Supplementary Information

for

A Nile Red/BODIPY-based bimodal probe sensitive to changes in micropolarity and microviscosity of endoplasmic reticulum†

Zhigang Yang,[a] Yanxia He,[a] Jae Hong Lee,[a] Weon-Sik Chae,[b] Wen Xiu Ren,[a] Joung Hae Lee,[c] Chulhun Kang,*[,d] and Jong Seung Kim*[,a]

[a]Department of Chemistry, Korea University, Seoul 136-701, Korea
[b]Gangneung Center, Korea Basic Science Institute, Gangneung 210-702, Korea
[c]Korea Research Institute of Standards and Science, Daejon 305-600, Korea
[d]The School of East-West Medical Science, Kyung Hee University, Yongin 446-701, Korea

Corresponding E-mail: jongskim@korea.ac.kr (JSK); kangch@khu.ac.kr (CK)

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General Methods:

Materials and reagents. All solvents and reagents used were reagent grade. Silica (230-400 mesh; Merck, Darmstadt, Germany) was used for flash column chromatography for purifications. Water used in all experiments was doubly purified by Milli-Q Systems equipment. All chemical reagents were purchased from Aldrich-Sigma, TCI, or Acros Company.

Sample Preparation. All solvents and reagents used were reagent grade. All reactions were carried out under argon atmosphere with dry, freshly distilled solvents under anhydrous conditions. Silica gel (100-200 mesh) was used for flash column chromatography for purifications. Water used in all experiments was doubly purified by Milli-Q Systems equipment. The solutions of compounds were typically prepared from 1.0 mM stock solutions in DMSO.

Viscosity Determination, Fluorescence Spectral Measurement and Fluorescence Lifetime Detection. The solvents were obtained by mixing methanol-glycerol and de-ionized water-glycerol systems in different proportions. Measurements were carried out with a NDJ-7 rotational viscometer at room temperature (25 ^{o}C), and each viscosity value was recorded. The solutions of **1** with different viscosity/ polarity were prepared by adding the stock solution (1.0 mM) to 10 mL of solvent mixture (methanol-glycerol or 1, 4-dioxane-water systems) to obtain the final concentration of the sensor (1.0 μM). These solutions were sonicated for 5 minutes to eliminate air bubbles. After standing for 1 hour at a constant temperature, the solutions were measured in a UV spectrophotometer and a fluorescence spectrophotometer. A Fluo Time 200 lifetime fluorometer was used to obtain the fluorescence lifetimes of compounds, with the excitation wavelength at 375 nm and detection at 516 nm for BODIPY moiety and 625 nm for Nile Red moiety, respectively.

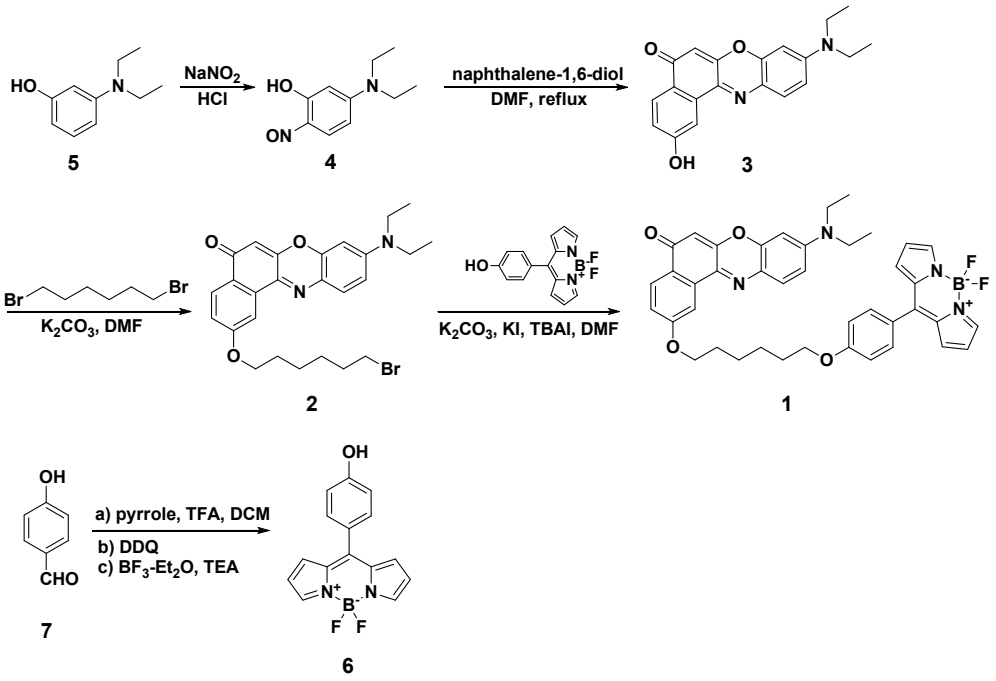
Cell Incubation and Imaging

HeLa cells were cultured in DEME (Invitrogen) supplemented with 10% FCS (Invitrogen). One day before imaging, cells were seeded into 24-well flat-bottomed plates. The next day, the cells were incubated with 1.0 μM dye for 0.5 h at 37 ºC under 5% CO_2 and washed with

phosphate-buffered saline (PBS) three times; or HeLa cells were treated with tunicamycin (10 μg/mL), a drug that can inhibit the glycosylation of nascent proteins, for 24 hours 37 ºC under 5% CO_2 atmosphere. Hela cell lines were incubated with **1**, and observed under a Leica TCS-SP2 confocal fluorescence microscope, 100 × objective lens. Then the fluorescence intensity and fluorescence lifetime of **1** in Hela cells were observed under a Leica TCS-SP2 confocal fluorescence microscope and FLIM equipment.

Experimental details for fluorescence lifetime imaging. Fluorescence lifetime imaging (FLIM) was performed using an inverted-type scanning confocal microscope (MicroTime-200, Picoquant, Germany) with a 60 x objective (NA = 1.2). A single-mode pulsed diode laser (375 nm with a pulse width of ~240 ps in full-width at half maximum and an average power of ~5 μW) was used as an excitation source. A dichroic mirror (Z375RDC, AHF), a long pass filter (HQ405lp, AHF), a 50 μm pinhole, a band-pass filter of 520 ± 5 nm, and an avalanche photodiode detector (PDM series, MPD) were used to collect emission from cells. FLIM measurement was performed multiple times for each sample. Time-resolved fluorescence decay curves were obtained from FLIM images and fluorescence lifetimes were evaluated according to non-linear least-squares iterative curve fitting using the SymPhoTime software (ver. 5.1.3). Two exponential decay model: $I(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$, where *I(t)* is the time-dependent fluorescence intensity, *A* is the amplitude, and τ is the lifetime, was used for the curve fitting. All lifetime values shown in this study mean amplitude-weighted average lifetime, $< \tau_{ave} >$, hereafter, which is defined as $< \tau_{ave} >= \sum_i A_i \tau_i \; / \sum_i A_i$.

Synthesis of Compound 1



Scheme S1. Synthetic route for the target compound **1**

5-(diethylamino)-2-nitrosophenol (4): Sodium nitrite (1.2 g, 18 mM) was dissolved in water (10 mL) and a solution of *N*, *N*-diethyl-3-aminophenol (2.0 g, 12 mM) in aqueous HCl (13 mL, 6 N) was added in several small portions at 0 ^{o}C. After stirring for 3 h at 0 ^{o}C, the resulting precipitate was filtered and dried to yield the product **4** as brown solid (2.0 g, 85%). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.29 (t, 6H, CH_3, J = 8 Hz), 3.51 (q, 4H, CH_2, J = 8 Hz), 5.71 (d, 1H, ArH, J = 4 Hz), 6.55 (m, 1H, ArH), 7.37 (d, 1H, ArH, J = 8 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): 13.47, 46.18, 96.28, 113.61, 135.75, 149.83, 157.31, 169.50.

9-(diethylamino)-2-hydroxy-5H-benzo[a]phenoxazin-5-one (3): To a solution of 5-(diethylamino)-2-nitrosophenol (2.0 g, 10.3 mM) in DMF (40 mL) was added 1,6-dihydroxy naphthalene (1.65 g, 10.3 mM) and the mixture was refluxed for 4 h under nitrogen atmosphere. After evaporating the solvent, the residue was purified by column chromatography (silica gel, Ethyl acetate: CH_2Cl_2 = 1:10, v/v) to yield a dark green colored product **3** (1.93 g, 56%). ^{1}H-NMR (400 MHz, MeOD and $CDCl_3$): 1.24 (t, 6H, CH_3, J = 8 Hz), 3.48 (q, 4H, CH_2, J = 8 Hz), 6.23 (s, 1H, ArH), 6.49 (d, 1H, ArH, J = 4 Hz), 6.72 (m, 1H, ArH), 7.07 (m, 1H, ArH), 7.57 (d, 1H, ArH, J = 8 Hz), 7.96 (d, 1H, ArH, J = 4 Hz), 8.07 (d, 1H, ArH, J = 8 Hz). ^{13}C-NMR (100 MHz, MeOD and $CDCl_3$): 10.79, 43.71, 94.64, 102.53, 107.47, 109.15, 116.82, 122.91, 123.76, 126.42, 129.93, 133.21, 137.51, 145.71, 150.09, 151.46, 159.61, 182.96.

2-((6-bromohexyl)oxy)-9-(diethylamino)-5H-benzo[a]phenoxazin-5-one (2): To a solution of intermediate (**3**) (1.9 g, 5.7 mmol) in dry DMF (5 mL) was added K_2CO_3 (2.3 g, 17.1 mmol) and 1,6-dibromohexane (6.9 g, 28.5 mmol). The solution was heated to 65 °C, and allowed to stir for 18 h under nitrogen atmosphere. Then water (100 mL) was added and extracted with 50 mL of ethyl acetate twice, dried over anhydrous Na_2SO_4, removal of the solvent and purified by silica gel column chromatography (1:1 hexanes: Ethyl acetate), the desired product **2** was obtained as a dark purple solid (2.2 g, 79%). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.18 (t, 6H, CH_3, J = 8 Hz), 1.51 (m, 4H, CH_2, J = 8 Hz), 1.84 (m, 4H, CH_2), 3.33 (m, 4H, CH_2), 3.41 (t, 2H, CH_2, J = 8 Hz), 4.05 (t, 2H, CH_2, J = 8 Hz), 6.14 (s, 1H, ArH), 6.25 (d, 1H, ArH, J = 4 Hz), 6.48 (m, 1H, ArH), 7.03 (m, 1H, ArH), 7.41 (d, 1H, ArH, J = 8 Hz), 7.86 (d, 1H, ArH, J = 4 Hz), 8.09 (d, 1H, ArH, J = 8 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.82, 12.90, 25.51, 28.15, 29.25, 32.88, 34.04, 45.20, 68.26, 96.17, 105.12, 106.50, 109.74, 118.21, 124.76, 125.60, 127.63, 131.05, 134.12, 139.74, 146.78, 150.79, 152.03, 161.77, 183.23.

5,5-difluoro-10-(4-hydroxyphenyl)-5H-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide (6): To 30 mL of dry dichloromethane, 4-hydroxybenzaldehyde (1.0 g, 8.2 mmol) and pyrrole (1.2 g, 18.0 mmol) were added. The solution was purged with argon and a drop of trifluoroacetic acid (TFA) was added. The reaction mixture was stirred for 4 h under argon at room temperature until TLC-control showed the complete consumption of 4-hydroxybenzaldehyde. At this point, a solution of 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 1.1 g, 8.2 mmol) in 20 mL absolute CH_2Cl_2 was added, stirring was continued for 1 h followed by the addition of 5 mL of Et_3N and 5 mL of BF_3-Et_2O respectively. After stirring for 2 h the reaction mixture was washed with water, dried over Na_2SO_4 and evaporated to dryness. The residue was purified with column chromatography (silica gel; Hexane/Ethyl acetate, 1:2, v/v) to give product **6** (0.23 g, 10 %) as a dark red solid. ^{1}H-NMR (400 MHz, $CDCl_3$): 6.55 (m, 2H, ArH), 6.97 (m, 4H, ArH), 7.45 (d, 2H, ArH, J = 8 Hz), 7.90 (s, 2H, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$): 115.88, 118.53, 126.18, 131.78, 132.93, 135.00, 143.39, 147.86, 159.18.

10-(4-((6-((9-(diethylamino)-5-oxo-5H-benzo[a]phenoxazin-2-yl)oxy)hexyl)oxy)phenyl)-5,5-difluoro-5H-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-4-ium-5-uide (1): To a solution of compound **2** (100 mg, 0.2 mmol) and **6** (57 mg, 0.2 mmol) in dry DMF (3 mL) was added K_2CO_3 (83 mg, 0.6 mmol), KI (33 mg, 0.2 mmol) and tetrabutylammonium iodide (TBAI, 15 mg, 0.04 mmol). The solution was heated to 50 °C, and allowed to stir for 3 days under nitrogen atmosphere. Then water (20 mL) was added and extracted with 30 mL of ethyl acetate twice, drying with Na_2SO_4, removal of the solvent and purified by silica gel column chromatography (100:3 CH_2Cl_2: Ethyl acetate), then evaporated the solvent to yield a dark red product **1** (28 mg, 20%). ^{1}H-NMR (400 MHz, $CDCl_3$): 1.25 (m, 6H, CH_3), 1.64 (m, 4H, CH_2), 1.91 (m, 4H, CH_2), 3.46 (q, 4H, CH_2, J = 8 Hz), 4.09

(m, 2H, CH_2), 4.20 (m, 2H, CH_2), 6.30 (s, 1H, ArH), 6.45 (d, 1H, ArH, J = 8 Hz), 6.54 (m, 2H, ArH), 6.63 (m, 1H, ArH), 6.97 (d, 2H, ArH, J = 8 Hz), 7.04 (m, 2H, ArH), 7.17 (m, 1H, ArH), 7.55 (m, 3H, ArH), 7.91 (s, 2H, ArH), 8.05 (d, 1H, ArH, J = 8 Hz), 8.22 (m, 1H, ArH). ^{13}C-NMR (100 MHz, $CDCl_3$): 12.79, 26.06, 26.09, 29.34, 29.90, 45.27, 68.37, 96.61, 105.41, 105.59, 106.95, 109.63, 114.72, 118.32, 124.87, 125.79, 126.34, 127.94, 131.55, 132.58, 132.73, 134.27, 135.01, 140.22, 143.41, 143.56, 147.06, 147.70, 150.94, 152.27, 161.99, 183.48.

Results of solution and biological tests:

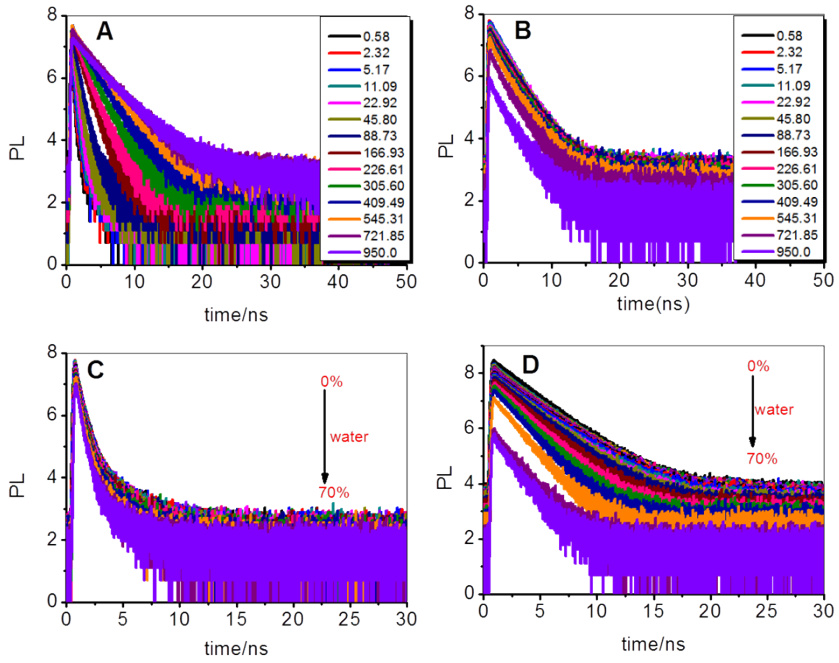


Figure S1. Fluorescence lifetime measurements of sensor **1** (1 μM) in different solvents excited at 375 nm detected at 516 and 635 nm, respectively; fluorescence decay of BODIPY moiety (A) and Nile Red unit (B) with the increase of solvent viscosity (methanol to 99% glycerol); Fluorescence decay of BODIPY moiety (C) and Nile Red unit (D) with the changes of solvent polarities (from 1, 4-dioxane to 70% water)

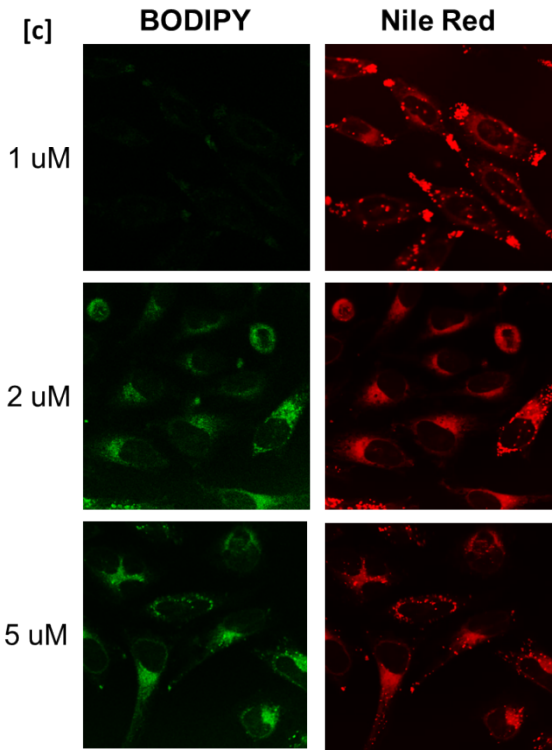


Figure S2. Fluorescence intensity changes of sensor **1** (BODIPY and Nile Red, respectively) in HeLa cells with the changes of concentrations from 1, 2 and 5 μM

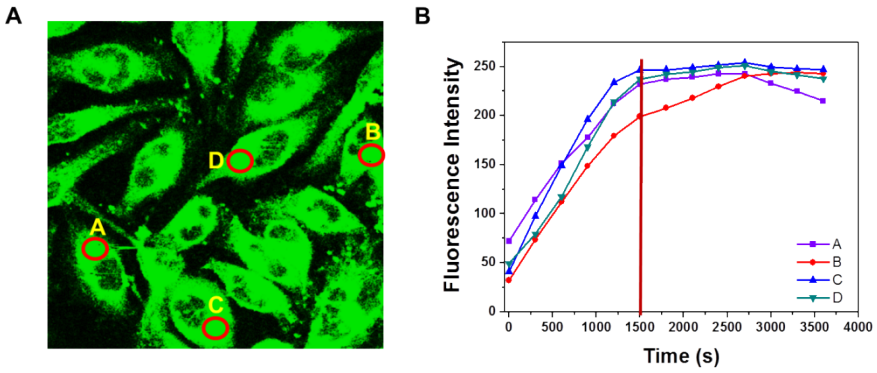


Figure S3 Confocal laser fluorescence microscopic images of HeLa cells incubated with sensor **1** (5 μM) for 1 h at 37 ^{O}C (A); Changes of Relative fluorescence intensity of sensor **1** in different areas with the variations of incubating time with HeLa cells, excited at 458 nm and collected at 480-680 nm

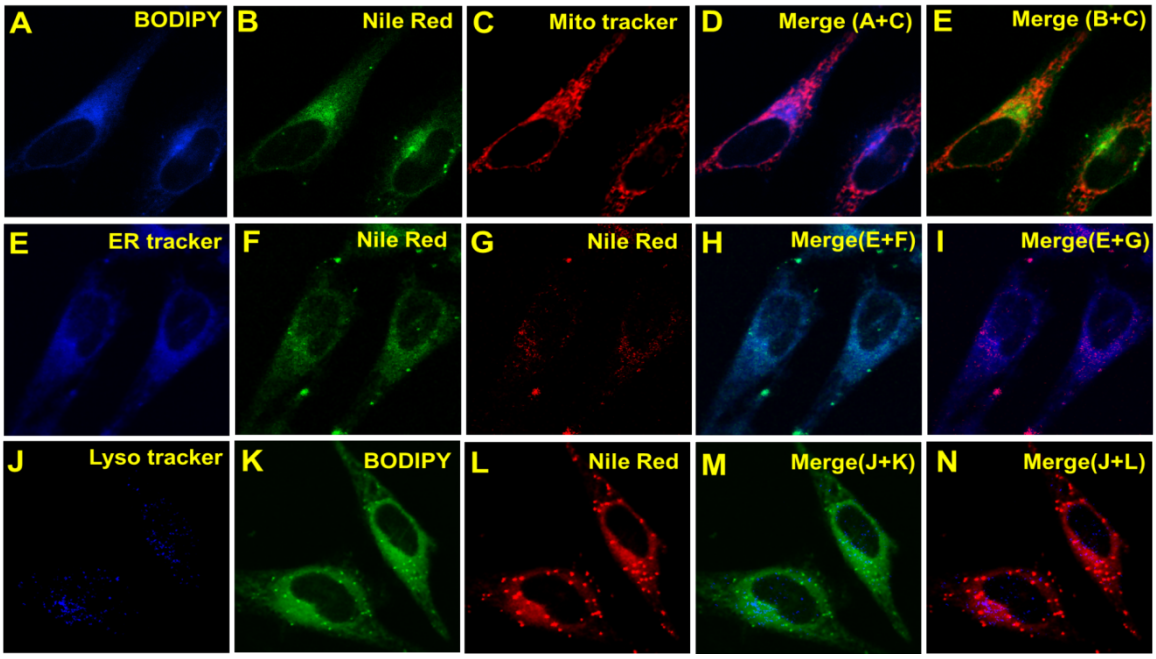


Figure S4. Colocalization experiment of sensor **1** (1μM) in HeLa cells, excited at 488 nm; A, fluorescence imaging of BODIPY (500-530 nm); B Fluorescence of Nile Red moiety (560-590 nm); C mitochondria Tracker; D merge image of A and C; E merge image of B and C; E, ER tracker, F, Nile Red emission I (560-590 nm); G, Nile Red emission II (620-650 nm); H merge image of E and F; I merge image of E and G; J lyso tracker; K, BODIPY fluorescence image (500-530 nm); L, Nile Red emission II (625-650 nm); M merge image of J and K; M merge image of J and L.

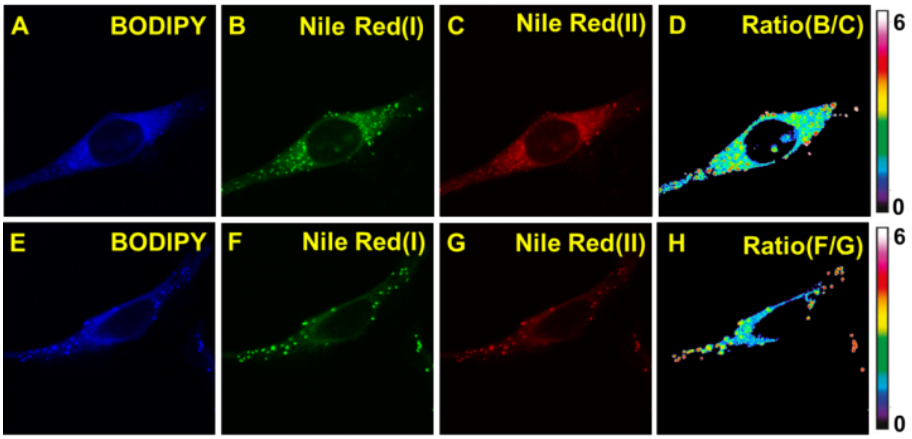


Figure S5. Fluorescence imaging of 1 (5.0 μM) in HeLa cells; (A), fluorescence of the BODIPY moiety (collected at 500–550 nm); (B), fluorescence of the Nile Red moiety (I) (collected at 560–590

nm); (C), fluorescence of Nile Red (II) (collected at 625–650 nm); (D), ratiometric image of Nile Red obtained from panels A and B untreated by tunicamycin; (D), fluorescence of the BODIPY moiety (collected at 500–550 nm) treated by tunicamycin for 24 hours; (E), fluorescence of the Nile Red moiety (I) (collected at 560–590 nm); (F), fluorescence of Nile Red (II) (collected at 625–650 nm); (G), ratiometric image of Nile Red obtained from panels E and F treated by tunicamycin (10 ug/mL) for 24 hours; excited at 488 nm, and fluorescence images were obtained using a Leica TCS SP2 confocal laser microscope.

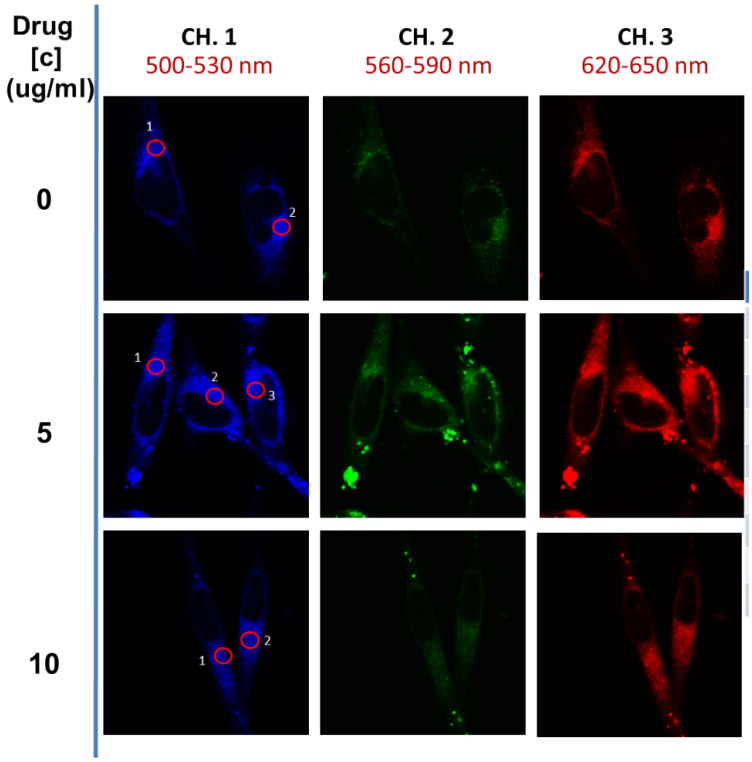


Figure S6. Fluorescence imaging of Sensor **1** (BODIPY and Nile Red, respectively) upon treated with different concentrations of tunicamycin (0, 5 and 10 μg/mL)

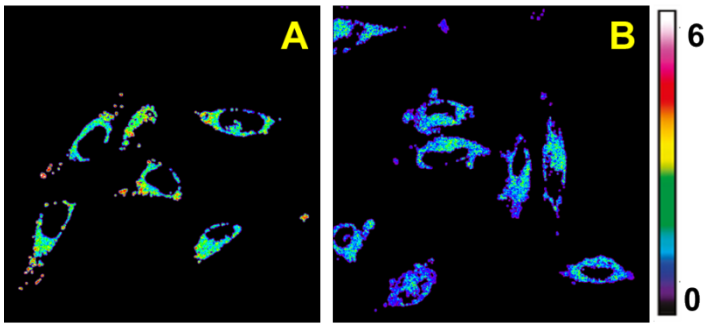


Figure S7 Fluorescence ratiometric imaging of **1** (5.0 μM) in HeLa cells; (A) ratiometric image of Nile Red moiety untreated by tunicamycin; (D), ratimetric image of Nile Red in HeLa cells treated by tunicamycin (5 ug/mL) for 24 hours; excited at 488 nm, and fluorescence images were obtained using a Leica TCS SP2 confocal laser microscope.

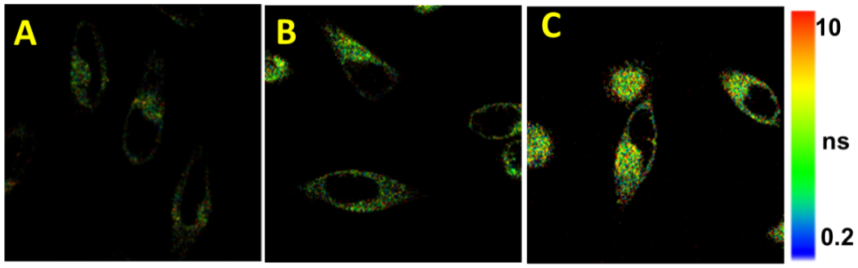


Figure S8. Fluorescence lifetime imaging (FLIM) of the BODIPY moiety in **1** (5.0 μM) in HeLa cells; (A), FLIM in HeLa cells untreated by tunicamycin; (B) FLIM in HeLa cells treated with tunicamycin (5 μg/mL) for 24 hours before incubation with **1**; (C) FLIM in HeLa cells treated with tunicamycin (10 μg/mL) for 24 hours before incubation with **1**; excited at 375 nm and emission was detected at 500–530 nm using an inverted-type scanning confocal microscope (MicroTime 200, PicoQuant, Germany) with a 60× objective.

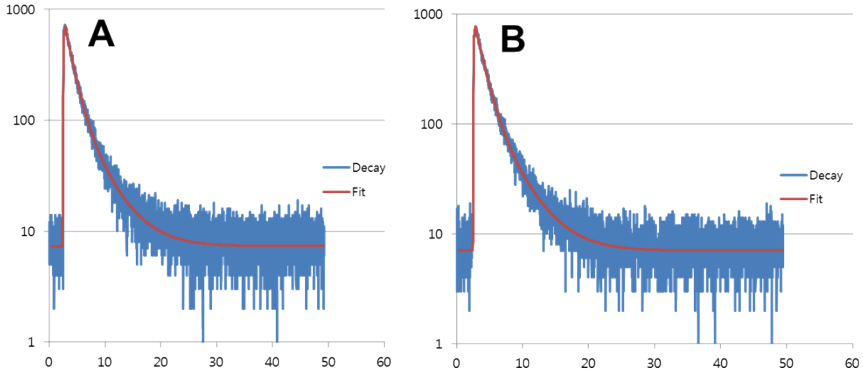


Figure S9. Fluorescence decay of BODIPY in sensor **1** with HeLa cells before and after treated by tunicamycin

(10 μg/mL), excited at 375 nm, and collected at the range of 500-530 nm, using an inverted-type scanning confocal microscope (MicroTime-200, Picoquant, Germany) with a 60 x objective (NA = 1.2).

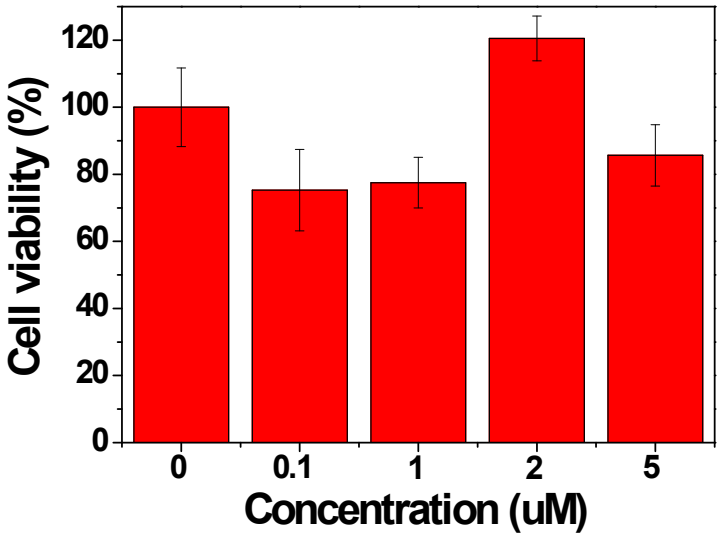


Figure S10. Cell viability experiment of **1**; HeLa cells were incubated with different concentration of **1** for 24 hours, and then MTT was used to stain the cells.

^{1}H NMR and ^{13}C NMR spectra

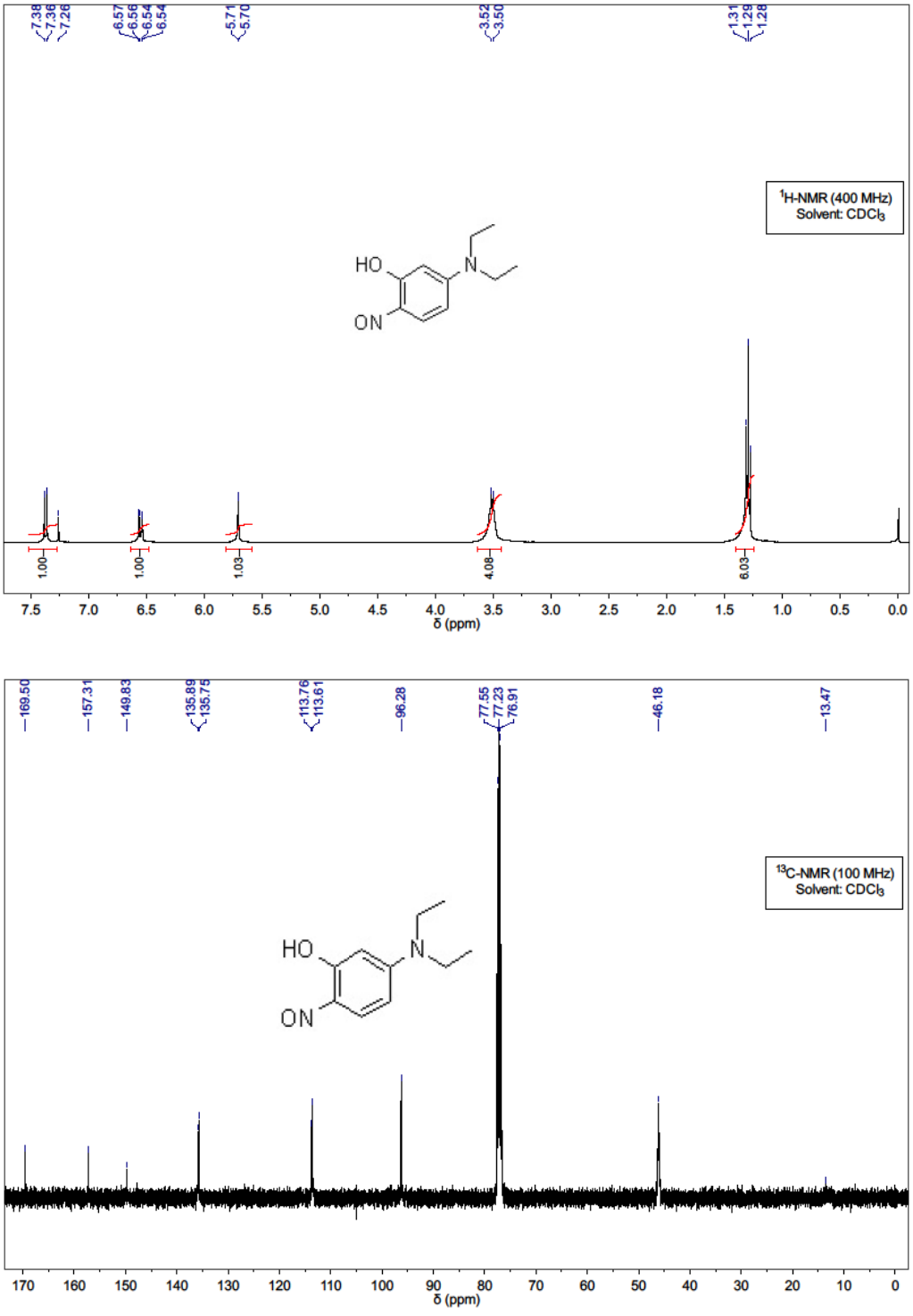


Figure S11. ^{1}H-NMR and ^{13}C-NMR spectra of **4**

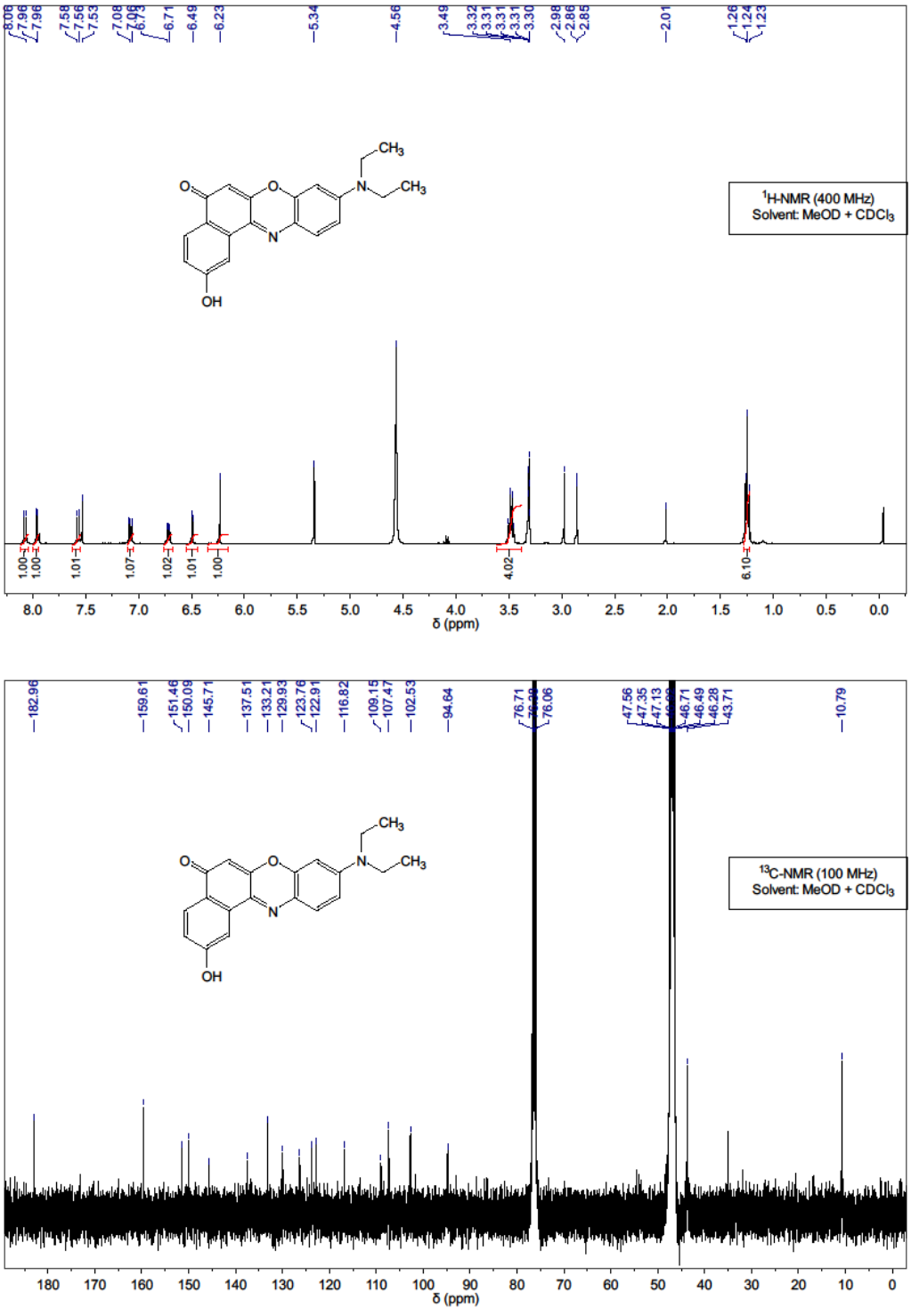


Figure S12. ^{1}H-NMR and ^{13}C-NMR spectra of **3**

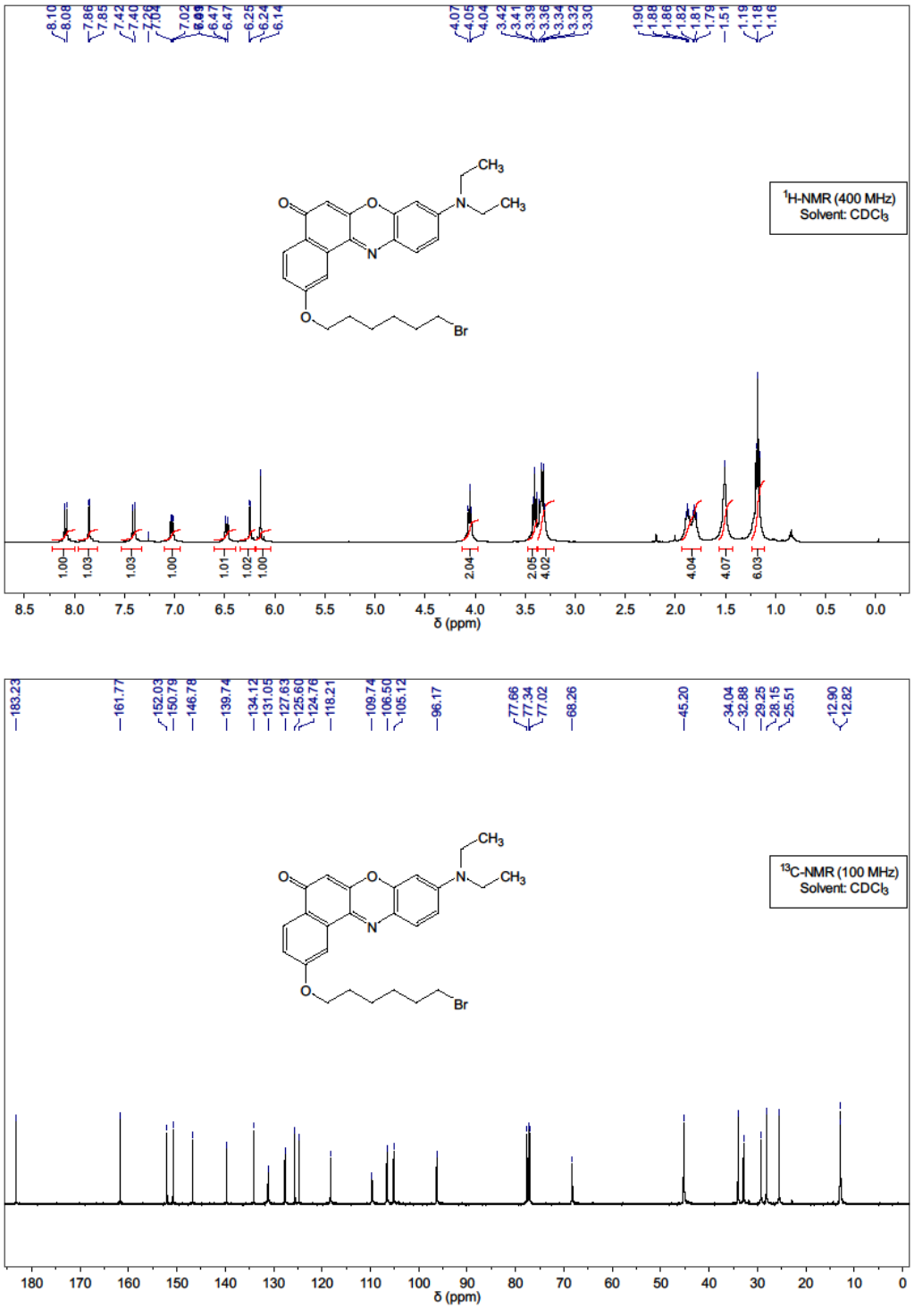


Figure S13. [1]H-NMR and [13]C-NMR spectra of **2**

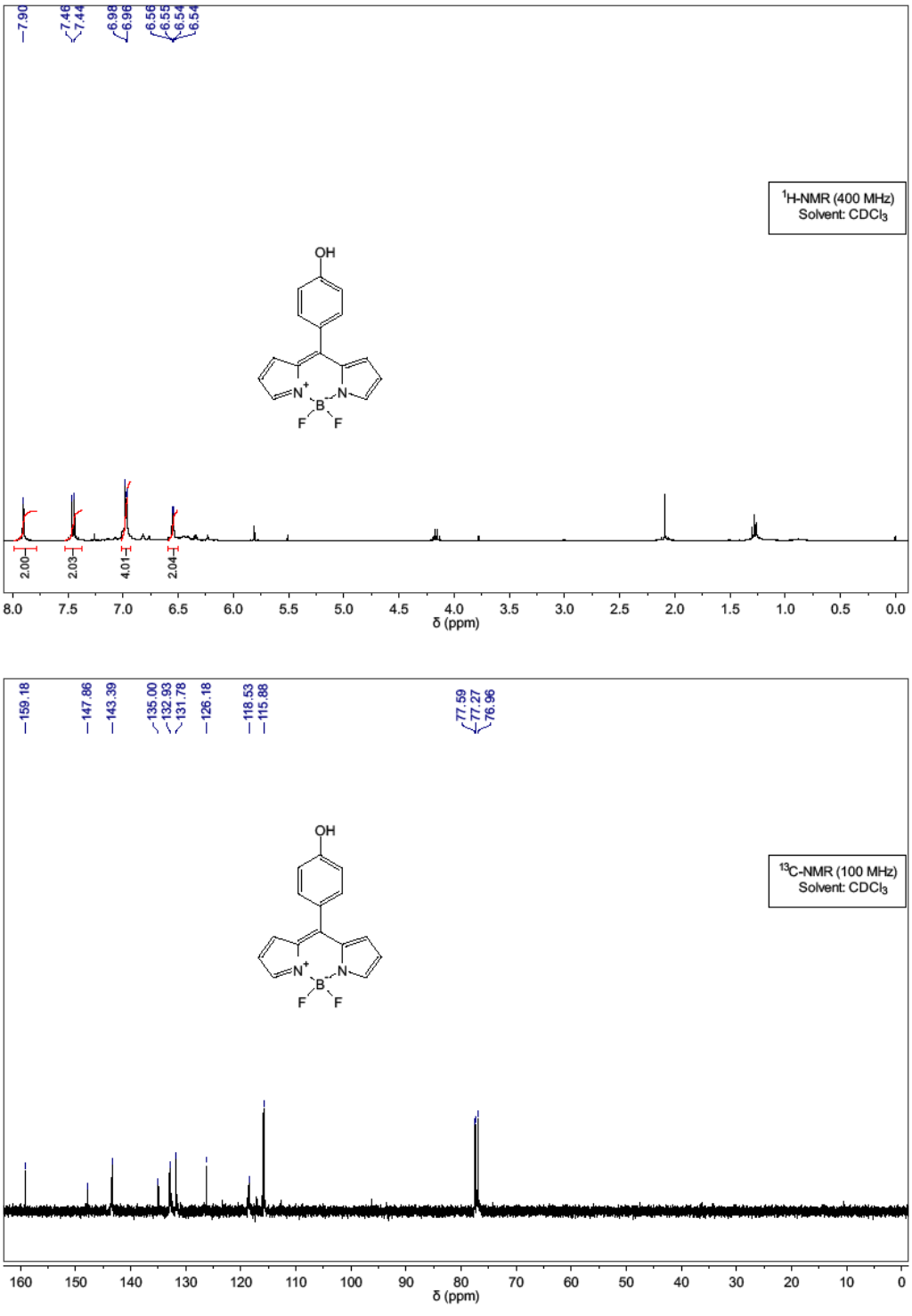
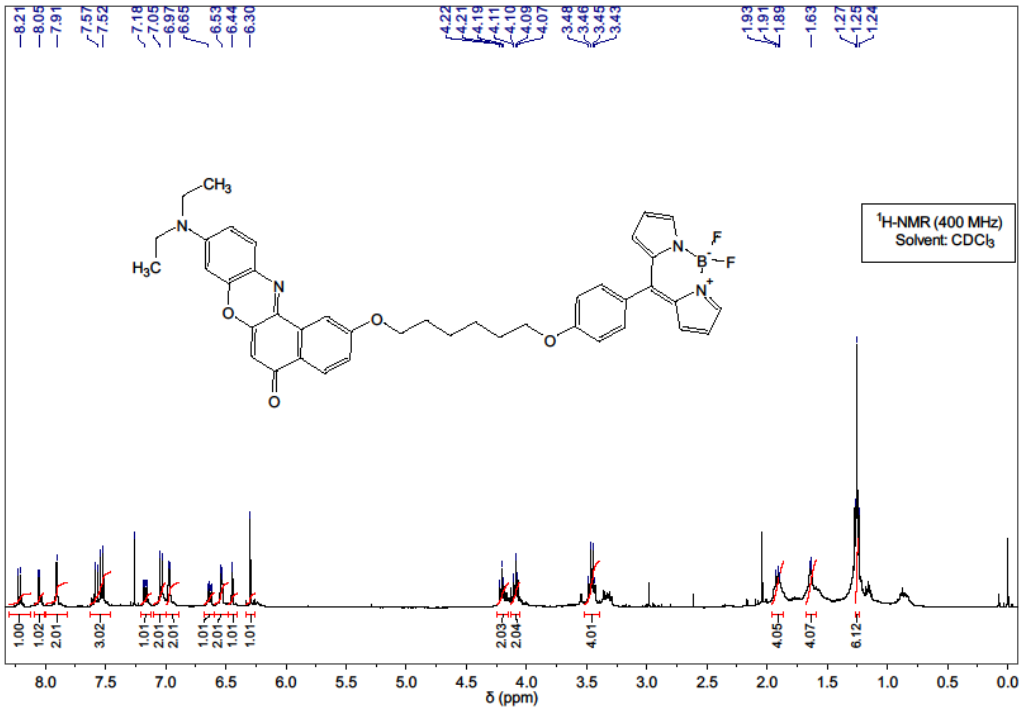


Figure S14. ^{1}H-NMR and ^{13}C-NMR spectra of **6**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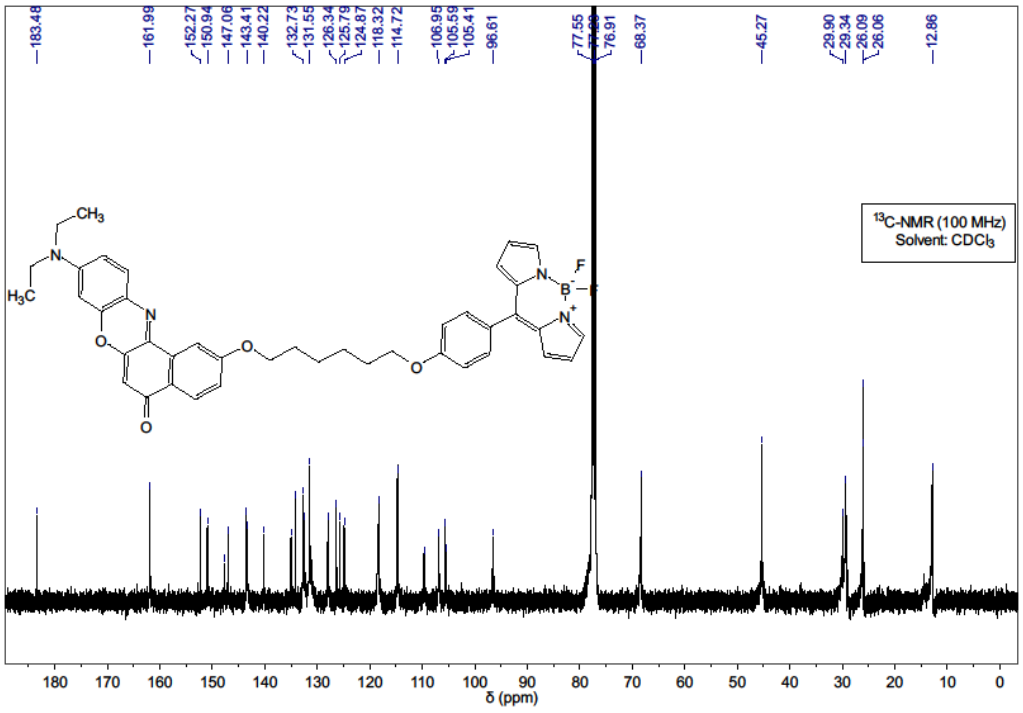
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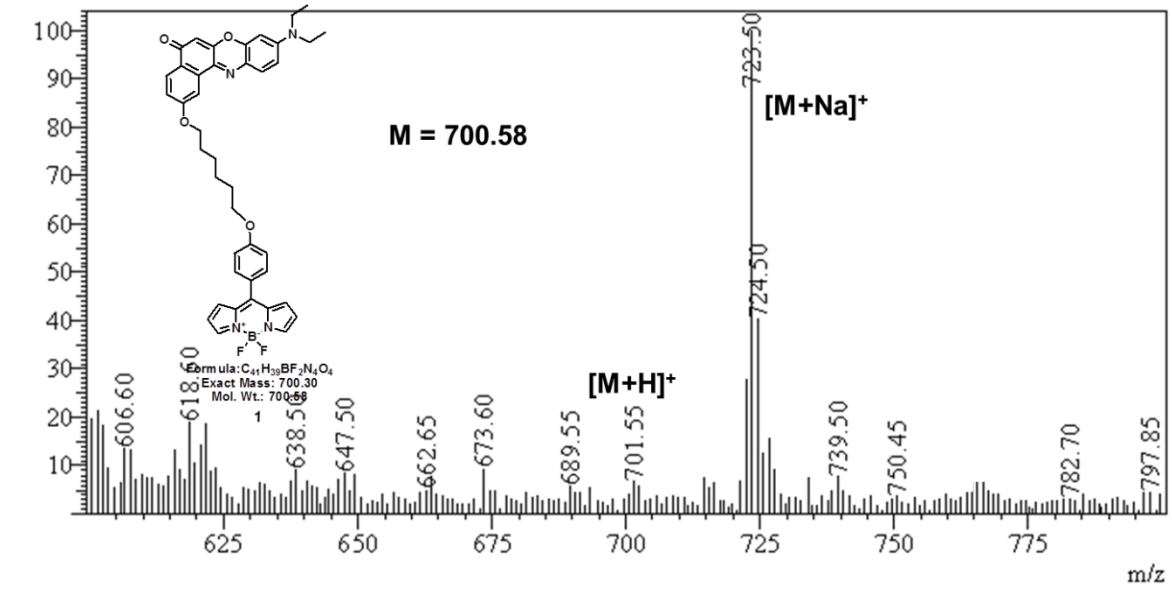

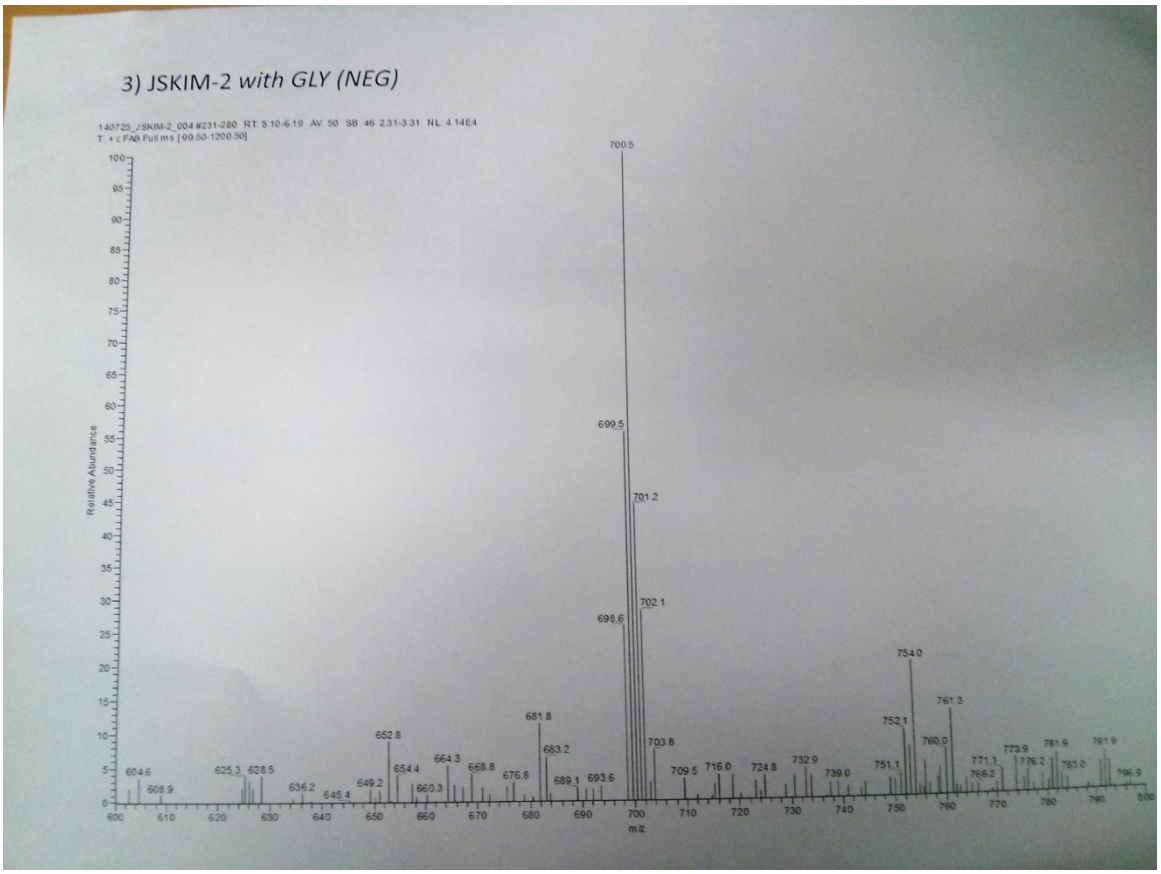

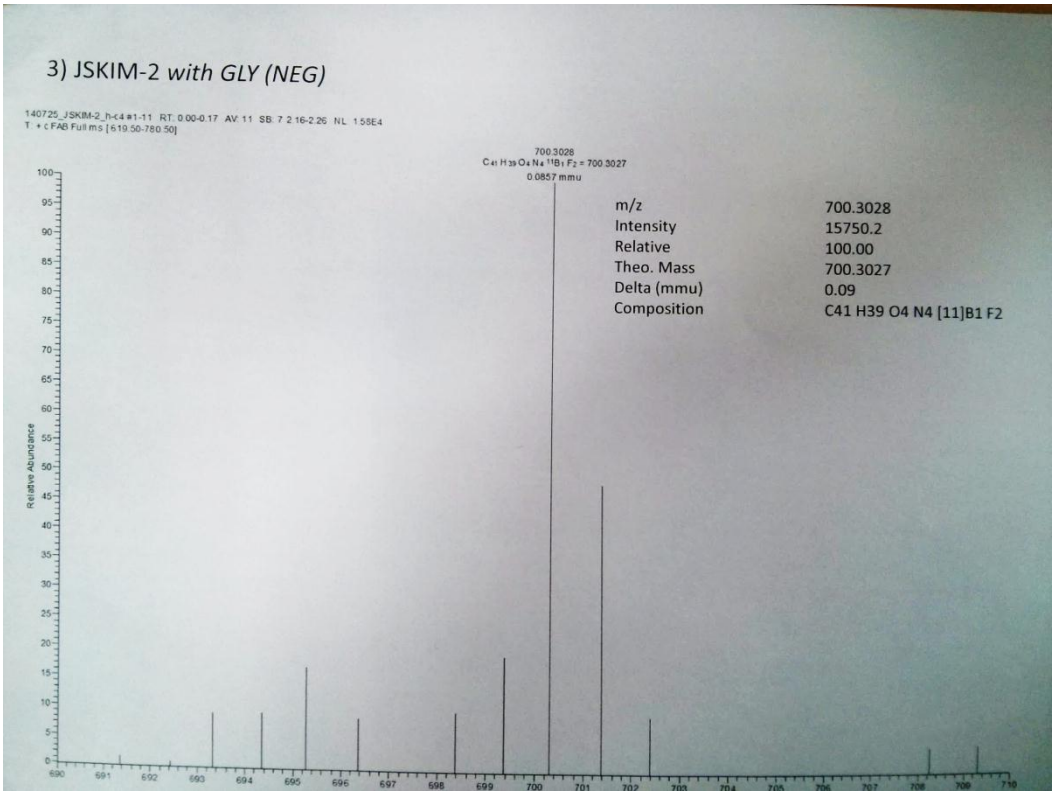

Figure S15. ^{1}H-NMR, ^{13}C-NMR and Mass and High resolution Mass spectra of **1**