Supporting Information for

Lysosome-Targeting 'turn-on' red/NIR BODIPY probes for hypoxic cells imaging

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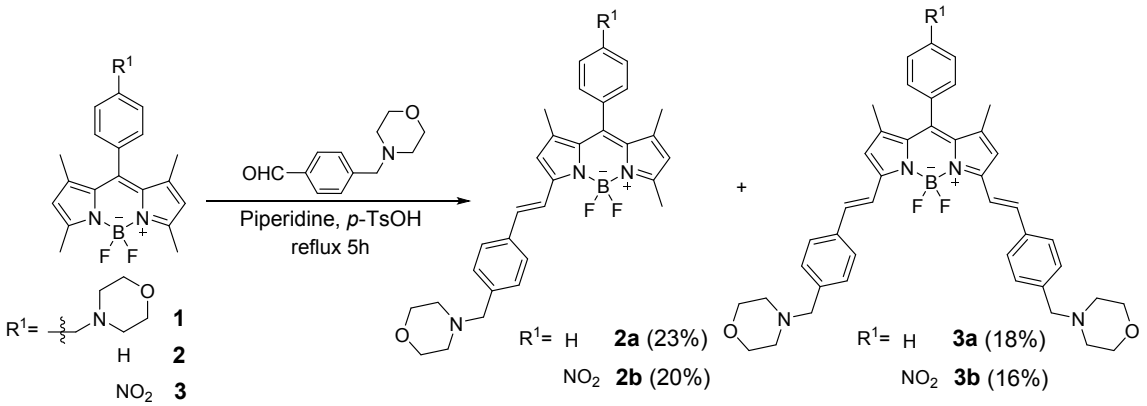
I. Experimental Section

I.1 Materials and instrumentations

All reagents were obtained from commercial suppliers and used without further purification unless otherwise indicated. All air and moisture-sensitive reactions were carried out under nitrogen atmosphere in oven-dried glassware. Glassware was dried in an oven at 120 °C and cooled under a stream of inert gas before use. Dichloromethane and triethylamine were distilled over calcium hydride. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX400 spectrometer and DRX500 spectrometer, and referenced to the residual proton signals of deuterated solvents. HR-MS were recorded on a Bruker Daltonics microTOF-Q II spectrometer. All the solvents employed for the spectroscopic measurements were of UV spectroscopic grade (Aldrich). Fluorescence imaging of cells was performed on a confocal laser scanning microscope (CLSM, LSM700, Zeiss, Germany). All images were digitized and analyzed by a ZEN imaging software.

I.2 Synthesis and characterization

1.2.1. Synthesis



Scheme S1. Synthesis of compound **1**, **2a**, **2b**, **3a** and **3b**.

2, 4-dimethylpyrrole (0.5 mL, 4.5 mmol) and 4-(morpholinomethyl)benzaldehyde (462 mg, 2.25 mmol) were dissolved in dry CH_2Cl_2 (50 mL) under nitrogen. One drop of trifluoroacetic acid (TFA) was added, and the solution was stirred for 5h at ambient temperature in the dark. 2,3-Dichloro-5,6-dicyanoquinone (DDQ, 0.5 g, 2.20 mmol) was added, and the mixture was stirred for an additional 30 min. The reaction mixture was then treated with triethylamine (1.5 mL) for 5 min. Boron trifluoride etherate (2.3 mL) was added and stirred for another 40 min, and the dark-brown solution was washed with water (2 × 20 mL) and brine (30 mL), dried over anhydrous magnesium sulfate, and concentrated at reduced pressure. The crude product was purified by silica-gel flash column chromatography (eluent: hexane/ethyl acetate= 4/1) to yield product as crystals in 26% yield (243 mg). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* =8.0 Hz, 2 H), 7.24 (d, *J* =8.0 Hz, 2 H), 5.97 (s, 2 H), 3.76 (s, 4 H), 3.65 (s, 2 H), 2.55 (s, 6 H), 2.52 (s, 4 H), 1.37 (s, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.4, 143.0, 141.7, 138.7, 133.9, 131.5, 129.8, 127.9, 121.2, 66.9, 63.1, 53.6, 14.6, 14.3; HRMS-ESI: m/z: calcd $[C_{24}H_{28}BF_2N_3O+H]^+$ m/z=424.2371; found m/z=424.2381, $[M+H]^+$.

Compound **2** was obtained as red solid in 35% yield by following a procedure similar to that of **1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (m, 3 H), 7.30 – 7.27 (m, 2 H), 5.98 (s, 2 H), 2.56 (s, 6 H), 1.37 (s, 6 H). HRMS-ESI: m/z: calcd$[C_{19}H_{19}BF_2N_2+Na]^+$ m/z=347.1505; found m/z=347.1517, $[M+Na]^+$.

Compound **3** was obtained as brick red solid in 31% yield by following a procedure similar to that of **1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.39 (d, *J* = 8.0 Hz, 2 H), 7.54 (d,

J = 8.0 Hz, 2 H), 6.02 (s, 2 H), 2.56 (s, 6 H), 1.36 (s, 6 H). HRMS-ESI: m/z: calcd[$C_{19}H_{18}BF_2N_3O_2$+H]$^+$ m/z=370.1536; found m/z=370.1554, [M+H]$^+$.

Synthesis of compound **2a** and **3a**

A solution of **2** (162 mg, 0.5 mmol), 4-(morpholinomethyl)benzaldehyde (153.8 mg, 0.75 mmol) and a crystal of *p*-TsOH in a mixture of toluene (50 mL) and piperidine (1 mL) was placed in a round bottom flask equipped with a Dean Stark trap, the mixture was heated at its boiling point until it had evaporated to dryness. The resulting solid was dissolved in dichloromethane and washed with water three times. The organic phase was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure, and the resulting crude residue was purified by silica-gel flash column chromatography (15% ethyl acetate/hexane) and recrystallized from CH_2Cl_2/Hexane to provide **2a** as a purple solid (59 mg, 23%) and the other product **3a** as dark blue solid in 18% yield.

2a: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 16.0 Hz, 1 H), 7.55 (d, *J* = 8.0 Hz, 2 H), 7.49 (m, 3 H), 7.35 (d, *J* = 8.0 Hz, 2 H), 7.30 (d, *J* = 4.0 Hz, 2 H), 7.22 (d, *J* = 16.0 Hz, 1 H), 6.60 (s, 1 H), 6.02 (s, 1 H), 3.75 (t, *J* = 4.0 Hz, 4 H), 3.57 (s, 2 H), 2.60 (s, 3 H), 2.50 (s, 4 H), 1.43 (s, 3 H), 1.39 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 151.6, 141.2, 138.0, 137.7, 134.9, 134.6, 134.1, 132.4, 128.6, 128.1, 128.0, 127.4, 126.5, 118.1, 116.8, 66.0, 62.1, 59.4, 52.6, 28.7, 20.0, 13.6, 13.2. HRMS-ESI: m/z: calcd[$C_{31}H_{32}BF_2N_3O$+H]$^+$m/z=512.2685; found m/z=512.2699, [M+H]$^+$.

3a: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J* = 16.0 Hz, 2 H), 7.59 (d, *J* = 8.0 Hz, 4 H), 7.50 (m, 3 H), 7.39 (d, *J* = 8.0 Hz, 4 H), 7.33 (d, *J* = 8.0 Hz, 2 H), 7.23 (d, *J* =

16.0 Hz, 2 H), 6.64 (s, 2 H), 3.76 (t, *J* = 4.0 Hz, 8 H), 3.58 (s, 4 H), 2.52 (s, 8 H), 1.45 (s, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 154.6, 151.5, 142.0, 141.4, 139.5, 134.8, 134.7, 134.6, 134.0, 131.7, 130.9, 128.6, 128.1, 128.0, 127.2, 127.1, 126.4, 124.5, 120.4, 118.0, 116.5, 65.8, 52.5, 33.2, 29.3, 28.7, 20.1, 13.7, 13.5, 13.4, 13.1. HRMS-ESI: m/z: calcd$[C_{43}H_{45}BF_2N_4O_2+H]^+$ m/z=699.3684; found m/z=699.3697, $[M+H]^+$.

Compound **2b** and **3b** were obtained by following a procedure similar to that of **2a** and **3a**.

2b: 20% yield, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (d, *J* =8.0 Hz, 2 H), 7.66 (d, *J* = 16.0 Hz, 1H), 7.57 (d, *J* = 4.0 Hz, 2H), 7.55 (d, *J* = 4.0 Hz, 2H), 7.36 (d, *J* = 8.0 Hz, 2 H), 7.25 (s, 1 H), 6.64 (s, 1 H), 6.05 (s, 1 H), 3.74 (s, 4 H), 3.55 (s, 2 H), 2.61 (s, 3 H), 2.48 (s, 4 H), 1.42 (s, 3 H), 1.38 (s, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.6, 153.2, 148.2, 142.4, 142.0, 141.8, 137.0, 135.8, 132.0, 131.1, 129.9, 124.4, 122.0, 118.2, 66.4, 62.8, 53.3, 30.0, 22.7, 15.0, 14.8, 14.1. HRMS-ESI: m/z: calcd$[C_{31}H_{31}BF_2N_4O_3+H]^+$ m/z=557.2536; found m/z=557.2545, $[M+H]^+$.

3b: 16% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (d, *J* = 12.0 Hz, 2 H), 7.72 (d, *J* = 16.0 Hz, 2 H), 7.60 (d, *J* = 4.0 Hz, 3 H), 7.58 (d, *J* = 4.0 Hz, 3 H), 7.39 (d, *J* = 8.0 Hz, 4 H), 7.28 (s, 2 H), 6.67 (s, 2 H), 3.75 (t, *J* = 4.0 Hz, 8 H), 3.56 (s, 4 H), 2.50 (s, 8 H), 1.44 (s, 6 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.4, 147.4, 141.1, 140.4, 135.8, 134.6, 131.6, 129.1, 128.8, 126.6, 123.3, 117.9, 117.4, 65.8,62.0, 52.5, 30.9, 28.9, 28.7, 28.4, 21.7, 14.0, 13.1. HRMS-ESI: m/z: calcd$[C_{43}H_{44}BF_2N_5O_4-F]^+$ m/z=725.3549; found m/z=725.3557, $[M-F]^+$.

1.2.2 Spectroscopic Measurements

Samples for absorption and emission measurements were contained in 1 cm × 1 cm quartz cuvettes. For all measurement, the temperature was kept constant at (298 ± 2) K. Dilute solution with an absorbance of less than 0.05 at the excited wavelength was used for the measurement of fluorescent quantum yields, The luminescence quantum yields in solution were measured by using fluorescein for **1** with excited wavelength 460 nm (Φ_F = 0.95 in 0.1M NaOH); methylene blue for **2b**, **3a**, **3b** with excited wavelength 530, 590, 600 nm, respectively (Φ_F = 0.03 in methanol); Rhodamin 6G for **2a** with excited wavelength 510 nm (Φ_F = 0.88 in ethanol) as reference. The quantum yield Φ as a function solvent polarity is calculated using the following equation.

$$\Phi_{sample}=\Phi_{std}\left[\frac{I\,sample}{I\,std}\right]\left[\frac{A\,std}{A\,\text{sample}}\right]\left[\frac{n\,sample}{n\,std}\right]^2 \quad (1)$$

Where subscript sample and std denote the sample and standard, respectively, Φ is quantum yield, I is the integrated emission intensity, A stands for the absorbance, n is refractive index.

The fluorescence lifetimes of the samples were measured on Horiba Jobin Yvon Fluorolog-3 spectrofluorimeter. The goodness of the fit of the single decays as judged by reduced chi-squared (χ2R) and autocorrelation function C(j) of the residuals was below χ2R <1.2.

When the fluorescence decays were monoexponential, the rate constants of radiative (kf) and nonradiative (knr) deactivation were calculated from the measured

fluorescence quantum yield (Φ_f) and fluorescence lifetime (τ) according to eqs 2 and 3:

$$k_f = \Phi_f/\tau \quad (2)$$

$$k_{nr} = (1-\Phi_f)/\tau \quad (3)$$

1.2.3 Computational details

The ground state structures of dyes were optimized using the density functional theory (DFT) method with the B3LYP functional and 6-31G(d) basis sets. The absorption properties were predicted by time dependent (TD-DFT) method by using the B3LYP functional with the same basis sets. All of the calculations were performed with the Gaussian09 program package.[S1]

1.2.4 Cell Culture

Human liver hepatocellular HepG2 was obtained from KeyGEN Biotech Co., Ltd (Nanjing, China). HepG2 cells were cultured in Dulbecco′s modified Eagle medium (DMEM) containing 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells were conditioned at 37 °C in a humidified atomosphere with 5% CO2 and 95% air. The medium was replenished every day and the cells were subcultured after reaching confluence.

1.2.5 HepG2 Cells Culture and Hypoxic Imaging

The requisite density of HepG2 cells were seeded into the imaging dish (35mm glass-bottom culture dishes). In the next 12 h, the cells were incubated under different oxygen level environments (the normoxic condition: 20% O_2; and the hypoxic condition: 1% O_2). Then the probe was added to the HepG2 cells, further incubated for another 20 min under respective conditions. After washing with PBS buffer (pH = 7.4) for three times, and the fluorescence images were acquired through a confocal

laser scanning microscope. Hoechst 33342 was excited at 405 nm with a violet laser diode and the emission was collected from 420 to 500 nm. LysoTracker Green and MitoTracker Green were excited at 488 nm with an argon ion laser, and the emission was collected from 505 to 535 nm. Compound **1** was excited at 445 nm, and the emission was collected from 480 to 545 nm. Compound **2a** and **2b** were excited at 555 nm, and the emission was collected from 565 to 600 nm. Compound **3a** and **3b** were excited at 633 nm, and the emission was collected from 640 to 675 nm.

1.2.6 Biocompatibility Assay

HepG2 cells growing in log phase were seeded into 96-well plates at a seeding density of 1×10^4 cells per well in 200 μL complete medium and allowed to attach for 24 h. After rinsing with PBS, HepG2 cells were incubated with 200 μL culture media containing serial concentrations of Probe **2a** or Probe **2b** for 12 h. Then 20 μL 5 mg/mL thiazolyl tetrazolium (MTT) was added to each well and followed by incubated for 4 h. Afterwards, the supernatants containing unreacted MTT were discarded carefully, and 150 μL DMSO was added to each well to dissolve the produced blue formazan. The absorbance was recorded at 490 nm using a microplate reader after 10 min of shaking. Cell viability was determined by the following formula: Cell viability (%) = ((OD value of treatment group – OD value of blank group)/ (OD value of control group – OD value of blank group)) × 100.

I.3 Spectroscopic measurements.

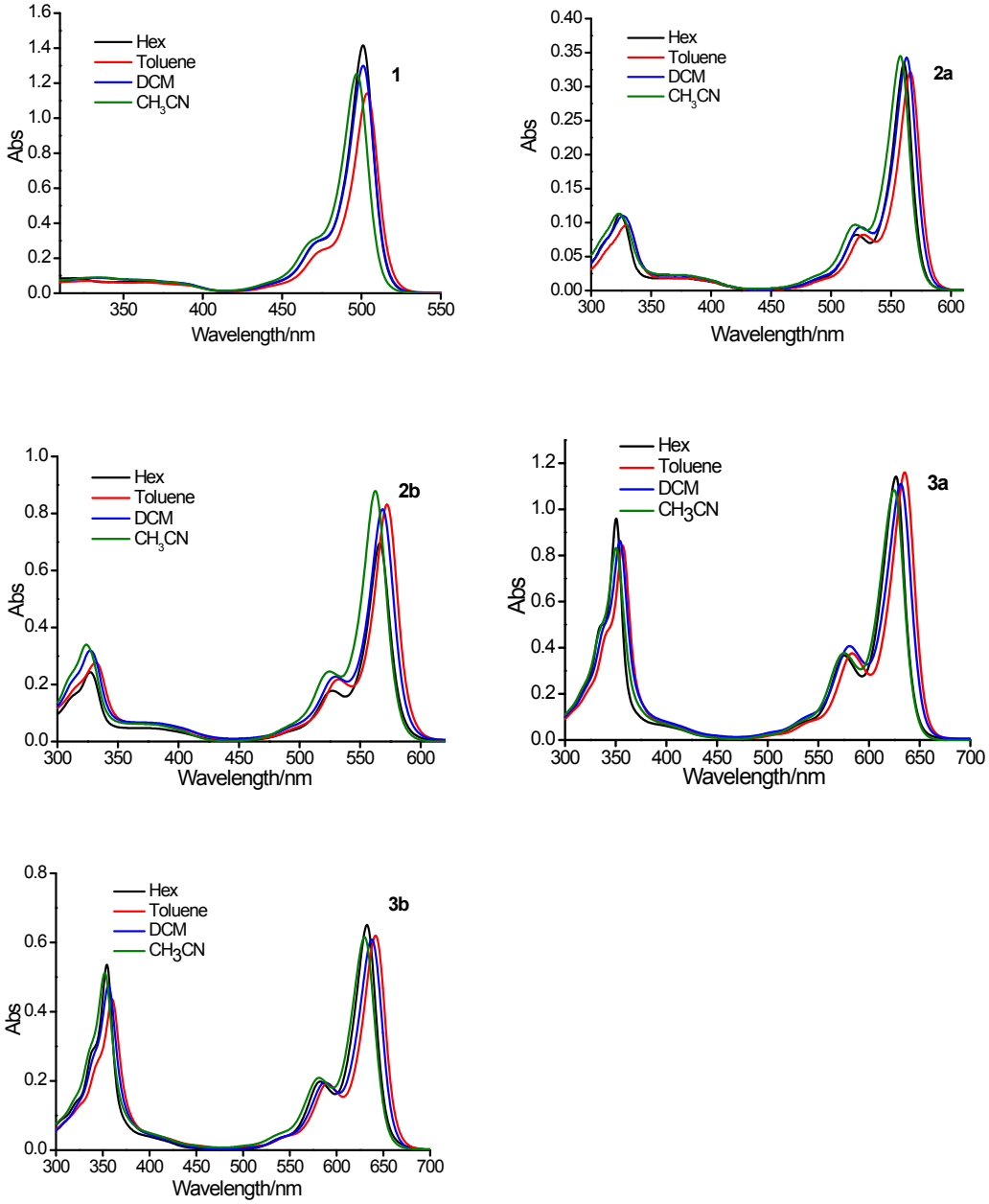


Figure S1. Absorption spectra of **1**, **2a, 2b, 3a, 3b** in kind of solvents with different polarity.

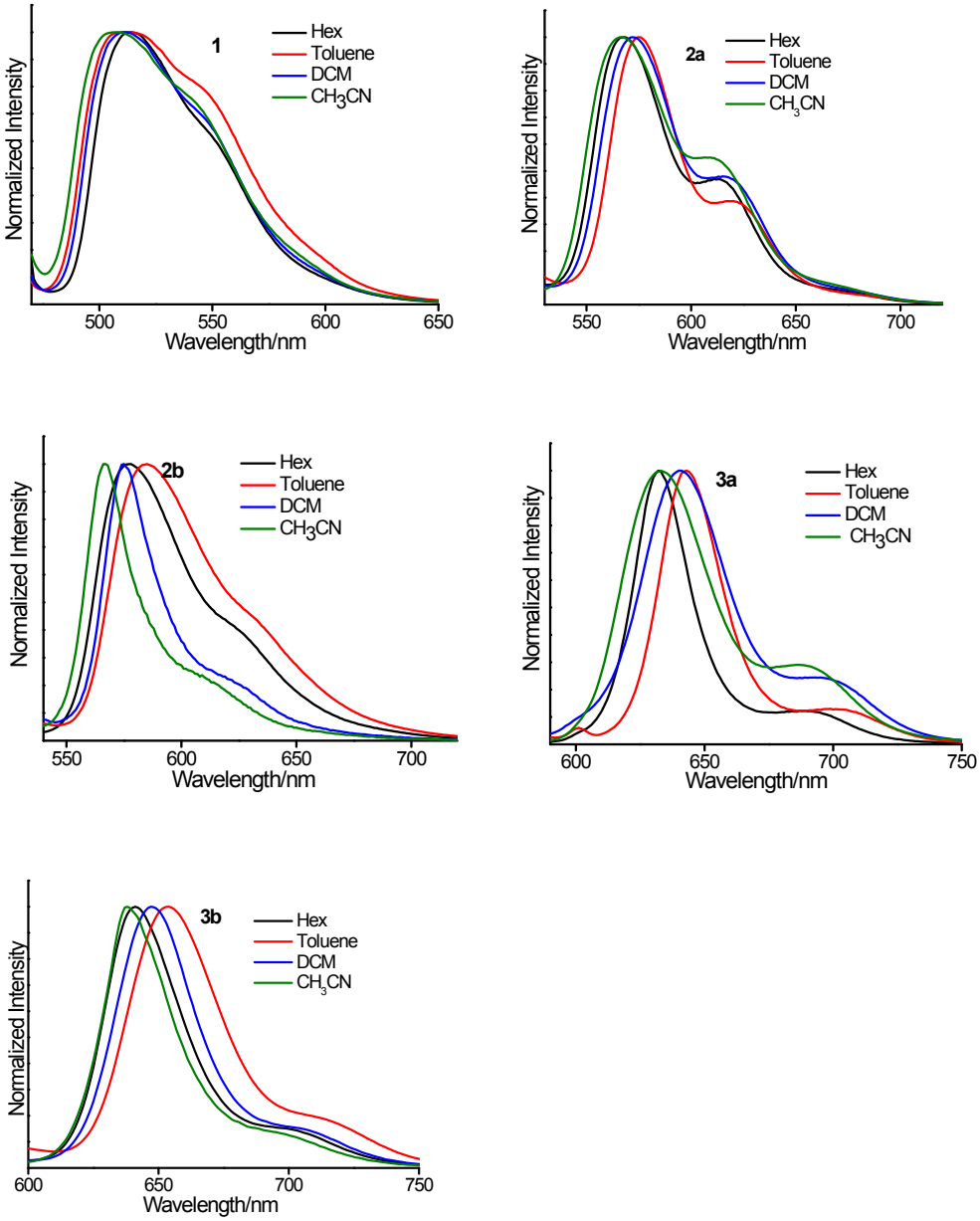


Figure S2. Emission spectra of **1**, **2a, 2b, 3a, 3b** in kind of solvents with different polarity.

I.4 DFT calculations.

Table S1. Lowest-energy absorption wavelengths (λ) and oscillator strengths (f) calculated at the B3LYP/6-31G(d) level

Compound	State [a]	Energy [eV]	λ [nm]	f [b]	Wave function [c]
1	S_2	3.03	409	0.46	H→L (94%)
2a	S_1	2.54	488	1.04	H→L (99%)
3a	S_1	2.14	581	0.78	H→L (98%)
2b	S_1	2.15	578	<0.01	H→L+1 (96%), H→L (3%)
	S_2	2.48	499	0.48	H-1→L (49%), H→L (49%)
	S_3	2.54	488	0.61	H-1→L (50%), H→L (48%)
amino-2b	S_1	2.54	487	1.06	H→L (100%)
3b	S_1	1.96	634	0.05	H→L (23%), H→L+1 (76%)
	S_2	2.12	585	0.72	H→L (77%), H→L+1 (23%)
amino-3b	S_1	2.14	580	0.78	H→L (98%)

[a] Excited state. [b] Oscillator strength. [c] MOs involved in the transitions; H and L means HOMO and LUMO of the system, respectively.

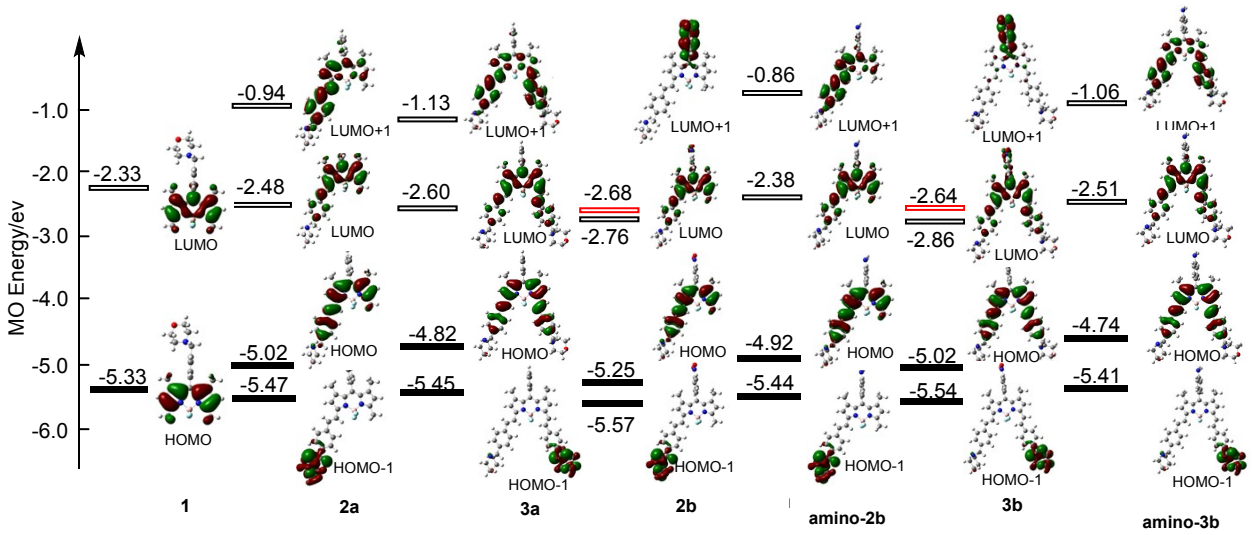


Figure S3. Frontier MOs and their energy levels of morpholinyl BODIPY **1**, **2a**-**2b, 3a**-**3b** and reduction **amino-2b**, **amino-3b**. Calculations were performed at the B3LYP/6-31G(d) level.

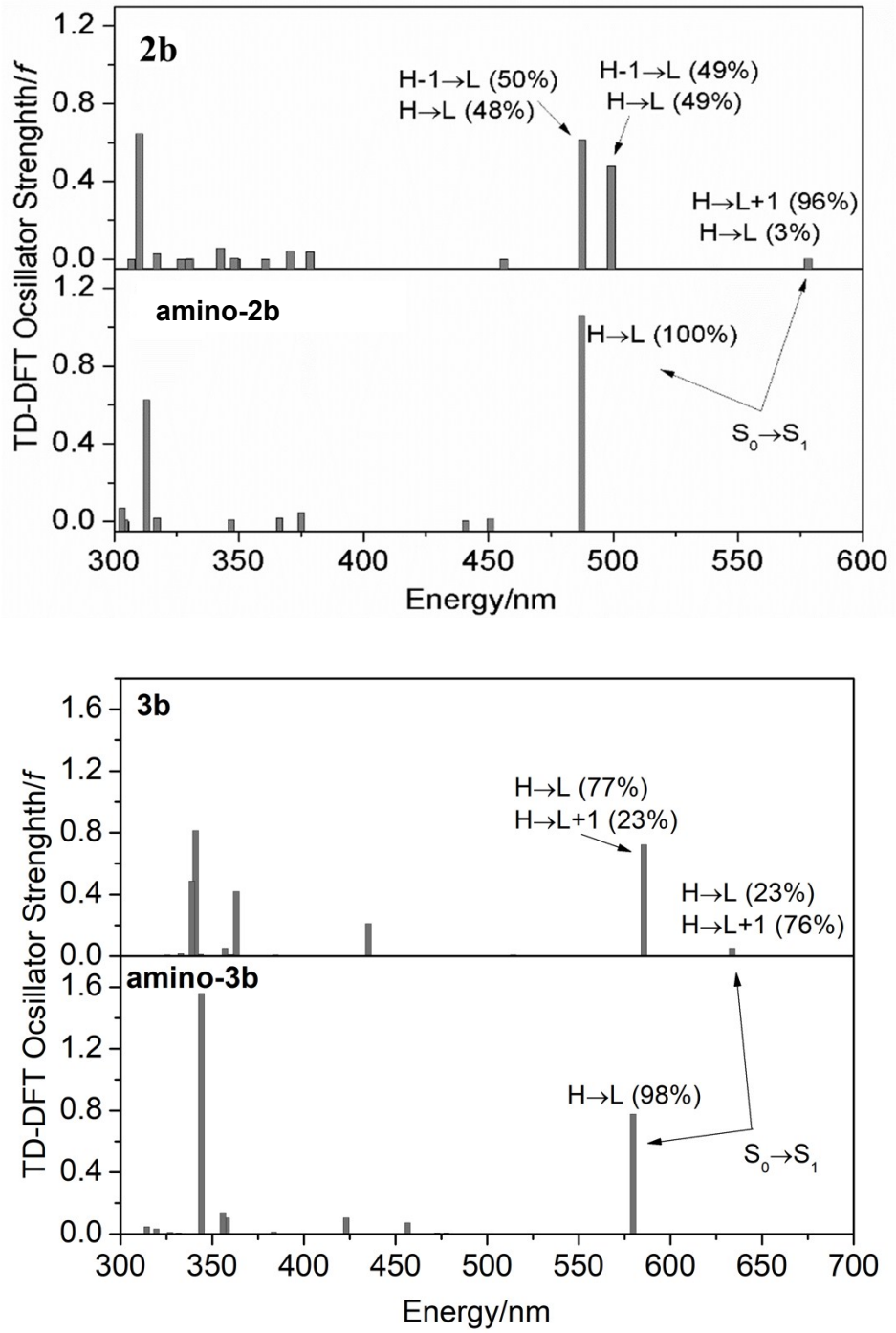


Figure S4. TD-DFT spectra of **2b, 3b** and their reduction **amino-2b**, **amino-3b** based on a DFT geometry optimization using the B3LYP functional with 6-31G(d)

1.5 Cell Culture, Confocal Imaging and Biocompatibility Assay

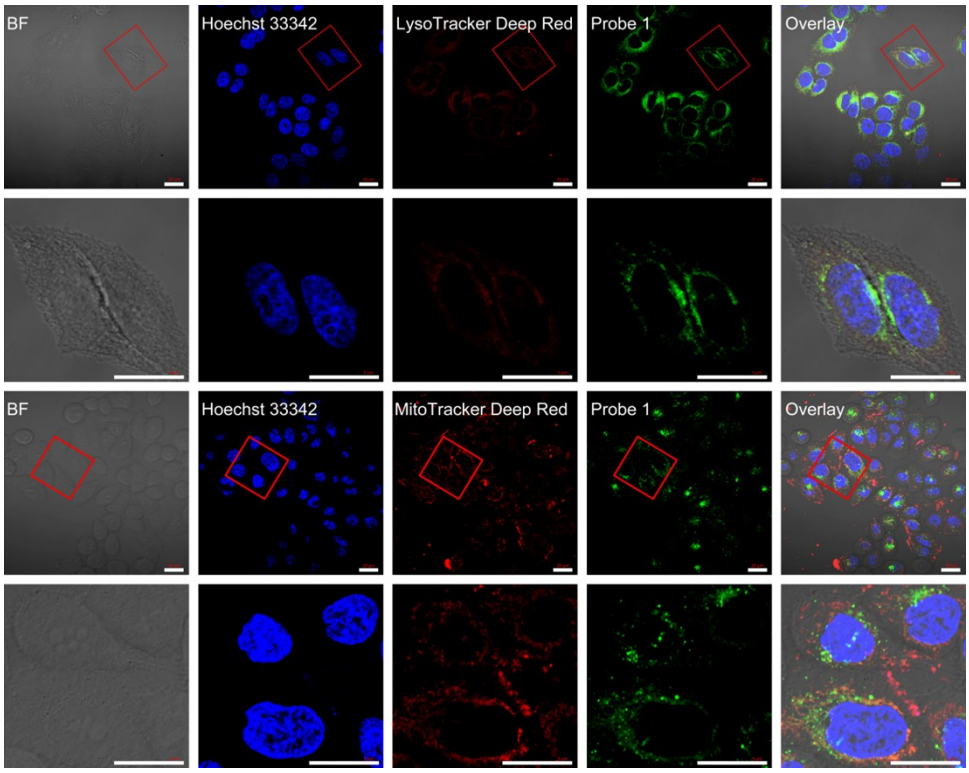


Figure S5. Confocal fluorescence and bright field (BF) images of HepG2 cells incubated with Probe **1** and then stained with Hoechst 33342 and LysoTracker Deep Red or MitoTracker Deep Red. Overlay is the merged image of the corresponding fluorescence and BF images. Scale bars: 20 μm.

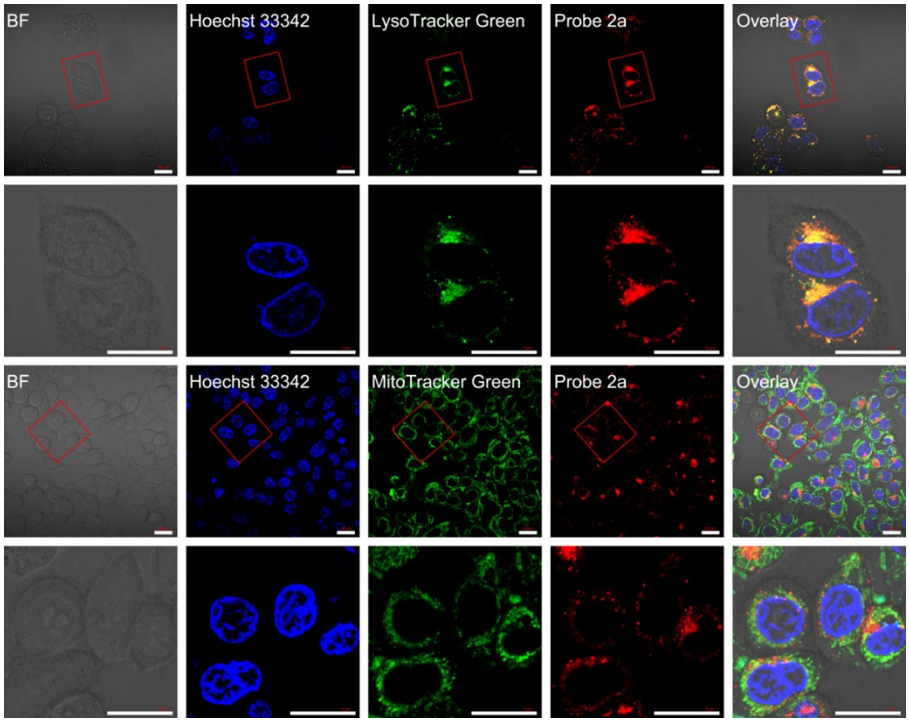


Figure S6. Confocal fluorescence and BF images of HepG2 cells incubated with Probe **2a** and then stained with Hoechst 33342 and LysoTracker Green or MitoTracker Green. Scale bars: 20 μm.

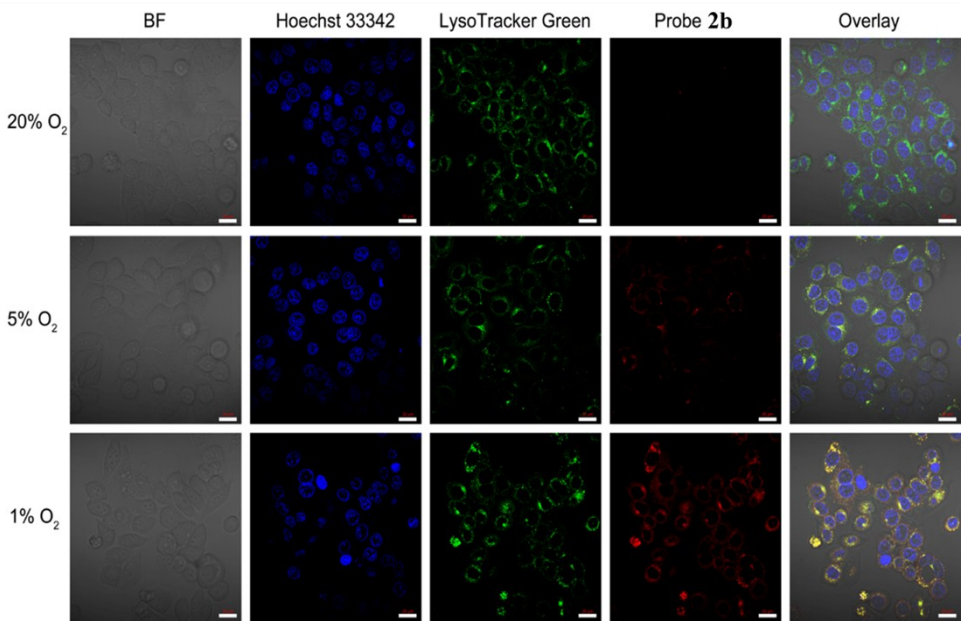


Figure S7. Confocal fluorescence and BF images of HepG2 cells incubated with 10 μM Probe **2b** at 37 °C under different oxygen concentrations and then stained with Hoechst 33342 and LysoTracker Green. Scale bars: 20 μm.

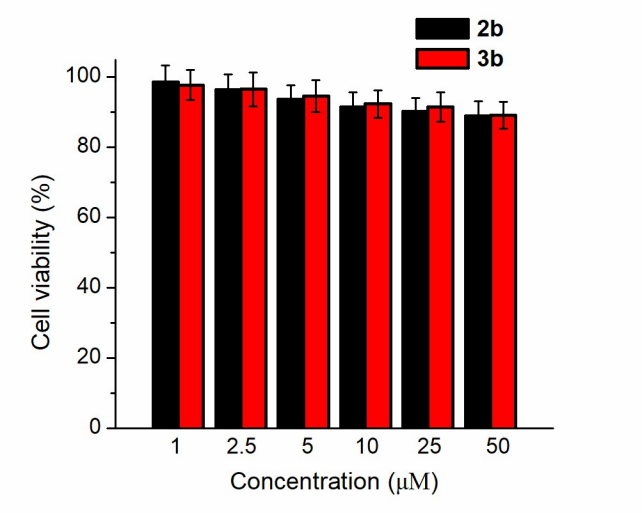


Figure S8. MTT assay for HepG2 cells after treatment with different concentrations of Probes **2b** and **3b**. Data are means ± SD (n=6).

1.6 Photostability

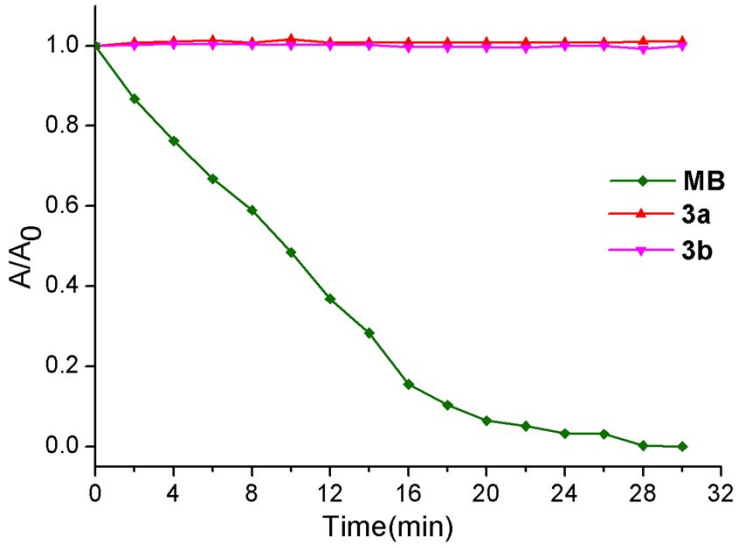


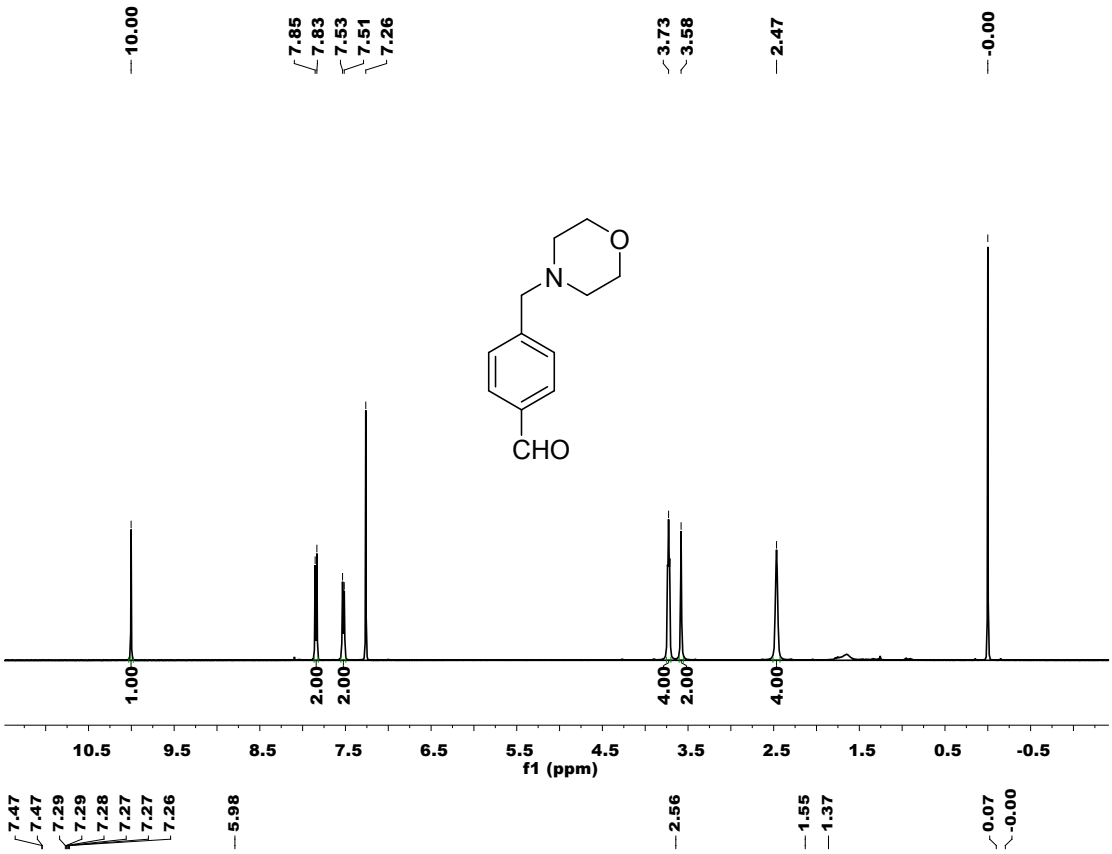
Figure S9. Photostability of **3a**, **3b** and **Methylene Blue** (MB) in dichloromethane at 2 min intervals determined using a laser beam (635 nm, 350 mW cm^{-2}) over an irradiation period of 30 min (c = 5.0 μM, 298 K).

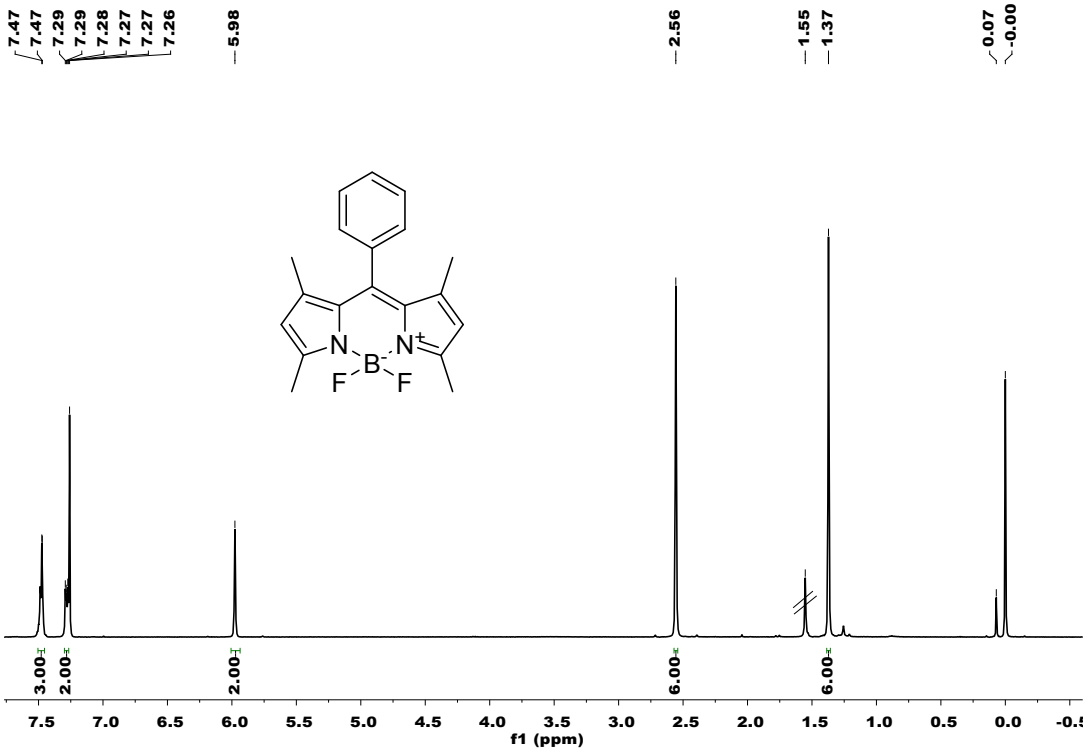
II. References

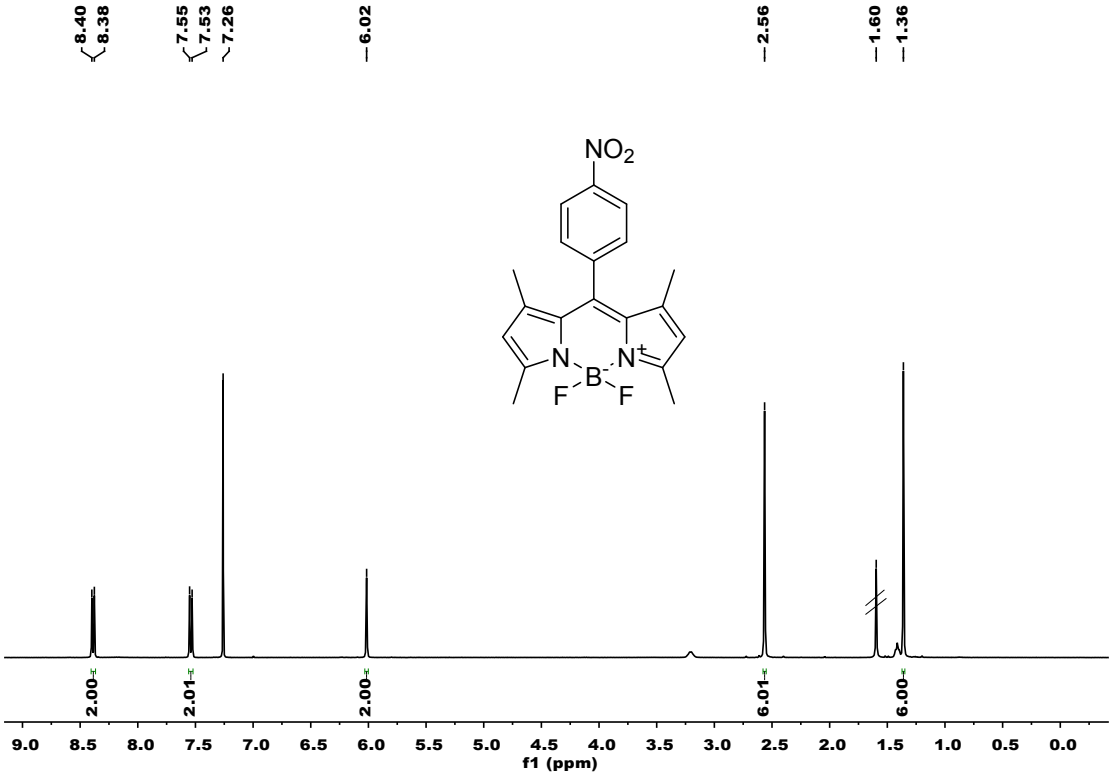
S1 Gaussian 09, Revision C.01, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, and D. J. Fox, Gaussian, Inc., Wallingford CT, **2009**.

III. NMR and HRMS spectra

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



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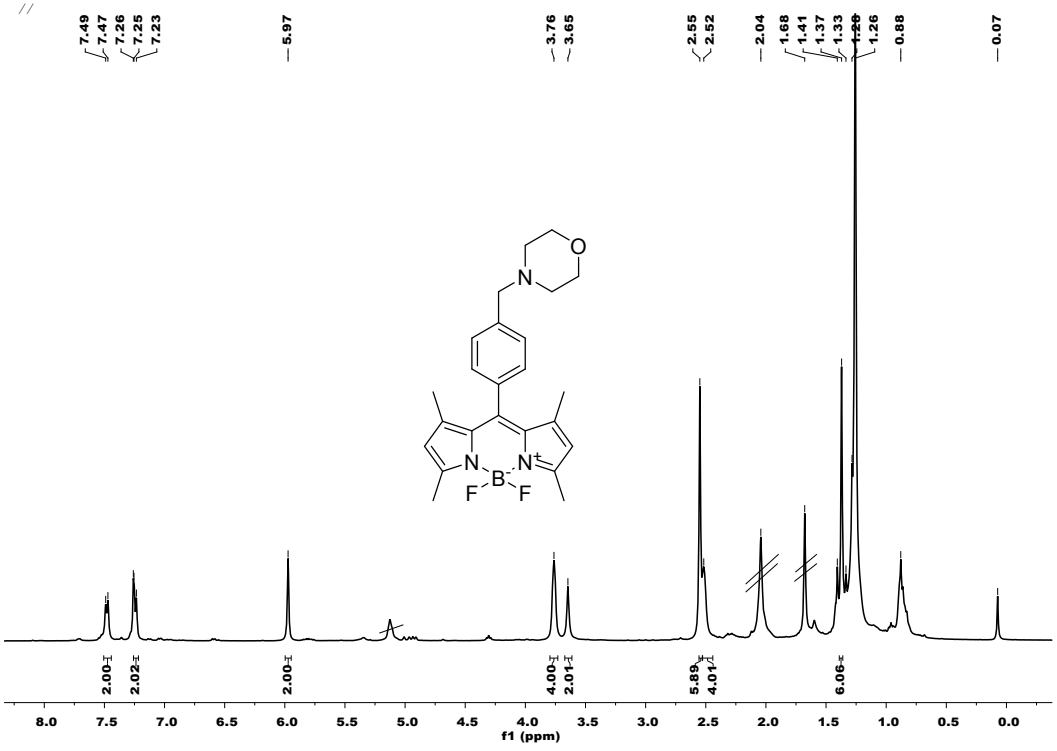
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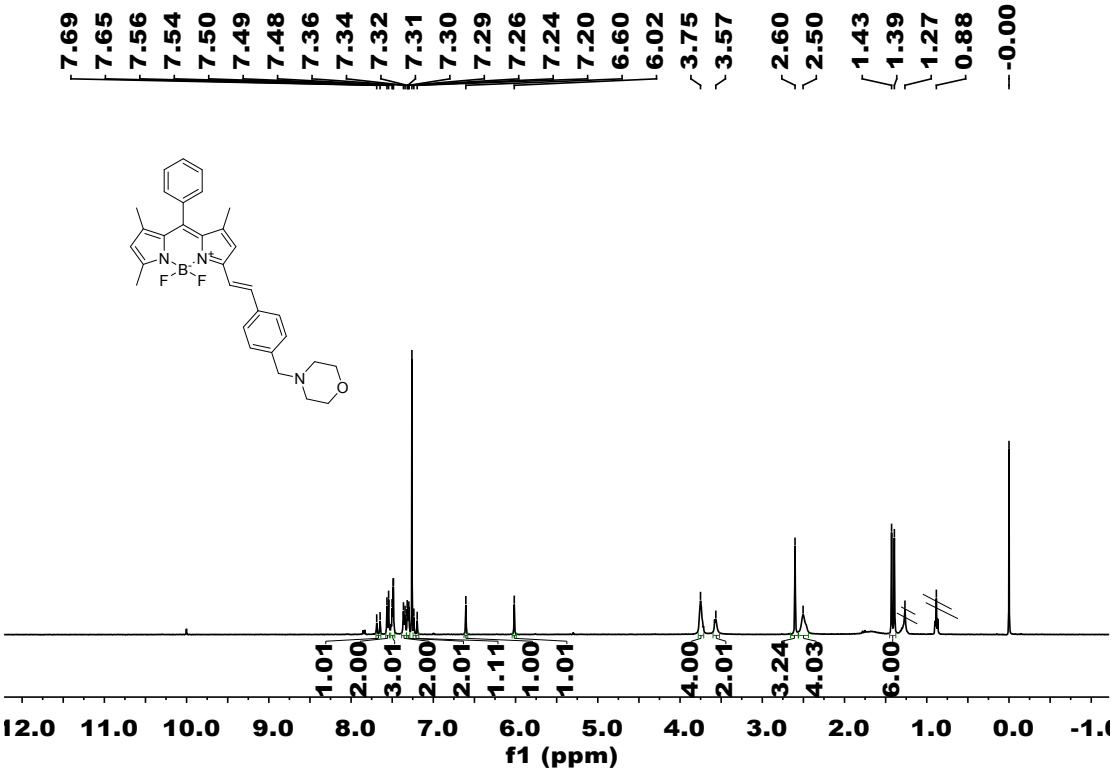
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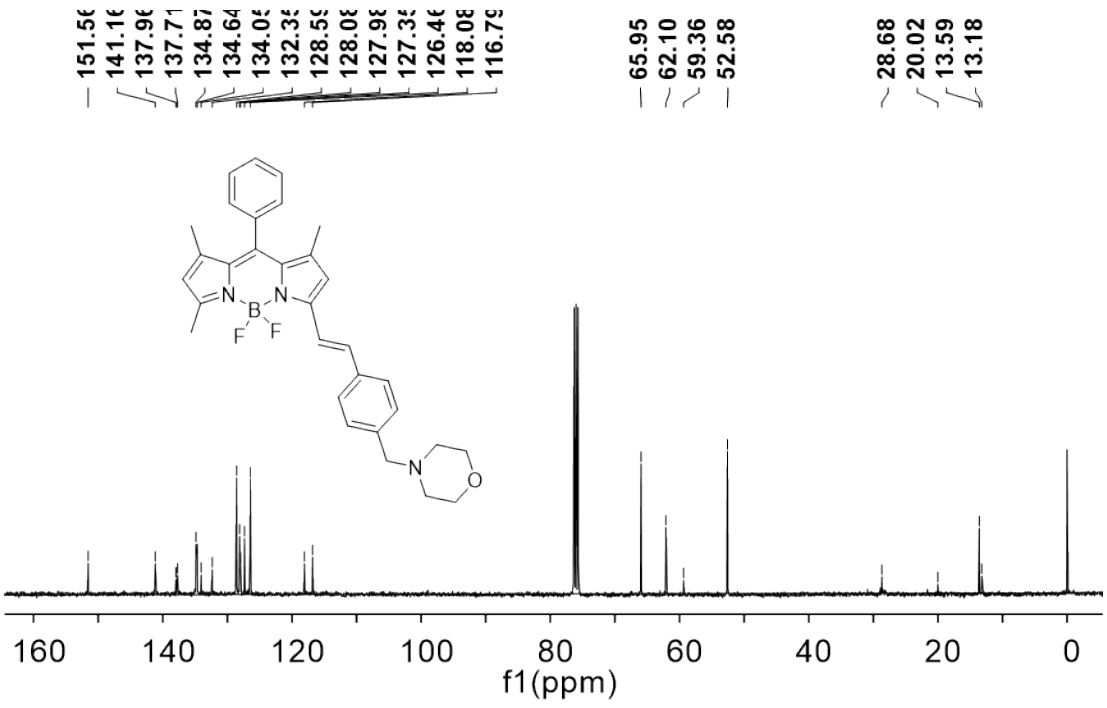
f1 (ppm)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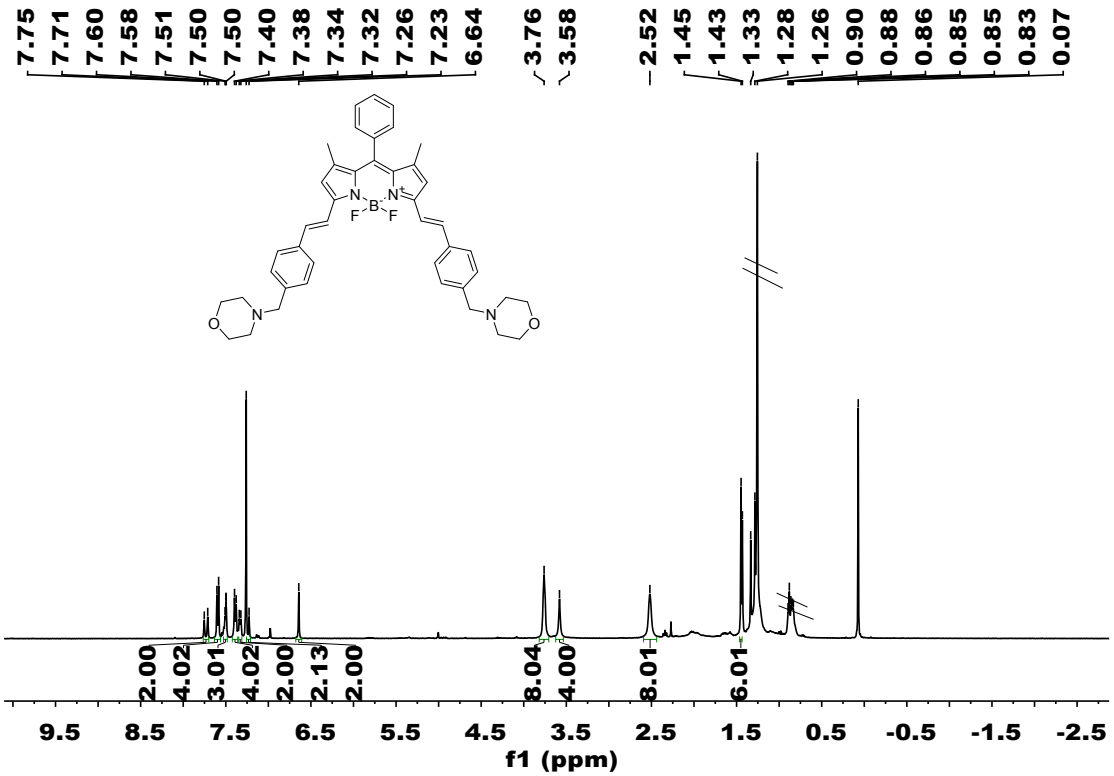
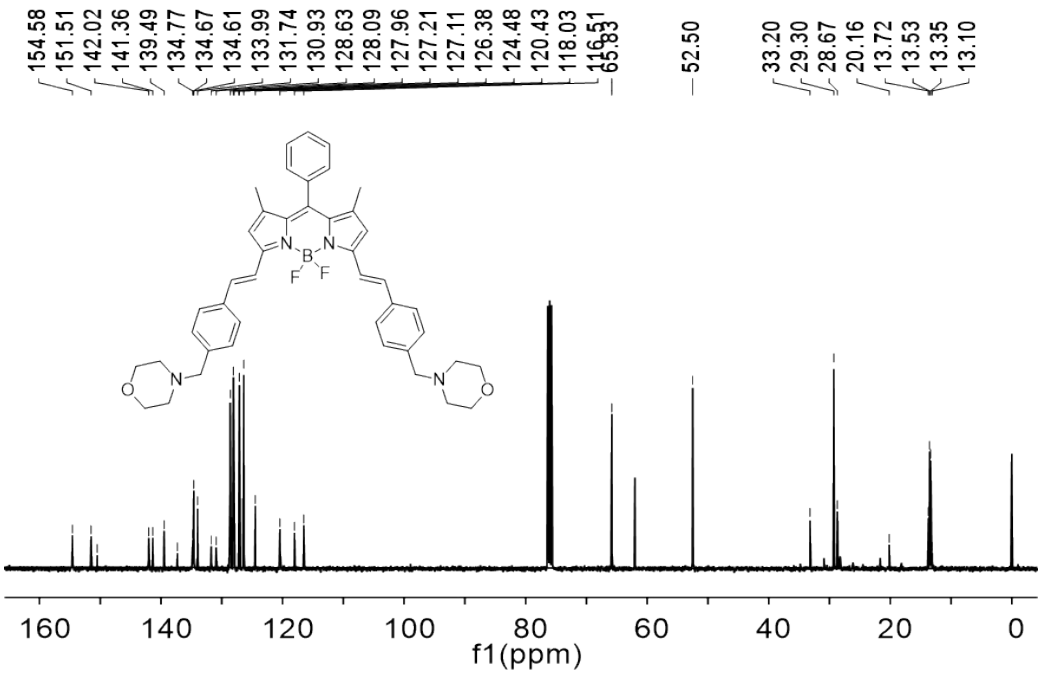
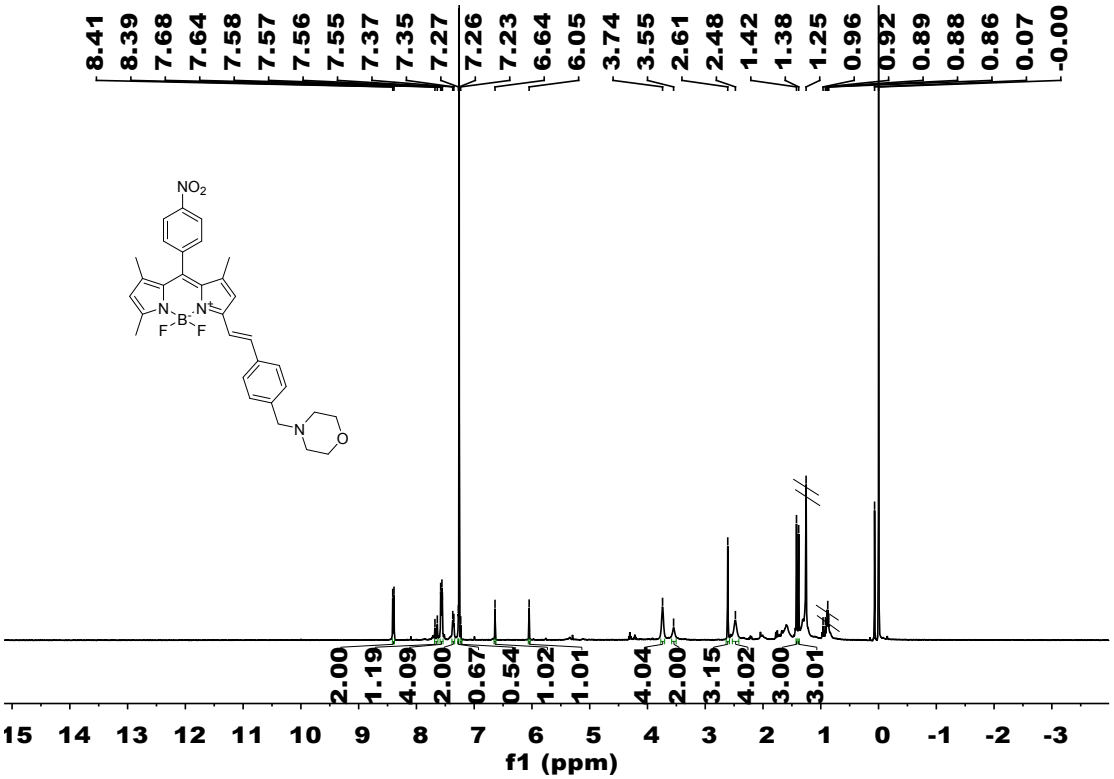
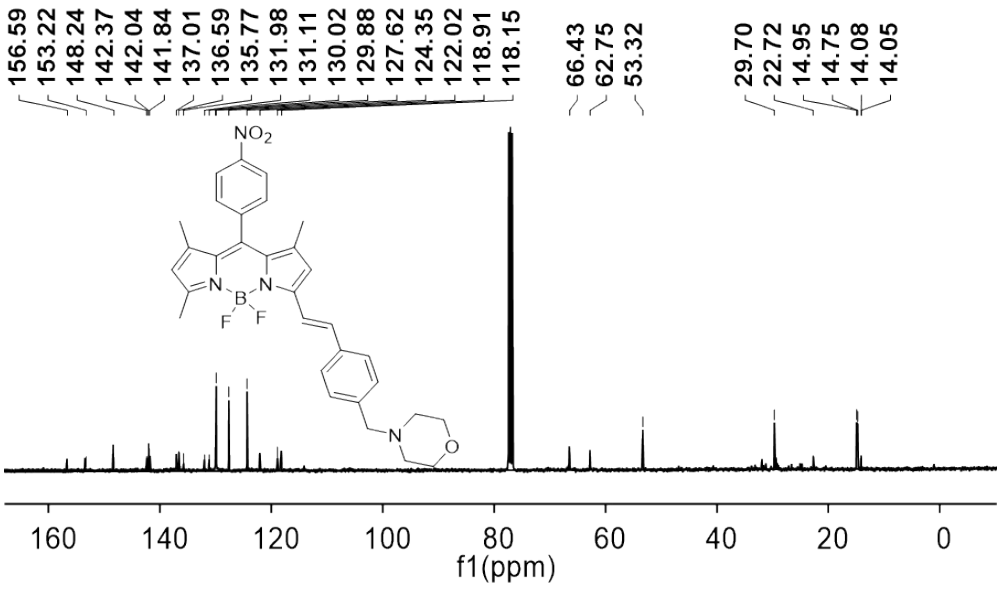
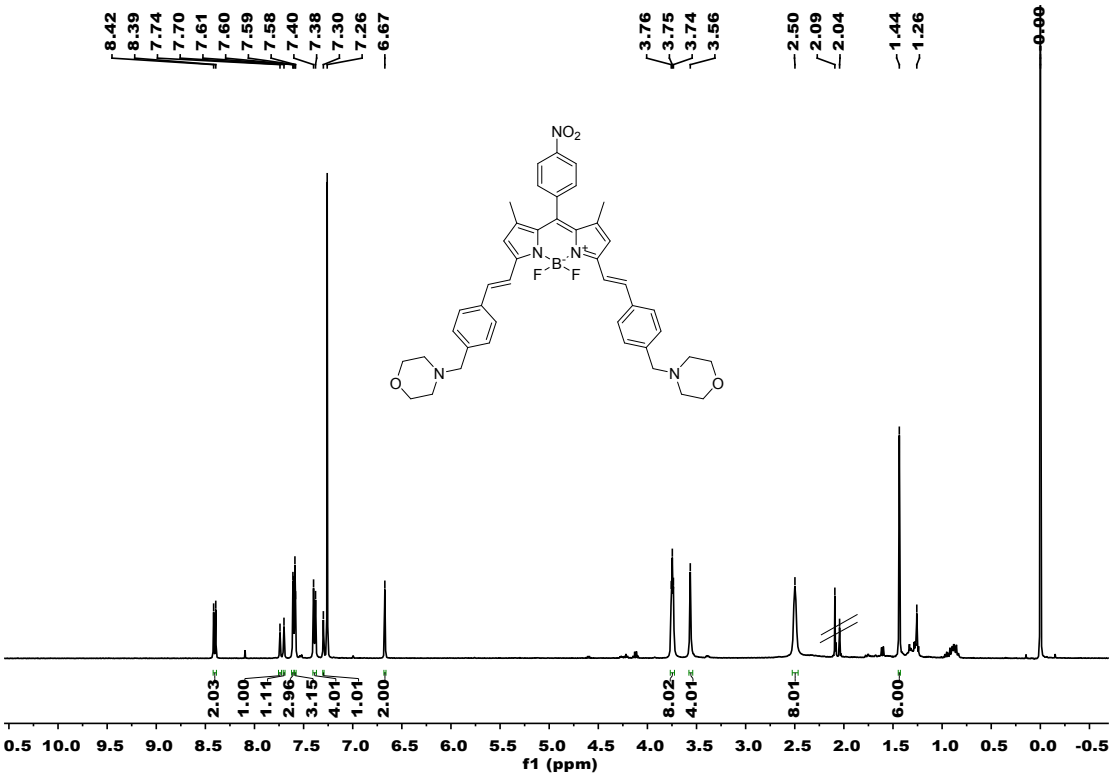
62.10
59.36
52.58
28.68
20.02
13.59
13.18
f1(ppm)

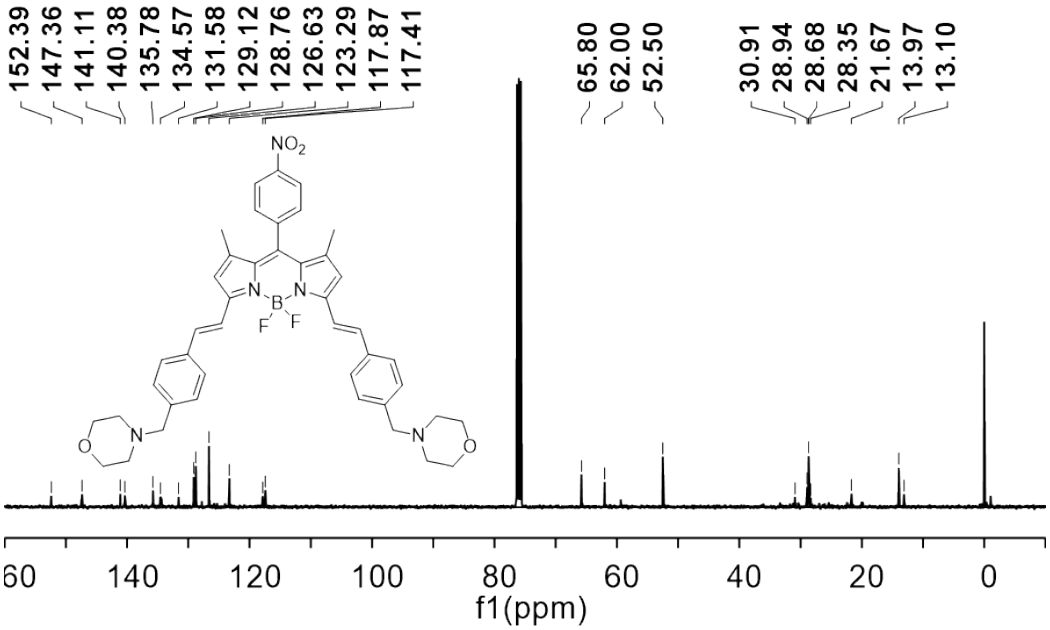

7.75
7.71
7.60
7.58
7.51
7.50
7.50
7.40
7.38
7.34
7.32
7.26
7.23
6.64
3.76
3.58
2.52
1.45
1.43
1.33
1.28
1.26
0.90
0.88
0.86
0.85
0.85
0.83
0.07
2.00
4.02
3.01
4.02
2.00
2.13
2.00
8.04
4.00
8.01
6.01
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
-0.5
-1.5
-2.5
f1 (ppm)


154.58
151.51
142.02
141.36
139.49
134.77
134.67
134.61
133.99
131.74
130.93
128.63
128.09
127.96
127.21
127.11
126.38
124.48
120.43
118.03
116.51
65.83
52.50
33.20
29.30
28.67
20.16
13.72
13.53
13.35
13.10
160
140
120
100
80
60
40
20
0
f1(ppm)

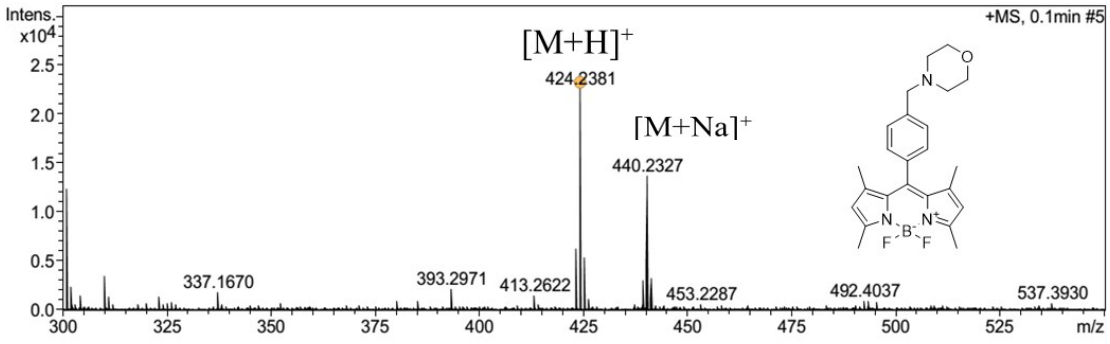

8.41
8.39
7.68
7.64
7.58
7.57
7.56
7.55
7.37
7.35
7.27
7.26
7.23
6.64
6.05
3.74
3.55
2.61
2.48
1.42
1.38
1.25
0.96
0.92
0.89
0.88
0.86
0.07
-0.00
NO2
N
B
F
F
O
2.00
1.19
4.09
2.00
0.67
0.54
1.02
1.01
4.04
2.00
3.15
4.02
3.00
3.01
15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0 -1 -2 -3
f1 (ppm)


156.59
153.22
148.24
142.37
142.04
141.84
137.01
136.59
135.77
131.98
131.11
130.02
129.88
127.62
124.35
122.02
118.91
118.15
66.43
62.75
53.32
29.70
22.72
14.95
14.75
14.08
14.05
NO2
N
B
F
F
O
160 140 120 100 80 60 40 20 0
f1(ppm)

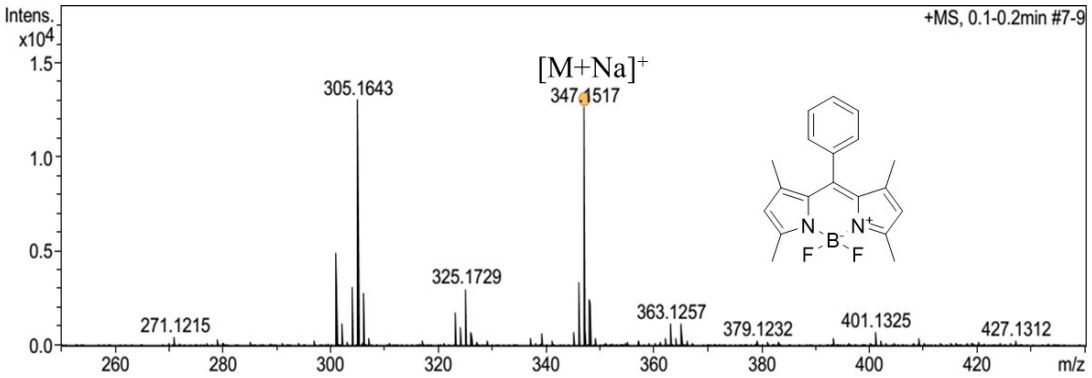




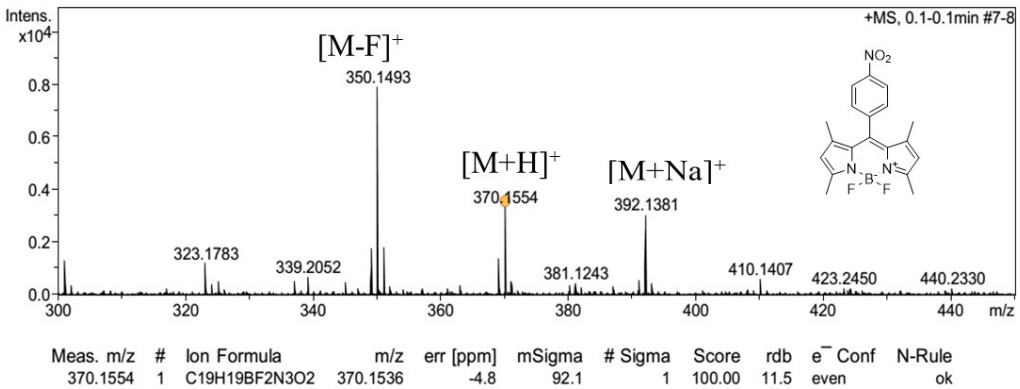
HR-MS spectra



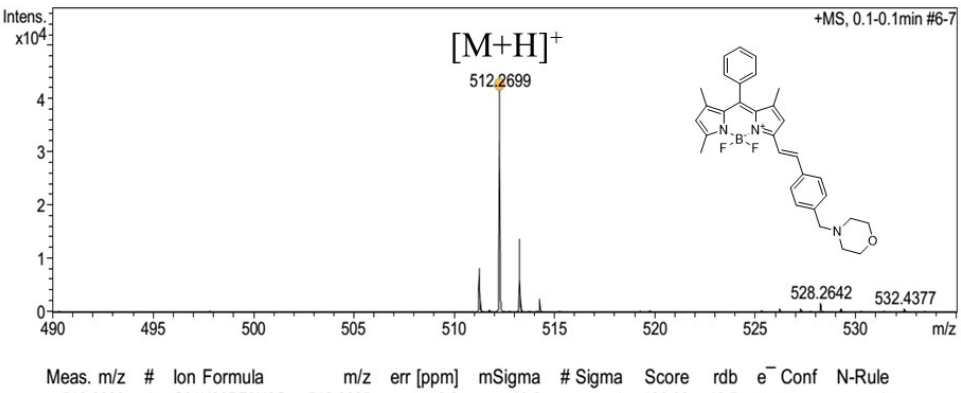
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
424.2381	1	C24H29BF2N3O	424.2371	2.5	27.5	1	100.00	11.5	even	ok



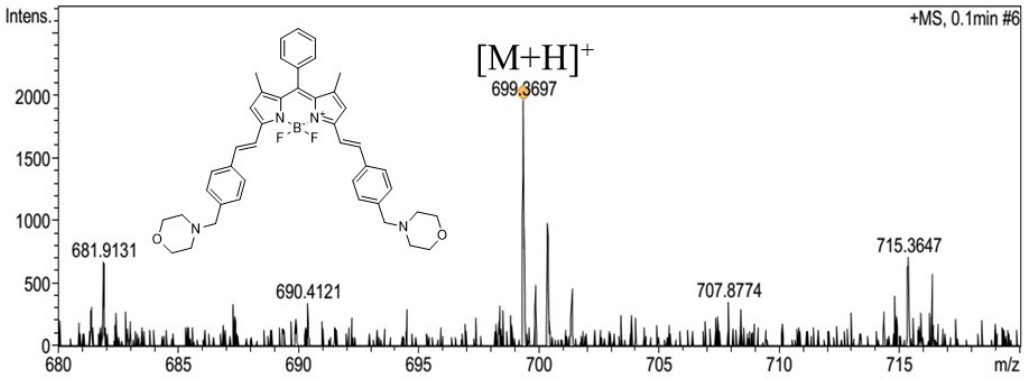
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
347.1517	1	C19H19BF2N2Na	347.1505	-3.5	16.6	1	100.00	10.5	even	ok



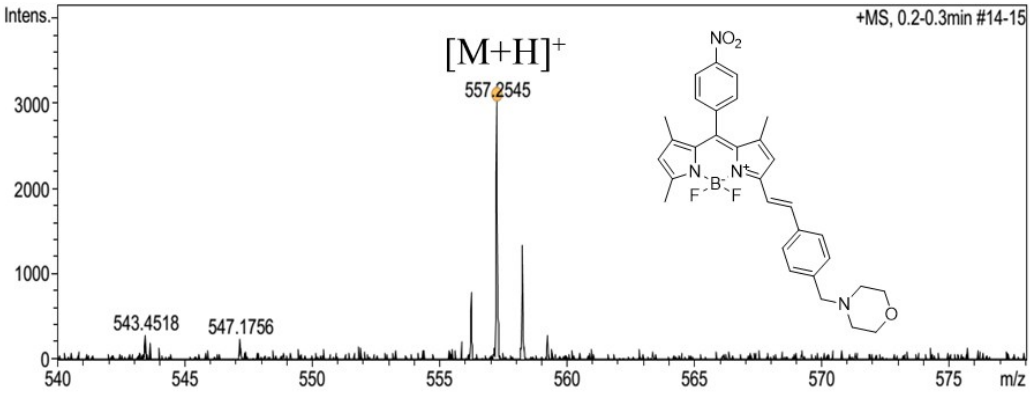
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
370.1554	1	C19H19BF2N3O2	370.1536	-4.8	92.1	1	100.00	11.5	even	ok



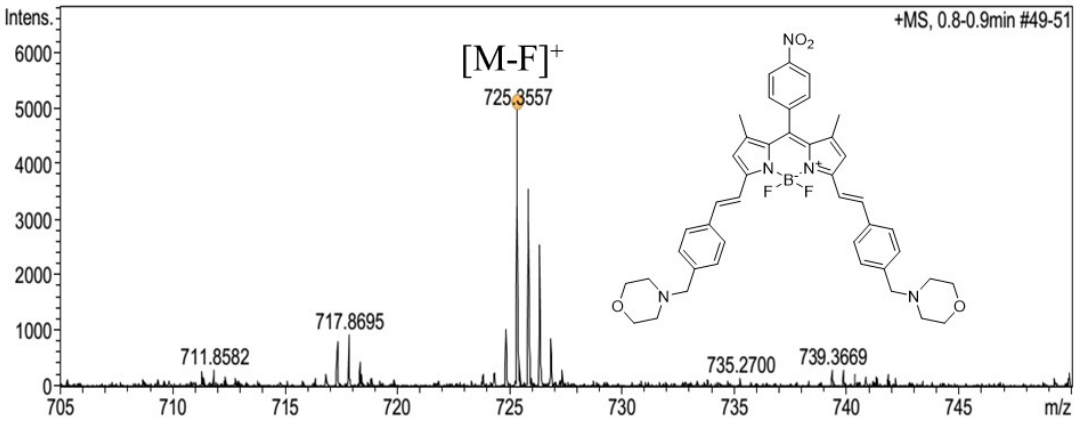
Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e⁻ Conf	N-Rule
512.2699	1	C31H33BF2N3O	512.2685	-2.8	13.2	1	100.00	16.5	even	ok



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e¯ Conf	N-Rule
699.3697	1	C43H46BF2N4O2	699.3684	1.9	352.8	1	100.00	22.5	even	ok



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e¯ Conf	N-Rule
557.2545	1	C31H32BF2N4O3	557.2536	1.8	49.7	1	100.00	17.5	even	ok



Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# Sigma	Score	rdb	e¯ Conf	N-Rule
725.3557	1	C43H44BFN5O4	724.3472	7.9	528.4	1	100.00	24.5	even	ok