Supporting Information

π-Spacer Modulation Unlock Tunable Photophysics and Organelle-Specificity in 2, 6 Disubstituted BODIPY-Cyanostilbene Fluorophores

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1. General Information

1.1 Materials and Measurements

All the commercially available chemicals and solvents were purchased and used as received. Toluene was dried over sodium, freshly distilled, and then used. The chlorinated solvents were dried over CaH_2 and subsequently stored over 4 Å molecular sieves. All the heating reactions were performed in a reaction block on a magnetic stirrer using a temperature-controlled probe. Moisture sensitive reactions were performed under a nitrogen atmosphere. The 500 MHz ^{1}H NMR, 126 MHz ^{13}C NMR, 471 MHz ^{19}F NMR and 160.4 MHz ^{11}B NMR spectra were recorded on a JEOL JNM-ECZ-500R/M1, 500 MHz NMR spectrometer. All solution ^{1}H and ^{13}C spectra were referenced internally to the solvent signal [TMS ($\delta = 0$)]. ^{11}B and ^{11}F spectra were referenced externally to $BF_3 \cdot Et_2O$ ($\delta = 0$) in C_6D_6. All the chemical shifts were reported in δ ppm, and the coupling constants (J) were given in Hertz (Hz). The HRMS was performed on a Waters Synapt XS spectrometer in the positive ion mode using acetonitrile as the solvent. The UV−Vis absorption spectra were measured using a UV-Vis spectrophotometer SHIMADZU-2600 with a slit width of 2. Fluorescence measurements were carried out on a PerkinElmer 6500 fluorescence spectrometer.

1.2 Fluorescence Quantum Yield

The fluorescence quantum yields of compounds **7** and **8** in solution were evaluated by using Rhodamine B in ethanol as a standard ($\Phi_F = 0.49$ in ethanol). The quantum yield Φ is calculated using the formula:

$$\Phi = \Phi_F \times (I/I_R) \times (A_R/A) \times (\eta/\eta_R)^2$$

where Φ = quantum yield of the compound, I = integral area of the emission peak, A = absorbance at λ_{ex}, η = refractive index of the solvent.

1.3 Cell based studies

HeLa cells were purchased from the American Type Culture Collection (ATCC). HeLa cells were maintained in a 100 mm dish to about 80-90% confluency. The cells were grown in complete media (DMEM, 10% FBS (Gibco), 1% Penicillin-Streptomycin antibiotic) at 37 °C, in a 5% CO_2 incubator. For fluorescence detection, Cells were seeded on glass bottom 35 mm confocal dishes, which were then maintained in a CO_2 incubator at 37 °C for 24 h, then cells were incubated with 500 nM of probes along with 200 nM of BODIPY 493/503 (Sigma Aldrich), 200 nM of Lysotracker Green (Invitrogen) and 200 nM of DAPI (Invitrogen) for 30

minutes in three separate setups of experiments. After incubation cells were washed thrice with 1X PBS and observed under confocal microscope.

1.4 MTT assay

$5x10^3$ cells/well were seeded in 96 well plate, after 24 h HeLa cells were treated with probes in different concentrations, and incubated for 24 h at 37 °C, CO_2-5% and humidity-controlled conditions. Following incubation, the cells were washed with 1X PBS, thrice. Then cells were treated with 0.5 mg/mL of MTT for 2 h and absorbance was measured at 590 nm.

1.5 Statistical Analyses

All the MTT data was acquired for three independent experiments and presented as the mean of three individual observations with the standard deviation. IC_{50} values were calculated using the AAT Bioquest IC_{50} calculator.

1.6 Live Cell Imaging and Image analysis

Confocal imaging was carried out using Nikon A1R MP + Ti-E confocal microscope. Images were acquired using Apochormat 100X 1.4 NA oil immersion-based objective lens. Imaging was performed at temperatures 37 °C, CO_2-5% and humidity-controlled conditions. 405 nm, 488 nm, and 560 nm lasers were used to excite the signals from Probes, BODIPY 493/503, Lysotracker Green and DAPI respectively, the emission signals were detected by an automated du4 detector. The visible puncta of the lipid droplets in HeLa cells were quantified by drawing manual ROI (Region of Interest) on individual cells using Nikon NIS Essentials analysis software. The Mean Fluorescent Intensity plots were plotted with the help of GraphPad Prism. The co-localization studies of the Probe and BODIPY 493/503 were quantified by drawing manual ROI (Region of Interest) on individual cells using Nikon NIS Essentials analysis software. The average for Pearson's co-efficient were plotted with the help of GraphPad Prism.

1.7 Oleic Acid (OA) Treatment

HeLa cells were maintained in a 100 mm dish to about 80-90% confluency. The cells were grown in complete media (DMEM, 10% FBS(Gibco), 1% Penicillin-Streptomycin antibiotic) at 37 °C, in a 5% CO_2 incubator. For fluorescence detection, Cells were seeded on glass bottom 35 mm confocal dishes, which were then maintained in a CO_2 incubator at 37 °C for 24 h, then cells were incubated with 100 μM of OA for 30 mins and washed with PBS, then with 500 nM of probes along with 200 nM of BODIPY 493/503. Finally, cells were observed under confocal microscope.

1.8 Chloroquine (CQ) Treatment

HeLa cells were maintained in a 100 mm dish to about 80-90% confluency. The cells were grown in complete media (DMEM, 10% FBS(Gibco), 1% Penicillin-Streptomycin antibiotic) at 37 °C, in a 5% CO_2 incubator. For fluorescence detection, Cells were seeded on glass bottom 35 mm confocal dishes, which were then maintained in a CO_2 incubator at 37 °C for 24 h, then cells were incubated with 50 μM of CQ for 30 mins and washed with PBS, then with 500 nM of probes along with 200 nM of Lysotracker-Green. Finally, cells were observed under confocal microscope.

1.9 Statistical Analyses

All statistical analyses were performed by using GraphPad Prism on the dataset of n≥25, of three independent individual experiment. An unpaired t-test was performed to compare the dataset of the two groups. All error bars indicate mean ± SEM. The significance values were depicted in the figures using the following key legend: *: p <0.05, **: p <0.01, ***: p <0.001, ****: p<0.0001.

2.0 Photostability

HeLa were maintained in a 100 mm dish to about 80-90% confluency. The cells were grown in complete media (DMEM, 10% FBS(Gibco), 1% Penicillin-Streptomycin antibiotic) at 37 °C, in a 5% CO_2 incubator. For fluorescence detection, Cells were seeded on glass bottom 35 mm confocal dishes, which were then maintained in a CO_2 incubator at 37 °C for 24 h, then cells were incubated with 500 nM of probes along with 200 nM of BODIPY 493/503. For the photostability test, cells incubated with the different probes were continuously irradiated with lasers (568 nm, 3% laser power). The images were captured at 5-minute time intervals.

2. Synthetic procedure for the precursors and target molecules

Synthesis of *(Z)-3-(4-bromophenyl)-2-phenylacrylonitrile* (1)

Phenyl acetonitrile (1. 5 mL, 13 mmol), and sodium hydroxide (1.7 g, 43.25 mmol) were added to 4-bromobenzaldehyde (2 g, 10.81 mmol) in 40 mL methanol. The reaction mixture was stirred at 40 °C for 4 h and was monitored using thin layer chromatography (TLC). Once the complete consumption of reactant was observed, the reaction mixture was allowed to cool to room temperature (RT), and the solid product was isolated by filtration. The solid product obtained was washed multiple times with methanol and dried under vacuum. The pure product was obtained as white solid powder (3.0 g, 98 %). The characterisation data matches well with

the literature reports.[1] ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.75 (d, *J* = 8.0 Hz, 2H), 7.67 (d, *J* = 7.1 Hz, 2H), 7.59 (d, *J* = 8.7 Hz, 2H), 7.48 – 7.39 (m, 4H).

Synthesis of *(Z)-2-phenyl-3-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl) acrylonitrile* (2)

Oven dried Schlenk flask was loaded with compound **1** (1 g, 3.51 mmol), bis(pinacolato)diboron (1.1 g, 4.41 mmol), potassium acetate (1.0 g, 10.6 mmol), and triphenylphosphine (37 mg, 0.14 mmol), followed by purging with N_2. The reagents were dissolved in 50 mL of dry toluene, and the mixture was degassed under a N_2 atmosphere for 30 minutes. Later, dichlorobis(triphenylphosphine)palladium(II) (50 mg, 0.07 mmol) was added as the catalyst, and the reaction mixture was refluxed at 110 °C for 24 hours. After completion, the volatiles were removed under reduced pressure. The residue was extracted multiple times with ethyl acetate and washed thoroughly with brine. The combined organic layers were dried over anhydrous sodium sulfate (Na_2SO_4), and the solvent was removed under reduced pressure to afford a brown solid as the crude product. Purification was carried out using silica gel column chromatography with hexane/ethyl acetate (95:5) as the eluent. The pure product was obtained as a white solid (1 g, 86 %). The NMR data matches well with the literature reported values.[1] ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.89 (q, *J* = 8.3 Hz, 4H), 7.69 (d, *J* = 7.1 Hz, 2H), 7.55 (s, 1H), 7.48 – 7.38 (m, 3H), 1.37 (s, 12H).

Synthesis of *(Z)-3-(5-bromothiophen-2-yl)-2-phenylacrylonitrile* (3)

Phenyl acetonitrile (1. 4 mL, 12.55 mmol), and potassium-*t*-butoxide (530 mg, 4.72 mmol) were added to 5-bromothiophene-2-carbaldehyde (1.2 mL, 10.47 mmol) in 50 mL ethanol and was stirred at RT for 4 h. The progress of the reaction was monitored using TLC and the complete consumption of reactant was observed. The solid product was filtered and washed multiple times with ethanol and dried under vacuum. The pure product was obtained as white solid powder (2.97 g, 98 %). The NMR data matches well with the literature reported values.[2] ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.62 (d, *J* = 8.4 Hz, 2H), 7.53 (s, 2H), 7.46 – 7.37 (m, 2H), 7.33 (d, *J* = 5.1 Hz, 1H), 7.11 (d, *J* = 4.1 Hz, 1H). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) δ: 140.0, 134.0, 133.9, 133.4, 131.1, 129.7, 129.6, 126.2, 118.8, 118.5, 109.1.

Synthesis of *(Z)-2-phenyl-3-(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)thiophen-2-yl)acrylonitrile* (4)

Oven-dried Schlenk flask was loaded with compound **3** (2 g, 6.89 mmol), bis(pinacolato)diboron (2.2 g, 8.66 mmol), potassium acetate (2 g, 20.38 mmol), and

triphenylphosphine (72 mg, 0.27 mmol). The flask was purged multiple times with N_2. The reactants were then dissolved in 50 mL of dry toluene, and the resulting reaction mixture was thoroughly degassed with N_2 for 30 minutes. Dichlorobis(triphenylphosphine)palladium(II) (97 mg, 0.14 mmol) was added as a catalyst, and the mixture was heated to reflux at 110 °C for 24 hours. After completion, the volatiles were removed under reduced pressure. The crude residue was extracted with ethyl acetate (50 mL, repeated 3–4 times) and washed well with brine. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure, yielding a brown solid crude product. This was purified via silica gel column chromatography using hexane/ethyl acetate (95:5) as the eluent. The pure product was obtained as a white solid powder (1.47 g, 63 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.74 (d, *J* = 3.7 Hz, 1H), 7.68 – 7.64 (m, 3H), 7.62 (d, *J* = 3.7 Hz, 1H), 7.46 – 7.35 (m, 3H), 1.36 (s, 12H). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) δ: 144.3, 137.9, 134.3, 134.1, 133.0, 129.6, 129.6, 126.3, 118.4, 110.1, 85.0, 84.0, 25.2. HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{19}H_{20}BNO_2S$, 338.1386; found, 338.1381.

Synthesis of *8-(4-Methoxyphenyl)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene* (5)

p-Anisaldehyde (893 µL, 7.34 mmol) and 2, 4 dimethyl pyrrole (1.8 mL, 17.63 mmol) were added to freshly distilled DCM (350 mL), stirred at room temperature under a N_2 atmosphere. The reaction mixture was thoroughly degassed using N_2 for at least 30 minutes, followed by the addition of a catalytic amount of trifluoroacetic acid (56.2 µL, 0.73 mmol). After 1 h, DDQ (1.9 g, 8.37 mmol) was added to the reaction mixture and was stirred for 5 h at room temperature. Triethylamine (10 mL, 73.45 mmol), and $BF_3 \cdot Et_2O$ (10 mL, 81.02 mmol) were then added to the resultant product and stirred at room temperature for overnight. The crude was purified by silica gel column chromatography (hexane/ethylacetate = 95:5) and yielded as an orange crystalline solid (0.79 g, 30 %). The NMR data matches well with the literature reported values.[3] ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.17 (d, *J* =8.7, 2H), 7.01 (d, *J* =8.7, 2H), 5.97 (s, 2H), 3.87 (s, 3H), 2.55 (s, 6H), 1.43 (s 6H).

Synthesis of Compound 6

Compound **5** (200 mg, 0.56 mmol) was dissolved in 40 mL of dry DCM and degassed the reaction mixture with N_2. N-iodo succinimide (318 mg, 1.41 mmol) was added and stirred the reaction mixture at RT for 3 h. The progress of the reaction was monitored using TLC and the complete consumption of reactant was observed. The volatiles were removed under reduced pressure, followed by extraction with DCM (40 mL for 3 to 4 times) and washed with brine

solution. The organic fraction was dried over anhydrous Na_2SO_4 and was evaporated under reduced pressure and kept under vacuum. The pure product was obtained as a red solid (335 mg, 98 %). The NMR data matches well with the literature reported values.[4] ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.14 (d, *J* =8.7, 2H), 7.03 (d, *J* =8.7, 2H), 3.89 (s, 3H), 2.64 (s, 6H), 1.44 (s 6H).

Synthesis of Compound 7

Oven dried Schlenk flask was charged with compound **5** (250 mg, 0.41 mmol), compound **2** (287 mg, 0.87 mmol), and potassium carbonate (2 M, 5 mL). The reactants were dissolved in 30 mL of THF, and the reaction mixture was thoroughly degassed with N_2 for 30 minutes. Following degassing, tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$] (40 mg, 0.03 mmol) was added as a catalyst. The mixture was then refluxed under a N_2 atmosphere for 24 hours. After completion, the volatiles were removed under reduced pressure, and the crude product was extracted with dichloromethane and washed with brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting crude product was purified by silica gel column chromatography using a 9:1 hexane/dichloromethane mixture as the eluent. The pure product was obtained as a red solid powder in moderate yield (169 mg, 54 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.94 (d, *J* = 8.4 Hz, 4H), 7.69 (d, *J* = 7.1 Hz, 4H), 7.55 (s, 2H), 7.48 – 7.37 (m, 7H), 7.32 – 7.26 (m, 5H), 7.06 (d, *J* = 8.8 Hz, 2H), 3.88 (s, 3H), 2.59 (s, 6H), 1.42 (s, 6H). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) δ: 160.8, 154.7, 142.2, 140.0, 136.7, 135.0, 133.2, 132.9, 132.4, 131.2, 129.8, 129.7, 129.6, 126.5, 118.6, 115.3, 111.9, 55.9, 14.0, 13.6. ^{11}B{^{1}H} NMR (160.4 MHz, $CDCl_3$) δ: 0.03 (t, *J* = 31.3 Hz). ^{19}F{^{1}H} NMR (471 MHz, $CDCl_3$) δ: -145.74 (q, *J* = 30.2 Hz). HRMS (ESI) m/z: $[M - F]^+$ calcd for $C_{50}H_{39}BF_2N_4O$, 741.3201; found 741.3221.

The synthesis of compound **8** was similar procedure of compound **7** and data as follows:
Compound **5** (250 mg, 0.41 mmol), compound **4** (334 mg, 0.99 mmol), potassium carbonate (2 M, 5 mL), and tetrakis(triphenylphosphine) palladium (0) $Pd(PPh_3)_4$ (40 mg, 0.03 mmol). The pure product was obtained as a red solid powder in moderate yield (153 mg, 48 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.67 – 7.60 (m, 8H), 7.47 – 7.33 (m, 6H), 7.24 (d, *J* = 8.7 Hz, 2H), 7.07 (d, *J* = 8.8 Hz, 2H), 6.92 (d, *J* = 4.0 Hz, 2H), 3.89 (s, 3H), 2.67 (s, 6H), 1.52 (s, 6H). ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) δ: 161.0, 155.6, 143.9, 141.6, 140.1, 138.8, 134.5, 134.4, 133.4, 132.5, 129.7, 129.6, 129.4, 128.9, 127.1, 126.1, 118.7, 115.4, 108.3, 55.9, 14.1, 13.9. ^{11}B{^{1}H} NMR (160.4 MHz, $CDCl_3$) δ: -0.25 (t, *J* = 32.4 Hz). ^{19}F{^{1}H} NMR (471 MHz, $CDCl_3$)

δ: -145.39 (q, J = 32.3 Hz). HRMS (ESI) m/z: $[M + H]^+$ calcd for $C_{46}H_{35}BF_2N_4OS_2$, 753.2329; found 753.2350.

3. NMR and HRMS spectra of precursors and target molecules

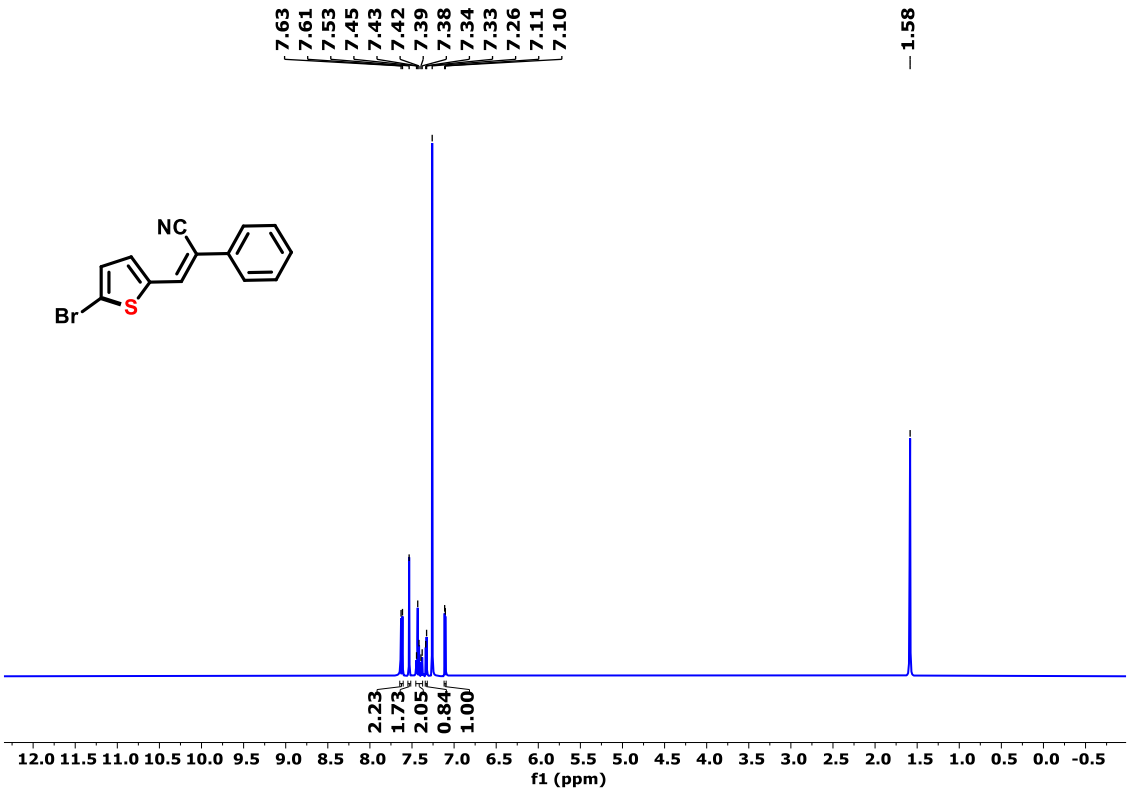

Figure S1. ^{1}H NMR spectrum (500 MHz, RT) of compound **3** in $CDCl_3$.

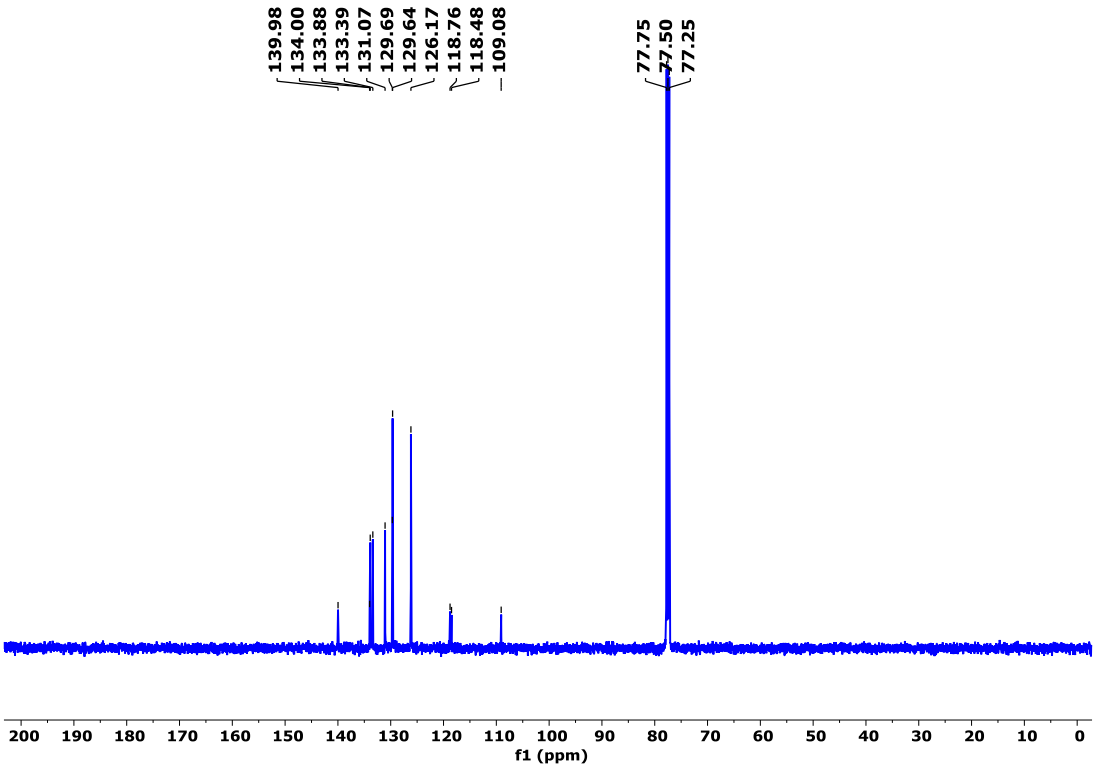

Figure S2. $^{13}C\{^{1}H\}$ NMR spectrum (126 MHz, RT) of compound **3** in $CDCl_3$.

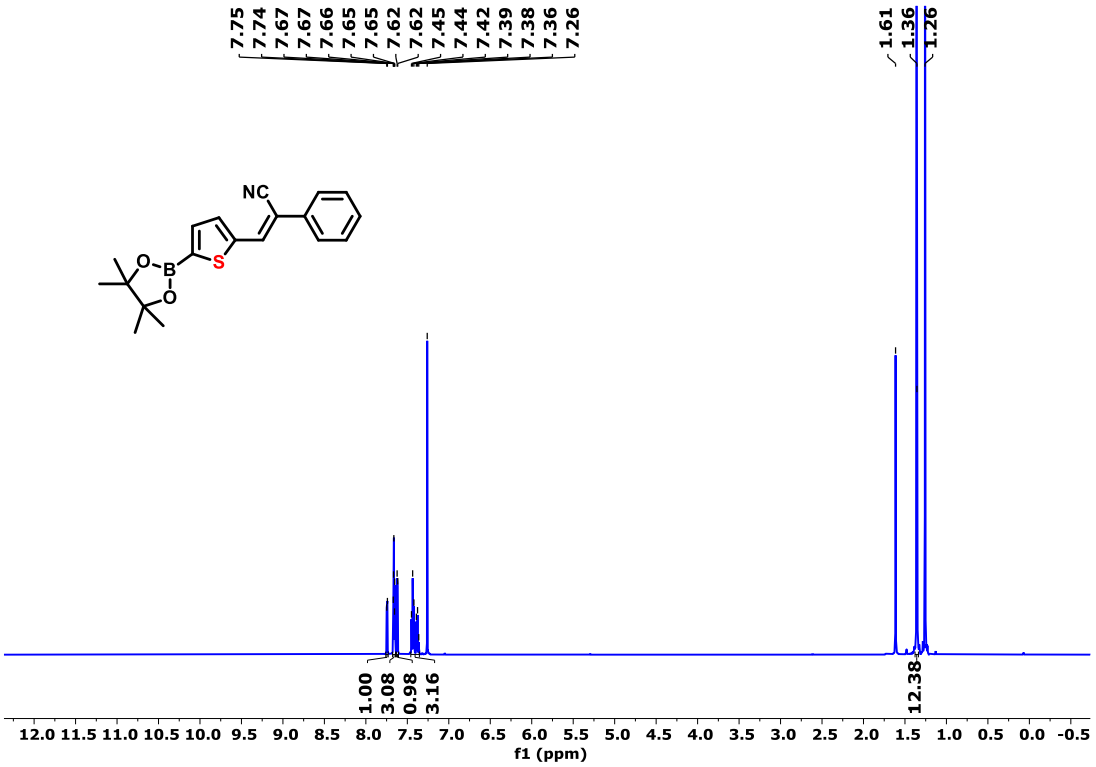

Figure S3. ^{1}H NMR spectrum (500 MHz, RT) of compound **4** in $CDCl_3$.

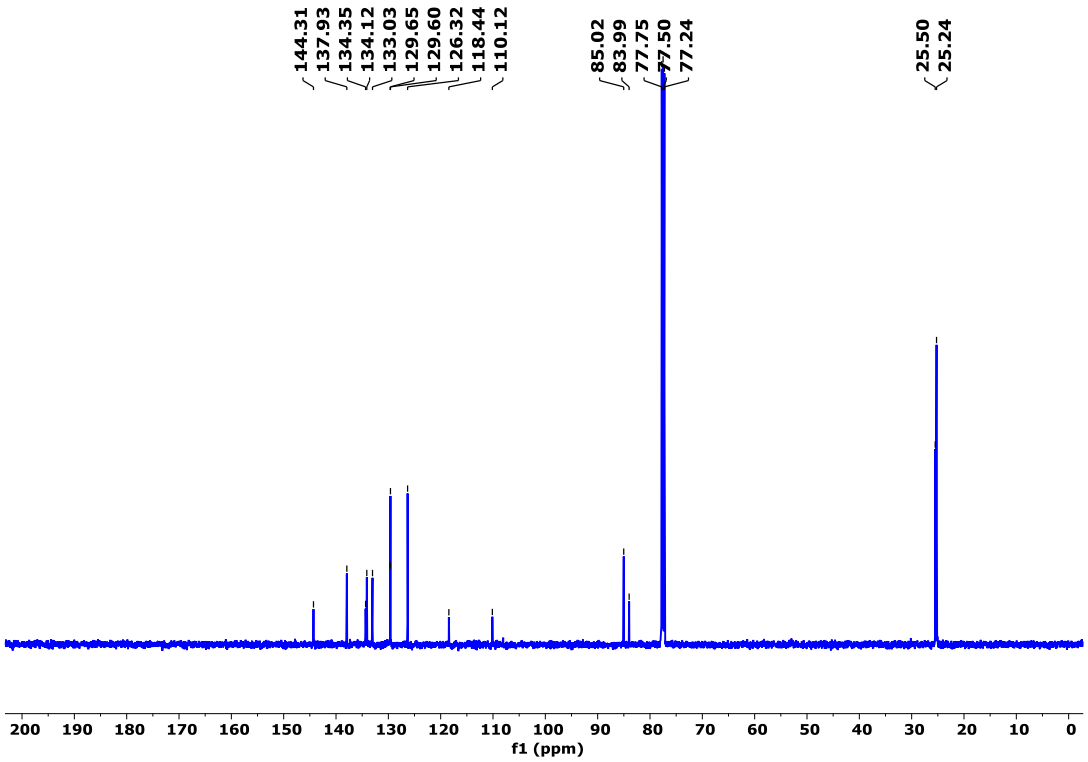

Figure S4. $^{13}C\{^{1}H\}$ NMR spectrum (126 MHz, RT) of compound **4** in $CDCl_3$.

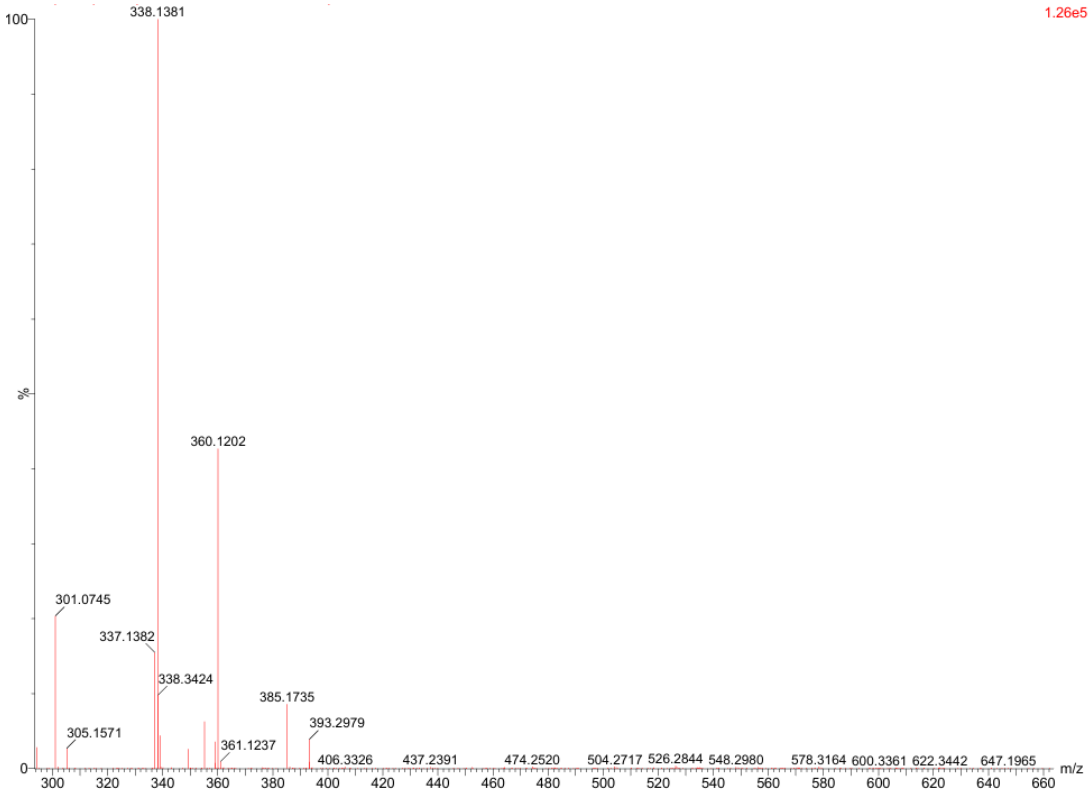

Figure S5. HRMS spectrum of compound **4**.

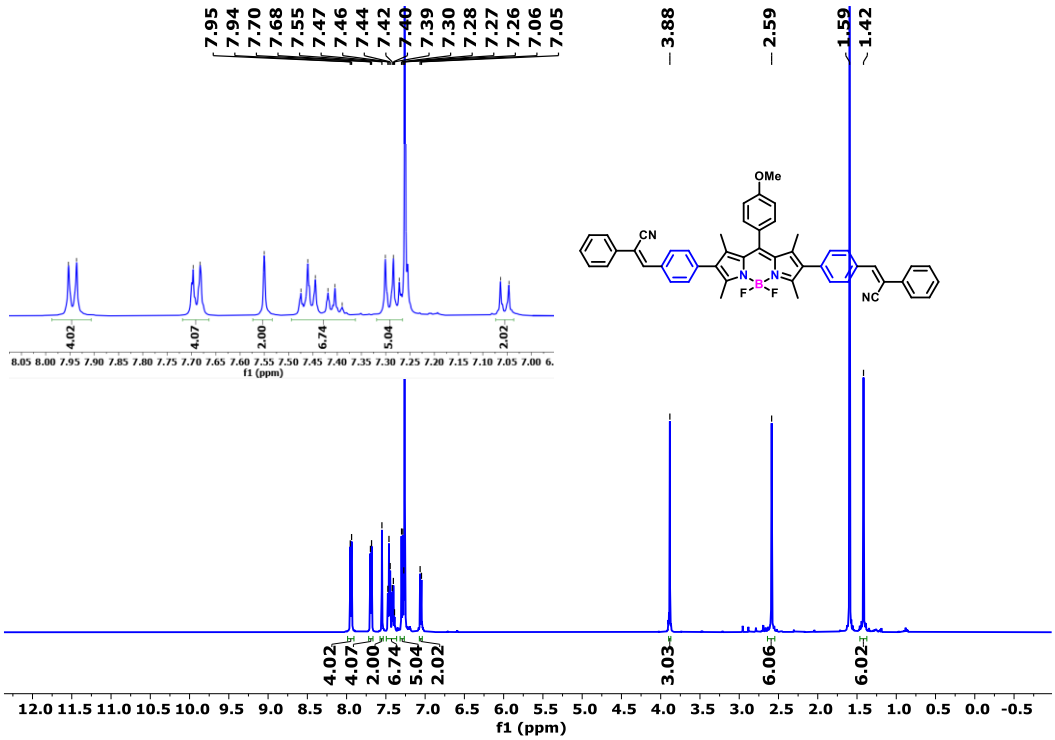

Figure S6. ^{1}H NMR spectrum (500 MHz, RT) of compound **7** in $CDCl_3$.

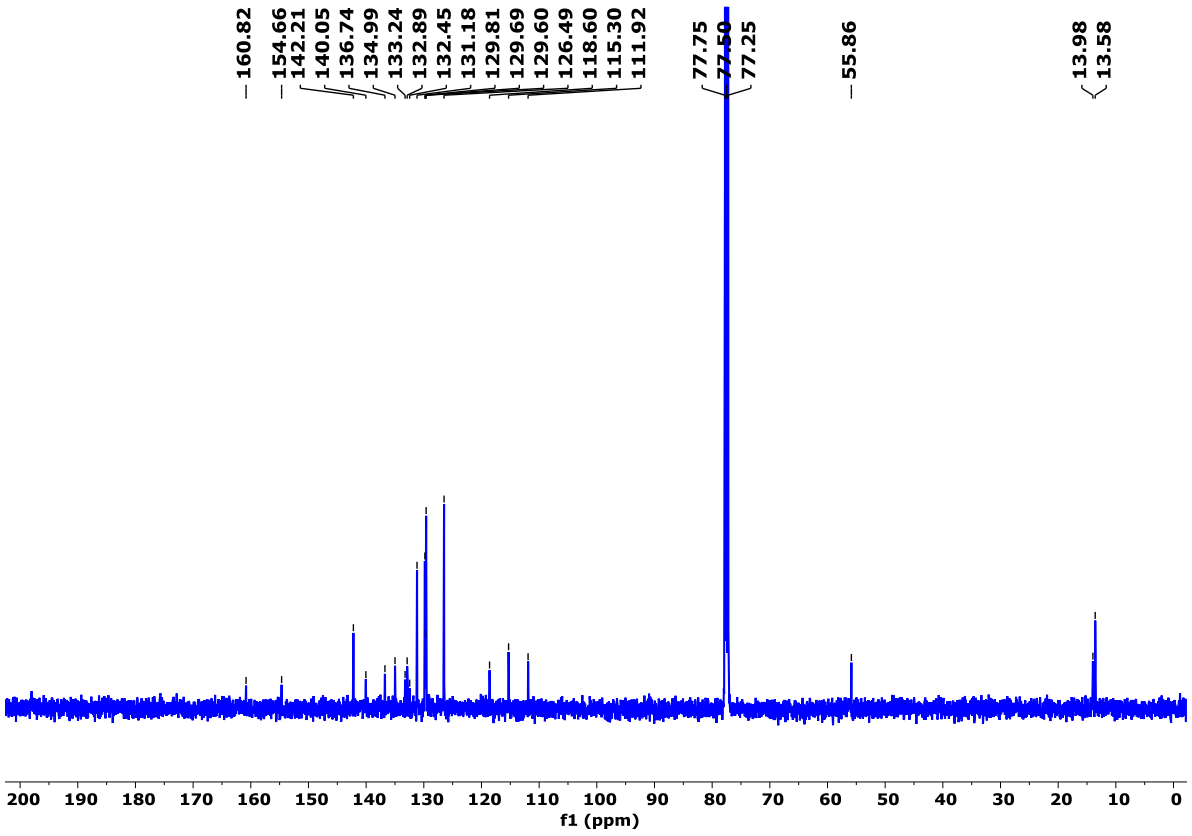

Figure S7. $^{13}C\{^{1}H\}$ NMR spectrum (126 MHz, RT) of compound **7** in $CDCl_3$.

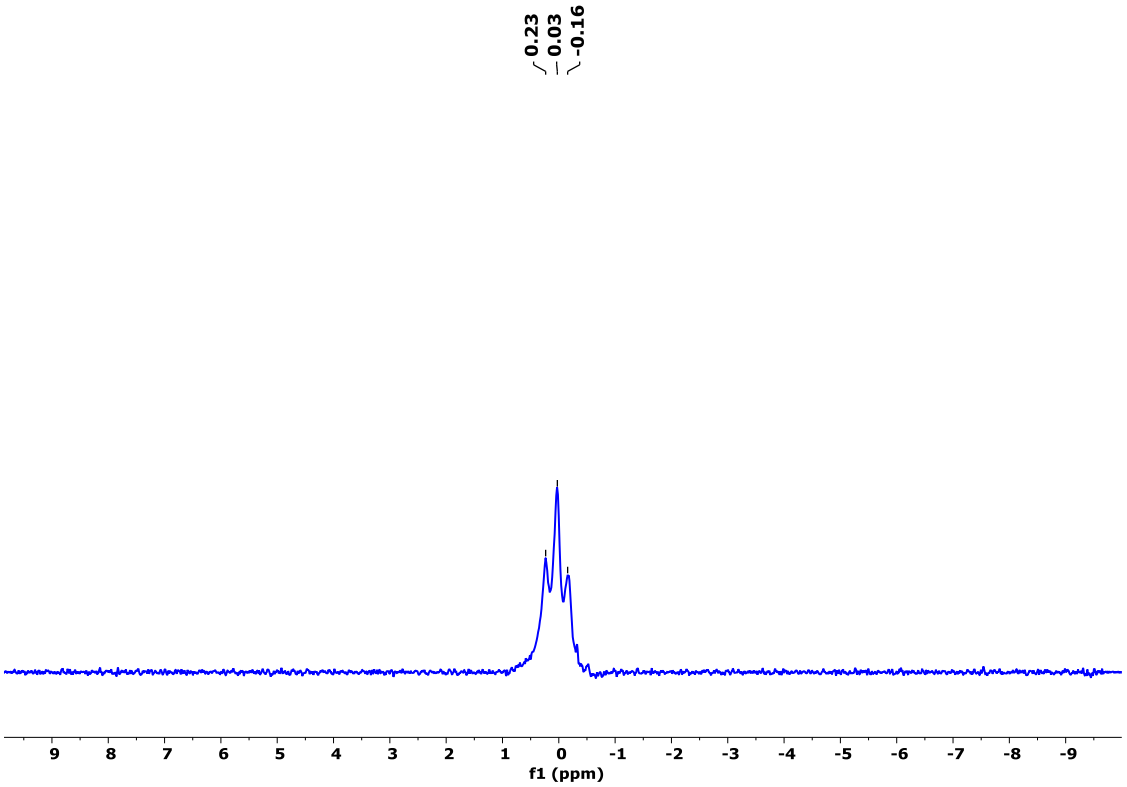

Figure S8. $^{11}B\{^{1}H\}$ NMR spectrum (160.4 MHz, RT) of compound **7** in $CDCl_3$.

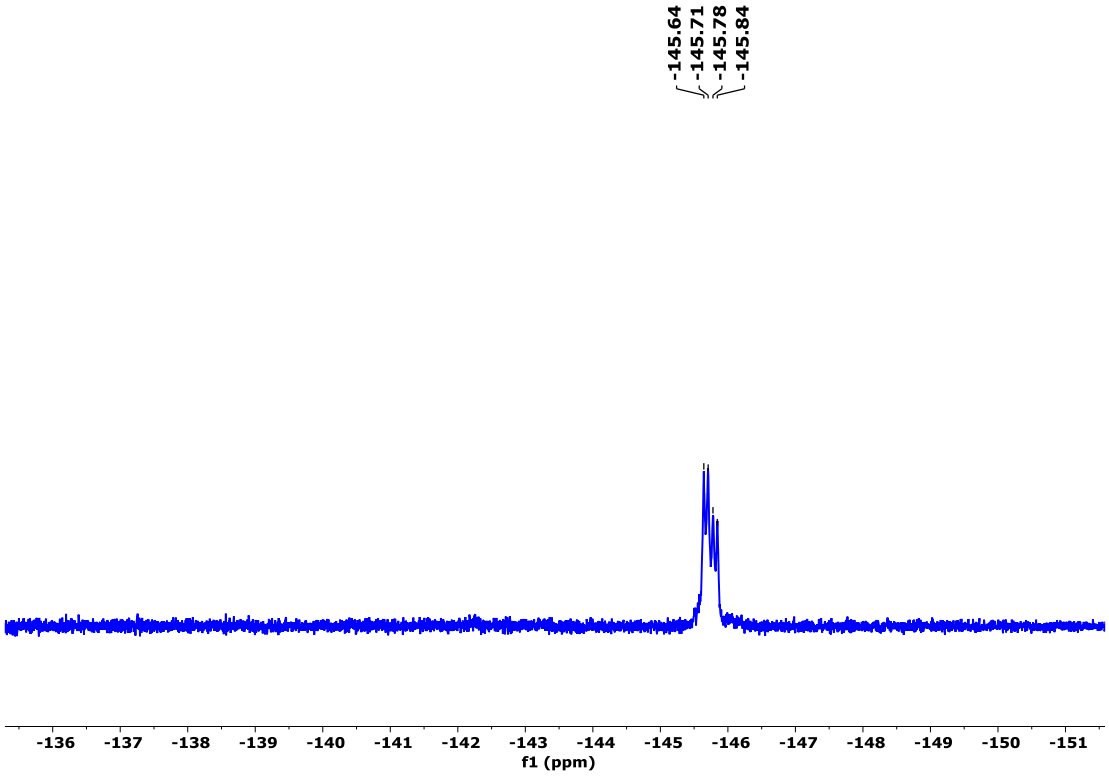

Figure S9. $^{19}F\{^{1}H\}$ NMR spectrum (471 MHz, RT) of compound **7** in $CDCl_3$.

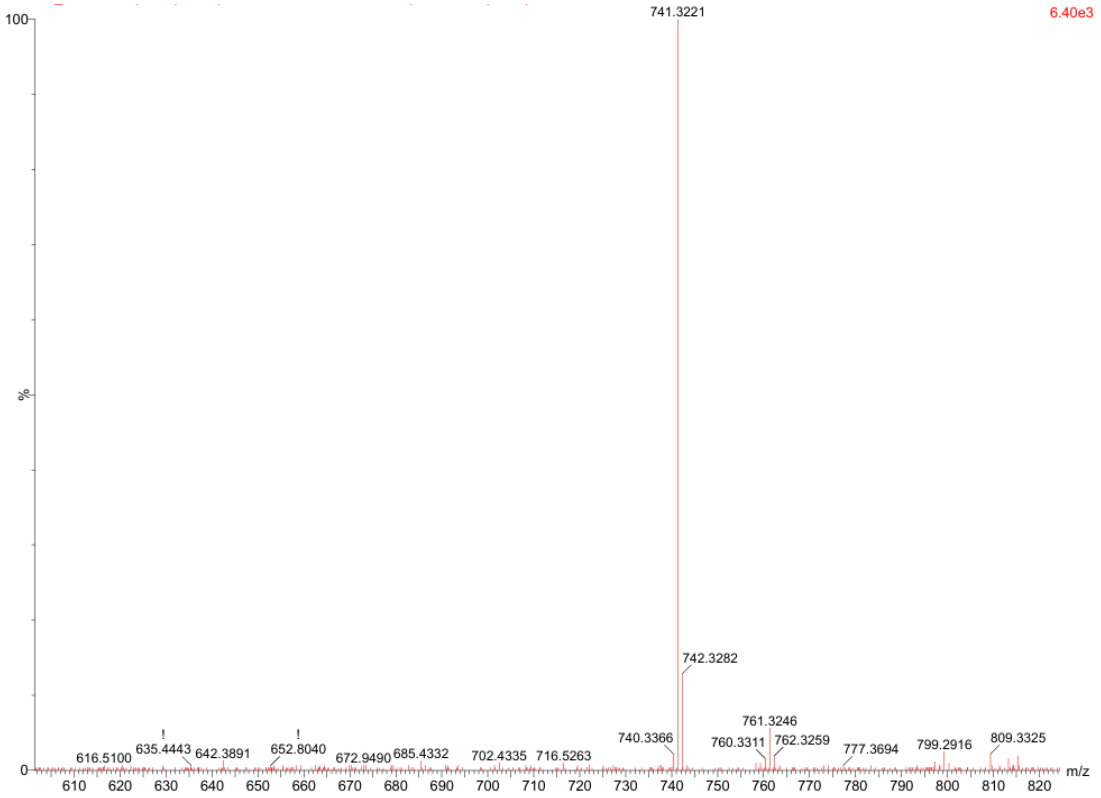

Figure S10. HRMS spectrum of compound **7**.

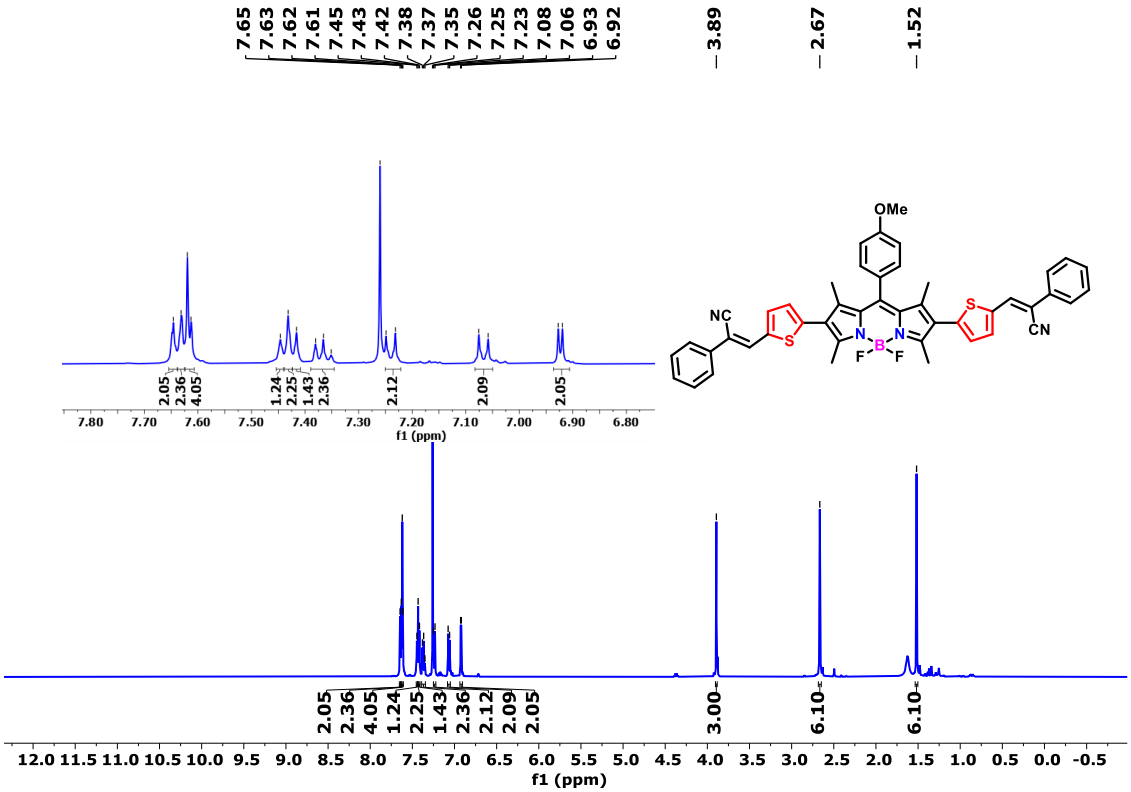

Figure S11. ^{1}H NMR spectrum (500 MHz, RT) of compound **8** in $CDCl_3$.

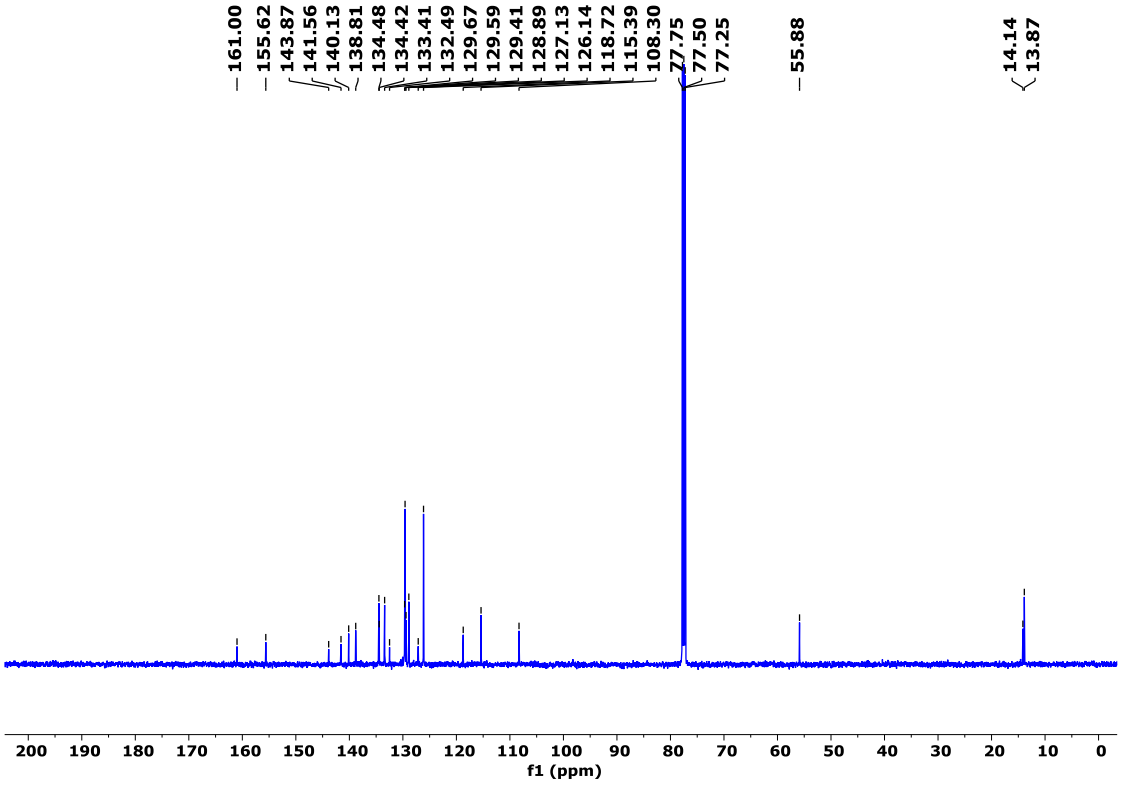

Figure S12. $^{13}C\{^{1}H\}$ NMR spectrum (126 MHz, RT) of compound **8** in $CDCl_3$.

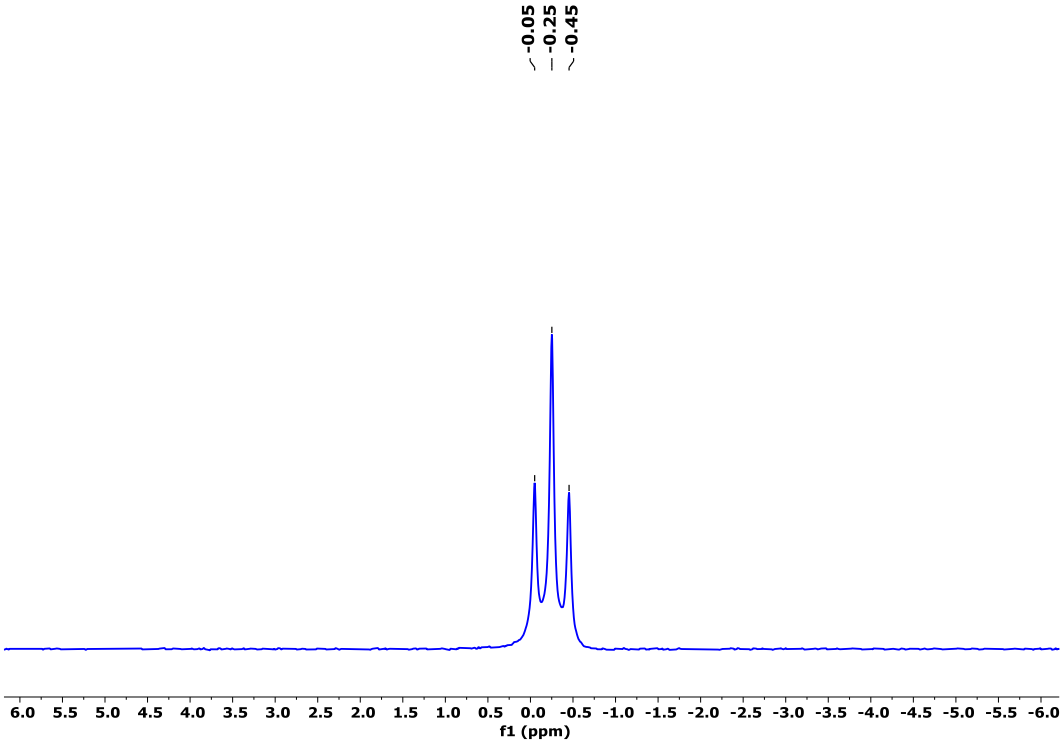

Figure S13. $^{11}B\{^{1}H\}$ NMR spectrum (160.4 MHz, RT) of compound **8** in $CDCl_3$.

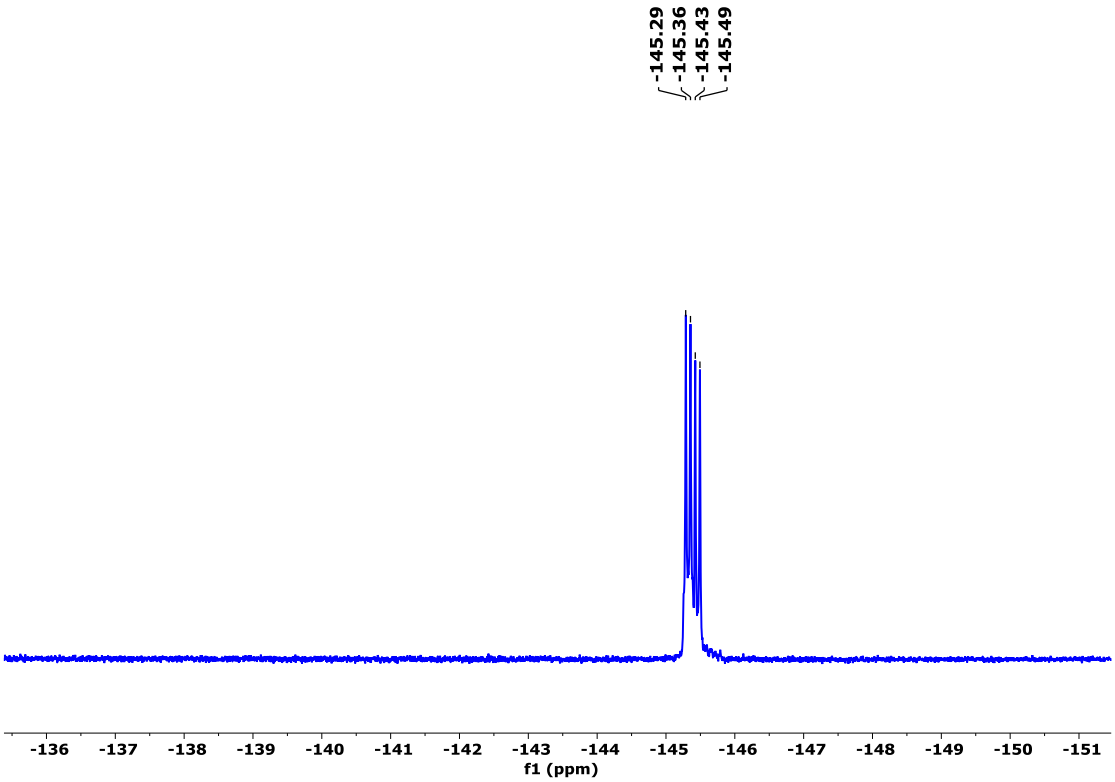

Figure S14. $^{19}F\{^{1}H\}$ NMR spectrum (471 MHz, RT) of compound **8** in $CDCl_3$.

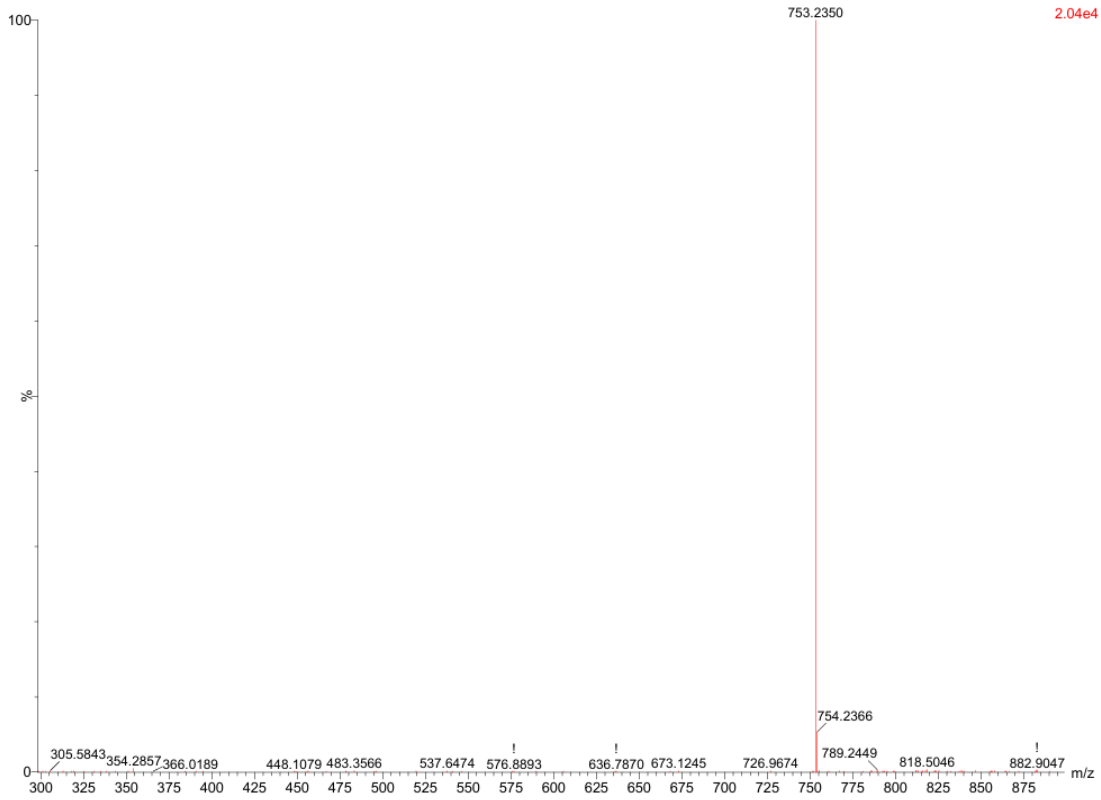

Figure S15. HRMS spectrum of compound **8**.

4. Photophysical studies of the target molecules

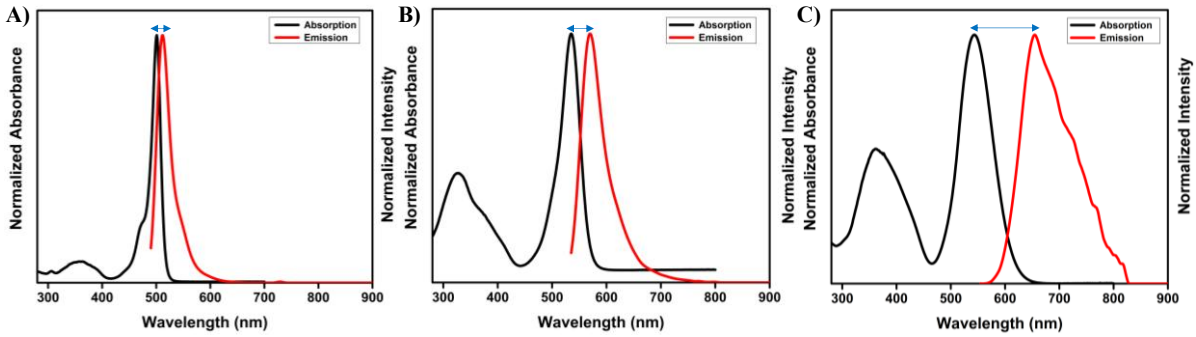

Figure S16. Merged absorption (10 μM) and emission spectra of compounds **Control** (A) (λ_{ex} = 485 nm), **7** (B), and **8** (C) in DCM (1.6 μM, λ_{ex} = 530 nm).

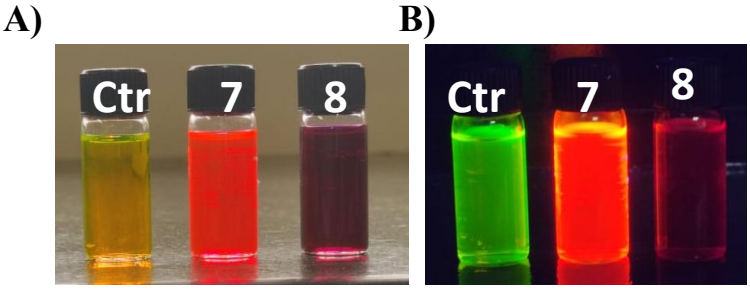

Figure S17. Fluorescence photographs of the compounds **Control**, **7,** and **8** taken under ambient light (a) and UV light (b).

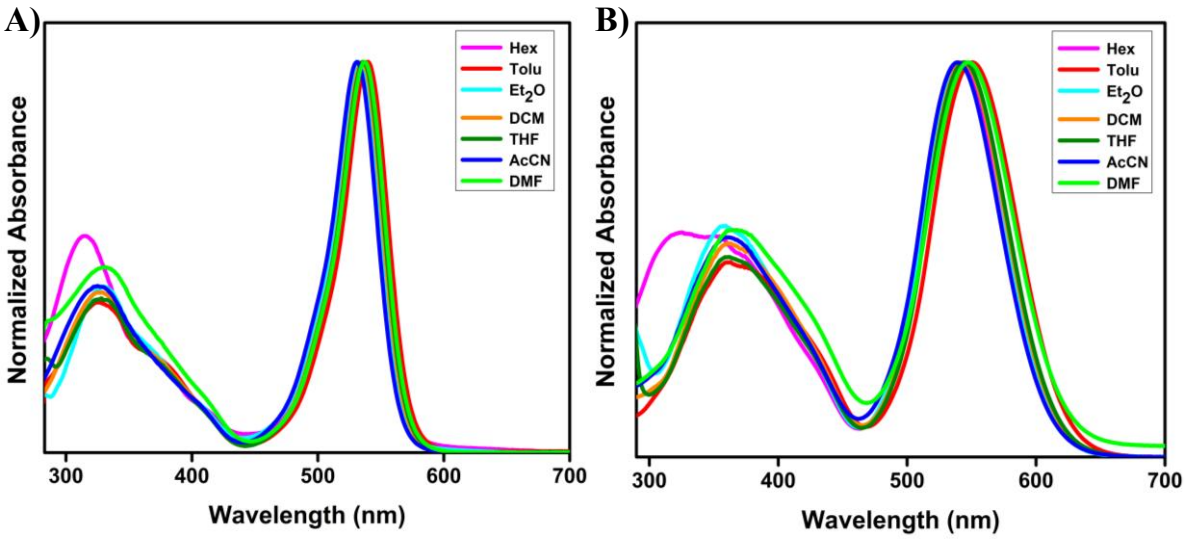

Figure S18. Normalized UV-Vis absorption spectra of compounds **7** (A) and **8** (B) in solvents of different polarity (10 μM).

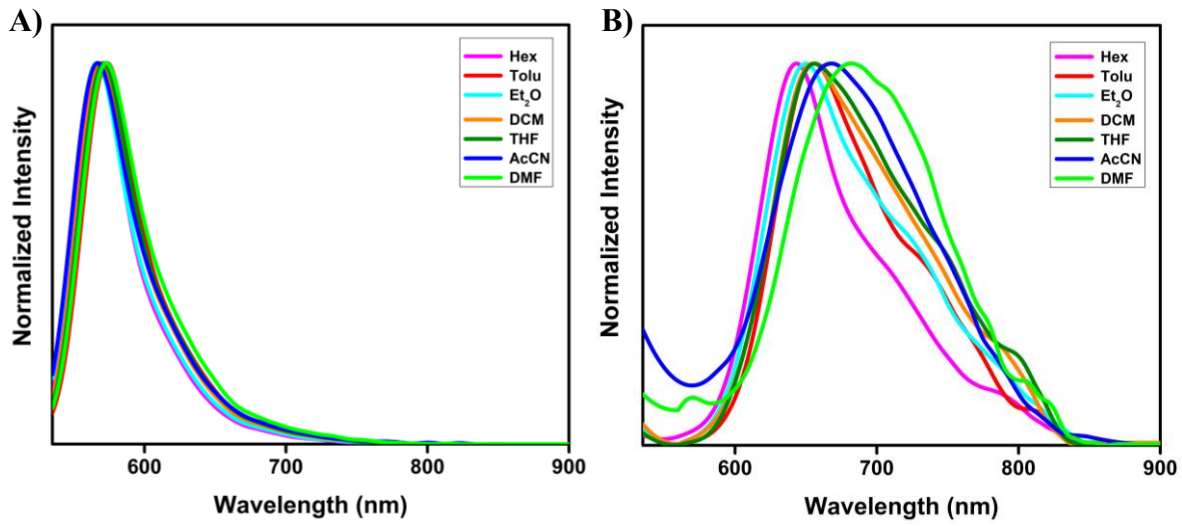

Figure S19. Normalized emission spectra of compounds **7** (A) and **8** (B) in solvents of different polarity (1.6 μM, λ_{ex} = 530 nm).

Compounds	**Solvents**	**λ_{max}[a] /ε (x 10^4)** (nm)	**λ_{max}[b]** (nm)	**Φ_F[c]**	**$\Delta\nu$[d]** (cm^{-1})
7	Hex	315 (1.1), 535 (1.9)	568	0.25	1086
	Toluene	325 (2.1), 539 (5.5)	574	0.18	1131
	Et_2O	330 (1.9), 532 (4.5)	567	0.20	1061
	DCM	329 (2.0), 535 (4.9)	570	0.19	1117
	THF	328 (1.3), 536 (3.4)	571	0.11	1144
	AcCN	325 (1.9), 531 (4.5)	567	0.09	1196
	DMF	331 (2.2), 537 (4.7)	572	0.13	1170
8	Hex	325 (2.2), 542 (3.7)	643	0.15	2898
	Toluene	363 (1.6), 550 (3.3)	656	0.09	2938
	Et_2O	359 (1.8), 544 (3.1)	650	0.07	2998
	DCM	361 (1.7), 543 (3.1)	655	0.04	3149
	THF	359 (1.6), 544 (3.2)	656	0.02	3138
	AcCN	362 (1.7), 539 (3.0)	668	0.01	3583
	DMF	364 (1.9), 547 (3.4)	682	0.01	3619

Table S1: Photophysical characteristics of compounds **7** and **8** in solvents of different polarity. [a] peak position of the absorption band maximum in nm. [b] peak position of the emission band maximum in nm. [c] Quantum yields were determined using Rhodamine B in ethanol as a standard (Φ_F = 0.49 in ethanol) and using the following formula $\phi = \phi_F \times I/I_R \times A_R/A \times \eta^2/\eta_R^2$ where ϕ = quantum yield, I = integral area of emission peak, A = absorbance at λ_{ex}, η = refractive index of solvent. [d] Stokes shift in cm^{-1}.

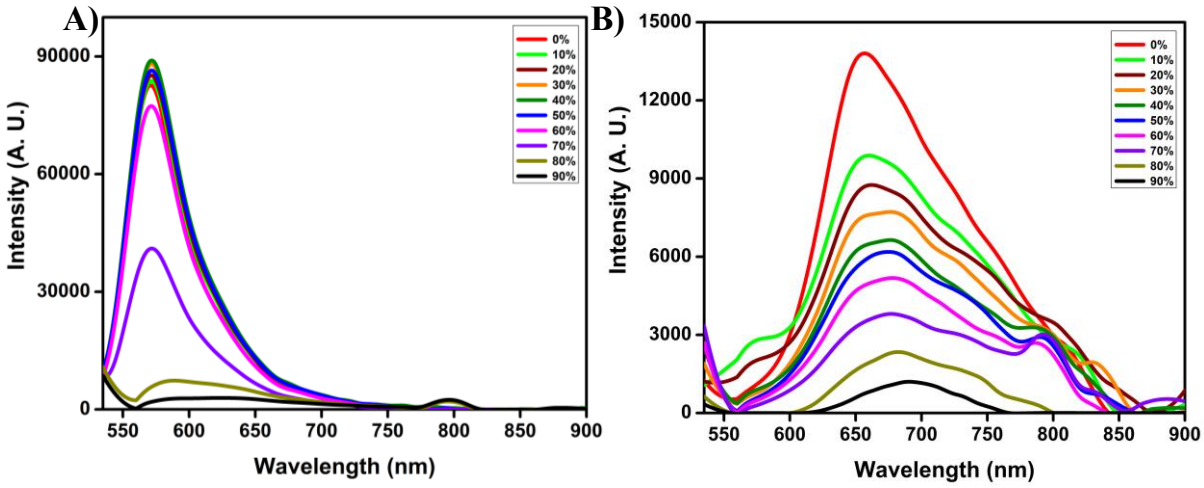

Figure S20. Emission spectra of compounds **7** (A) and **8** (B) in THF-water mixtures with increasing concentration of water from 0% to 90% (1.6 μM, λ_{ex} = 530 nm).

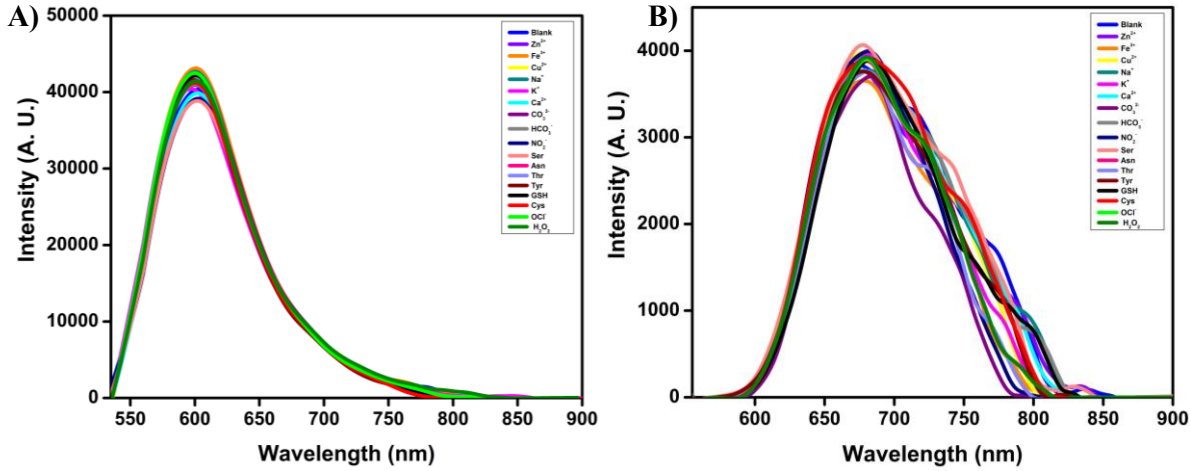

Figure S21. Emission spectra of compounds (A) **7** and (B) **8** in a mixture of THF/PBS (pH 7.4, 1 mM) (1:4 ratio) with various analytes (100 μM) (Blank, $Zn(OAc)_2$, $FeCl_3$, $Cu(OAc)_2$, NaCl, KCl, $CaCl_2$, K_2CO_3, $NaHCO_3$, $NaNO_2$, Ser, Asn, Thr, Tyr, GSH, Cys, OCl^-, and H_2O_2. (1.6 μM, λ_{ex} = 530 nm).

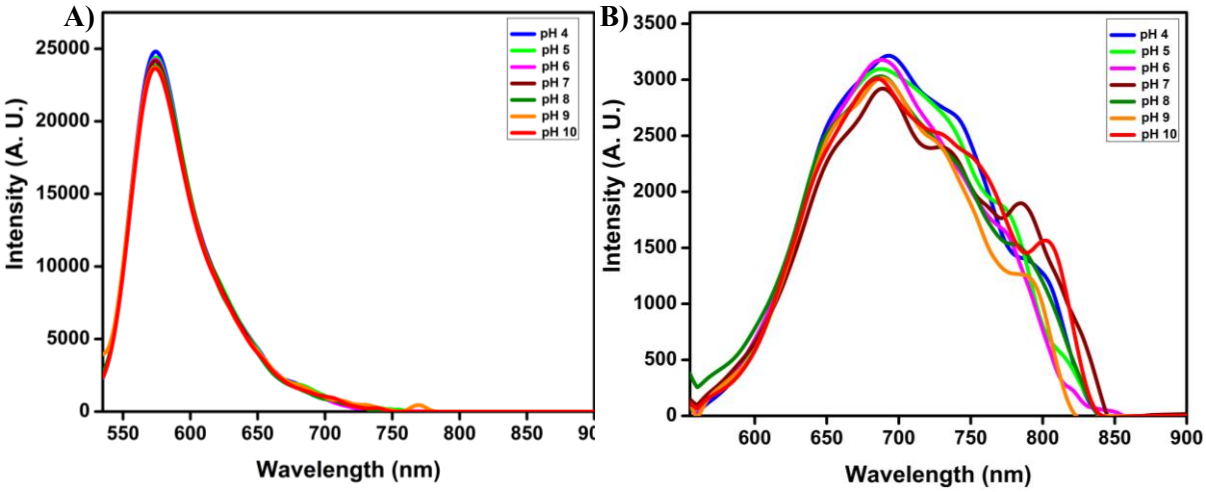

Figure S22. Fluorescence spectra of compound **7** (A) and **8** (B) at different pH values (4-10) in DMSO solvent. (1.6 μM, λ_{ex} = 530 nm)

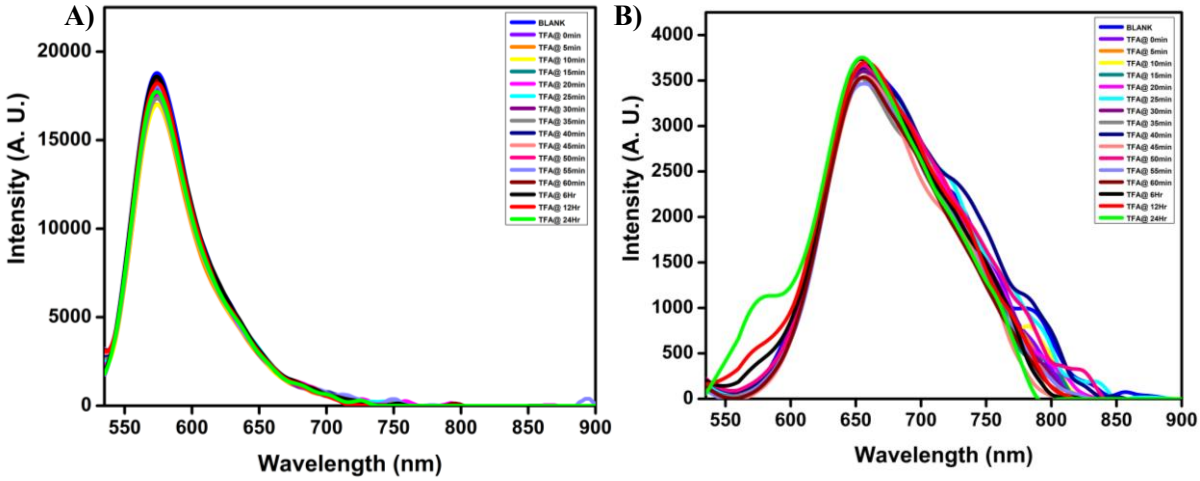

Figure S23. Emission spectra of compounds A) **7** and B) **8** in THF (1.6 μM, λ_{ex} = 530 nm) upon addition with 10 equivalents of TFA over a 24 h. Spectra were recorded at 5-min intervals from 0 to 60 minutes, followed by measurements at 6 h, 12 h, and 24 h.

5. DFT and TD-DFT Calculations

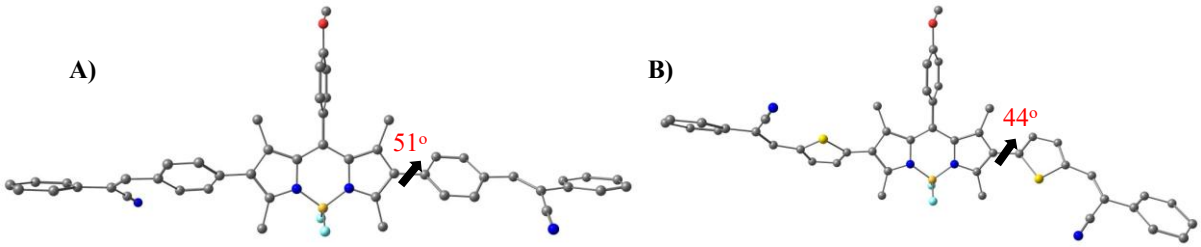

Figure S24. Optimized geometries of compounds **7** (A) and **8** (B).

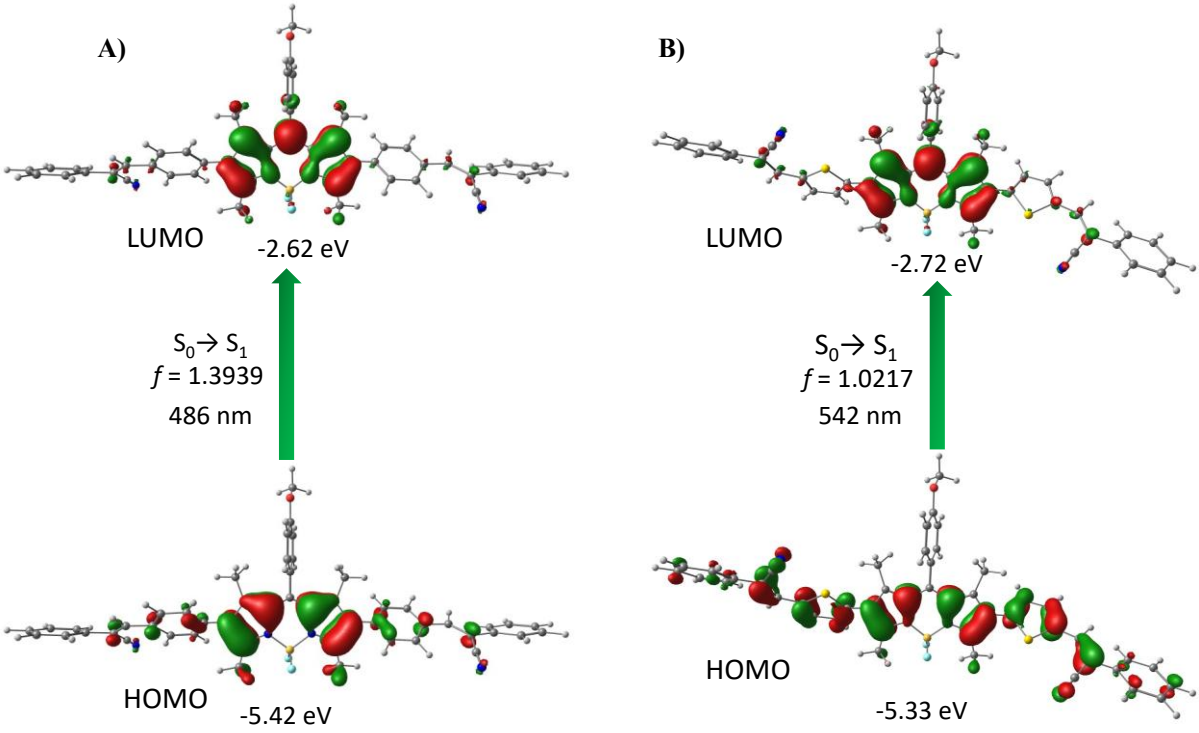

Figure S25. The frontier molecular orbitals (FMOs) involved in the vertical excitations of compounds **7** (A) and **8** (B).

6. Cytotoxicity Assay

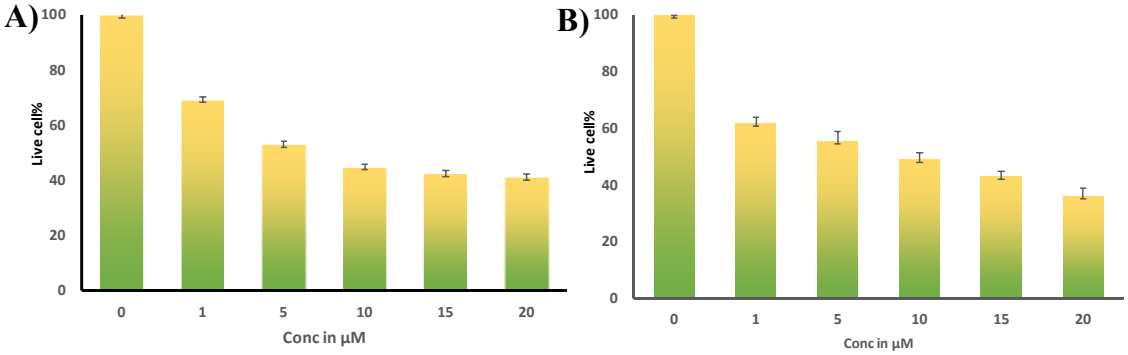

Figure S26. Cytotoxicity data of compounds **7** (A) and **8** (B) in HeLa cells.

Color-mixing prevention experiment:

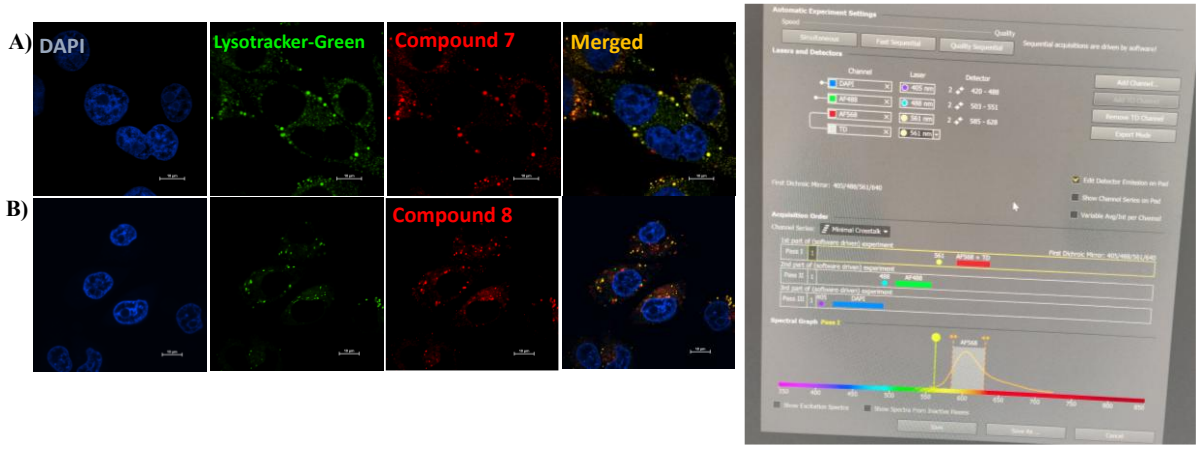

Figure S27. Confocal images of HeLa cells stained with 500 nM of Compound **7** (A), Compound **8** (B), 200 nM LysoTracker Green and 200 nM DAPI for 30 minutes, Scale bar, 10 µm and 3% laser power. To eliminate the possibility of color mixing, confocal images were acquired using a Nikon Ti2E AX R NSPARC confocal laser scanning microscope under sequential scanning mode with minimal laser power. This approach effectively prevents spectral bleed-through and ensures the accuracy of co-localization analysis.

7. Photostability Studies

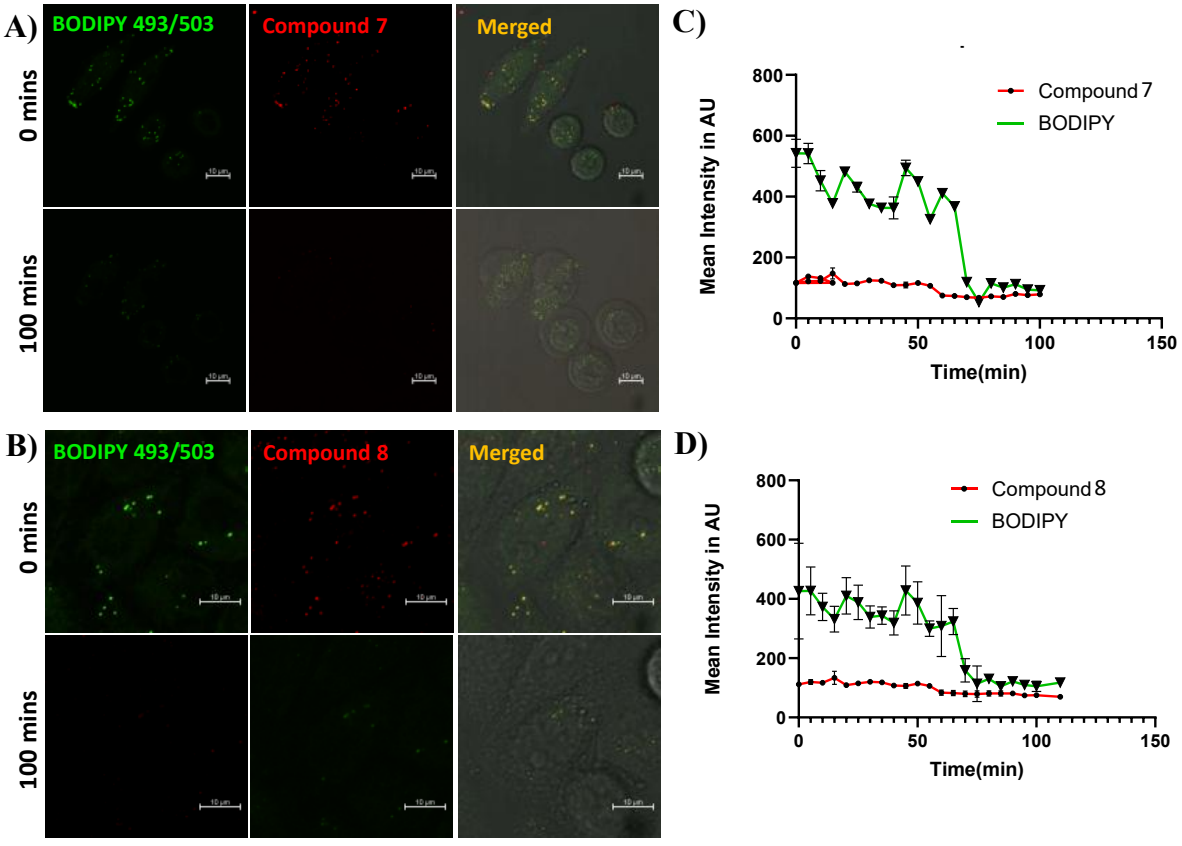

Figure S28. Photostability of probes. Confocal fluorescence microscopy images of HeLa cells incubated with compounds **7** (A) and **8** (B) (middle), commercially available BODIPY 493/503 (left), and the merged images (right) under continuous laser irradiation with laser (568 nm, 3% laser power) from 0 to 100 minutes until cell rupture occurred. The line graph shows mean fluorescence intensity of compounds **7** (C) and **8** (D) (± SEM, n = [insert n]) over time. Cells were pre-incubated with 500 nM of target probes and 200 nM BODIPY 493/503 for 30 min prior to imaging. Scale bar, 10 µm.

8. DFT Coordinates

Table S2: The coordinates of compounds 7 (DFT/B3LYP/6-31G(d) level with Gaussian 09)

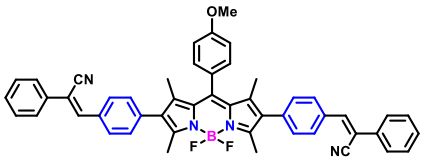

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Table S3: The coordinates of compounds 8 (DFT/B3LYP/6-31G(d) level with Gaussian 09)

6	-2.165944774	-1.892718956	-1 .042620651
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9	0.618534770	-2.915671570	-0.166272669
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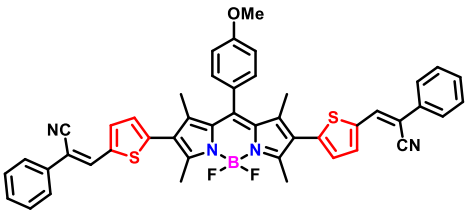

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3. M. Wang, Y. Zhang, T. Wang, C. Wang, D. Xue, and J. Xiao, *Org. Lett*., 2016, **18**, 1976–1979.
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