

# Supporting Information

## A Ratiometric pH Reporter For Imaging Protein-dye Conjugates In Living Cells

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## 1. General Procedures

Bovine serum albumin (BSA) was purchased from Calbiochem. Sephadex™ G-25 (PD-10) was bought from GE healthcare. Pep-1 (Chariot) was purchased from Active Motif. Et<sub>3</sub>N were distilled from CaH<sub>2</sub>. Unless otherwise mentioned, other solvents and reagents were used as received. NMR spectra were recorded on a VXP-300 MHz and Inova-500 MHz spectrometers (<sup>1</sup>H at 300 MHz or 500 MHz, <sup>13</sup>C at 75 or 125 MHz) at room temperature unless otherwise mentioned. Chemical shifts of <sup>1</sup>H NMR spectra were recorded and reported in ppm from the solvent resonance (CDCl<sub>3</sub> 7.26 ppm, CD<sub>3</sub>OD 3.30 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants, and number of protons. Proton decoupled <sup>13</sup>C NMR spectra were also recorded in ppm from solvents resonance (CDCl<sub>3</sub> 77.0, CD<sub>3</sub>OD 49.1ppm). Analytical thin layer chromatography (TLC) was performed on EM Reagents 0.25 mm silica-gel 60-F plates, and visualized with UV light. Flash chromatography was performed using silica gel (230–600 mesh). UV/Visible and fluorescence spectra were taken in pH 7.4 PBS buffer unless otherwise mentioned. MS were measured under ESI or MALDI conditions.

## 2. Determination of Quantum Yields and Extinction Coefficients.

UV/Vis absorbance spectra were recorded on a Cary 100 Bio spectrophotometer. Steady-state fluorescence spectroscopic studies were performed on a Cary Eclipse fluorometer. The slit width was 5 nm for both excitation and emission. The excitation wavelength for the test sample and the standard is the same. Fluorescence spectra were corrected for detector sensitivity. The relative quantum yields of the samples were obtained by comparing the area under the corrected emission spectrum of the test sample with that of a solution of standard. The quantum efficiencies of fluorescence were average of two measurements with the following equation:

$$F_x = F_{st} (I_x/I_{st}) (A_{st}/A_x) (\eta_x^2/\eta_{st}^2)$$

Where  $F_{st}$  is the reported quantum yield of the standard,  $I$  is the area under the emission spectra,  $A$  is the absorbance at the excitation wavelength and  $\eta$  is the refractive index of the solvent used, measured on a pocket refractometer from ATAGO.  $X$  subscript denotes unknown, and  $st$  means standard.

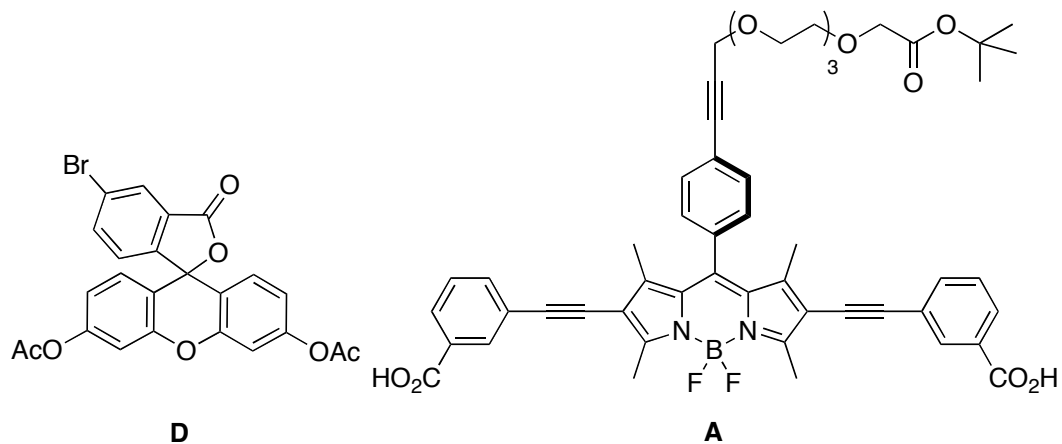
Fluorescein ( $\phi = 0.92$  in 0.1M NaOH)<sup>1</sup> and Rhodamine 6G ( $\phi = 0.95$  in EtOH)<sup>2</sup> were used as the standards for measurements of quantum yields of **BSA-1** in pH 4.1 and 8.8 buffers respectively, and the excitation wavelength was 488 nm.

### 3. Cell Culture

COS-7 cells (American Type Culture Collection) were cultured as subconfluent monolayers on 75 cm<sup>2</sup> culture flask with vent caps in DMEM supplemented with 10 % fetal bovine serum (FBS) in a humidified incubator at 37 °C with 5 % CO<sub>2</sub>. Cells grown to subconfluence were enzymatically dissociated from the surface with trypsin and plated 2-3 days prior to the experiments in Lab-Tek two well chambered coverglass slides (Nunc).

### 4. Fluorescence Microscopy

Cells were washed several times with PBS buffer and then put on the stage of the Bio-Rad 2000MP system (Bio-Rad Laboratories, Hercules, CA) equipped with a Nikon T300 inverted microscope with a 60x (NA1.2) water immersion objective lens and an Argon laser tuned to 488 nm wavelength. Through Bond Energy Transfer data in COS-7 cells loaded with 5-bromo-fluorescein diacetate (**D**, control donor) alone (2 min at 37 °C), BODIPY derivative (**A**, control acceptor) (30 min at 37 °C) alone or with **BSA-1** (1:20 mol ratio of **BSA-1**:pep-1 for 1h at 37 °C; the complex **BSA-1**:pep-1 was preformed by mixing both reagents and incubating them at room temperature for 30 min) were collected using 488 nm excitation wavelength. Both **D** and **A** were excited at 488 nm and emission of **D** (FITC channel; donor signal) was collected using a 560 DCLP XR dichroic mirror and a HQ 528/50 –nm emission filter whereas emission of **A** (FRET channel; acceptor signal) was collected using a HQ 600/50-nm filter. Donor bleed through signal to the FRET channel was calculated by measuring the FRET channel signal resulting from COS cells loaded only with the donor. Acceptor bleed through to the FRET channel was calculated by measuring the FRET channel signal resulting from COS cells loaded with **A** alone. Accumulated images (N=6, F=1) at a 1024x1024 resolution were captured.



For the experiment done at 4 °C, the cells were pre-incubated at 4 °C for 30 min, before the addition of the complex. After addition of the complex, the cells were incubated for another hour at 4 °C.

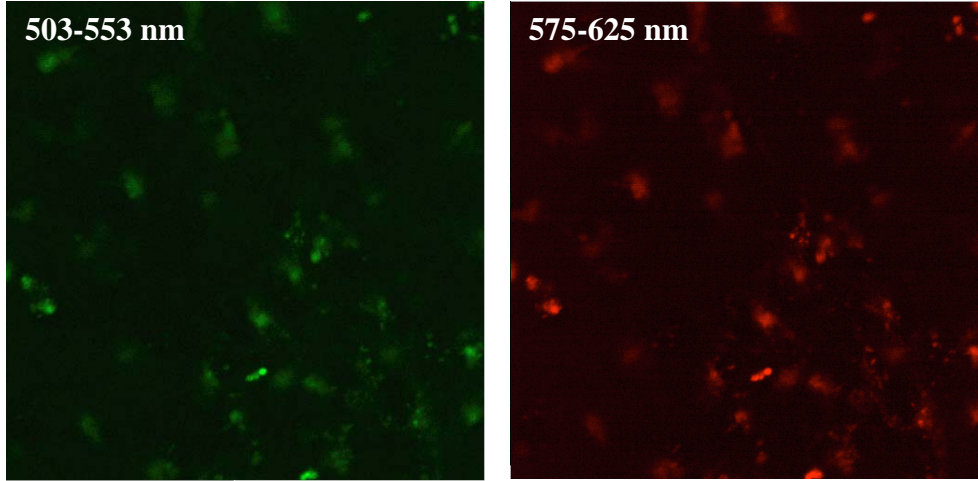
### *Quantitative Analysis of Energy Transfer*

Background was subtracted from images prior to analysis.

Then: Red signal (corrected) = Red signal - (Df/Dd)\*Green

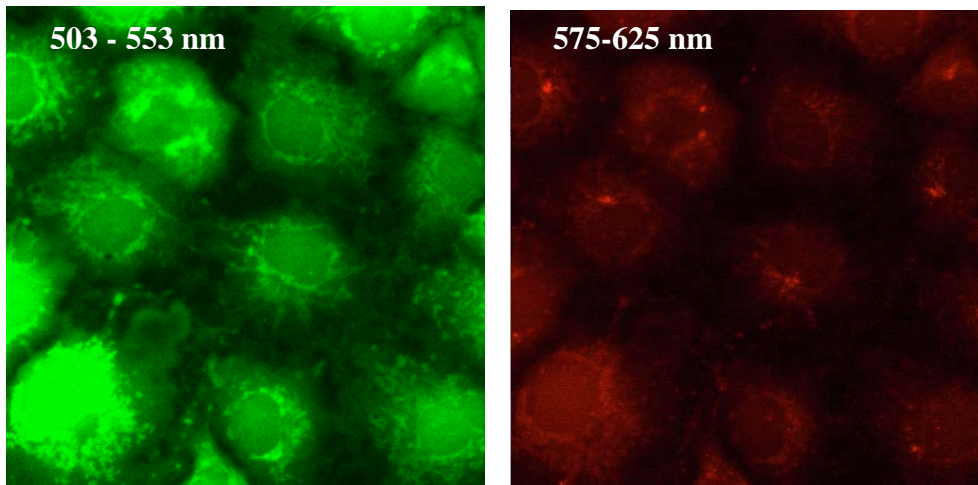
The constant Df/Dd is the bleedthrough constants describing donor emission visible in FRET channel.<sup>3</sup>

***COS-7 Cell Autofluorescence*** (excitation at 488 nm)



***Control Donor*** (excitation at 488 nm)

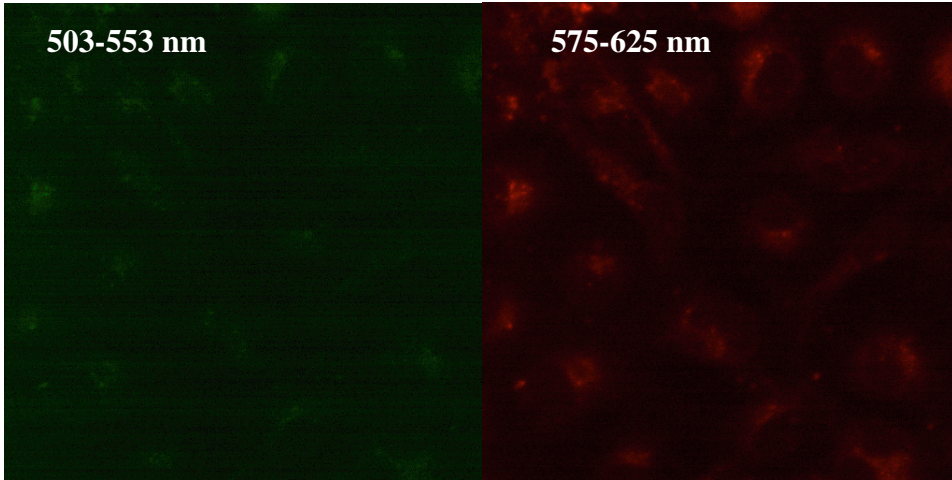
COS 7 cells were incubated with 10 nM of 5-bromo-fluorescein diacetate (stock solution in DMSO) (Donor, D) for 2 min at 37 °C or 4 °C in ACAS medium. After being washed, the cells were irradiated at 488 nm, and emissions at 503-553 nm and 575-625 nm were measured. Diffuse fluorescence was observed in the cytosol, and more markedly in the mitochondria. Fast photobleaching was observed.



***Control Acceptor*** (excitation at 488 nm)

COS 7 cells were incubated with 100 nM of BODIPY derivative (stock solution in DMSO) (Acceptor, A) for 30 min at 37 °C or 4 °C in ACAS medium. After being washed, the cells were irradiated at 488 nm, and emissions at 503-553 nm and 575-625

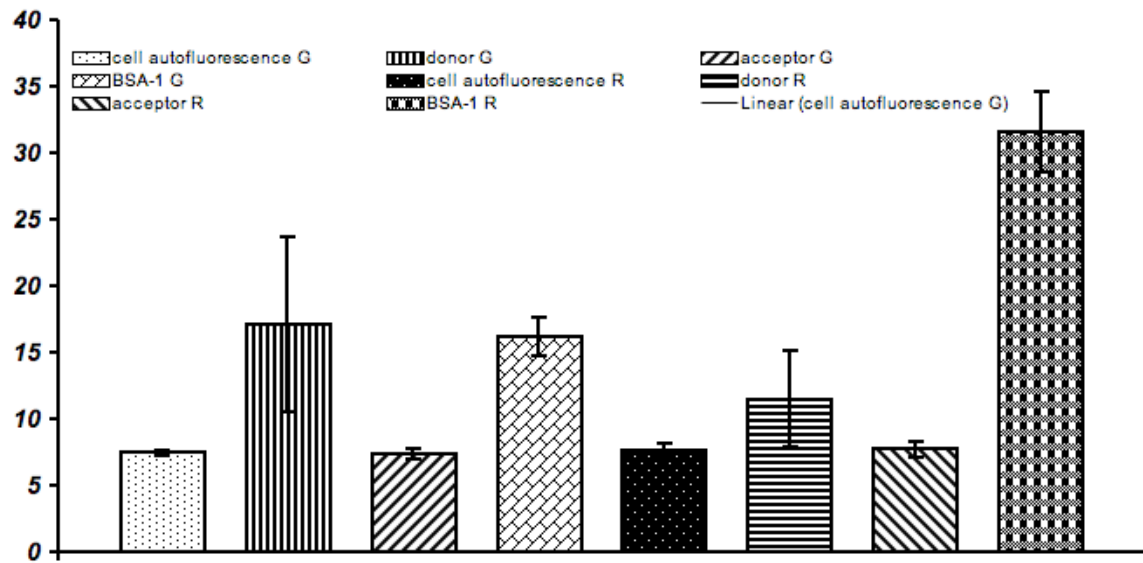
nm were measured. Diffuse fluorescence was observed in the cytosol, and more markedly in the Golgi.



**Summary of Uptake at 37 °C**

G indicates fluorescence intensity detected in the green channel, *i.e.* 503-553 nm.

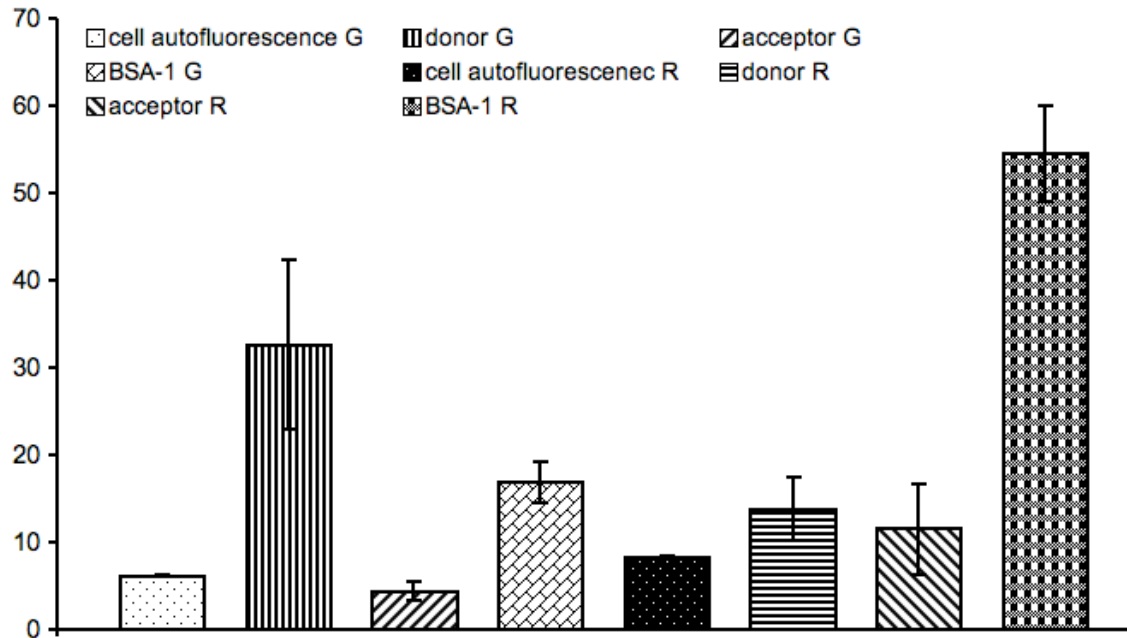
R indicates fluorescence intensity detected in the red channel, *i.e.* 575-625 nm.



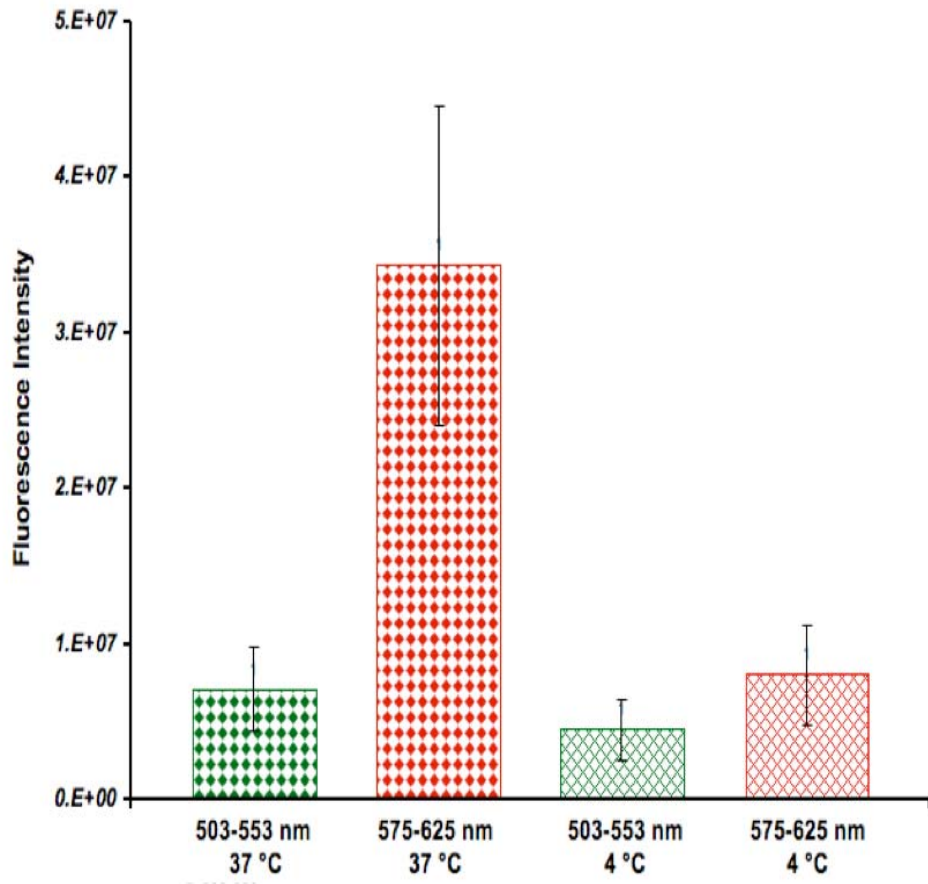
**Summary of Uptake at 4 °C**

G indicates fluorescence intensity detected in the green channel, *i.e.* 503-553 nm.

R indicates fluorescence intensity detected in the red channel, *i.e.* 575-625 nm.



*Summary of Uptake at 37 and 4 °C – After correction for cell autofluorescence and donor bleedthrough*





## 5. Measurement of $\text{pH}_i$ using BSA-1

COS-7 cells (American Type Culture Collection) were cultured as subconfluent monolayers on 75 cm<sup>2</sup> culture flask with vent caps in DMEM supplemented with 10 % fetal bovine serum (FBS) in a humidified incubator at 37 °C with 5 % CO<sub>2</sub>. Cells grown to subconfluence were enzymatically dissociated from the surface with trypsin and plated 2-3 d prior to the experiments in Lab-Tek two well chambered coverglass slides (Nunc) in 1 mL DMEM. To measure  $\text{pH}_i$  with **BSA-1**, cells were incubated with the pre-formed **BSA-1:pep-1** complex (1  $\mu\text{M}$  **BSA-1**:20  $\mu\text{M}$  pep-1) for 60 min at 37 or 4 °C. When the experiment was performed at 4 °C, the cells were pre-incubated at 4 °C for 30 min before the addition of the complex. After incubation, the cells were washed and analyzed on a Bio-Rad 2000MP system (Bio-Rad Laboratories, Hercules, CA) equipped with a Nikon T300 inverted microscope with a 60x (NA1.2) water immersion objective lens and an Argon laser tuned to 488 nm wavelength (as described in the section *Fluorescence Microscopy* above).

*Ex vivo* calibration curve was obtained as follows. Briefly, after incubation with **BSA-1**, the cells were washed in ACAS medium of varying pH values (pH was adjusted by adding small amounts of 0.2 N solution of NaOH or 0.1 N solution of HCl). 1  $\mu\text{g}/\text{mL}$  of nigericin (Aldrich) was added to the medium to allow a rapid exchange of K<sup>+</sup> for H<sup>+</sup> which resulted in a rapid equilibration of external and internal pH. The cells were then analyzed by fluorescence microscopy as described above.

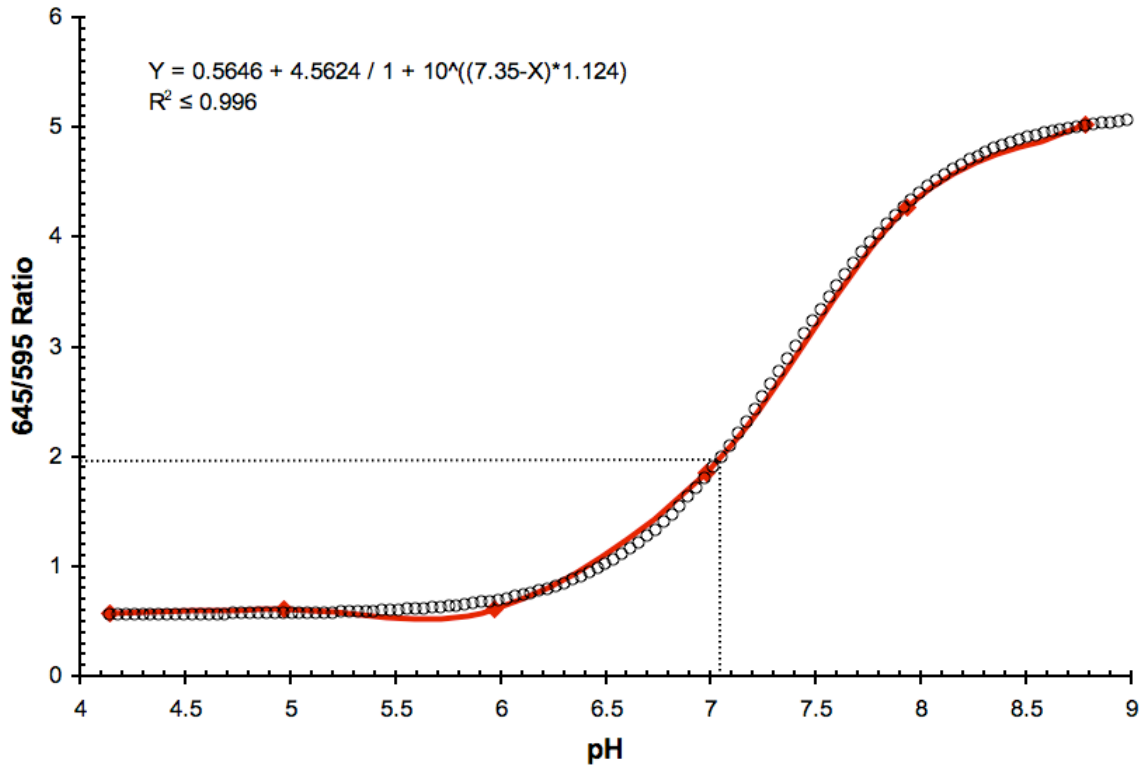
The  $\text{pH}_i$  calibration was fit to a linear regression curve using Excel. The fitted parameters were used to generate an equation that converted ratio channel (red/green) number to  $\text{pH}_i$ .

## 6. Measurement of $\text{pH}_i$ using SNARF-1

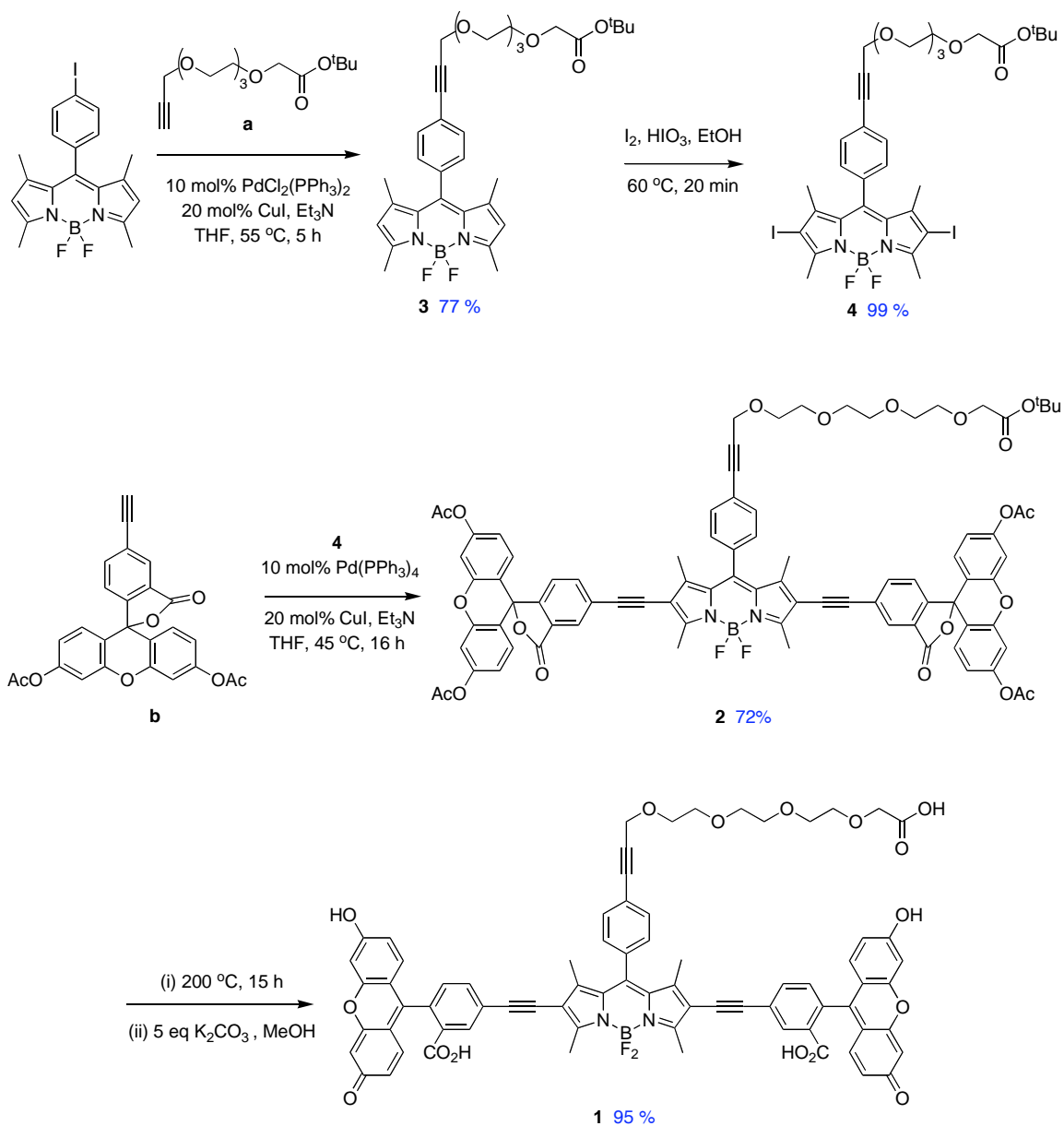
COS-7 cells (American Type Culture Collection) were cultured as subconfluent monolayers on 75 cm<sup>2</sup> culture flask with vent caps in DMEM supplemented with 10 % fetal bovine serum (FBS) in a humidified incubator at 37 °C with 5 % CO<sub>2</sub>. Cells grown to subconfluence were enzymatically dissociated from the surface with trypsin and plated 2-3 d prior to the experiments in a 12 wells tissues culture plate (Falcon) in 1 mL DMEM. To measure  $\text{pH}_i$  with SNARF-1, cells were placed in 1 mL ACAS medium and 10  $\mu\text{M}$  (2  $\mu\text{L}$ ) SNARF-1/AM (Invitrogen) was added from a 5 mM stock solution in DMSO and the sample was incubated for 60 min at 37 °C or 30 min at 4 °C. When the experiment was performed at 4 °C, the cells were pre-incubated at 4 °C for 30 min before the addition of SNARF-1/AM. After incubation, the cells were washed and analyzed on a BioTek Synergy 4 plate reader. Emission spectra were obtained upon excitation at 530 nm.

A calibration curve was generated by staining the cells in high K<sup>+</sup> buffers of varying pH values, and adding 5  $\mu\text{g}/\text{mL}$  nigericin (Aldrich) to equilibrate the intracellular/extracellular pH. High K<sup>+</sup> buffers contained 125 mM KCl, 20 mM NaCl, 0.5 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 25 mM of one of the buffers, including acetate (4.14, 4.97), Mes (5.97), Mops (6.98) and HEPES (7.93).

The  $\text{pH}_i$  calibration was fit to a sigmoid. The fitted parameters were used to generate an equation that converted ratio channel ( $\lambda_{645}/\lambda_{595}$ ) number to  $\text{pH}_i$ .

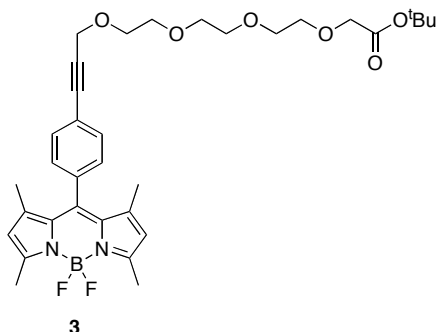


## 7. Synthetic Scheme for Cassette 1

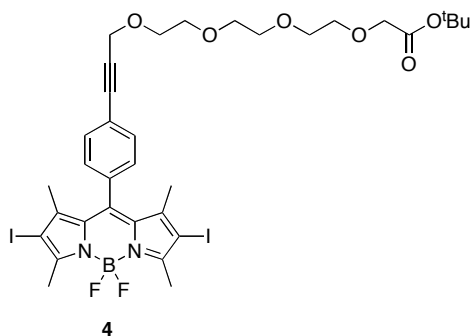


Compounds **a**<sup>4</sup> and **b**<sup>5,6</sup> were synthesized according to literature procedures.

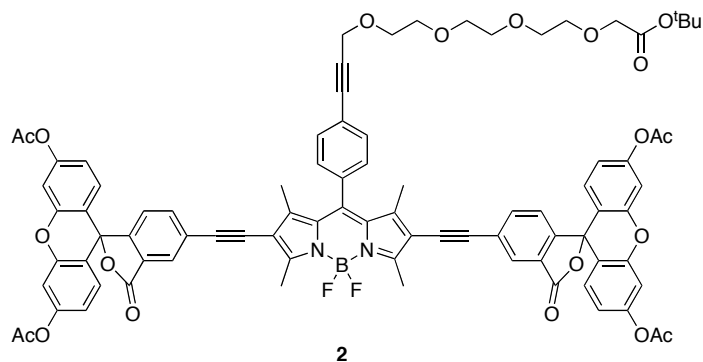
## 8. Syntheses and Characterization of Compounds 1 - 4 and BSA-1



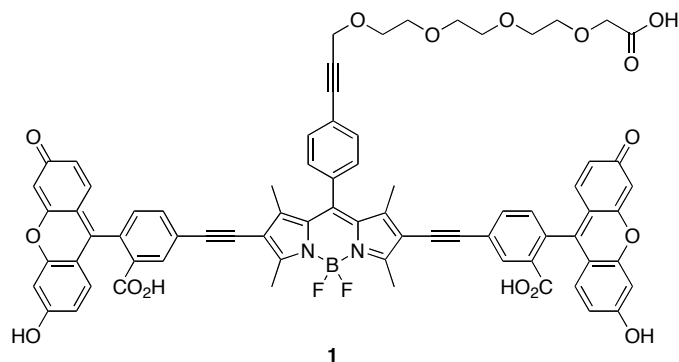
**(3)** Iodophenyl BODIPY<sup>7</sup> (142 mg, 0.316 mmol), **a** (100 mg, 0.331 mmol), PdCl<sub>2</sub>(PPh<sub>3</sub>)<sub>2</sub> (24 mg, 0.032 mmol, 10 mol %), CuI (12 mg, 0.064 mmol, 20 mol %), Et<sub>3</sub>N (0.44 ml, 3.16 mmol) and 5 ml THF were added into a 50 mL round bottom flask. The solvent was degassed three times to remove oxygen, and then the reaction was kept at 55 °C for 5 h. The reaction solvent was removed under reduced pressure. The crude product was purified by flash column chromatography eluting with 30 % hexane/ethyl acetate to give the desired product as an orange solid (151 mg, 77 %). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>), δ7.53 (d, *J* = 8.1, 2H), 7.20 (d, *J* = 8.1 Hz, 2H), 5.95 (s, 2H), 4.42 (s, 2H), 3.98 (s, 2H), 3.72-3.78(m, 2H), 3.63-3.69(m, 10H), 2.51(s, 6H), 1.45(s, 9H), 1.36(s, 6 H) <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>), δ169.5, 155.6, 142.8, 140.6, 135.0, 132.3, 131.0, 128.0, 123.4, 121.2, 86.5, 85.4, 81.4, 70.6, 70.5, 70.4, 70.3, 69.2, 68.9, 59.0, 27.9, 14.4. MS (ESI) calcd for C<sub>34</sub>H<sub>43</sub>BF<sub>2</sub>N<sub>2</sub>O<sub>6</sub> (M+H)<sup>+</sup>, 624.32, found 624.13. TLC (1:1 EtOAc/Hexane), R<sub>f</sub> = 0.42.



**(4)** A mixture of **3** (104 mg, 0.165 mmol), I<sub>2</sub> (100 mg, 0.412 mmol), HIO<sub>3</sub> (58 mg, 0.33 mol) and 10 mL EtOH in a 50 mL flask were warmed up to 60 °C for 20 min, and then it was cooled to room temperature. The reaction was quenched by addition of Na<sub>2</sub>SO<sub>3</sub> (2 mL 1M). Water (20 mL) was added to the reaction mixture, and the product was extracted from water with CH<sub>2</sub>Cl<sub>2</sub> (25 mL x 3). The combined organics were concentrated under reduced pressure, and the resulting crude product was purified by flash chromatography eluting with hexane and ethyl acetate (1:1) to give **4** (145 mg, 99%) as a red solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>), δ 7.60 (d, *J* = 8.4 Hz, 2H), 7.21 (d, *J* = 8.4 Hz, 2H), 4.47 (s, 2H), 4.02 (s, 2H), 3.78-3.81 (m, 2H), 3.70-3.75 (m, 10H), 2.64 (s, 6H), 1.47 (s, 9H), 1.40 (s, 6 H). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>), δ 169.9, 157.3, 145.4, 140.6, 135.0, 133.0, 131.3, 128.2, 124.4, 87.3, 86.1, 85.5, 81.8, 71.0, 70.9, 70.9, 70.8, 70.7, 69.7, 69.3, 59.4, 28.4, 17.4, 14.4. MS (MALDI) calcd for C<sub>34</sub>H<sub>41</sub>BF<sub>2</sub>N<sub>2</sub>NaO<sub>6</sub><sup>+</sup> (M+Na)<sup>+</sup>, 899.10, found 898.91. TLC (1:1 EtOAc/Hexane), *R<sub>f</sub>* = 0.45.

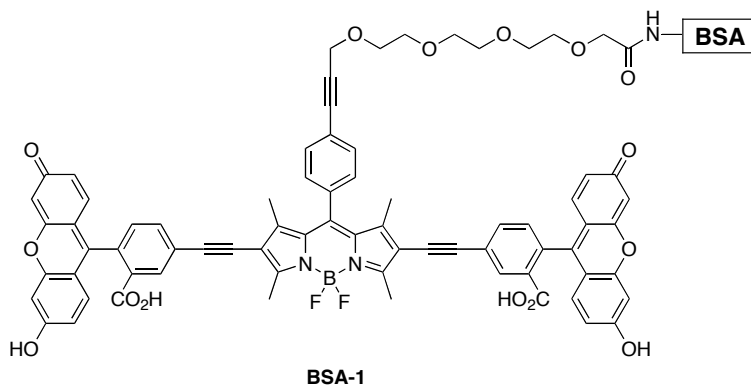


**(2)** A mixture of **4** (65 mg, 0.074 mmol), diacetylfluoresceinalkyne **b**, (82 mg, 0.186mmol), Et<sub>3</sub>N (0.11 mL, 0.74 mmol), Pd(PPh<sub>3</sub>)<sub>4</sub> (8 mg, 0.007 mmol), CuI (3 mg, 0.014 mmol) were dissolved in THF (2 mL). After the solution was degassed three times via the freeze-thawed method, the mixture was heated up to 45 °C for 16 h. The reaction solvent was removed under reduced pressure and the crude product was purified by flash column eluting with 50% hexane:ethyl acetate to give the desired product as a light yellow solid (80 mg, 72%). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>), δ 8.08 (m, 2H), 7.73 (dd, J = 8.0, 1.5 Hz, 2H), 7.65(d, J = 8.0 Hz, 2H), 7.29 (d, J = 8.5 Hz, 2H), 7.15 (d, J = 8.2 Hz, 2H), 7.10 (d, J = 2.0 Hz, 4H), 6.83 (bs, 4H), 6.83 (d, J = 2.0 Hz, 4H), 4.48 (s, 2H), 4.02 (s, 2H), 3.80-3.82 (m, 2H), 3.70-3.75 (m, 10H), 2.75 (s, 6H), 2.32 (s, 12H), 1.58 (s, 6H), 1.47(s, 9H). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>), δ 169.6, 168.8, 168.2, 159.1, 152.1, 151.8, 151.5, 144.6, 142.1, 137.9, 134.1, 132.8, 131.1, 128.9, 127.9, 127.7, 126.6, 125.8, 124.3, 124.2, 117.8, 116.0, 115.6, 110.5, 94.7, 87.2, 85.3, 84.1, 81.8, 81.5, 70.7, 70.6, 70.6, 70.5, 69.5, 69.0, 59.2, 28.1, 21.1, 13.8, 13.7 MALDI MS calcd for C<sub>86</sub>H<sub>71</sub>BF<sub>2</sub>N<sub>2</sub>NaO<sub>20</sub><sup>+</sup> (M+Na)<sup>+</sup> 1523.46, found 1523.26. TLC (1:1 EtOAc/Hexane), R<sub>f</sub> = 0.20.



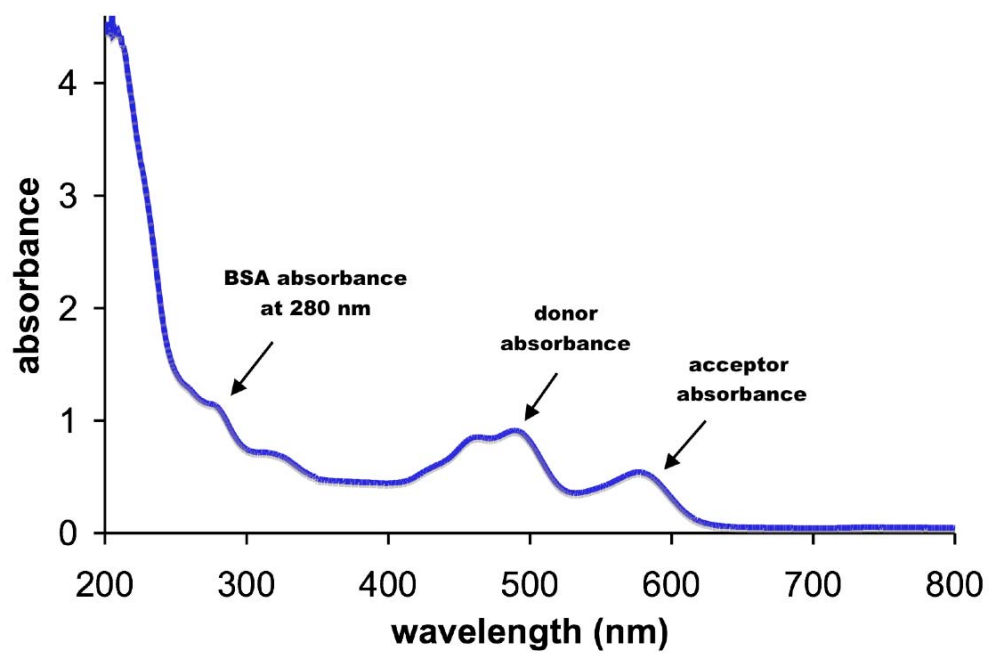
(1) Compound **2** (6.1 mg, 0.004 mmol) in 5 mL ground bottom flask was heat up to 200 °C in a sand bath for 15 h. Then the crude product was dissolved in 1 mL MeOH, followed by addition of K<sub>2</sub>CO<sub>3</sub> (3 mg, 0.022 mmol). The mixture was stirred at room temperature for overnight. Then 10 mL water was added to the mixture and the non-water-soluble impurity was extracted out of the aqueous solution with CH<sub>2</sub>Cl<sub>2</sub> (5 mL x 2). The aqueous solution was carefully neutralized with 0.1M HCl and the product was extracted out of the water with 75% CHCl<sub>3</sub>/iPrOH (10 mL x 3) to afford a dark red solid (5 mg, 95 %) after removing the solvent under reduced pressure. <sup>1</sup>H NMR (500 MHz, 75% CD<sub>3</sub>OD/CDCl<sub>3</sub>), δ 8.06 (s, 2H), 7.78 (dd, *J* = 8.5 Hz, 1.3 Hz, 2H), 7.68 (d, *J* = 8.5Hz, 2H), 7.36 (d, *J* = 8.5 Hz, 2H), 7.19 (d, *J* = 8.5 Hz, 2H), 6.74 (s, 4H), 6.67 (d, *J* = 7.5 Hz, 4H), 6.58 (d, *J* = 7.5 Hz, 4H), 4.49 (s, 2H), 4.13 (s, 2H), 3.81-3.83(m, 2H), 3.69-3.75 (m, 10H), 2.74 (s, 6H), 1.61 (s, 6H). <sup>13</sup>C NMR (125 MHz, 75% CD<sub>3</sub>OD/CDCl<sub>3</sub>), δ 180.8, 173.4, 169.8, 159.6, 154.1, 154.0, 153.9, 145.3, 143.0, 138.1, 134.8, 133.4, 131.8, 129.9, 128.7, 128.5, 128.4, 126.1, 125.5, 124.8, 116.4, 113.8, 111.0, 103.3, 95.6, 87.4, 86.0, 84.3, 71.2, 71.1, 71.0, 70.9, 70.8, 69.8, 68.8, 59.5, 30.2, 14.0. <sup>19</sup>F NMR (300 MHz, CD<sub>3</sub>OD), 30.6 (q, *J* = 36.0 Hz). MS (MALDI) calcd for C<sub>76</sub>H<sub>55</sub>BF<sub>2</sub>N<sub>2</sub>O<sub>16</sub><sup>-</sup> (M-H)<sup>-</sup> 1275.35, found 1275.31.



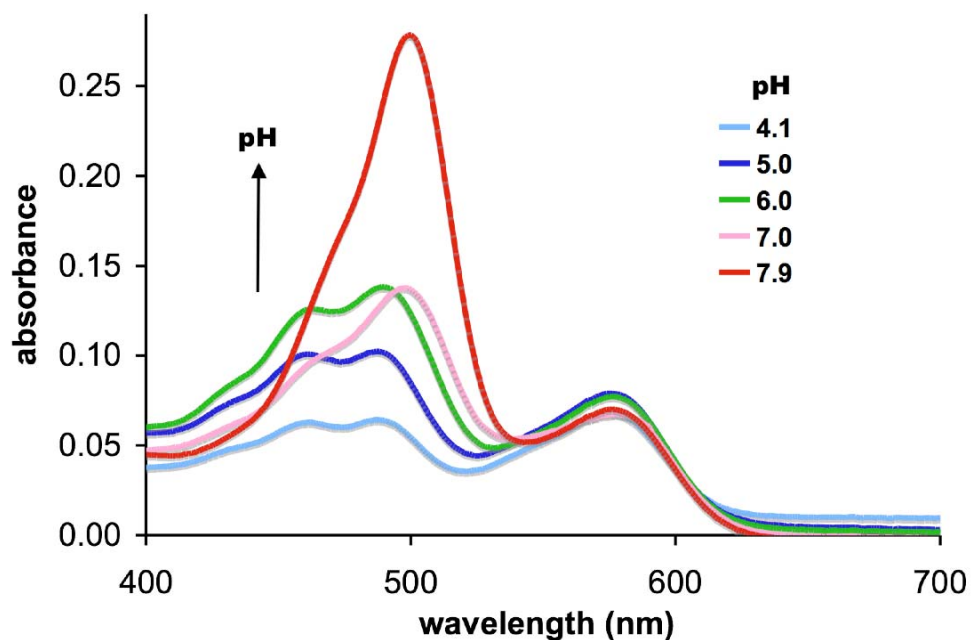


**BSA-1.** Cassette **1** (1.5 mg) was dissolved in dry DMF (0.15 mL) and *N*-hydroxysuccinimide (0.4 mg), diisopropyl carbodiimide (DIC, 0.7  $\mu$ L) were added. The reaction mixture was stirred at room temperature for 24 h.

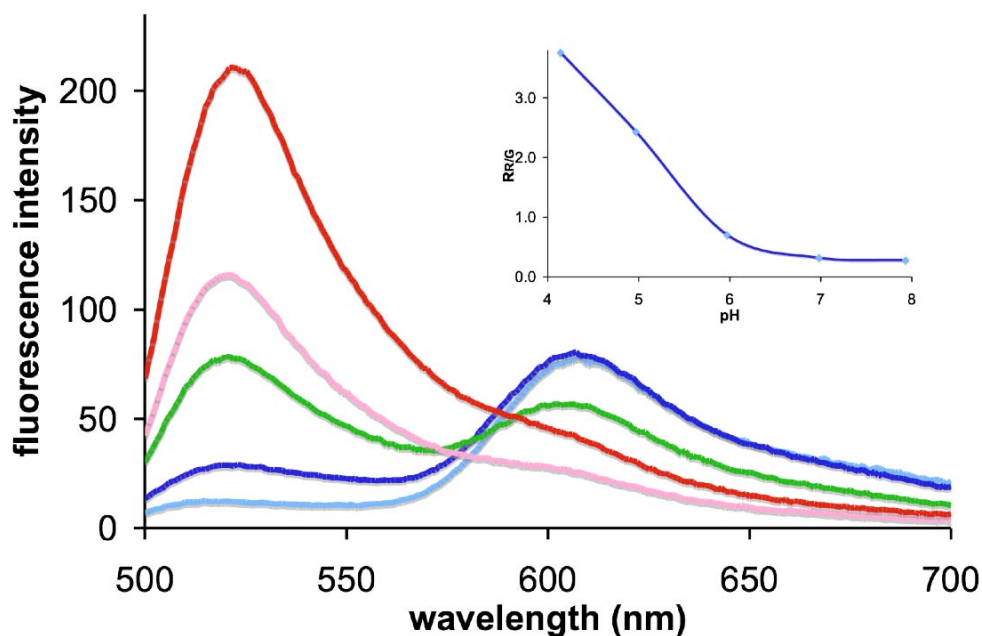
The activated cassette **1** solution (100  $\mu$ L, 6 eq.) was added to the solution of bovine serum albumin (13 mg, 1 eq.) in 1.5 mL freshly prepared sodium bicarbonate (0.1 M, pH 8.3). The solution was stirred at room temperature in the dark for 1 h. The desired product was purified by Sephadex<sup>TM</sup> G-25 (PD-10) desalting column eluting with DI-water. The UV-vis spectra show the absorbance peak of avidin at 280 nm and the two maximum absorbance peaks of the cassettes **1** at 490 nm and 570 nm (data are shown here).



*Figure S1* UV absorbance spectra of **BSA-1** conjugate in DI water.



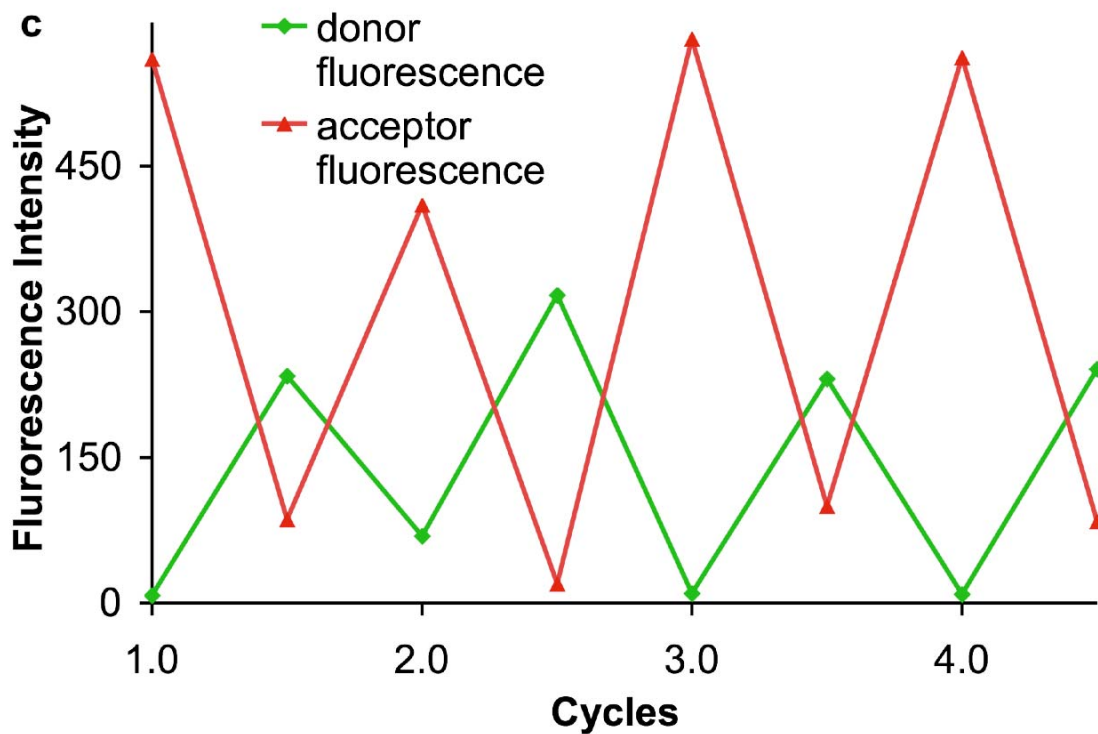
**Figure S2** Absorbance spectra of **BSA-1** ( $0.75 \times 10^{-6}$  M) in aqueous solutions containing 125 mM KCl, 20 mM NaCl, 0.5 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 25 mM of one of the buffers, including acetate (4.1, 5.0), Mes (6.0), Mops (7.0) and HEPES (7.9).



**Figure S3** Fluorescence spectra of **BSA-1** ( $0.75 \times 10^{-6}$  M) in aqueous solutions containing 125 mM KCl, 20 mM NaCl, 0.5 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 25 mM of one of the buffers, including acetate (4.1, 5.0), Mes (6.0), Mops (7.0) and HEPES (7.9).

Inset: Ratio of fluorescence integral for the red channel (575-625 nm) relative to the green channel (503–553 nm) at different pH values.

## 9. Fluorescence responses of **1** to acid/base cycles



**Figure S4** Fluorescence responses of **1** ( $8.0 \times 10^{-7}$  M) to acid/base cycles in 1:1 water-ethanol solutions. The pH values used were 3.8 to 7.2, 6.0 to 8.0, 3.5 to 7.5, and 3.4 to 7.6, corresponding to cycles 1 to 4 respectively. The fluorescence intensities of the donor at 525 nm and acceptor at 600 nm were monitored (excitation at 488 and 565 nm respectively).

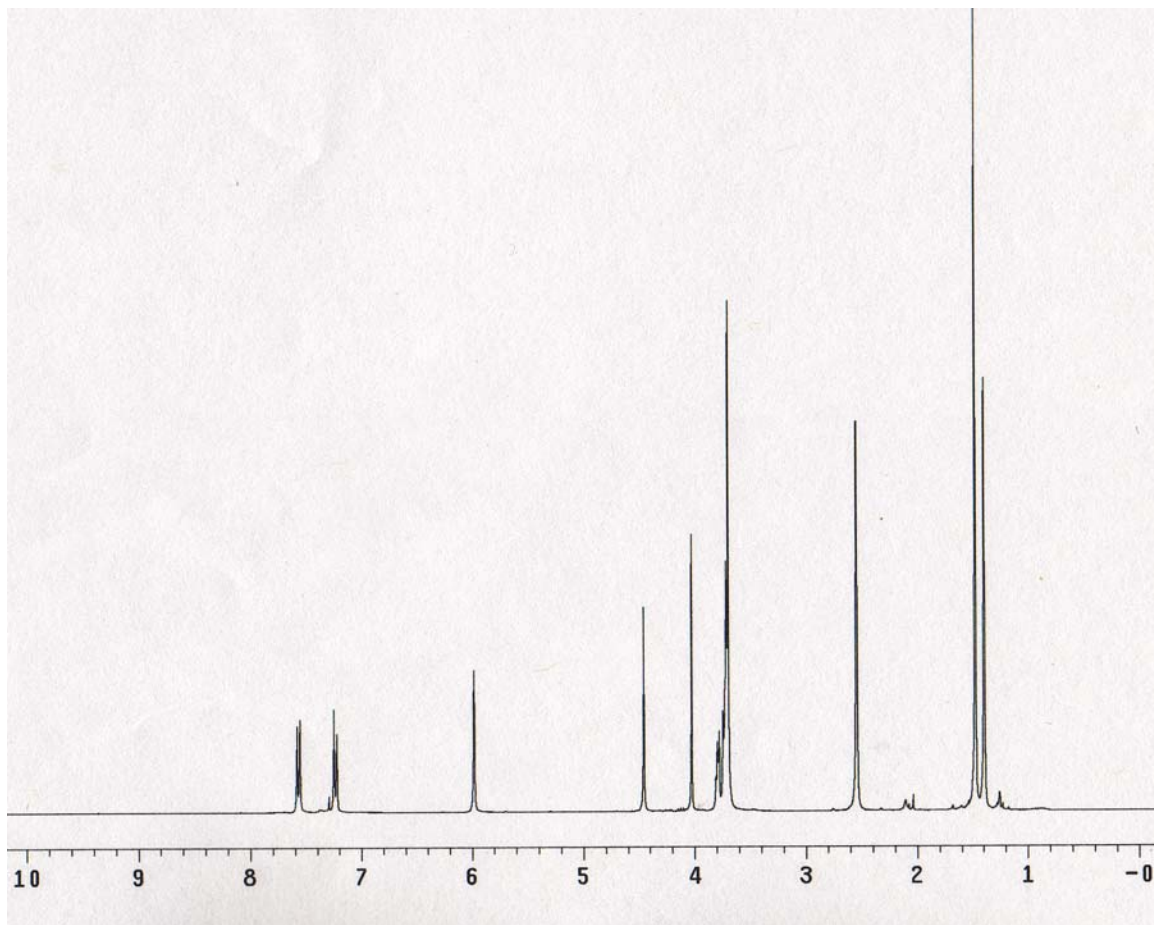
## 10. Fluorescence Picture of **1** in Different pH Aqueous Solutions



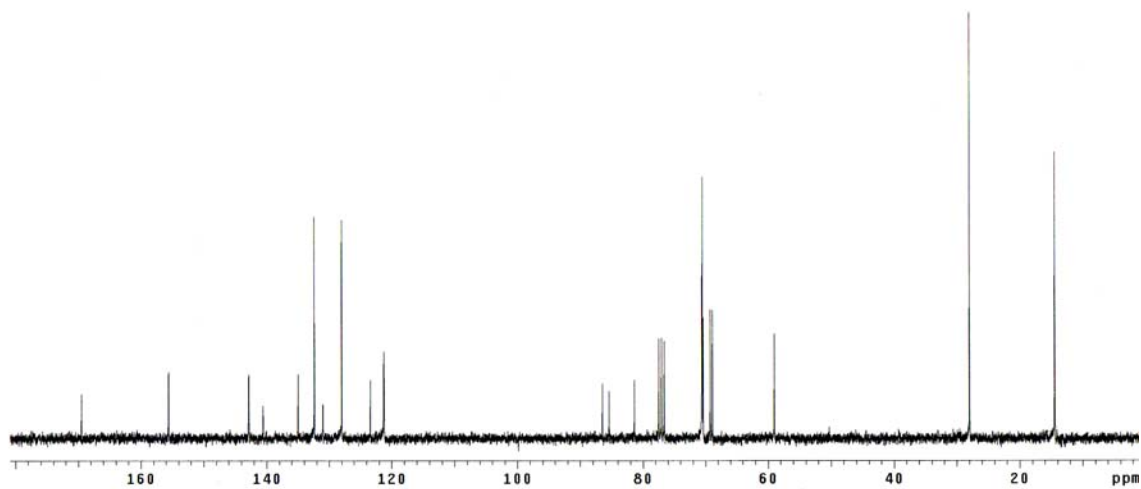
**Figure S5** Photograph of **1** in basic (a), neutral (b) and acidic (c) 50:50 water and ethanol solution under UV light radiation.

## 11. $^1\text{H}$ , $^{13}\text{C}$ and $^{19}\text{F}$ NMR of Compounds 1-4

$^1\text{H}$  NMR of compound **3** ( $\text{CDCl}_3$ , 300 MHz)

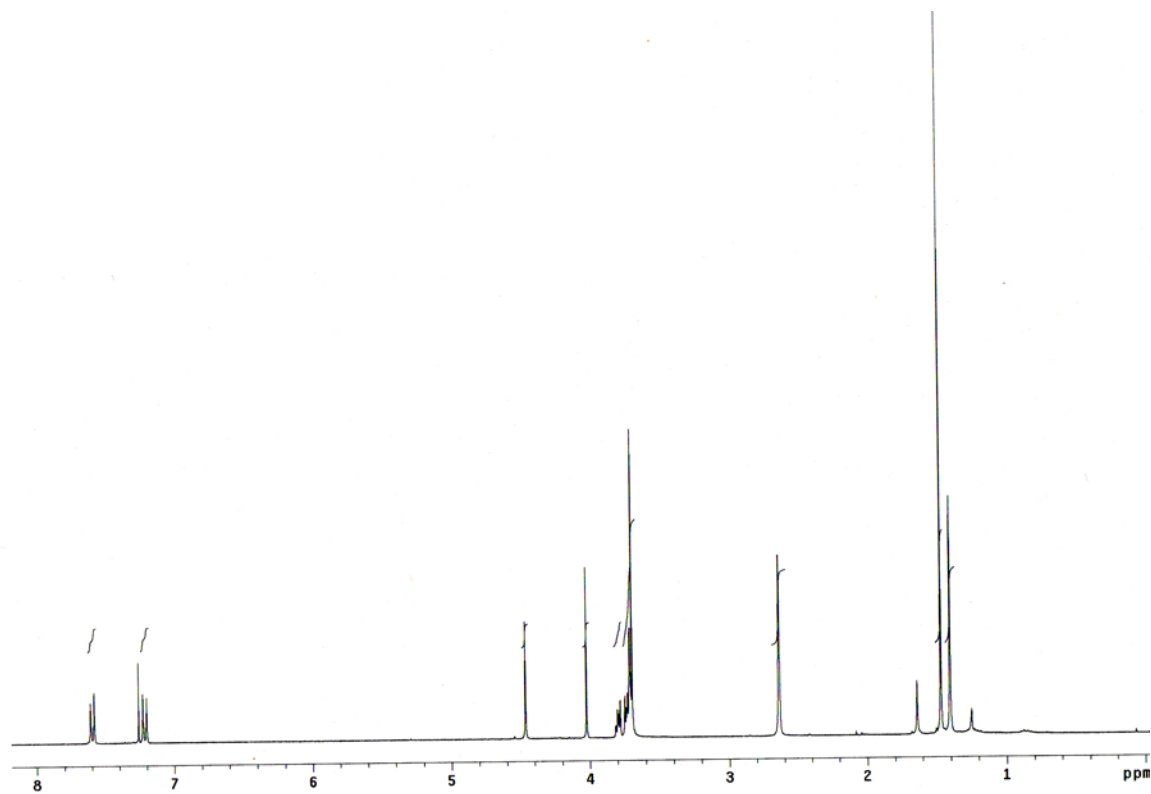


$^{13}\text{C}$  NMR of compound **3** ( $\text{CDCl}_3$ , 125 MHz)

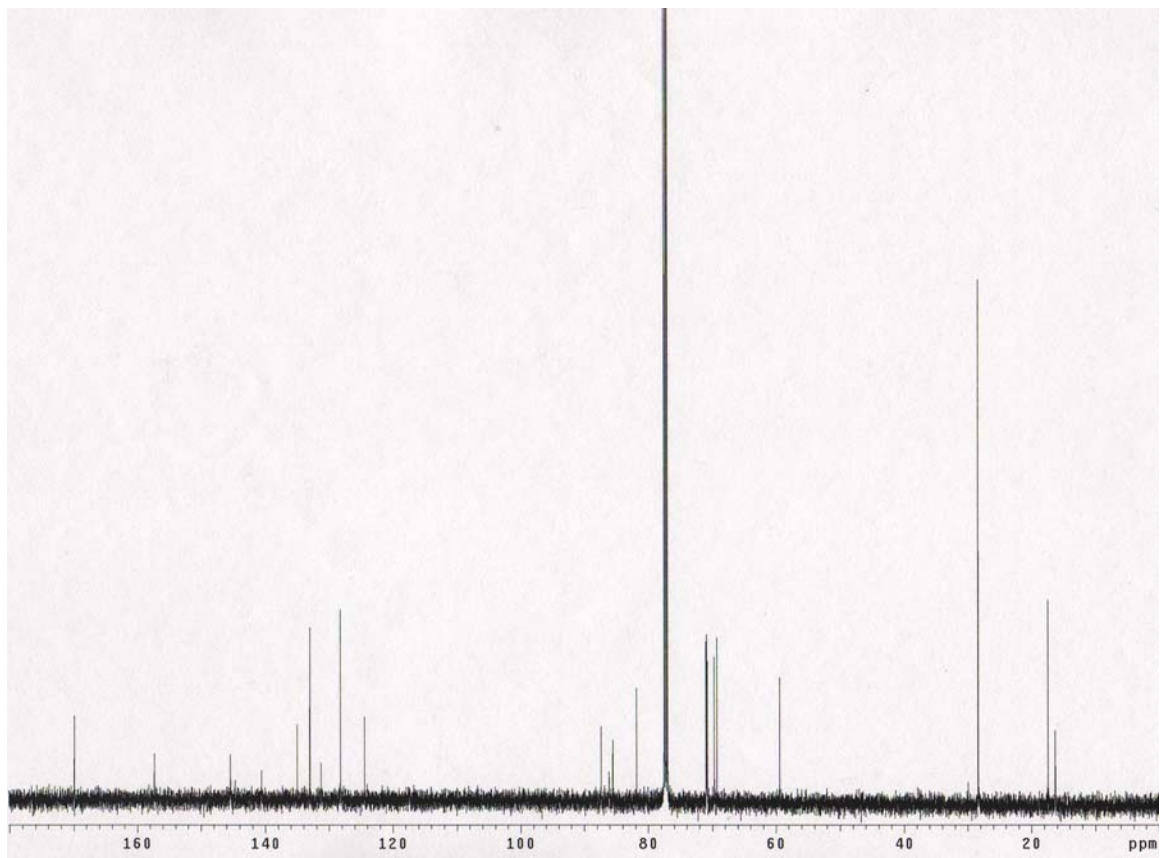




$^1\text{H}$  NMR of compound **4** ( $\text{CDCl}_3$ , 300 MHz)

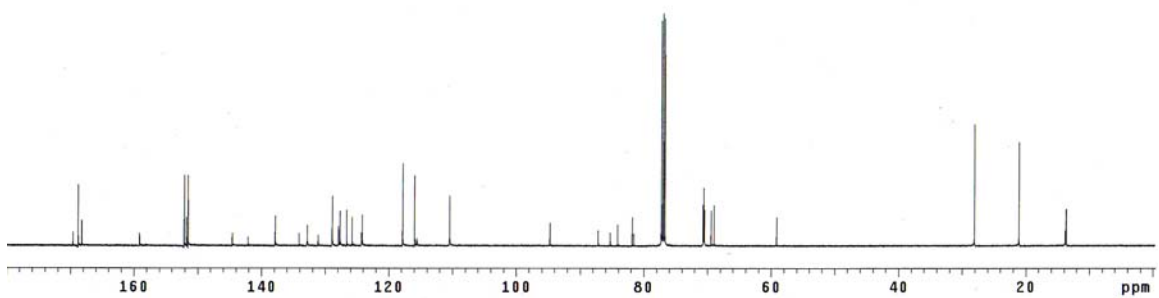


$^{13}\text{C}$  NMR of compound **4** ( $\text{CDCl}_3$ , 125 MHz)

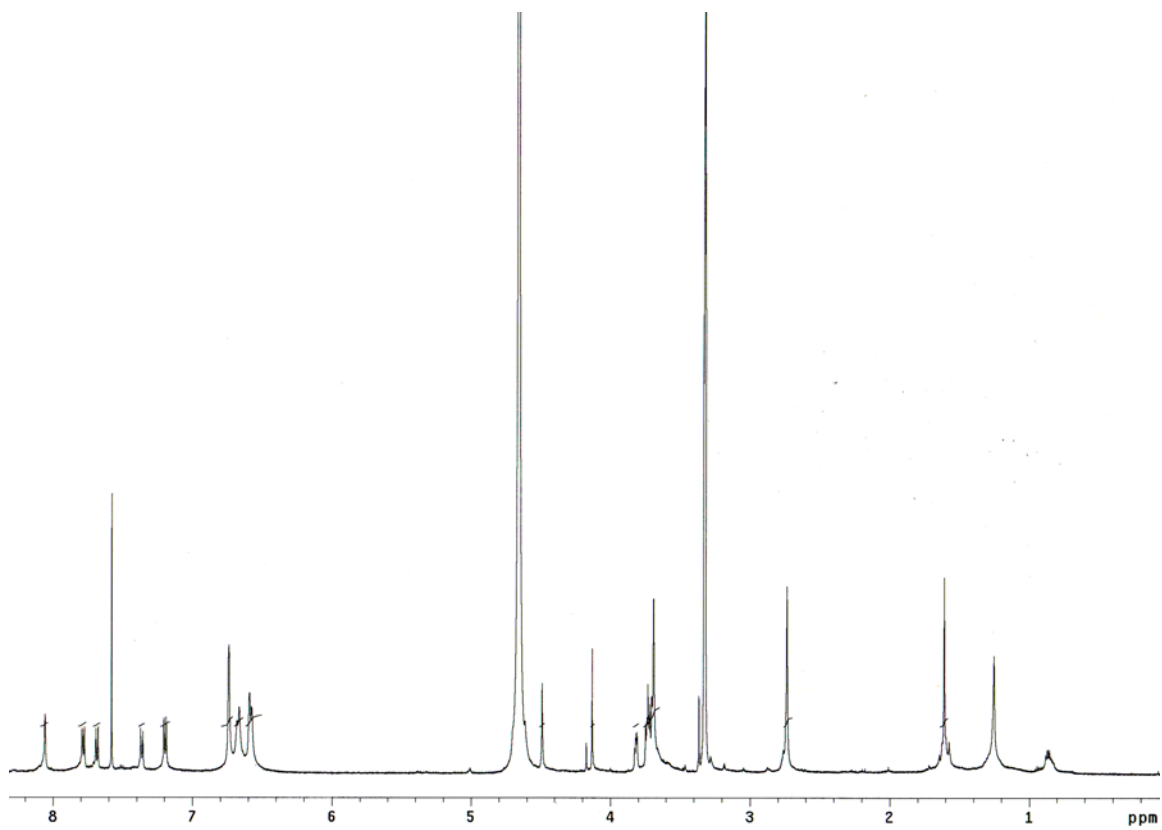


$^1\text{H}$  NMR of compound **2** ( $\text{CDCl}_3$ , 500 MHz)

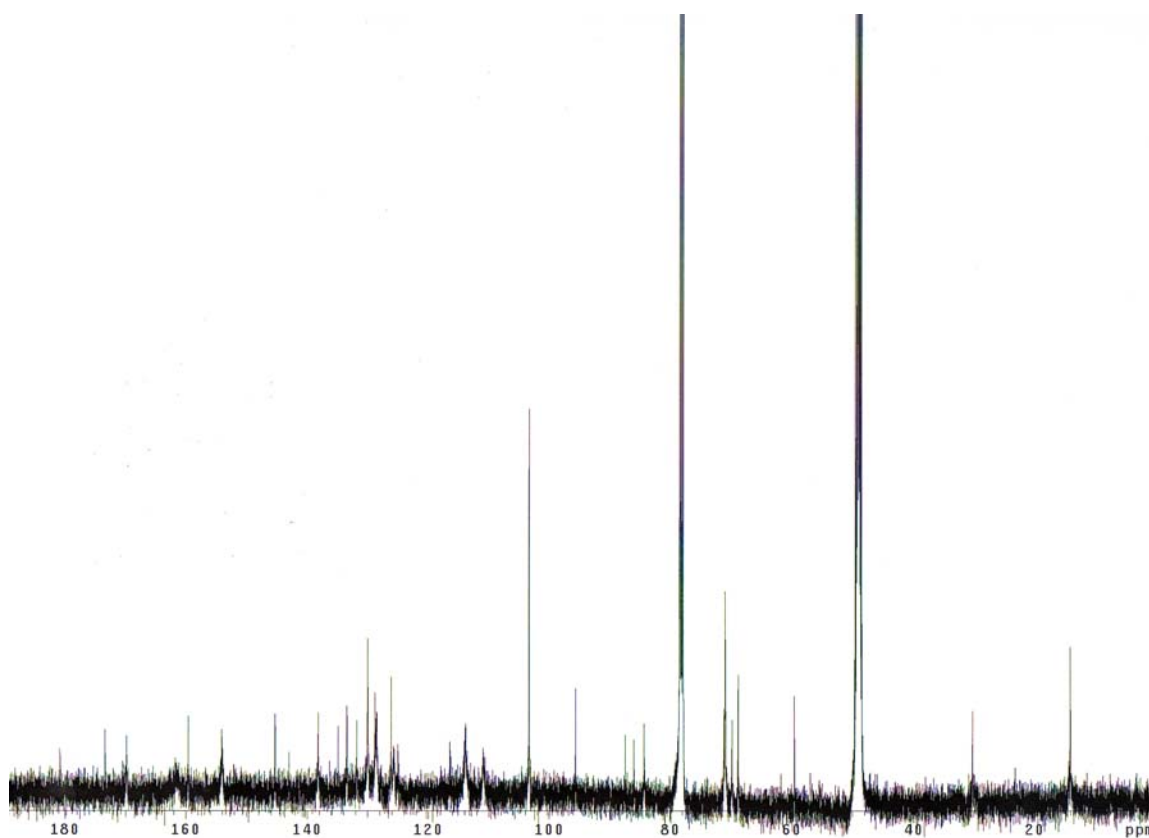
$^{13}\text{C}$  NMR of compound **2** ( $\text{CDCl}_3$ , 125 MHz)



$^1\text{H}$  NMR of compound **1** (3:1  $\text{CD}_3\text{OD}/\text{CDCl}_3$ , 500 MHz)



$^{13}\text{C}$  NMR of compound **1** (3:1  $\text{CD}_3\text{OD}/\text{CDCl}_3$ , 125 MHz)



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