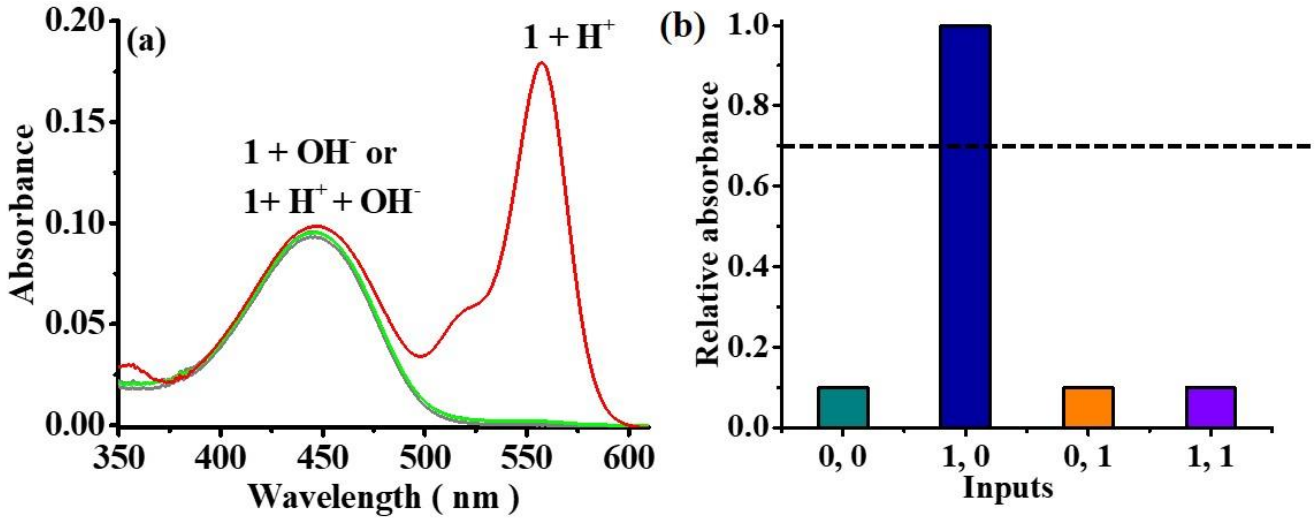


Figure S15. Absorption spectra and bar diagram of probe **1** (10 μM) upon applying H^+ (In_1) and OH^- (In_2) in Britton−Robinson (B−R) buffer (5% EtOH) at RT.

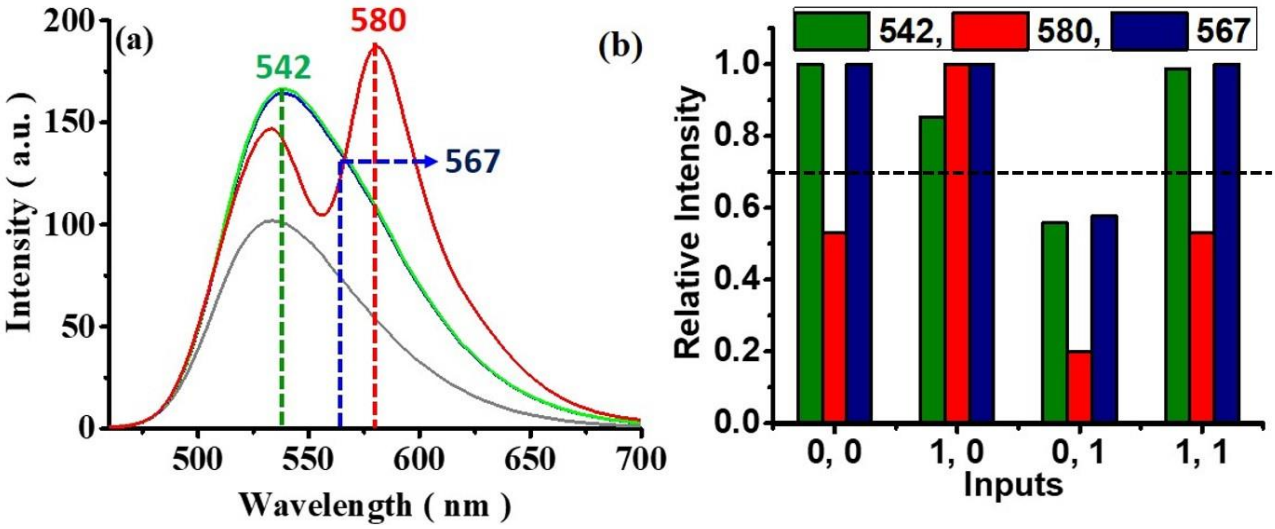


Figure S16. Emission spectra and bar diagrams of probe **1** (10 μM) at λ = 542, 580 and 567 nm showcasing their relative intensities upon applying H^+ (In_1) and OH^- (In_2) in Britton−Robinson (B−R) buffer (5% EtOH) at RT.

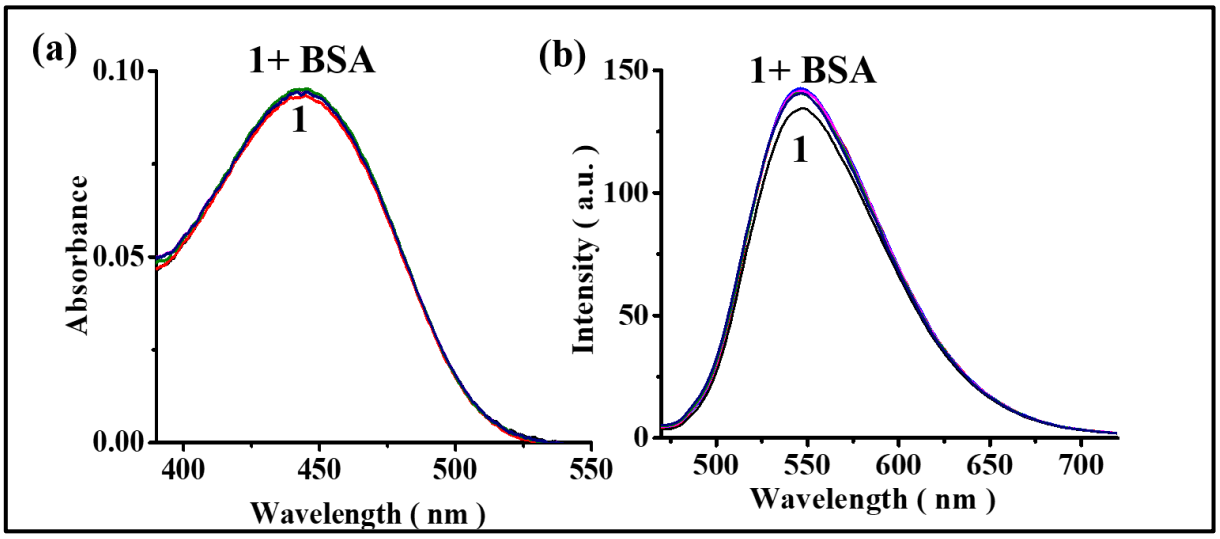


Figure S17. (a) Absorption (b) emission spectra of Probe **1** (10 μM; λ_{ex}= 450 nm) with increasing concentration of BSA (0 – 30 μM) in 10 mM phosphate buffer (5% EtOH; pH 7.0) at RT (slit width = 5 nm).

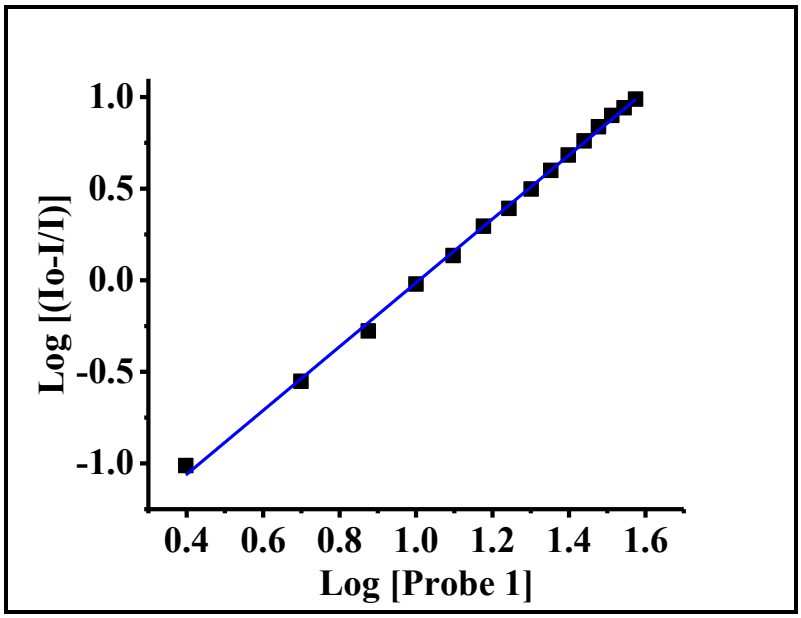


Figure S18. Scatchard plot of BSA with probe **1** (0 – 40 µM) in 10 mM phosphate buffer (5% EtOH; pH 7.0) at RT and λ_{ex}= 295 nm, slit width = 5 nm.

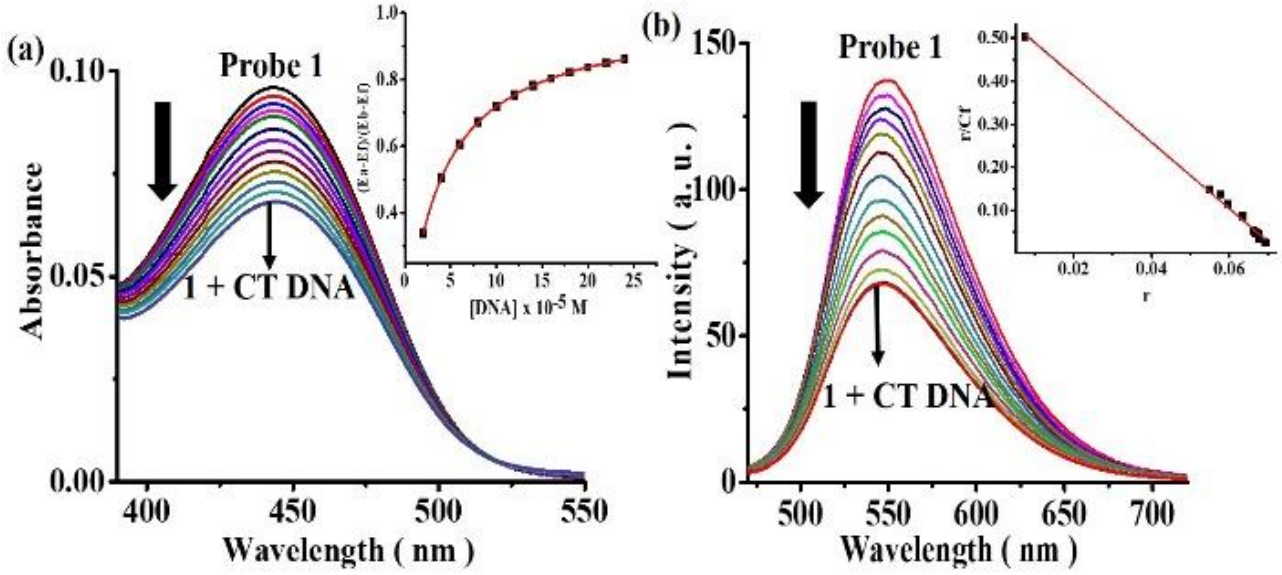


Figure S19. (a) Absorption (b) emission spectra of probe **1** (10 µM) with increasing concentration of CT-DNA (0- 150 µM) in 10 mM phosphate buffer (5% EtOH; pH 7.0) and λ_{ex}= 450 nm (slit width = 5 nm, 298 K). Inset: (a) plot of $(\varepsilon_a-\varepsilon_f)/(\varepsilon_b-\varepsilon_f)$ vs. [DNA] (b) Scatchard plot of probe **1** with CT-DNA where r = Cb/DNA, Cb (bound probe concentration) = [(If-I)/(If-Ib)]C (total probe concentration and C_f= free probe concentration.

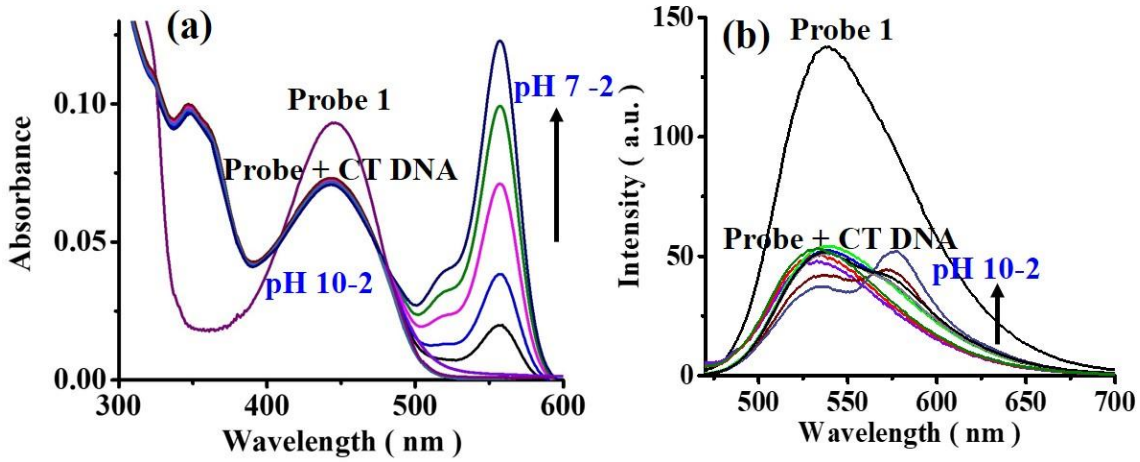


Figure S20. (a) Absorption and (b) emission spectra (λ_{ex}= 450 nm; slit width = 5 nm, 298 K) of probe **1** (10 µM) + CT-DNA (150 µM) with changing pH 2 to 10 in phosphate buffer (5% EtOH).

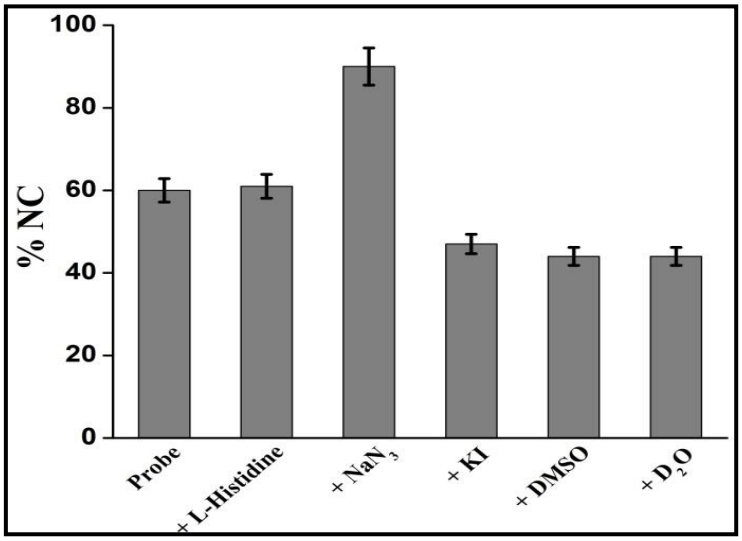


Figure S21. Bar diagram displaying the photocleavage of SC pUC19 DNA by probe **1** (10 µM) on photo exposure at 312 nm for 90 min in the presence of different additives in 10 mM phosphate buffer (5% EtOH; pH 7.0) at RT.