Development of pH-activatable fluorescent probes for rapid visualization of metastatic tumors and fluorescence-guided surgery via topical spraying

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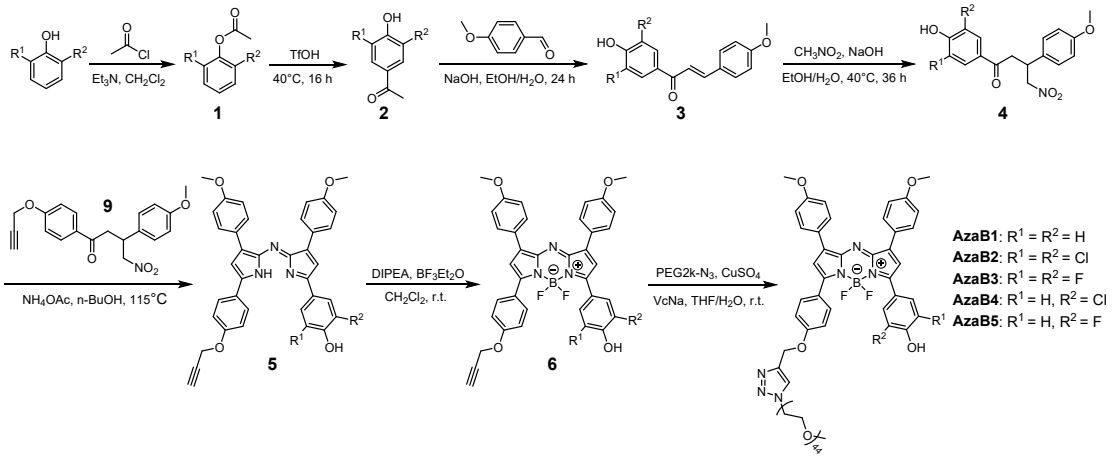
Supporting Information

Contents

1. General information

All reagents and solvents were purchased from Aladdin, Bidepharm, and J&K and used without further purification. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased form Tianjin Kaimeihong Biological Technology Co., Ltd. LysoTracker Green was purchased from Yingwei Jieji (Shanghai) Trading Co., Ltd. The ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra were recorded on a Bruker AM 400 MHz and 100 MHz spectrometer in $CDCl_3$ or DMSO-d_6. The absorbance and fluorescence spectra were recorded on the UV-vis spectrophotometer (Shimadzu, UV-2600) and Hitachi F4700 fluorometer, respectively. Cell viability was measured by CCK-8 assay on a Tecan Infinite M Plex microplate reader. The fluorescence images of cells were obtained on a NIKON A1+ confocal. The whole body and *ex vivo* organ fluorescence imaging was performed on an IVIS Lumina System. All the images were analyzed using ImageJ.

2. Supplemental figures and tables



Scheme S1. Synthetic route of pH-activatable aza-BODIPY probes.

Table S1. Photophysical properties and p*K*a values of **AzaB1-5**.

Probe	$\lambda_{abs\text{-}acid}$ (nm)	$\lambda_{abs\text{-}base}$ (nm)	$\lambda_{em\text{-}acid}$ (nm)	ε (M^{-1} cm^{-1})	p*K*a value
AzaB1	702	756	728	28300	7.9
AzaB2	697	767	723	30500	5.0
AzaB3	700	760	724	26600	5.9
AzaB4	702	764	729	28150	6.6
AzaB5	701	759	728	29500	6.7

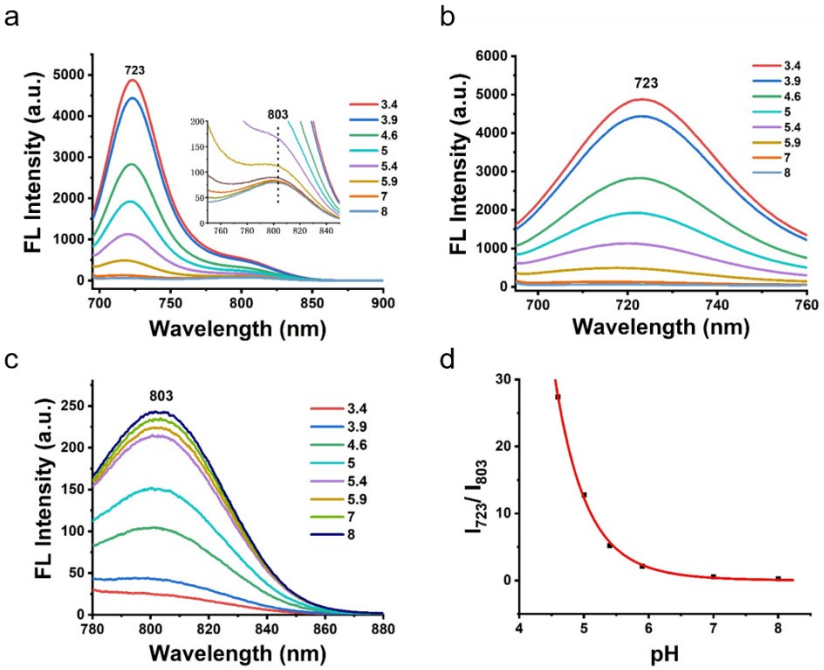


Figure S1. (a) FL spectra of **AzaB2** in citrate buffer containing 1% Triton X-100 with the enlarged view at 803 nm. λ_{ex}: 680 nm. (b, c) FL spectra of **AzaB2** in citrate buffer with excitation at 680 nm (b) and 760 nm (c). (d) Ratio of fluorescence intensity at 723 nm and 803 nm at different pH. Because all five probes exhibited similar fluorescence spectra, we herein only presented the fluorescence intensity variations of **AzaB2**.

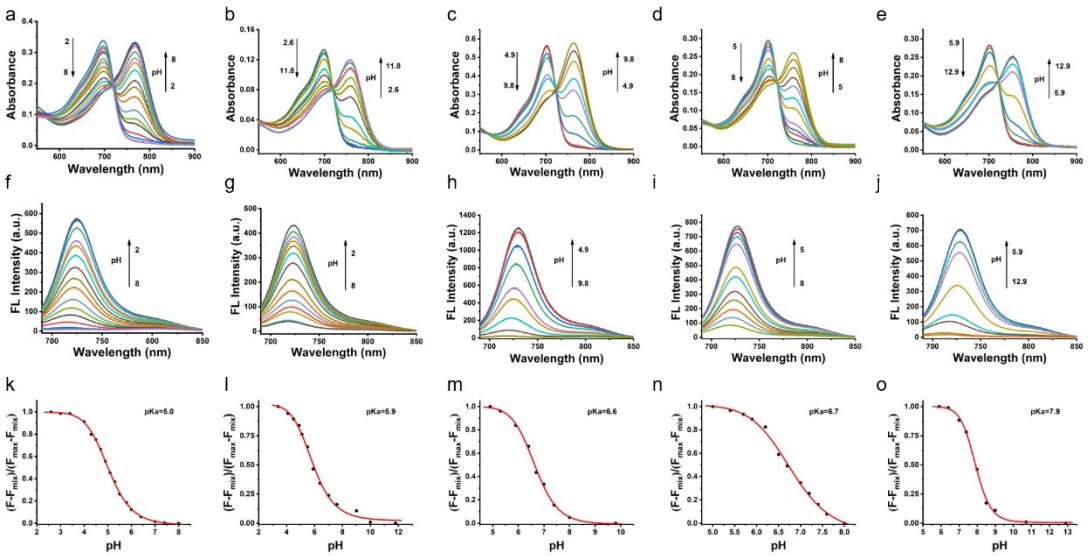


Figure S2. The UV-vis absorbance, fluorescence spectra, and normalized pH titration profiles of **AzaB2** (a, f, k), **AzaB3** (b, g, l), **AzaB4** (c, h, m), **AzaB5** (d, I, n), and **AzaB1** (e, j, o) in different citrate buffers containing 1% Triton X-100.

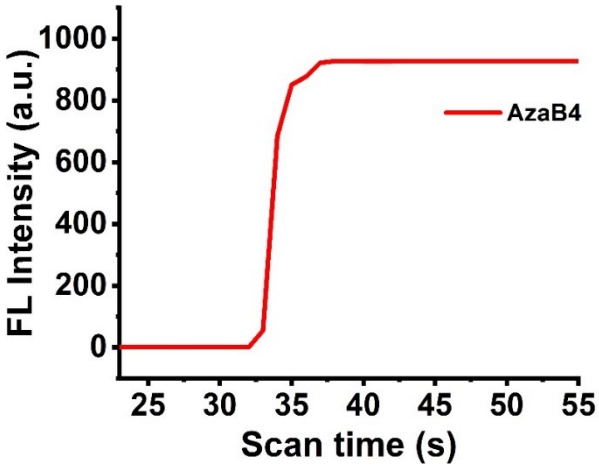


Figure S3. Real-time records of fluorescence intensity at 729 nm of 5 μM **AzaB4** in pH 9.3 buffer (2 mL) after addition of 1 N HCl (50 μL).

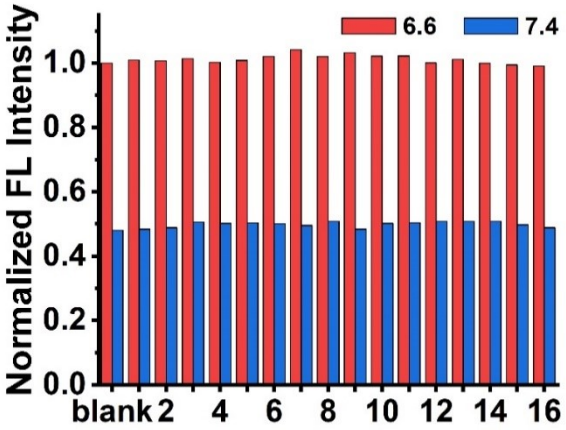


Figure S4. Normalized fluorescence intensity at 729 nm of 5 μM **AzaB4** in the presence of various species at pH 6.6 and 7.4, respectively. 1: BSA (20 mg/mL), 2-4: H_2O_2, $O_2^{\bullet-}$, •OH (50 μM), 5-10: GSH, L-Arginine, L-Cysteine, L-Cystine, L-Glutamic acid, L-Tyrosine, 11: vitamin C (50 μM), 12-16: NO_3^-, Cu^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+} (50 μM).

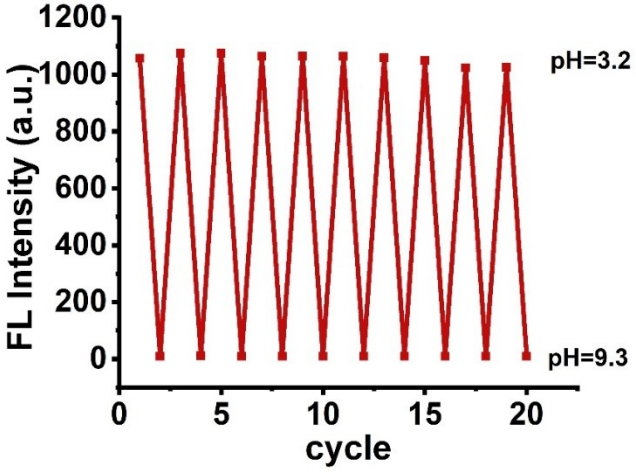


Figure S5. Reversible fluorescence response profile of **AzaB4** (4 μM) in citrate buffers for 10 cycles.

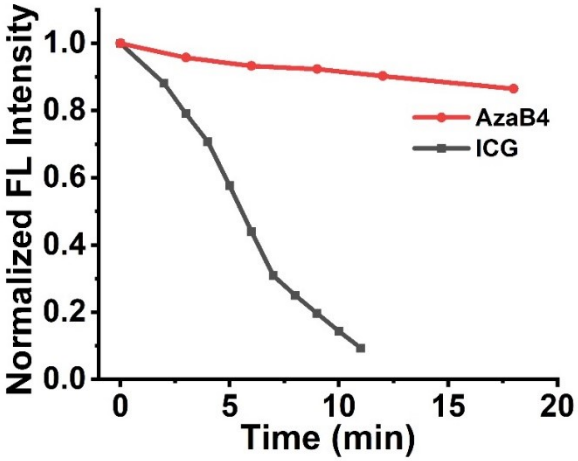


Figure S6. Photostability curves of **AzaB4** (5 μM) in pH 3.0 buffer and ICG (5 μM) in PBS solution upon continuous laser irradiation at 650 nm (100 mW/cm^2).

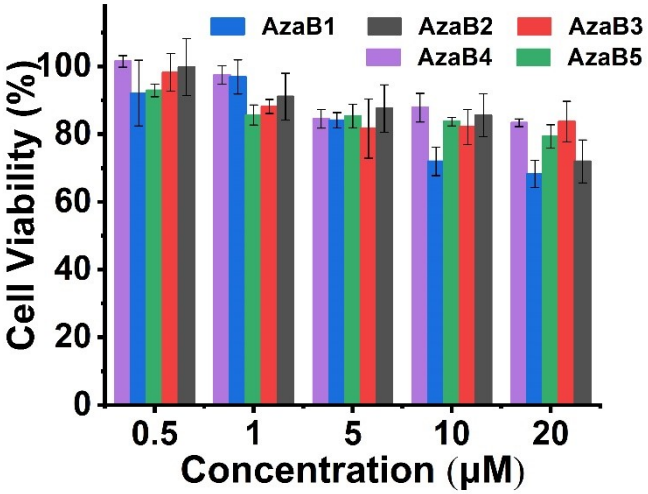


Figure S7. Cell viability of Hela cells incubated with different concentrations of **AzaB1-5** by CCK-8 assay (n = 3).

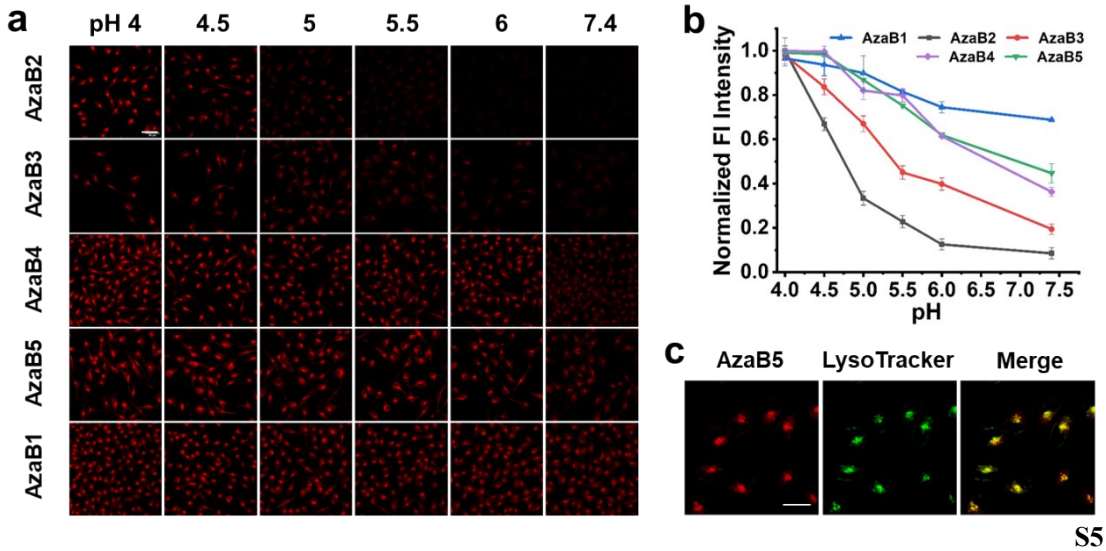


Figure S8. (a) Fluorescence images of HeLa cells incubated with 5 μM probes in different pH citrate buffers. λ_{ex}: 637 nm, channel: Cy5.5. Scale bar: 50 μm. (b) Normalized fluorescence intensity profiles of cells in (a). (c) Colocalization images of **AzaB5** with LysoTracker Green in HeLa cells. Scale bar: 20 μm.

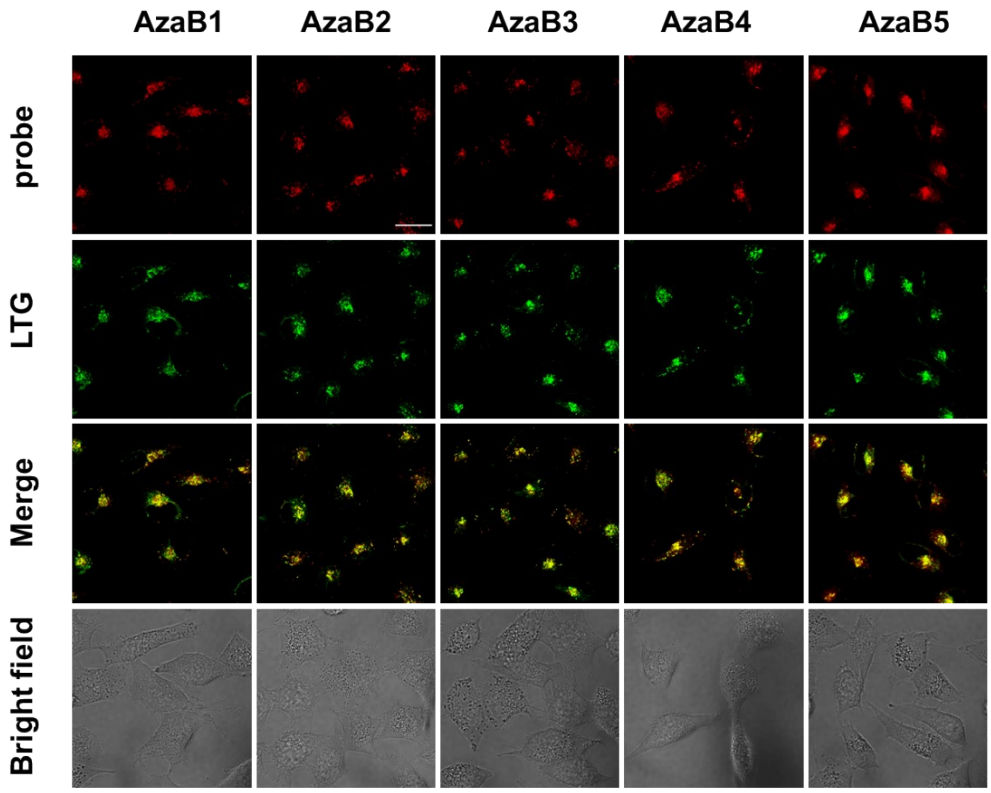


Figure S9. Colocalization of **AzaB1-5** (5 μM) with LysoTracker Green (1 μM) in HeLa cells. λ_{ex}: 680 nm, channel: Cy5.5, scale bar: 20 μm.

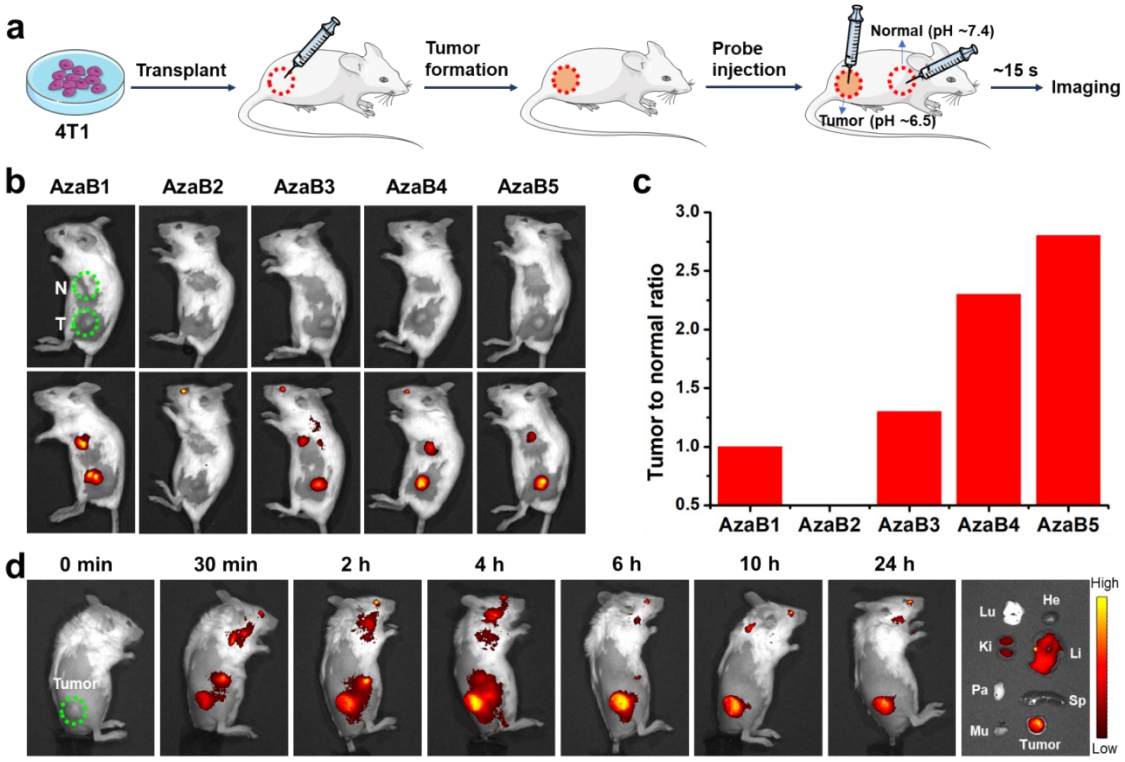


Figure S10. (a) Illustration of mouse model for imaging tumor and normal microenvironment pH. (b) In vivo NIR fluorescence imaging of mice bearing subcutaneous 4T1 breast cancer tumors after injection of **AzaB1−5** (3 μM, 20 μL) into tumors and normal tissues, respectively. Channel: Cy5.5 filter, λex = 640 nm. (c) Tumor-to-normal ratios of relative AzaB1−5 fluorescence intensity at ~15 s postinjection using ROI analysis. (d) Time-dependent NIR fluorescence images of 4T1 tumor-bearing mice after intravenous injection of 200 μL **AzaB2** in PBS (10 μM). Representative fluorescence image of harvested organs and tumor at 24 h postinjection. He: heart, Lu: lung, Li: liver, Ki: kidneys, Sp: spleen, Pa: pancreas, Mu: muscle.

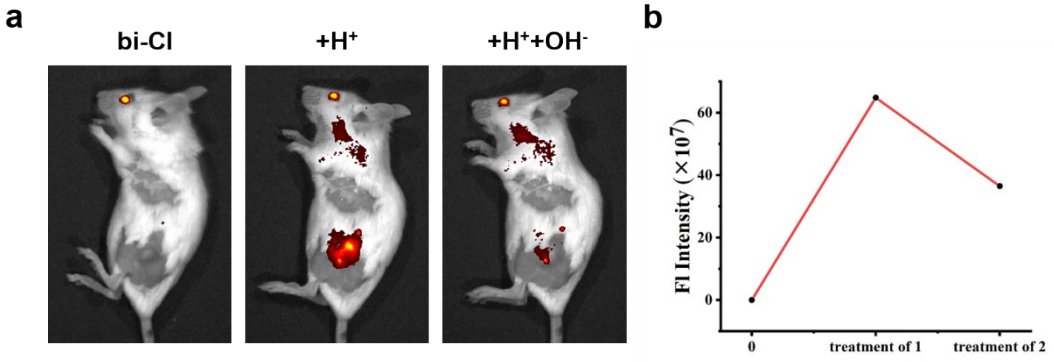
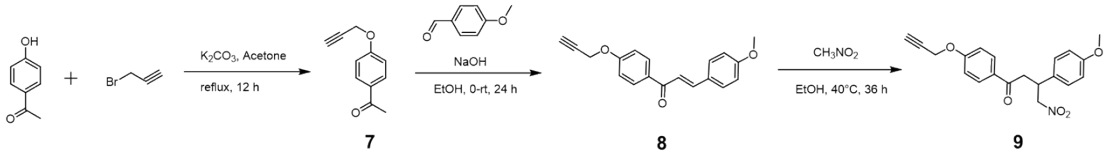


Figure S11. (a) FL imaging of 4T1 tumor-bearing mice after injection of **AzaB2** (3 μM, 20 μL) into tumors and normal tissues (left), followed by injection of acidic buffer (pH 5.0, 20 μL) into tumor site (middle), and saturated sodium bicarbonate solution (20 μL) into tumor (right) in turn. (b) FL intensity of tumor site upon the treatment.

3. Synthesis of probes AzaB1-5

Scheme S1. Synthetic route[1, 2] of **7-9**



7 **8** **9**

Synthesis of **7**: A mixture of p-Hydroxyacetophenone (2 g, 14.7 mmol), propargyl bromide (2.27 g, 19 mmol), and potassium carbonate (2.62 g, 19 mmol) in acetone was stirred at 60 ℃ for 12 h under a nitrogen atmosphere. After cooling to room temperature, the solvent was removed in vacuo and solid residue obtained was extracted with EtOAc (200 mL) and H_2O (200 mL). The organic layer was separated, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The obtained product **1** was used without further purification. (2.37 g, 93%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.92 (d, *J* = 8.8 Hz, 2H), 6.99 (d, *J* = 8.8 Hz, 2H), 4.73 (d, *J* = 2.0 Hz, 2H), 2.54-2.53 (m, 4H).

Synthesis of **8**: **7** (2 g, 11.5 mmol) and p-anisaldehyde (1.56 g, 11.5 mmol) were dissolved in ethanol (40 mL). A aq. solution of NaOH (8 mL, 51.7 mmol) was added dropwise under the ice bath. After stirring at room temperature for 24 h, the reaction solution was directly filtered to afford the product as a yellow solid. (3.23 g, 96%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.04 (d, *J* = 8.8 Hz, 2H), 7.78 (d, *J* = 15.6 Hz, 1H), 7.60 (d, *J* = 8.8 Hz, 2H), 7.42 (d, *J* = 15.6 Hz, 1H), 7.06 (d, *J* = 8.8 Hz, 2H), 6.94 (d, *J* = 8.8 Hz, 2H), 4.78 (d, *J* = 2.4 Hz, 2H), 3.86 (s, 3H), 2.56 (t, *J* = 2.4 Hz, 1H).

Synthesis of **9**: To a solution of **8** (2 g, 6.85 mmol) and nitromethane (4.2 g, 68.5 mmol) in EtOH (10 mL), a aq. solution of NaOH (2 mL, 13.7 mmol) was added under an ice bath. The reaction was stirred at 40 ℃ for 20 h and then quenched with 0.02 M HCl solution. The reaction mixture was extracted with EtOAc (3×). The combined organic fractions were dried over Na_2SO_4, filtered, concentrated, and purified via silica gel column chromatography (1:3 v/v EtOAc/petroleum ether) to afford the product as a yellow oil or solid. (1.29 g, 53%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.91 (d, *J* = 8.8 Hz, 2H), 7.19 (d, *J* = 8.8 Hz, 2H), 7.00 (d, *J* = 9.2 Hz, 2H), 6.85 (d, *J* = 8.4 Hz, 2H), 4.82-4.75 (m, 3H), 4.66-4.61 (m, 1H), 4.19-4.12 (m, 1H), 3.77 (s, 3H), 3.42-3.30 (m, 2H), 2.55 (t, *J* = 2.4 Hz, 1H).

Synthesis of **1**: To a solution of substituted phenol (6.1 mmol, 1 equiv) and TEA (15.25 mmol, 2.5 equiv) in anhydrous DCM (15 mL), acetylchloride (7.4 mmol, 1.2 equiv) was slowly added under the ice bath. Then the mixture was warmed to room temperature and stirred for 5 h. The reaction was quenched with saturated $NaHCO_3$ solution and was extracted with DCM (3×). The combined organic fractions were dried over anhydrous Na_2SO_4, filtered, concentrated, and purified via silica gel column chromatography (1:8 v/v EtOAc/petroleum ether) to afford the product as a colorless oil.

1 (**AzaB2**): Yield: 87%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.35 (d, *J* = 8.0 Hz, 2H), 7.13 (t, *J* = 8.0 Hz, 1H), 2.40 (s, 3H).

1 (**AzaB3**): Yield: 83%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.16-7.14 (m, 1H), 6.99-6.94 (m, 2H), 2.37 (s, 3H).

1 (**AzaB4**): Yield: 93%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.57 (d, *J* = 8.0 Hz, 1H), 7.40 (t, *J* = 8.0 Hz, 1H), 7.31 (t, *J* = 7.6 Hz, 1H), 7.26 (d, *J* = 8.0 Hz, 1H), 2.47 (s, 3H).

1 (**AzaB5**): Yield: 73%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.32-7.26 (m, 4H), 2.46 (s, 3H).

Synthesis of **2**: Trifluoromethanesulfonic acid (TfOH, 7 mL) was added to **1** (20 mmol) slowly under an ice bath and nitrogen atmosphere. The reaction was stirred at 40 ℃ for 16 h and then quenched with $NaHCO_3$ aqueous solution. The reaction mixture was extracted with EtOAc (3×). The combined organic fractions were dried over anhydrous Na_2SO_4, filtered, concentrated, and purified via silica gel column chromatography (1:3 v/v EtOAc/petroleum ether) to afford the product as a white oil.

2 (**AzaB2**): Yield: 83%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.90 (s, 2H), 6.29 (s, 1H), 2.55 (s, 3H).

2 (**AzaB3**): Yield: 73%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 11.25 (s, 1H), 7.64-7.62 (m, 2H), 2.51 (s, 3H).

2 (**AzaB4**): Yield: 93%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.98 (d, *J* = 2.0 Hz, 1H), 7.82 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 1H), 2.55 (s, 3H).

2 (**AzaB5**): Yield: 94%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.74-7.68 (m, 2H), 7.06 (t, *J* = 8.4 Hz, 1H), 2.55 (s, 3H).

Synthesis of **3**: **2** (11.5 mmol, 1 equiv) and p-anisaldehyde (11.5 mmol, 1 equiv) were dissolved in ethanol (40 mL). A aq. solution of NaOH (51.7 mmol, 4.5 equiv) was added dropwise under the ice bath. The reaction was stirred at room temperature for 24 h and then quenched with 0.02 M HCl solution. The reaction mixture was extracted with EtOAc (3×). The combined organic fractions were dried over Na_2SO_4, concentrated, and purified via silica gel column chromatography (1:3 v/v EtOAc/petroleum ether) to afford the product.

3 (**AzaB1**): Yellow solid, yield: 62%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 10.34 (s, 1H), 8.05 (d, *J* = 8.8 Hz, 2H), 7.82 (d, *J* = 8.8 Hz, 2H), 7.76 (d, *J* = 15.6 Hz, 1H), 7.65 (d, *J* = 15.6 Hz, 1H), 7.01 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 8.4 Hz, 2H), 3.82 (s, 3H).

3 (**AzaB2**): Yellow solid, yield: 79%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.19 (s, 2H), 7.89 (d, *J* = 8.8 Hz, 2H), 7.84 (d, *J* = 15.6 Hz, 1H), 7.72 (d, *J* = 15.6 Hz, 1H), 7.01 (d, *J* = 8.8 Hz, 2H), 3.83 (s, 3H).

3 (**AzaB3**): Yellow solid, yield: 89%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 7.77 (d, *J* = 8.8 Hz, 2H), 7.65 (d, *J* = 15.6 Hz, 1H), 7.51-7.44 (m, 3H), 6.96 (d, *J* = 8.8 Hz, 2H), 3.80 (s, 3H).

3 (**AzaB4**): Yellow solid, yield: 71%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 11.18 (s, 1H), 8.19 (d, *J* = 2.0 Hz, 1H), 8.00 (dd, *J* = 8.8, 2.4 Hz, 1H), 7.86 (d, *J* = 8.8 Hz, 2H), 7.80 (d, *J* = 15.6 Hz, 1H), 7.68 (d, *J* = 15.6 Hz, 1H), 7.10 (d, *J* = 8.4 Hz, 1H), 7.01 (d, *J* = 8.8 Hz, 2H), 3.82 (s, 3H).

3 (**AzaB5**): Yellow solid, yield: 78%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 10.86 (s, 1H), 7.97 (dd, *J* = 12.4, 2.0 Hz, 1H), 7.90 (dd, *J* = 8.4, 2.0 Hz, 1H), 7.85 (d, *J* = 8.8 Hz, 2H), 7.79 (d, *J* = 15.2 Hz, 1H), 7.68 (d, *J* = 15.6 Hz, 1H), 7.08 (t, *J* = 8.4 Hz, 1H), 7.01 (d, *J* = 8.8 Hz, 2H), 3.82 (s, 3H).

Synthesis of **4**: To a solution of **3** (5.85 mmol, 1 equiv) and nitromethane (68.5 mmol, 10 equiv) in EtOH (10 mL), a aq. solution of NaOH (2 equiv) was added under the ice bath. The reaction was stirred at 40-70 ℃ for 24 h and then quenched with 0.02 M HCl solution. The reaction mixture was extracted with EtOAc (3×). The combined organic fractions were dried over Na_2SO_4, concentrated, and purified via silica gel column chromatography (1:3 v/v EtOAc/petroleum ether)

to afford the product.

4 (**AzaB1**): Yellow oil, yield: 80%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.93 (d, *J* = 8.8 Hz, 2H), 7.28 (d, *J* = 8.8 Hz, 2H), 6.96 (d, *J* = 8.8 Hz, 2H), 6.94 (d, *J* = 8.8 Hz, 2H), 4.91-4.86 (m, 1H), 4.75-4.70 (m, 1H), 4.28-4.21 (m, 1H), 3.86 (s, 3H), 3.51-3.39 (m, 2H).

4 (**AzaB2**): Yellow solid, yield: 35%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 11.22 (s, 1H), 7.90 (s, 2H), 7.27 (d, *J* = 8.4 Hz, 2H), 6.84 (d, *J* = 8.4 Hz, 2H), 4.92-4.87 (m, 1H), 4.77-4.71 (m, 1H), 3.97-3.91 (m, 1H), 3.90 (s, 3H), 3.70-3.38 (m, 2H).

4 (**AzaB3**): Yellow oil, yield: 35%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.50 (d, *J* = 8.4 Hz, 2H), 7.17 (d, *J* = 8.8 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 2H), 4.78-4.74 (m, 1H), 4.66-4.61 (m, 1H), 4.17-4.10 (m, 1H), 3.78 (s, 3H), 3.38-3.26 (m, 2H).

4 (**AzaB4**): Yellow oil, Yield: 25%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 11.19 (s, 1H), 7.91 (d, *J* = 2.0 Hz, 1H), 7.75 (dd, *J* = 8.4, 2.4 Hz, 1H), 7.26 (d, *J* = 8.4 Hz, 2H), 7.03 (d, *J* = 8.4 Hz, 1H), 6.84 (d, *J* = 8.8 Hz, 2H), 4.93-4.88 (m, 1H), 4.79-4.74 (m, 1H), 3.98-3.91 (m, 1H), 3.70 (s, 3H), 3.47-3.31 (m, 2H).

4 (**AzaB5**): Yellow oil, Yield: 34%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.70-7.64 (m, 2H), 7.18 (d, *J* = 7.6 Hz, 2H), 7.04 (t, *J* = 8.8 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 2H), 4.80-4.75 (m, 1H), 4.66-4.61 (m, 1H), 4.16-4.13 (m, 1H), 3.78 (s, 3H), 3.40-3.28 (m, 2H).

Synthesis of **5** and **6**: A suspension of **4** (2.1 mmol, 1 equiv), **9** (6.3 mmol, 3 equiv), and NH_4OAc (31.5 mmol, 15 equiv) in *n*-butanol (20 mL) was stirred at 115 ℃ for 5 h under a nitrogen atmosphere. Then, the reaction was cooled, concentrated via rotary evaporation, and extracted with EtOAc. The organic layer was washed with brine (5×), dried, concentrated, and purified by silica gel column chromatography (50:1 v/v CH_2Cl_2/MeOH) to obtain the raw product as a dark blue solid. To a solution of **5** (1.0 equiv) in anhydrous CH_2Cl_2 (50 mL), *N,N*-diisopropylethylamine (10 equiv) and boron trifluoride diethyl etherate (10 equiv) were added successively. The reaction mixture was stirred under a nitrogen atmosphere at room temperature for 5 h. Then the reaction solution was concentrated via rotary evaporation and purified by silica gel column chromatography (50:1 v/v CH_2Cl_2/MeOH) to obtain the product as a black purple solid.

6 (**AzaB1**): Yield: 34%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 10.47 (s, 1H), 8.18-8.08 (m, 8H), 7.51 (s, 1H), 7.41 (s, 1H), 7.18-7.12 (m, 6H), 6.94 (d, *J* = 8.8 Hz, 2H), 4.94 (d, *J* = 2.0 Hz, 2H), 3.87 (m, 6H), 3.64 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.5, 161.2, 160.9, 159.7, 159.0, 155.9, 145.2, 144.1, 142.8, 141.4, 132.6, 131.6, 131.1, 131.0, 125.2, 124.90, 124.89, 122.1, 118.8, 117.9, 116.3, 115.5, 114.85, 114.83, 79.4, 79.1, 56.1, 55.9, 55.8. LRMS-ESI (*m/z*): Exact mass calcd for $C_{37}H_{29}BF_2N_3O_4^+$ $[M+H]^+$: 628.21; Found: 628.2230.

6 (**AzaB2**): Yield: 38%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.18-8.15 (m, 8H), 7.55-7.53 (m, 2H), 7.20-7.12 (m, 6H), 4.96 (d, *J* = 2.4 Hz, 2H), 3.87 (m, 6H), 3.65 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.4, 161.1, 160.5, 159.2, 153.5, 151.8, 145.6, 144.3, 143.5, 141.7, 132.2, 131.3, 131.0, 130.0, 125.1, 124.7, 124.24, 124.21, 122.8, 119.2, 118.1, 115.6, 114.85, 114.80, 79.2, 56.3, 55.9, 55.8. LRMS-ESI (*m/z*): Exact mass calcd for $C_{37}H_{27}BCl_2F_2N_3O_4^+$ $[M+H]^+$: 696.14; Found: 696.1454.

6 (**AzaB3**): Yield: 36%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.20-8.14 (m, 6H), 7.89 (d, *J* = 10.4 Hz, 2H), 7.56 (s, 1H), 7.53 (s, 1H), 7.21 (d, *J* = 9.2 Hz, 2H), 7.16-7.13 (m, 4H), 4.96 (d, *J* = 2.0 Hz, 2H), 3.88 (m, 6H), 3.65 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.9, 160.6, 160.0, 158.5, 153.7, 151.8 (dd, *J* = 8, 240 Hz), 145.0, 143.9, 142.9, 141.3, 136.6 (t, *J* = 170

Hz), 131.7, 130.8, 130.6, 124.5, 124.3, 123.8, 121.1 (t, J = 9 Hz), 118.6, 117.7, 115.2, 114.39, 114.36, 78.7, 55.8, 55.4, 53.6. ^{19}F NMR (376 MHz, DMSO-d_6, δ): -130.52 (q, J = 33.8 Hz, 2H), -132.96 (s, 2H). LRMS-ESI (*m/z*): Exact mass calcd for $C_{37}H_{27}BF_4N_3O_4^+$ $[M+H]^+$: 664.20; Found: 664.2040.

6 (**AzaB4**): Yield: 25%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 11.16 (s, 1H), 8.20-8.13 (m, 7H), 8.00 (dd, J = 8.4, 2.4 Hz, 1H), 7.52 (s, 1H), 7.48 (s, 1H), 7.19-7.13 (m, 7H), 4.96 (d, J = 2.0 Hz, 2H), 3.88 (m, 6H), 3.64 (t, J = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.2, 160.1, 157.4, 156.6, 156.3, 144.84, 144.78, 142.40, 142.37, 131.91, 131.87, 131.1, 130.54, 130.49, 125.0, 124.6, 123.4, 120.8, 118.55, 118.47, 117.2, 115.5, 114.9, 79.3, 79.2, 56.2, 55.9. LRMS-ESI (*m/z*): Exact mass calcd for $C_{37}H_{28}BClF_2N_3O_4^+$ $[M+H]^+$: 662.18; Found: 662.1843.

6 (**AzaB5**): Yield: 19%. ^{1}H NMR (400 MHz, DMSO-d_6, δ): 10.89 (s, 1H), 8.18-8.14 (m, 6H), 8.02 (dd, J = 13.2, 2.0 Hz, 1H), 7.88 (dd, J = 8.4, 1.6 Hz, 1H), 7.52 (s, 1H), 7.49 (s, 1H), 7.20-7.08 (m, 7H), 4.95 (d, J = 2.0 Hz, 2H), 3.88 (m, 6H), 3.65 (t, J = 2.4 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 161.1, 160.0, 157.3, 156.6, 151.1 (d, J = 239.5 Hz), 149.0, 148.8, 144.8, 142.3, 131.9, 131.1, 127.7, 125.00, 124.96, 124.6, 122.64, 122.57, 118.44, 118.36, 118.3, 118.0, 117.8, 115.5, 114.8, 79.3, 79.2, 56.2, 55.8. ^{19}F NMR (376 MHz, DMSO-d_6, δ): -130.56 (q, J = 32.7 Hz, 2H), -136.45 (s, 1H). LRMS-ESI (*m/z*): Exact mass calcd for $C_{37}H_{28}BF_3N_3O_4^+$ $[M+H]^+$: 646.20; Found: 646.2137.

Synthesis of **AzaB5**: **6** (**AzaB5**, 19.4 mg, 0.03 mmol), methoxy-poly(ethylene glycol)-azide (PEG-N_3, average M_n =2000, 64 mg, 0.032 mmol), $CuSO_4$ (2.0 mg, 0.012 mmol), and sodium ascorbate (VcNa, 20.0 mg, 0.077 mmol) were added to a 10 mL schlenk flask. The vessel was evacuated and back-filled with N_2. Then 4 mL THF (degassed) and 1 mL DI water (degassed) were added. The reaction mixture was stirred overnight. After evaporation of THF, the residue was dialyzed in DI water for 6 h and lyophilized to obtain a blue powder. The collected product was then purified by column chromatography through a Sephadex LH-20 resin using MeOH as the eluent, affording **AzaB5** as dark blue solid.

Other probes (**AzaB1**, **AzaB2**, **AzaB3** and **AzaB4**) were synthesized by the same method.

AzaB1: ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.26 (s, 1H), 8.20-8.08 (m, 8H), 7.57-7.56 (m, 2H), 7.23-7.11 (m, 8H), 5.28 (s, 2H), 4.57 (t, J = 5.6 Hz, 2H), 3.88-3.87 (m, 6H), 3.85-3.83 (m, 2H), 3.51-3.48 (m, 172H), 3.24 (s, 3H).

AzaB2: ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.37 (s, 1H), 8.25-8.23 (m, 4H), 8.08-7.99 (m, 4H), 7.65-7.63 (m, 2H), 7.17-7.06 (m, 6H), 5.26 (s, 2H), 4.56 (t, J = 5.2 Hz, 2H), 3.87-3.82 (m, 8H), 3.50-3.43 (m, 172H), 3.24 (s, 3H).

AzaB3: ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.24-8.06 (m, 9H), 7.98-7.97 (m, 2H), 7.18-7.10 (m, 4H), 7.06 (d, J = 8.0 Hz, 2H), 5.25 (s, 2H), 4.56 (t, J = 5.2 Hz, 2H), 3.87-3.84 (m, 6H), 3.69-3.67 (m, 2H), 3.50-3.48 (m, 172H), 3.24 (s, 3H).

AzaB4: ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.29-8.07 (m, 9H), 7.65-7.52 (m, 2H), 7.22-7.12 (m, 6H), 6.88 (s, 1H), 5.28 (s, 2H), 4.57-4.56 (m, 2H), 3.87-3.86 (m, 8H), 3.50 (m, 172H), 3.23 (s, 3H).

AzaB5: ^{1}H NMR (400 MHz, DMSO-d_6, δ): 8.25 (s, 1H), 8.19-8.06 (m, 7H), 7.93-7.92 (m, 1H), 7.59 (m, 1H), 7.37 (m, 1H), 7.23 (d, J = 8.8 Hz, 2H), 7.14-7.04 (m, 5H), 5.28 (s, 2H), 4.60 (t, J = 5.2 Hz, 2H), 3.88-3.87 (m, 6H), 3.85-3.82 (m, 2H), 3.51-3.47 (m, 172H), 3.24 (s, 3H).

4. Spectroscopic measurements

The UV-vis absorbance and fluorescence emission spectra of the probes were measured in

different aqueous solutions (citrate buffer, containing 1% Triton X-100). A series of standard pH buffer solutions were prepared by mixing 200 mM Na_2HPO_4 and citrate at varied volume ratios, and the calibrated pH values were measured by a Mettler Toledo FE28 pH meter. The p*K*a values were calculated from fluorescence emission for each probe based on a sigmoid curve analyzed by nonlinear regression analyses in the program Origin. The selectivity of each probe was examined in the presence of protons and various other species (BSA, H_2O_2, $O_2^{\bullet-}$, $^{\bullet}OH$, GSH, L-Arginine, L-Cysteine, L-Cystine, L-Glutamic acid, Tyrosine, VcNa, NO_3^-, Cu^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}). For reversible fluorescence response experiment, the pH values were adjusted by 1 M NaOH and HCl solutions.

5. Cell culture and cytotoxicity assay

Hela cells were cultured in DMEM medium containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin−streptomycin in a humidified incubator with 5% CO_2 at 37 °C. The cytotoxicities of **AzaB1**-**5** were evaluated by CCK-8 assay.[3] The cells were plated in 96-well plates, and the number of cells was about 10000 in each well. After 24 h, five probes were added to each well at a final concentration of 0, 0.5, 1, 5, 10, and 20 μM, respectively, and incubated for 24 h. Then remove the old medium and wash the wells three times with PBS buffer. Subsequently, 100 μL fresh medium and 10 μL of CCK-8 was added and incubated for another 1 h. Finally, the absorbance was recorded at 450 nm using a Tecan Infinite M Plex microplate Reader. n = 3.

6. Colocalization experiments

Hela cells were seeded in confocal culture dishes and allowed to attach for 24 h. After culturing overnight at 37°C, the old medium was removed and replaced with fresh DMEM containing probes (5 μM). After 2 h, the cells were washed three times with PBS and incubated with DND-26 (LysoTracker Green, 1 μM) for another 30 min. Finally, the cells were washed with PBS before imaging. Cells images were obtained on a NIKON A1+ confocal using a 60× oil immersion objective lens.

7. Fluorescence imaging in cells

Attached Hela cells were incubated with probes (5 μM) for 2 h, and then fixed by 4% PFA (200 μL) for 5 min. After that, cells were washed three times with PBS and incubated with fresh buffers at different pH values (4.0, 4.5, 5.0, 5.5, 6.0, 7.4) for 30 min before fluorescence imaging. Images were analyzed using ImageJ.

8. Fluorescence imaging in subcutaneous tumor model

Female BALB/C mice (6-8 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All experiments were approved by the Institutional Animal Care and Use Committees of Nankai University. 4T1-luc tumor cells in 100 μL PBS/Matrigel (v/v, 50/50) were injected subcutaneously into each flank of mice. After four days when the tumors reached adequate size (~6 mm), each probe (3 μM) in 20 μL deionized water was injected into tumors and normal tissues, respectively. After 15 s, mice were anesthetized with 2.5% isoflurane in oxygen and the whole body NIR fluorescence images (the Cy5.5 filter was used) were captured using an IVIS Lumina imaging system. Images were analyzed using ImageJ.

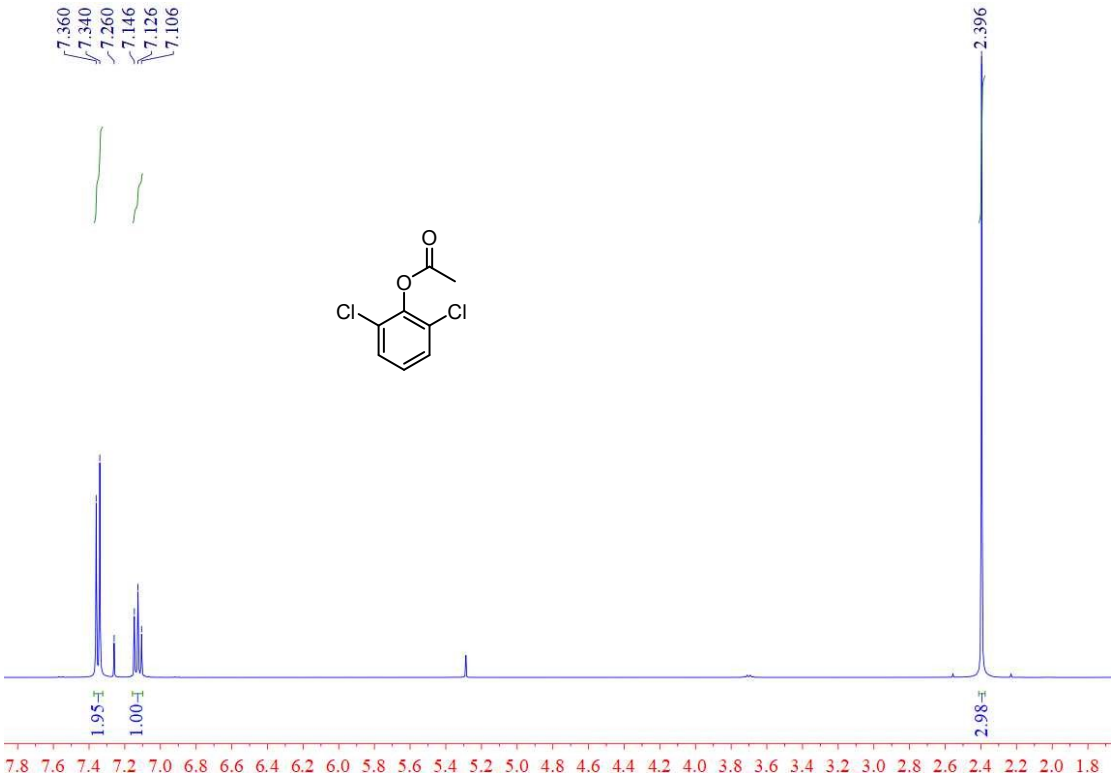
9. Fluorescence imaging in metastatic lung tumor model via topical spraying

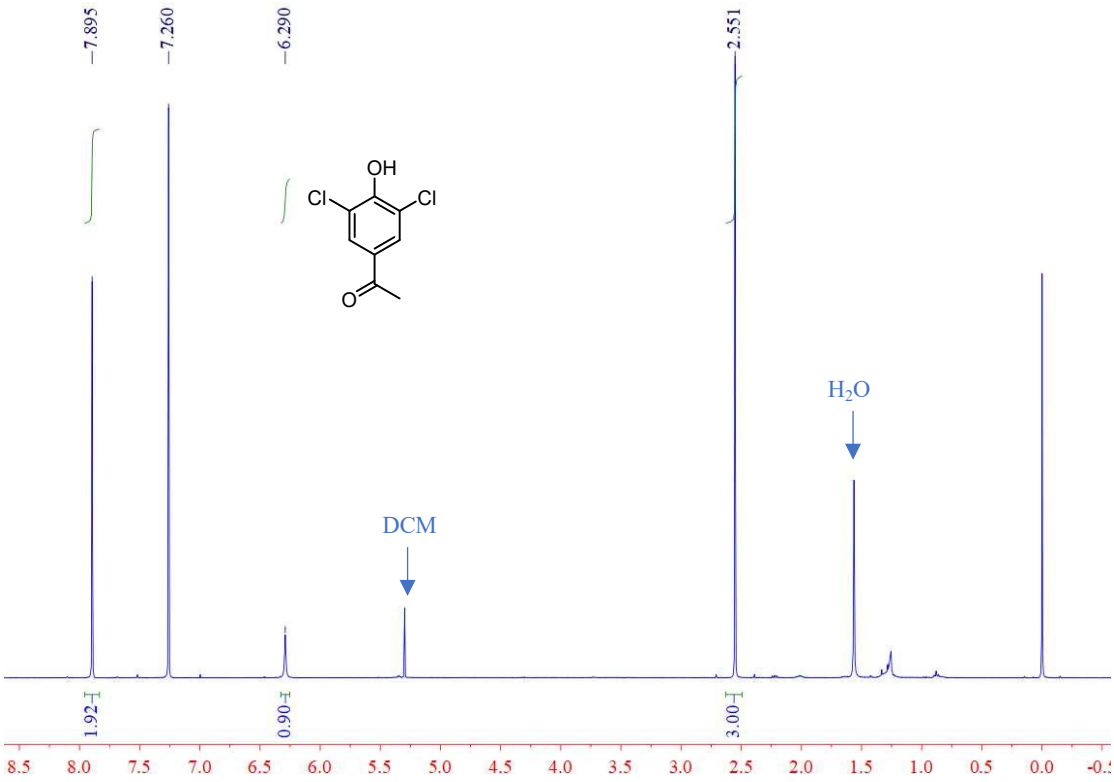
4T1-luc cells (2×10^5) in 100 μL PBS were intravenously injected into the BALB/C mice (6-8 weeks). The generation of metastatic lung tumors was monitored by bioluminescence imaging, 100 μL of 40 g/L luciferin PBS solution was injected subcutaneously, and 3 min later, the bioluminescence signals were measured. Five days later, strong bioluminescence signals in the lungs were observed, indicating that the metastatic lung tumor model was successfully generated. Then the mice were euthanized and the chest was opened. After spraying the probe **AzaB5** (3 μM) for 30 s, fluorescence imaging was collected on the IVIS Lumina system.

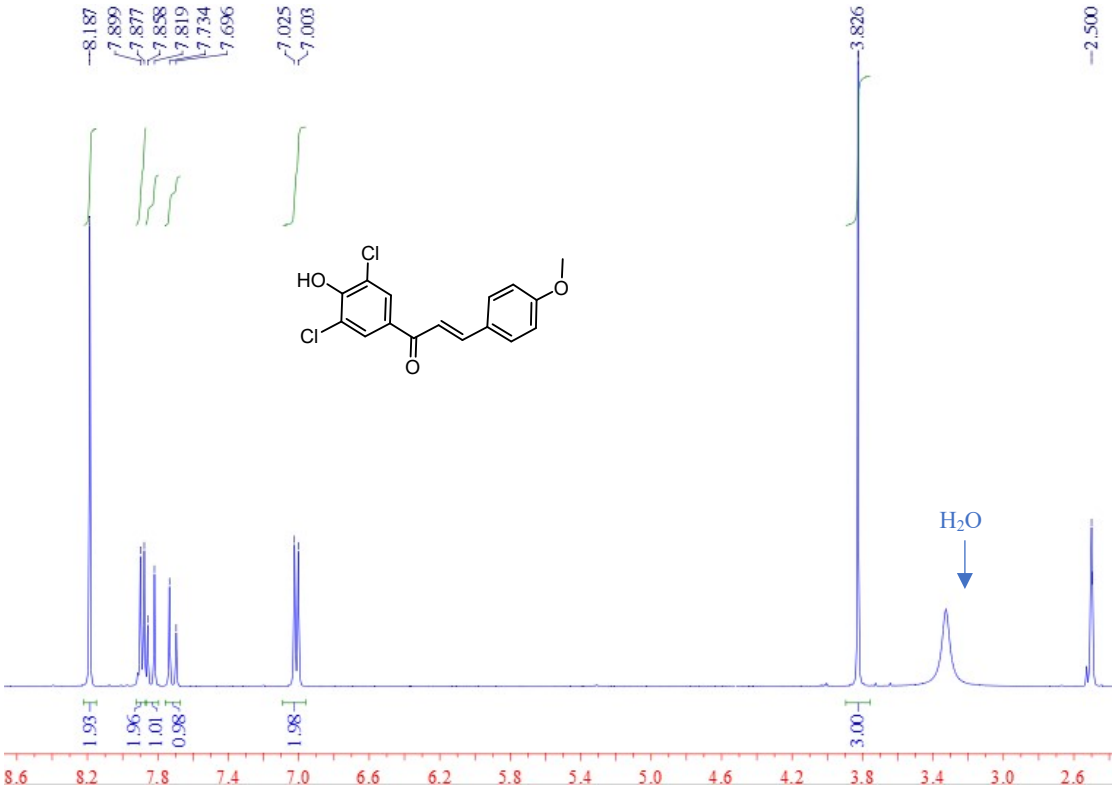
10. Fluorescence imaging in abdominal metastatic tumors via topical spraying

4T1-luc cells (2×10^5) in 100 μL PBS were intraperitoneally injected into the BALB/C mice (6-8 weeks).[4] The formation of metastatic intraperitoneal tumors was monitored using bioluminescence imaging. Ten days later, intense bioluminescence signals at the abdomen were detected, indicating that the abdominal metastases were successfully established. Then the mice were euthanized and the peritoneum was opened. After spraying probe **AzaB5** in the belly for 30 s, fluorescence imaging was performed on the IVIS Lumina system.

11. ^{1}H NMR, ^{13}C NMR, and ^{19}F NMR spectra



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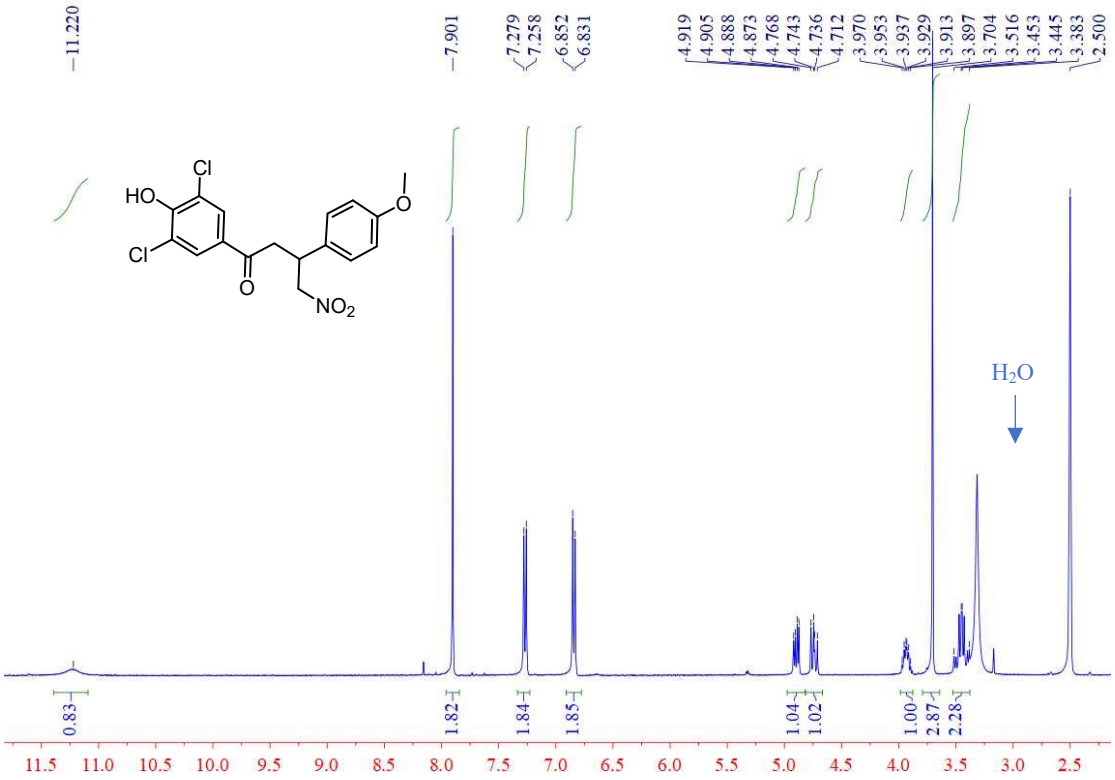
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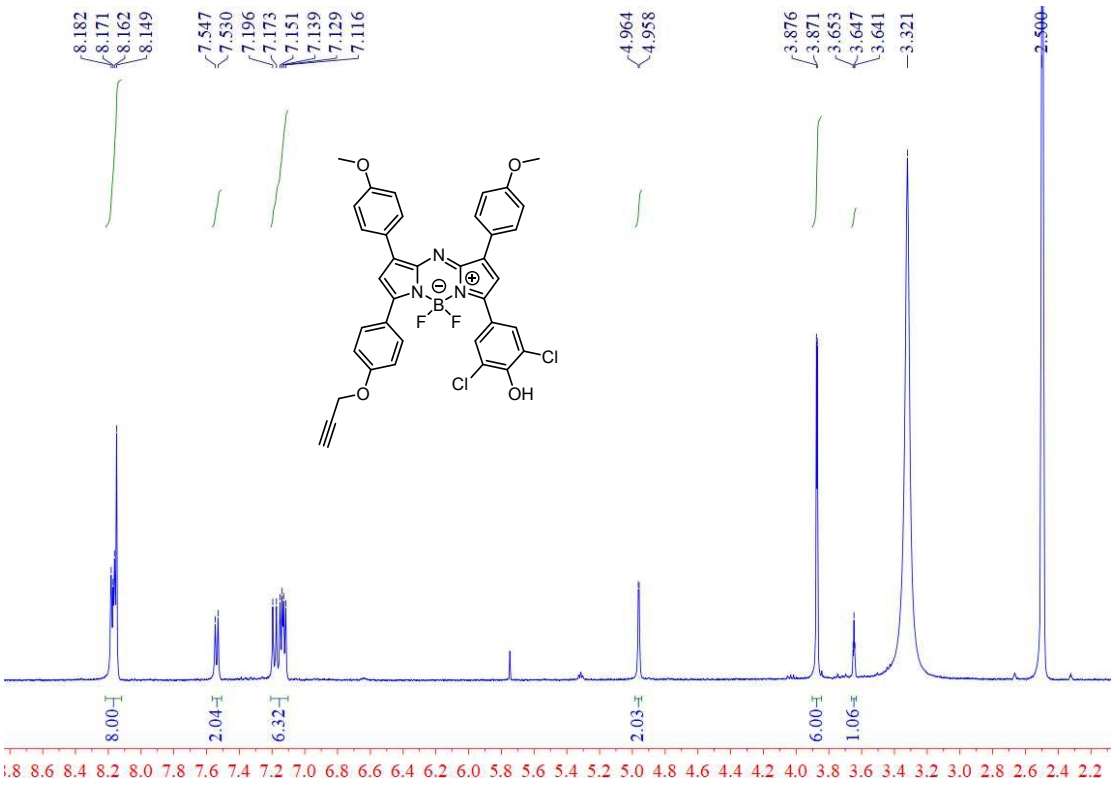
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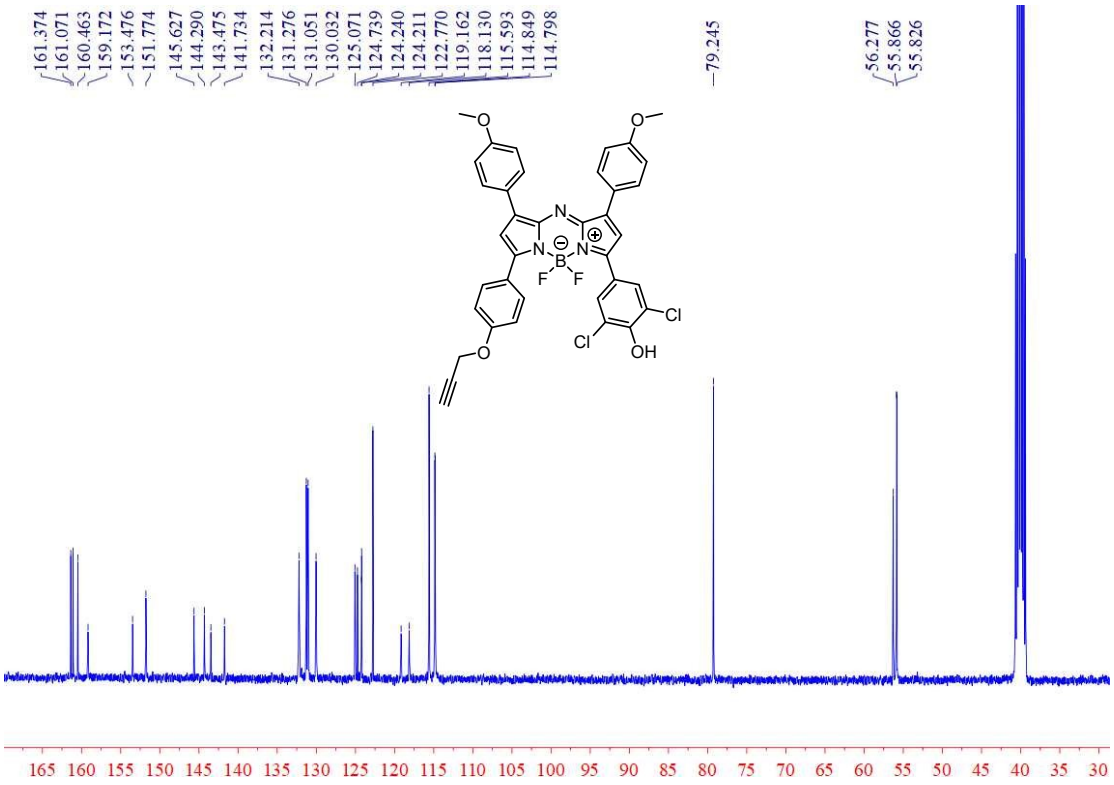
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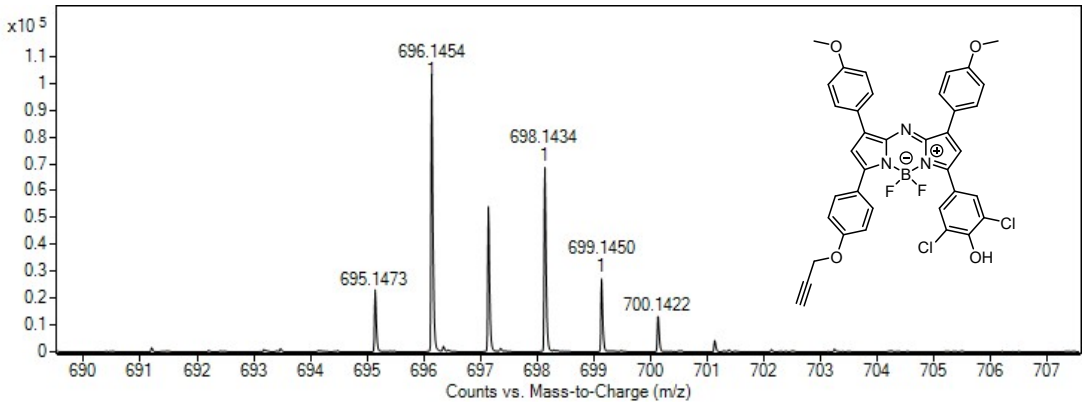
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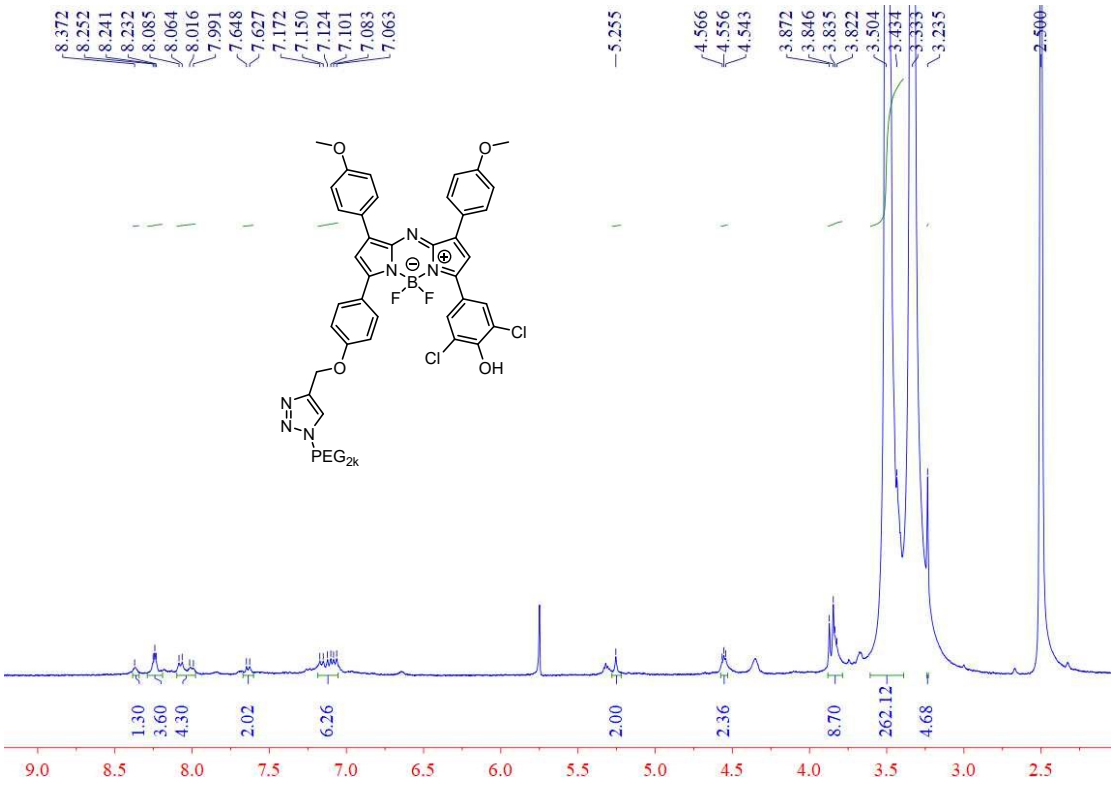
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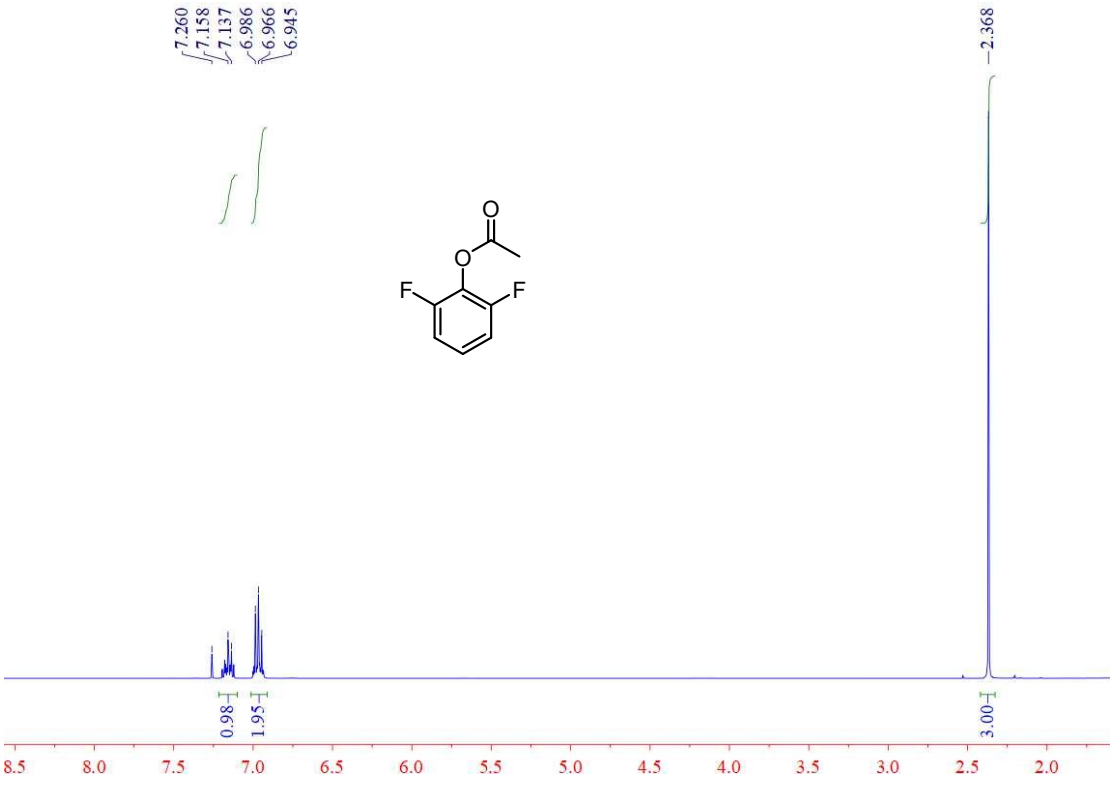
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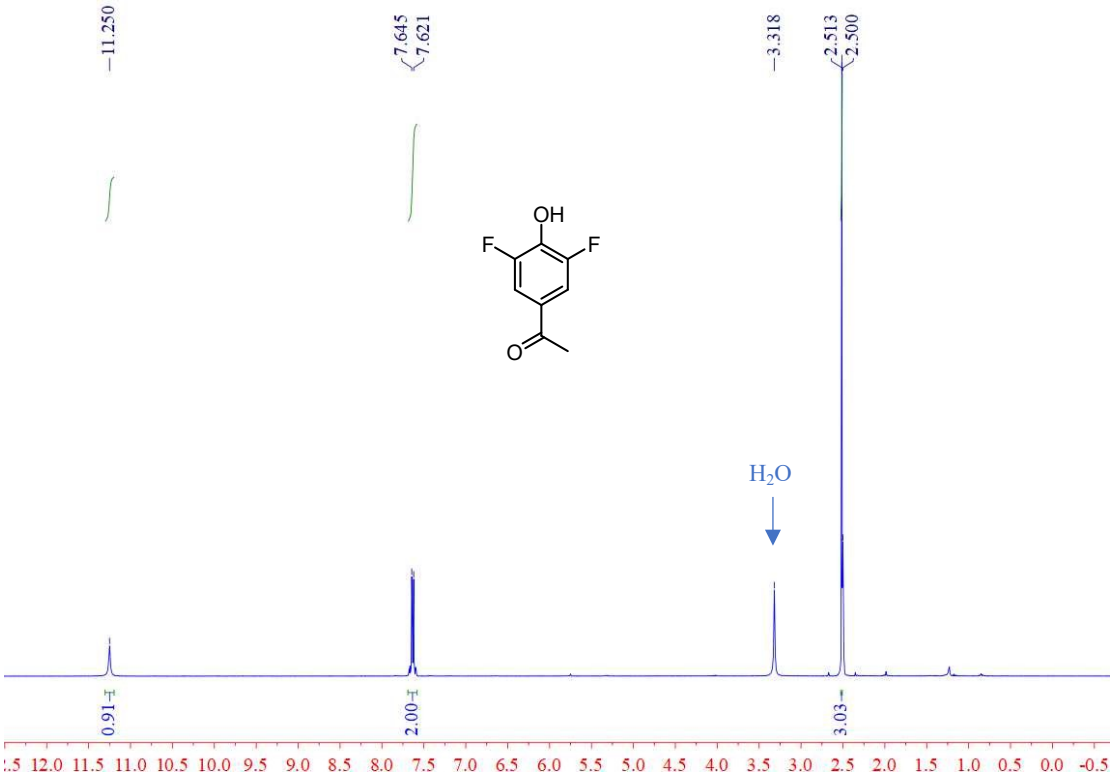
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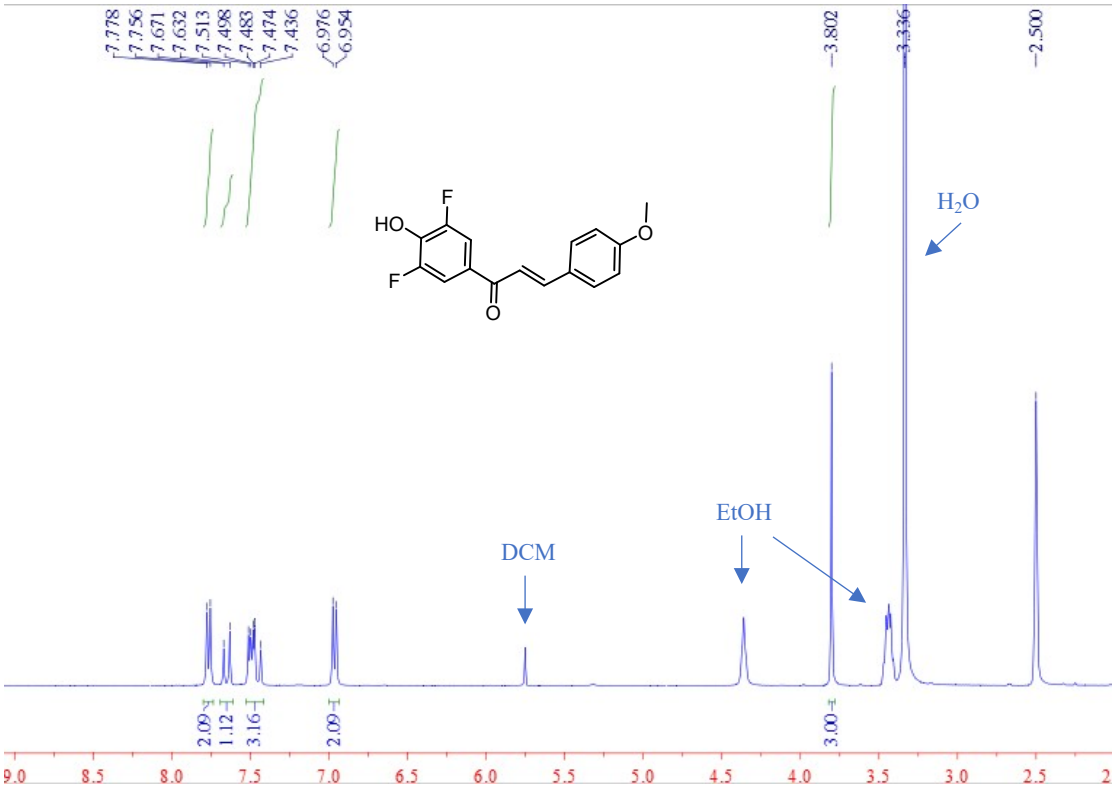
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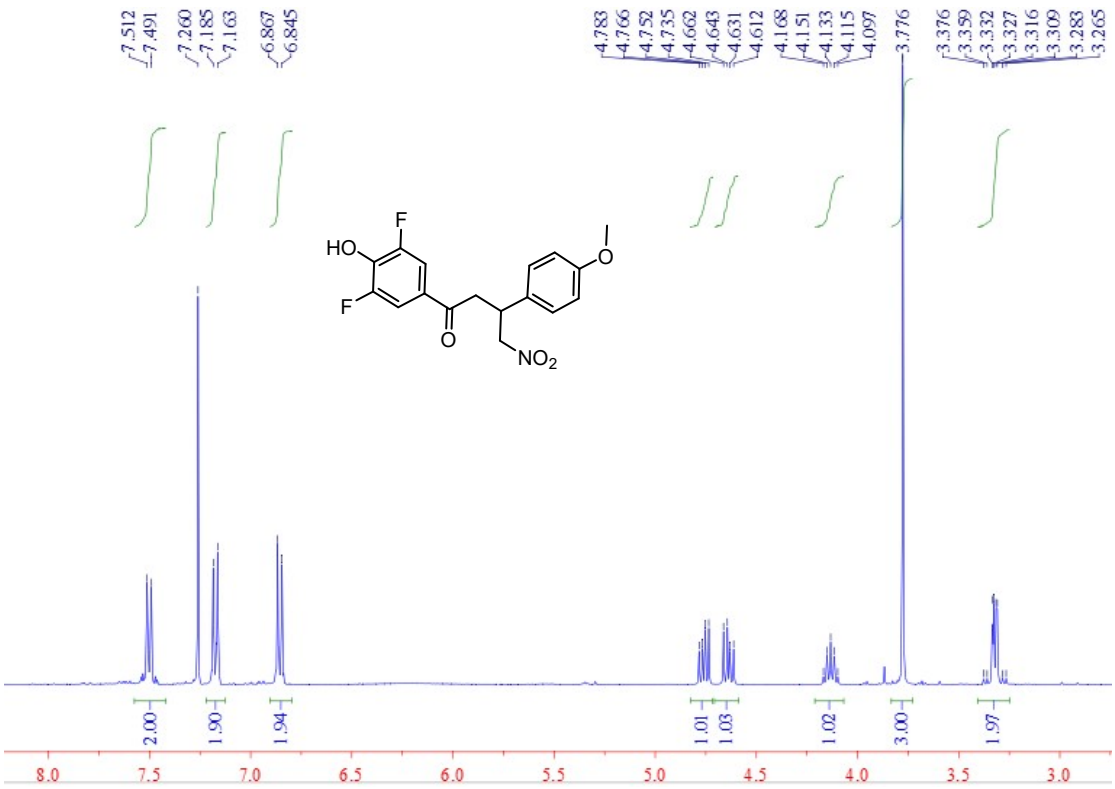
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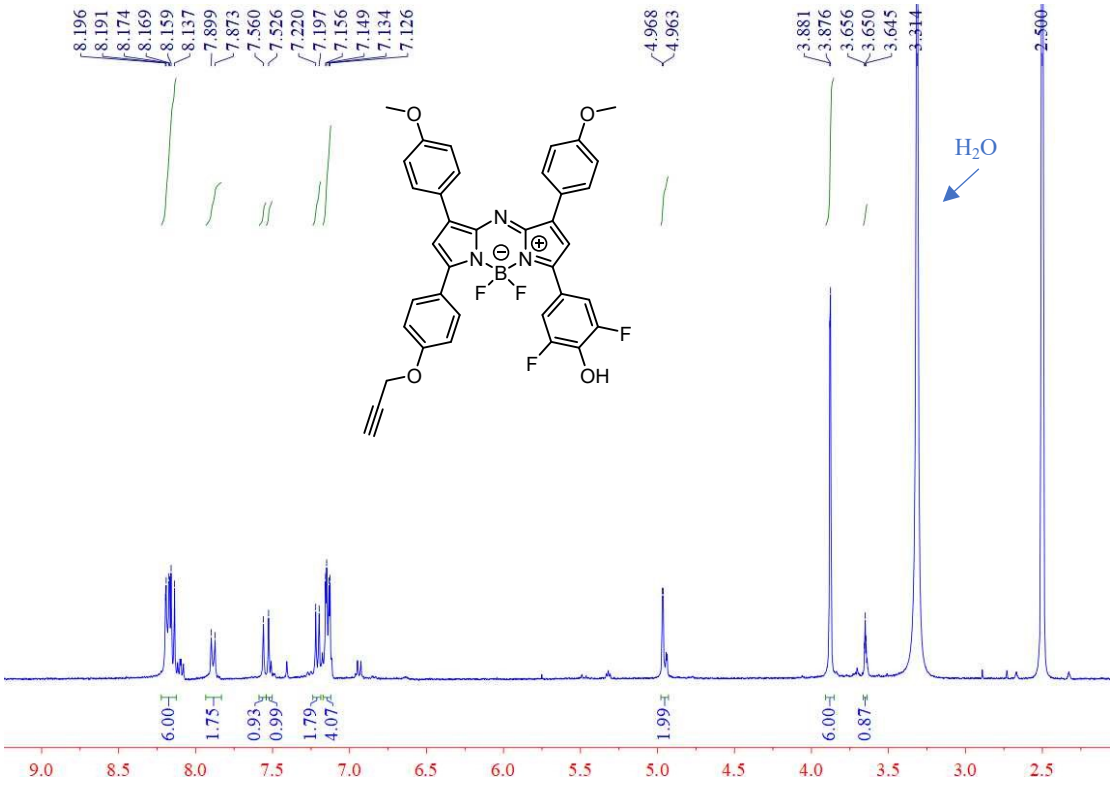
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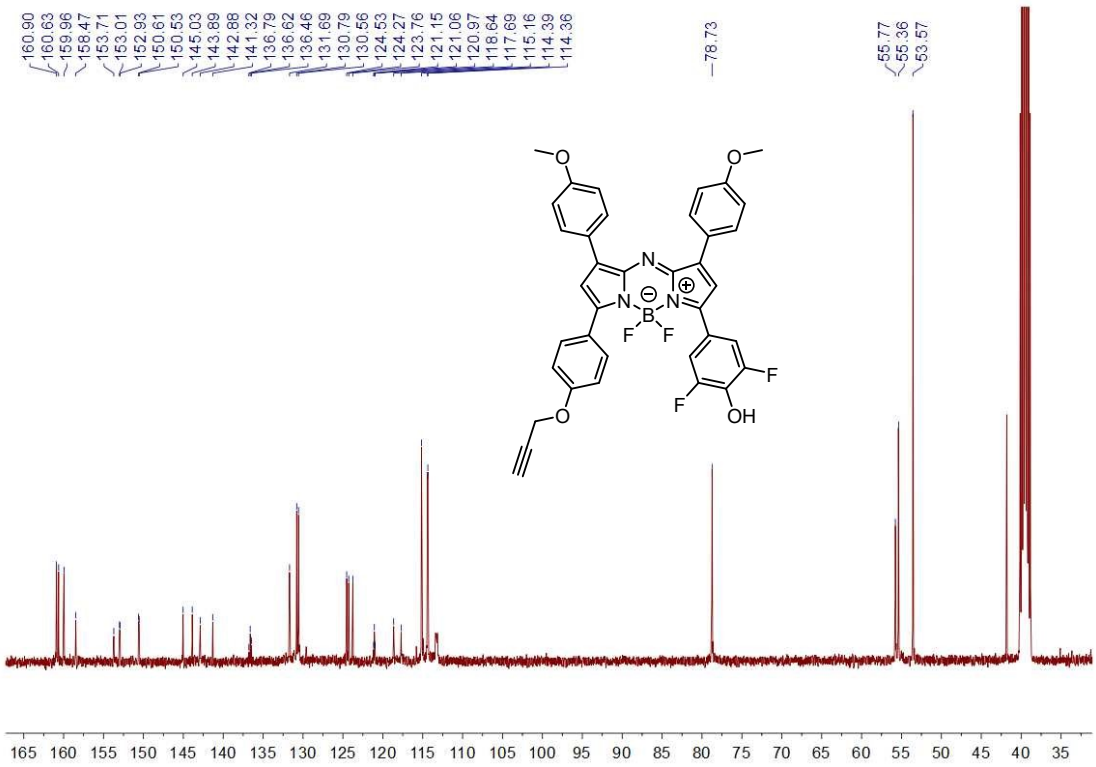
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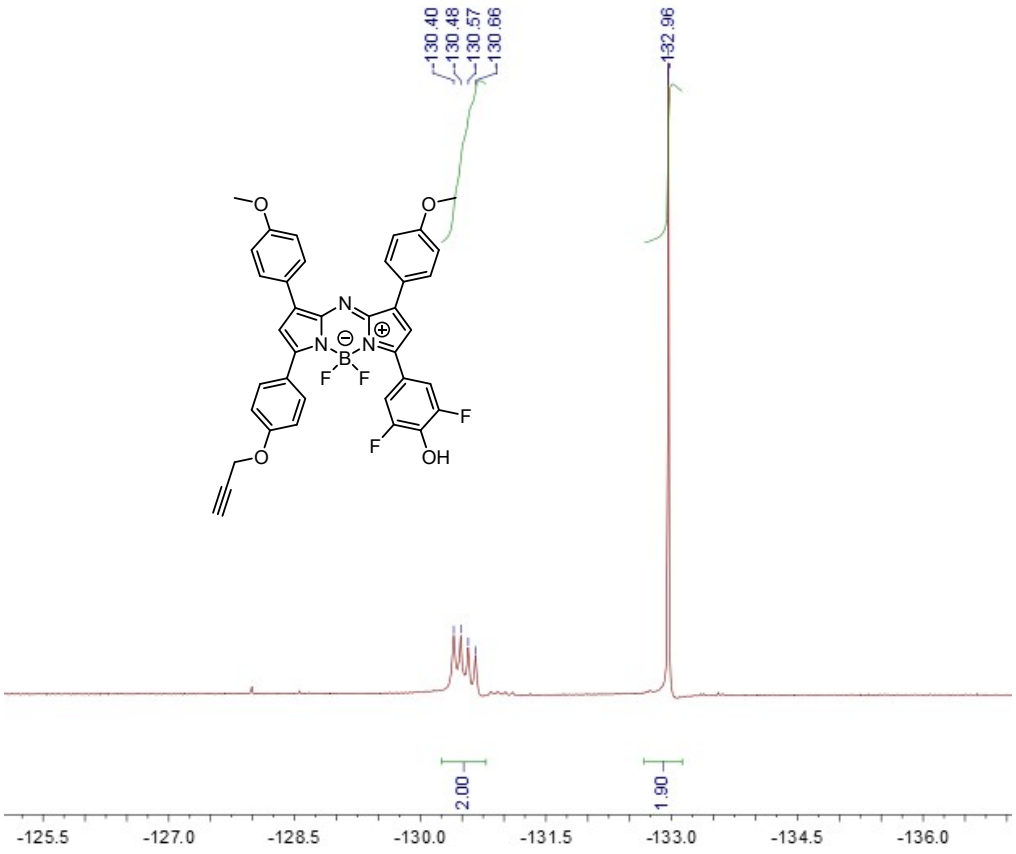
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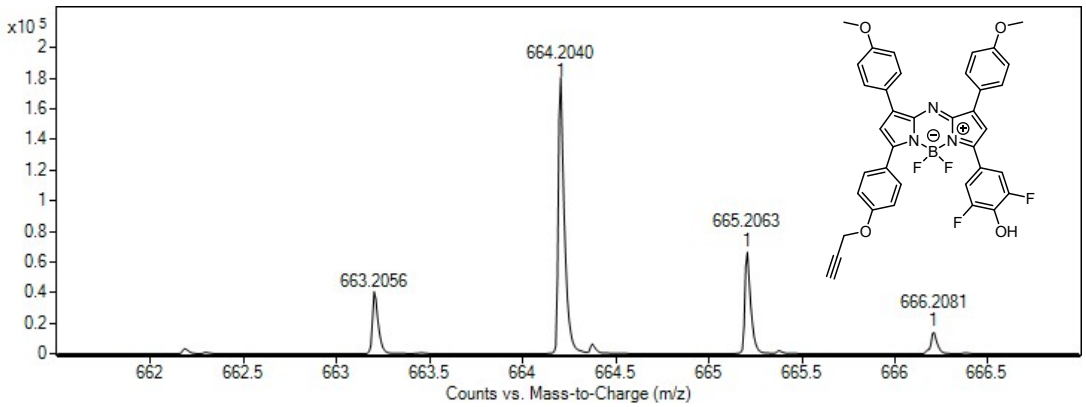
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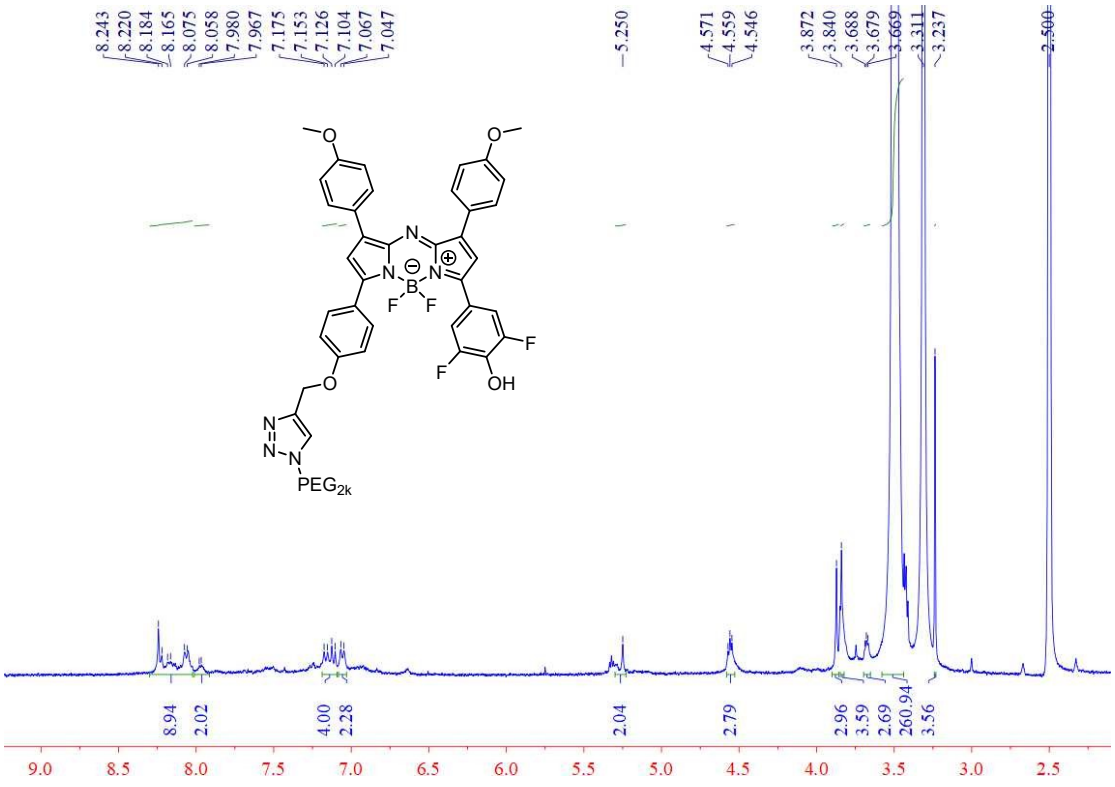
664.2040
665.2063
663.2056
666.2081
Counts vs. Mass-to-Charge (m/z)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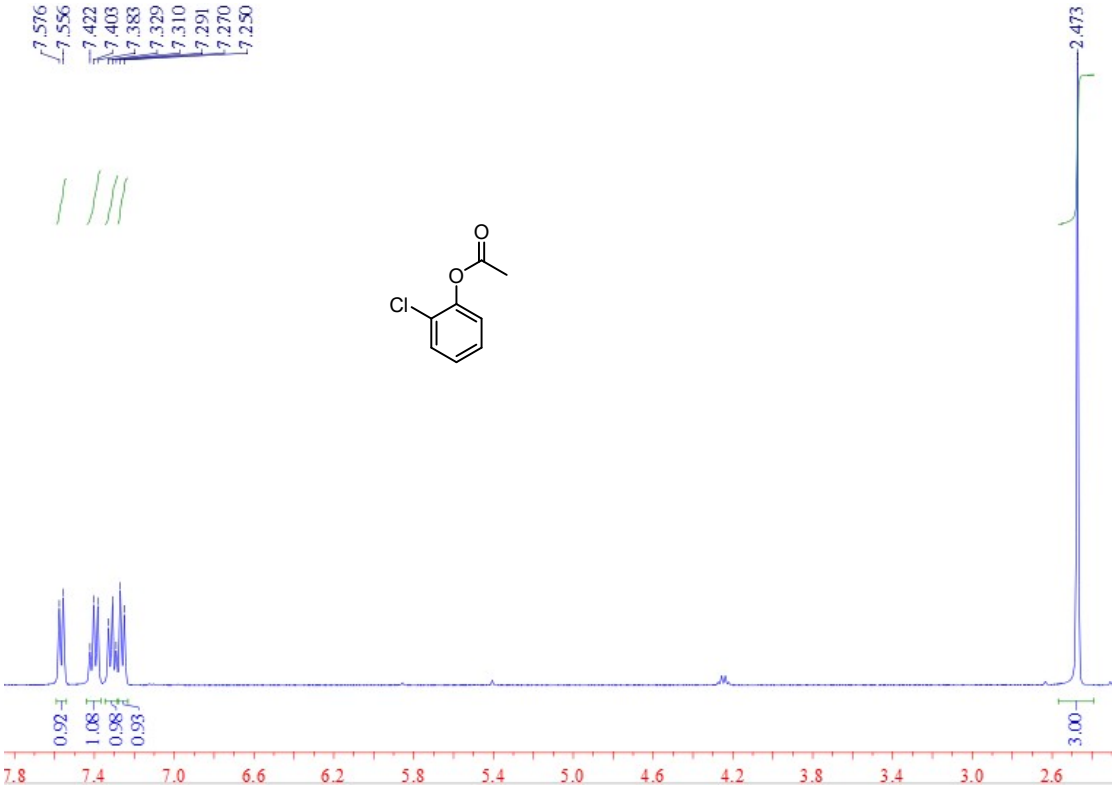
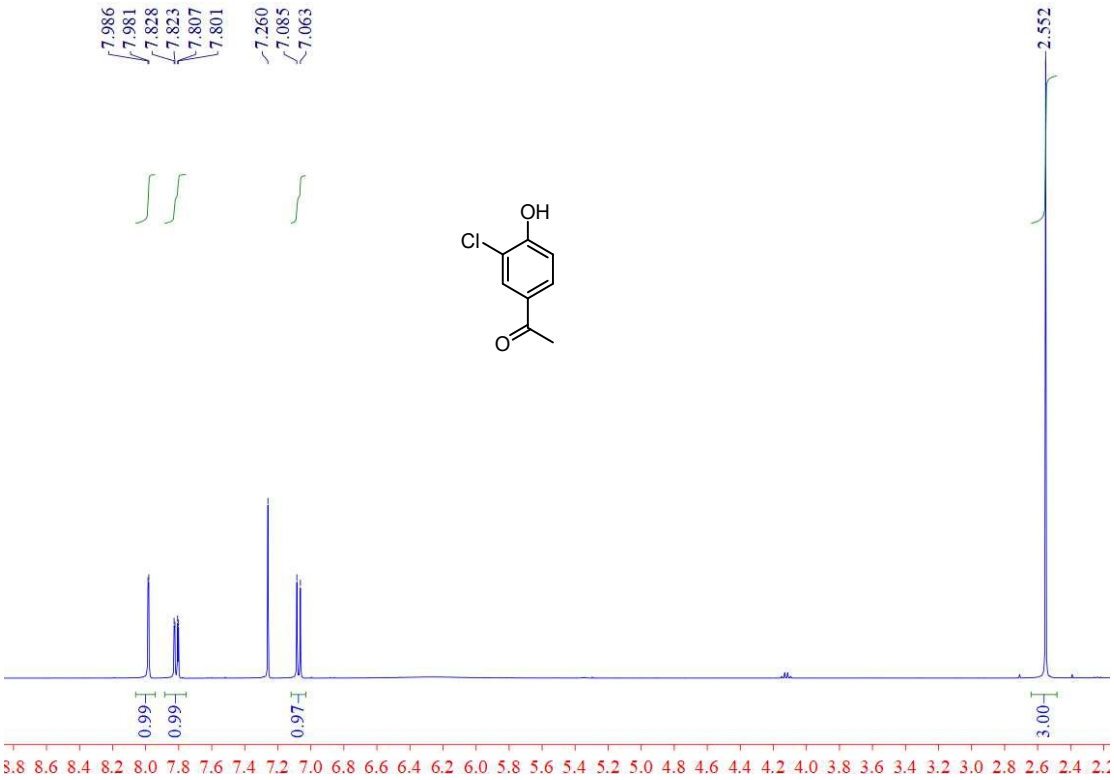
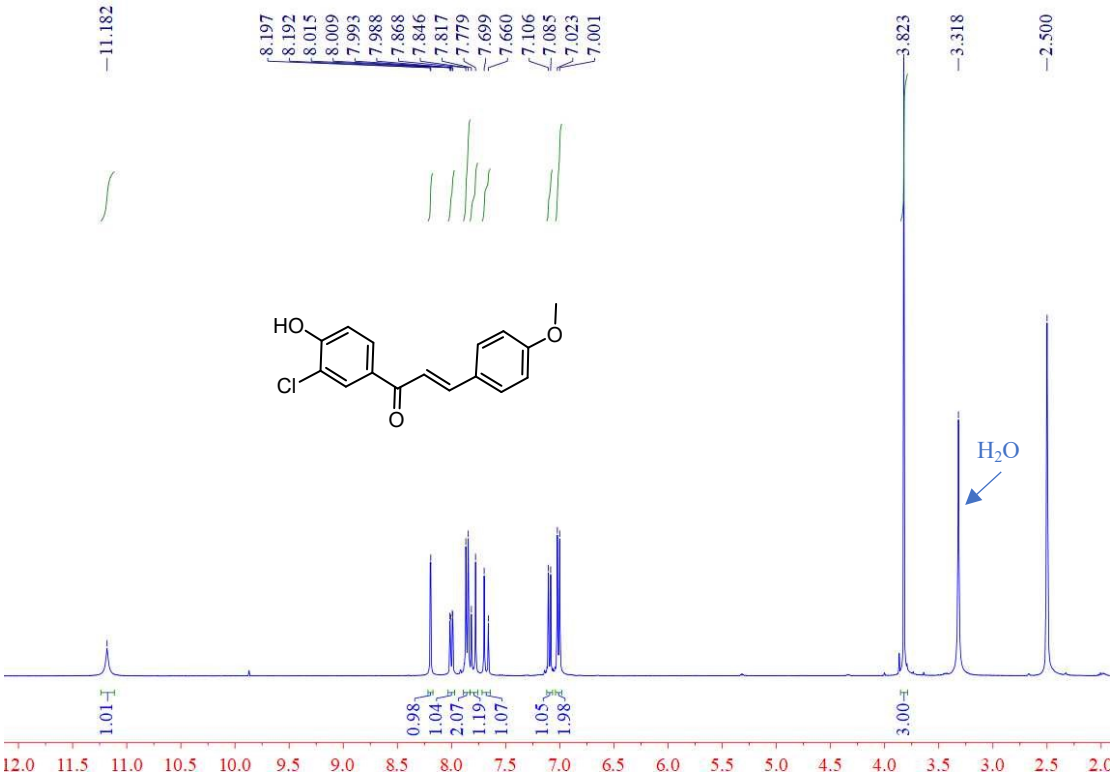
8.220
8.184
8.165
8.075
8.058
7.980
7.967
7.175
7.153
7.126
7.104
7.067
7.047
5.250
4.571
4.559
4.546
3.872
3.840
3.688
3.679
3.669
3.311
3.237
2.500
O
O
N
N
B
F
F
F
F
OH
O
N
N
N
PEG2k
8.94
2.02
4.00
2.28
2.04
2.79
2.96
3.59
2.69
260.94
3.56
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5


7.576
7.556
7.422
7.403
7.383
7.329
7.310
7.291
7.270
7.250
2.473
O
O
Cl
0.92
1.08
0.98
0.93
3.00
7.8
7.4
7.0
6.6
6.2
5.8
5.4
5.0
4.6
4.2
3.8
3.4
3.0
2.6


7.986
7.981
7.828
7.823
7.807
7.801
7.260
7.085
7.063
2.552
OH
Cl
O
0.99
0.99
0.97
3.00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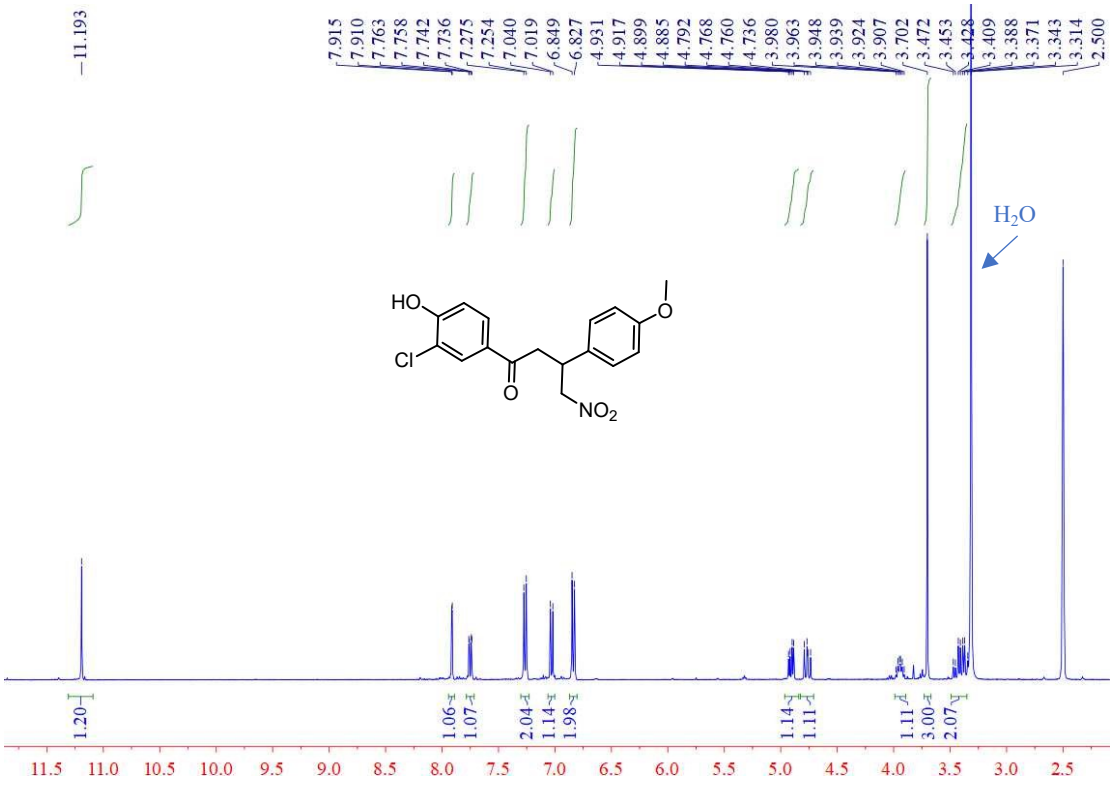
8.197
8.192
8.015
8.009
7.993
7.988
7.868
7.846
7.817
7.779
7.699
7.660
7.106
7.085
7.023
7.001
3.823
3.318
2.500
HO
Cl
O
O
H2O
1.01
0.98
1.04
2.07
1.19
1.07
1.05
1.98
3.00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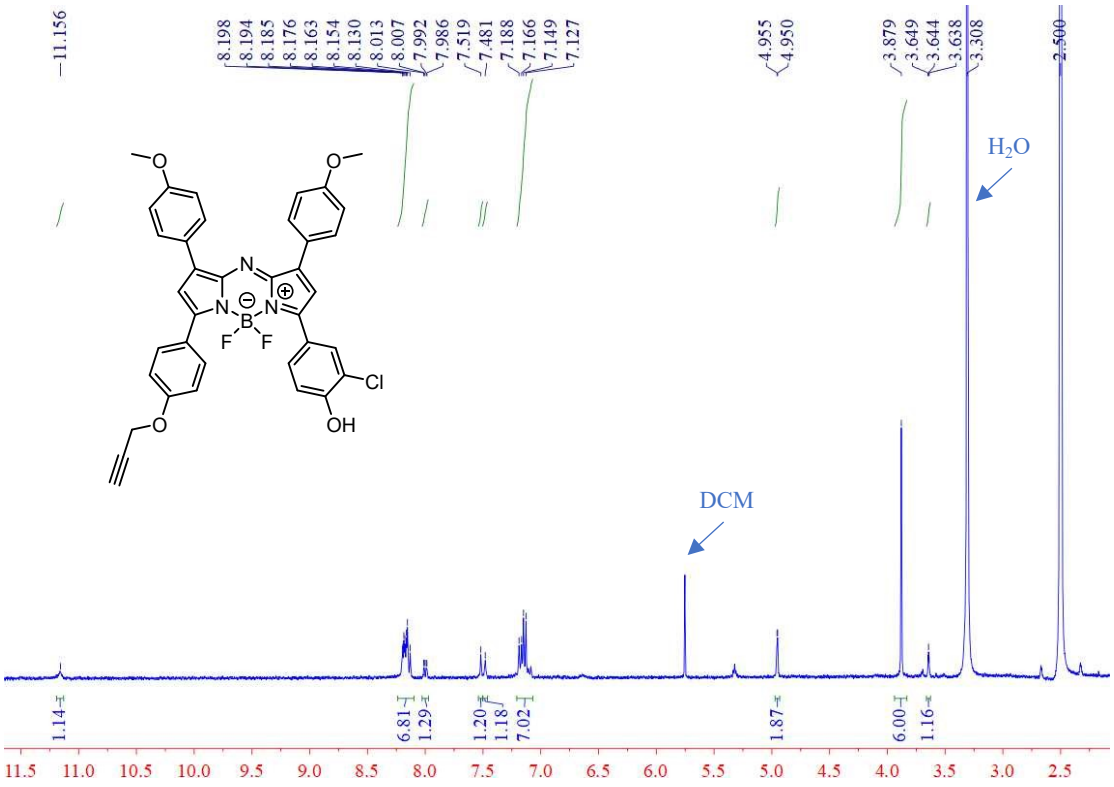
7.915
7.910
7.763
7.758
7.742
7.736
7.275
7.254
7.040
7.019
6.849
6.827
4.931
4.917
4.899
4.885
4.792
4.768
4.760
4.736
3.980
3.963
3.948
3.939
3.924
3.907
3.702
3.472
3.453
3.428
3.409
3.388
3.371
3.343
3.314
2.500
H2O
HO
Cl
O
O
NO2
1.20
1.06
1.07
2.04
1.14
1.98
1.14
1.11
1.11
3.00
2.07
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5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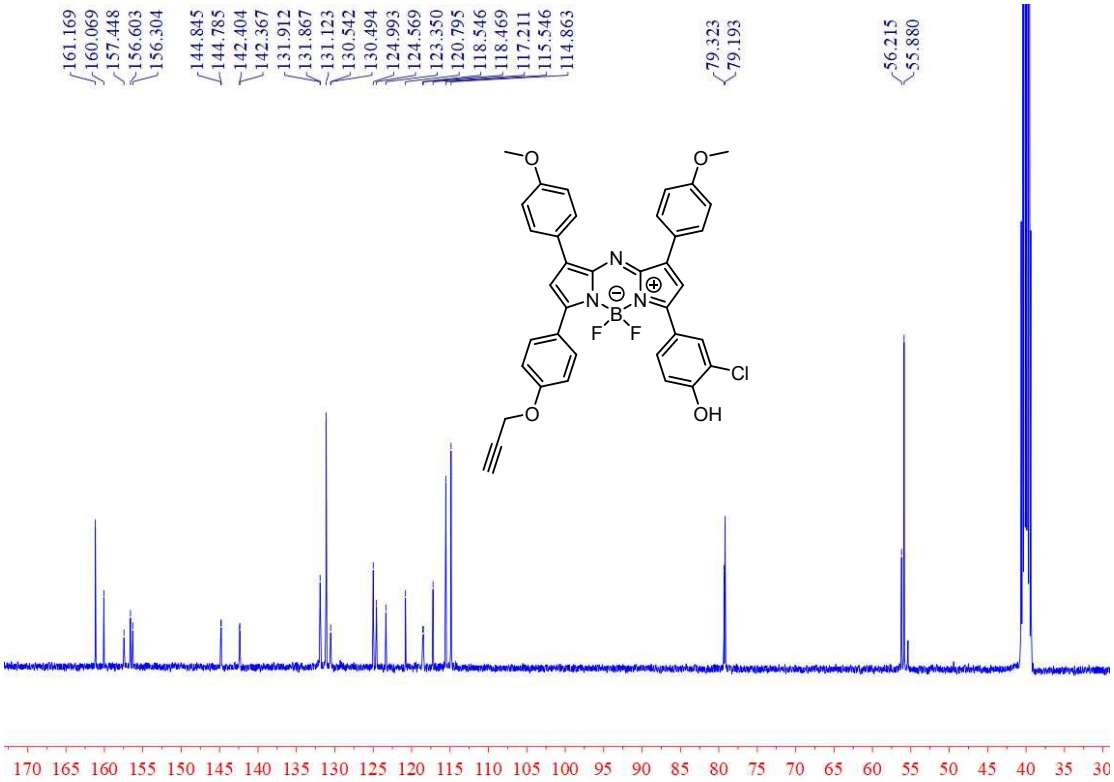
8.198
8.194
8.185
8.176
8.163
8.154
8.130
8.013
8.007
7.992
7.986
7.519
7.481
7.188
7.166
7.149
7.127
4.955
4.950
3.879
3.649
3.644
3.638
3.308
2.500
H2O
DCM
O
O
N
N
B
F
F
N
Cl
OH
O
1.14
6.81
1.29
1.20
1.18
7.02
1.87
6.00
1.16
11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5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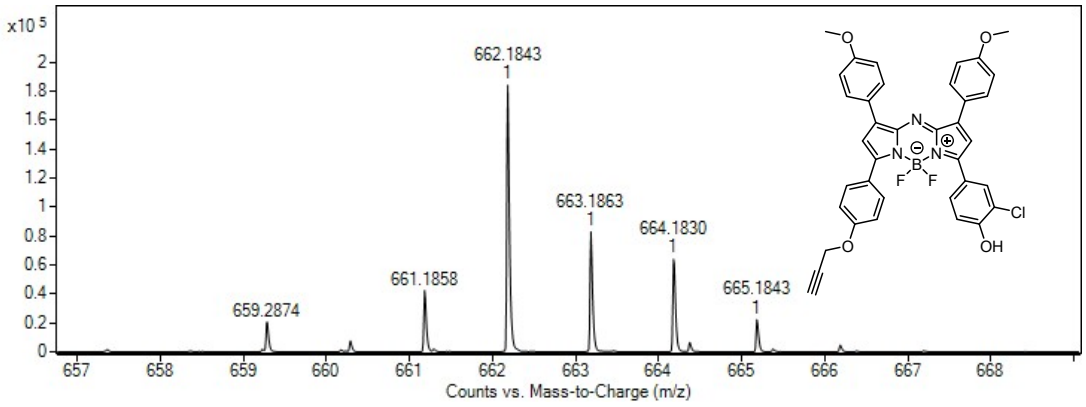
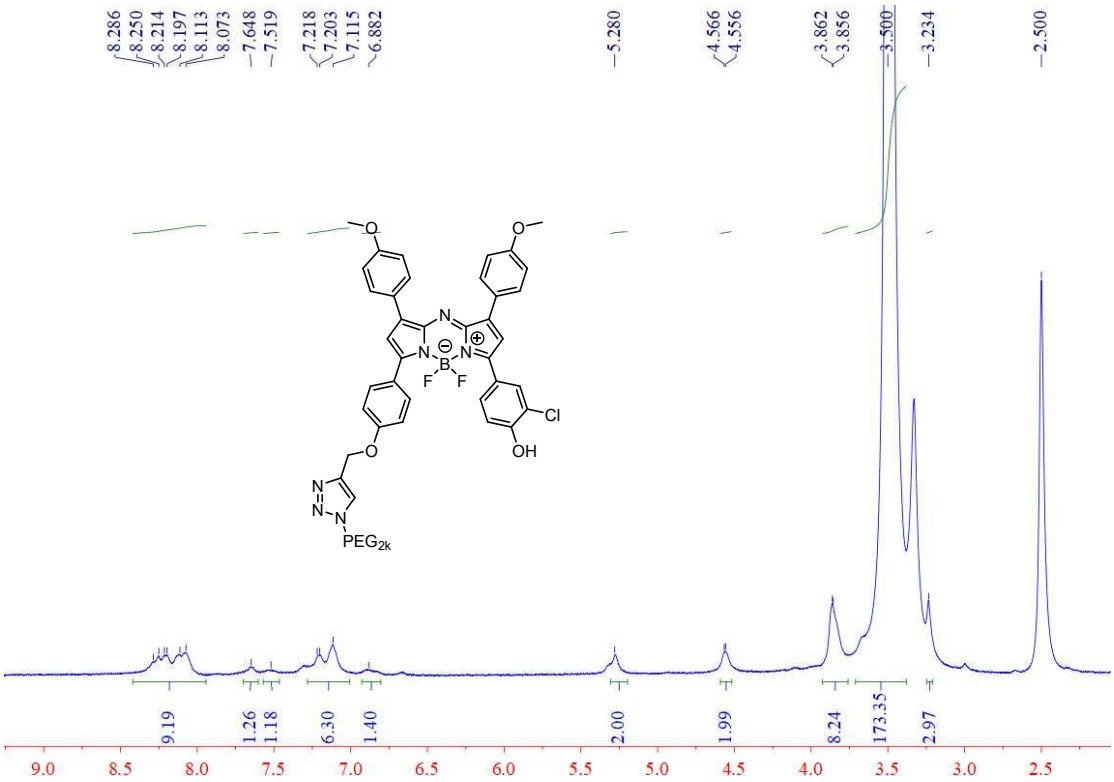
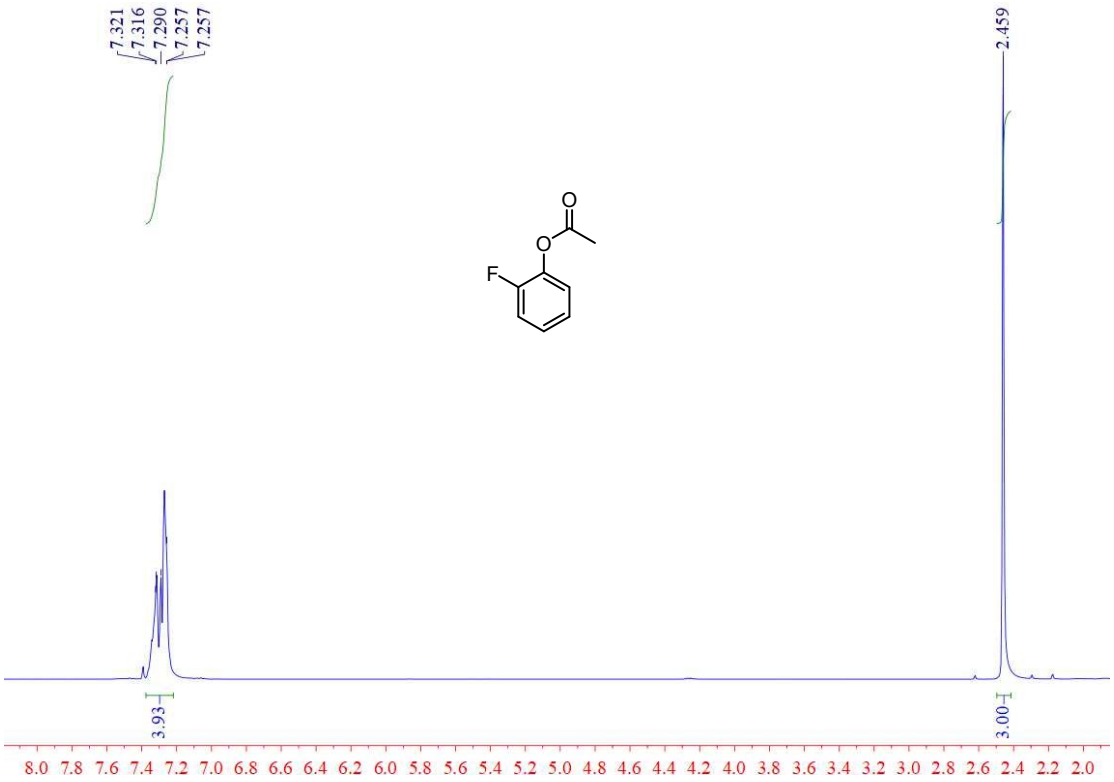
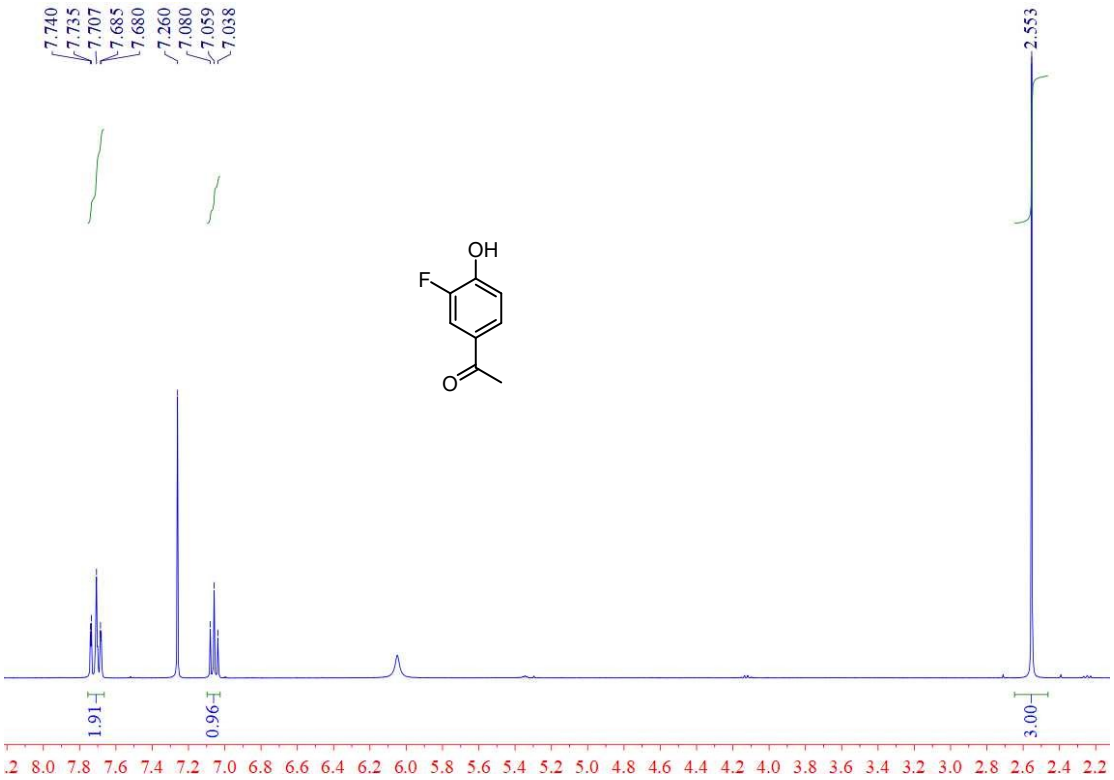
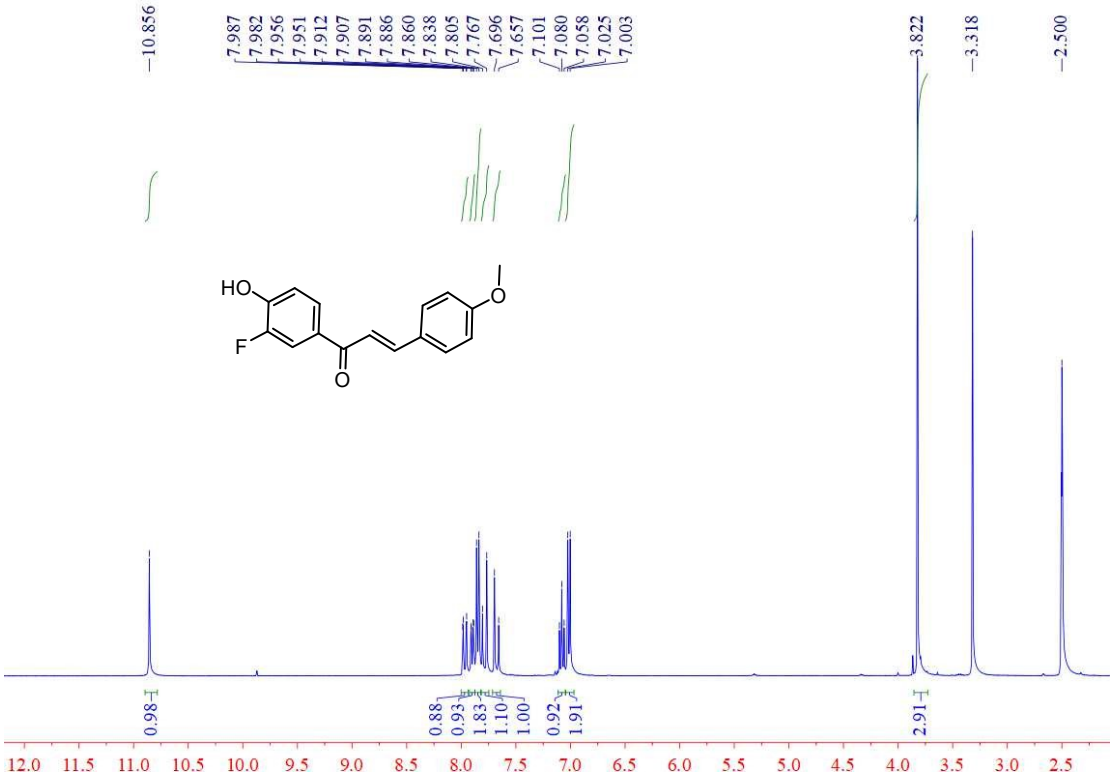
160.069
157.448
156.603
156.304
144.845
144.785
142.404
142.367
131.912
131.867
131.123
130.542
130.494
124.993
124.569
123.350
120.795
118.546
118.469
117.211
115.546
114.863
79.323
79.193
56.215
55.880
O
O
N
N
B
F
F
Cl
OH
O
170 165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30

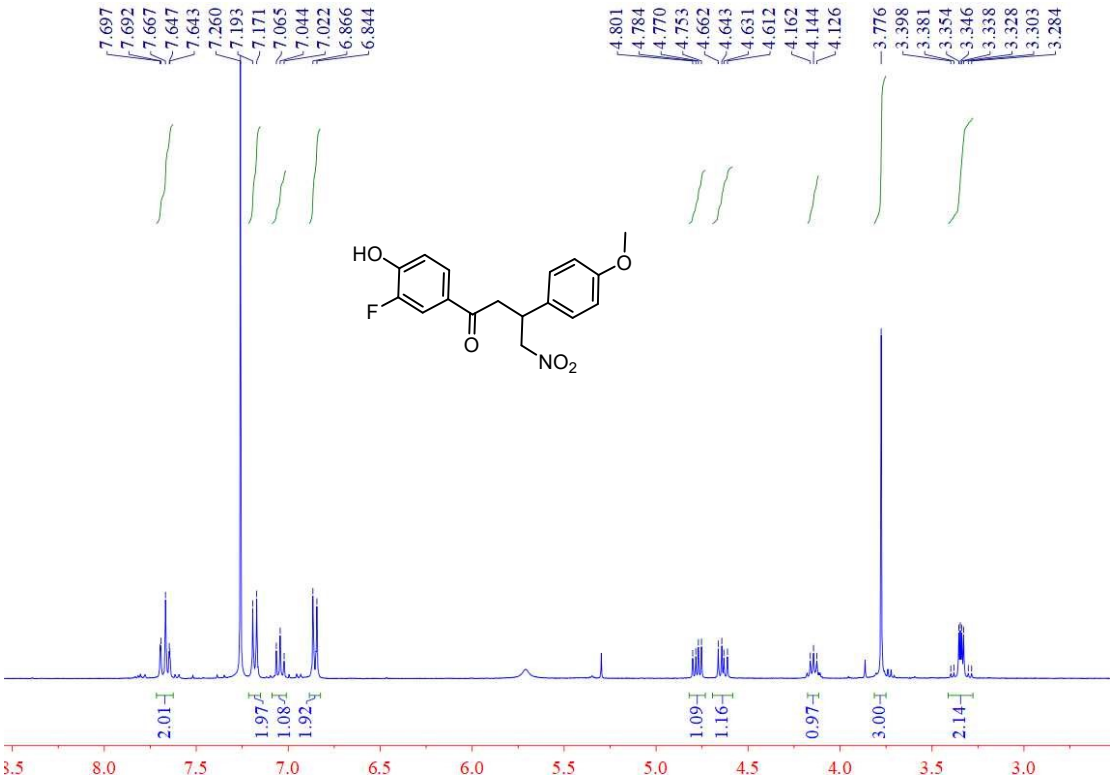

x10 5
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
662.1843
1
663.1863
1
664.1830
1
661.1858
665.1843
1
659.2874
657
658
659
660
661
662
663
664
665
666
667
668
Counts vs. Mass-to-Charge (m/z)
O
O
N
N
B
F
F
Cl
OH


8.286
8.250
8.214
8.197
8.113
8.073
7.648
7.519
7.218
7.203
7.115
6.882
5.280
4.566
4.556
3.862
3.856
3.500
3.234
2.500
O
O
N
N
B
F
F
N
N
Cl
OH
O
N
N
N
PEG2k
9.19
1.26
1.18
6.30
1.40
2.00
1.99
8.24
173.35
2.97
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5


7.321
7.316
7.290
7.257
7.257
2.459
O
O
F
3.93
3.00
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0


7.740
7.735
7.707
7.685
7.680
7.260
7.080
7.059
7.038
2.553
OH
F
O
1.91
0.96
3.00


10.856
7.987
7.982
7.956
7.951
7.912
7.907
7.891
7.886
7.860
7.838
7.805
7.767
7.696
7.657
7.101
7.080
7.058
7.025
7.003
3.822
3.318
2.500
HO
F
O
O
0.98
0.88
0.93
1.83
1.10
1.00
0.92
1.91
2.91


7.697
7.692
7.667
7.647
7.643
7.260
7.193
7.171
7.065
7.044
7.022
6.866
6.844
4.801
4.784
4.770
4.753
4.662
4.643
4.631
4.612
4.162
4.144
4.126
3.776
3.398
3.381
3.354
3.346
3.338
3.328
3.303
3.284
HO
F
O
O
NO2
2.01
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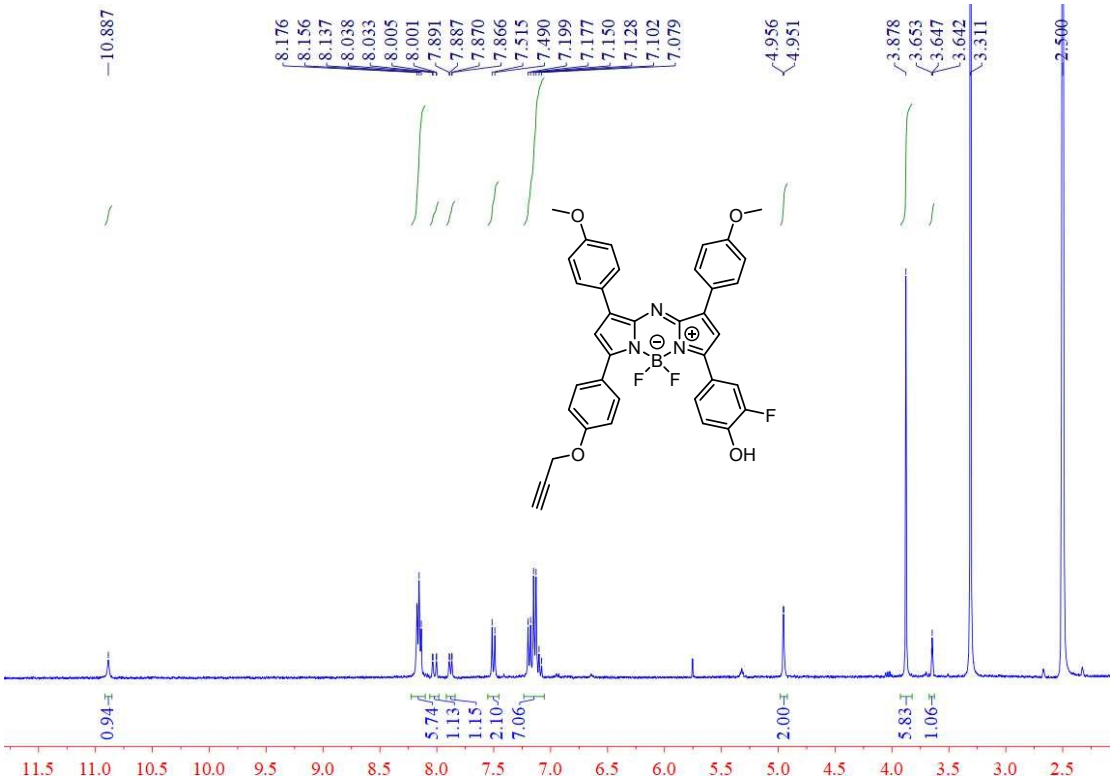
1.08
1.92
1.09
1.16
0.97
3.00
2.14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8.176
8.156
8.137
8.038
8.033
8.005
8.001
7.891
7.887
7.870
7.866
7.515
7.490
7.199
7.177
7.150
7.128
7.102
7.079
4.956
4.951
3.878
3.653
3.647
3.642
3.311
2.500
O
O
N
N
B
F
F
F
OH
O
0.94
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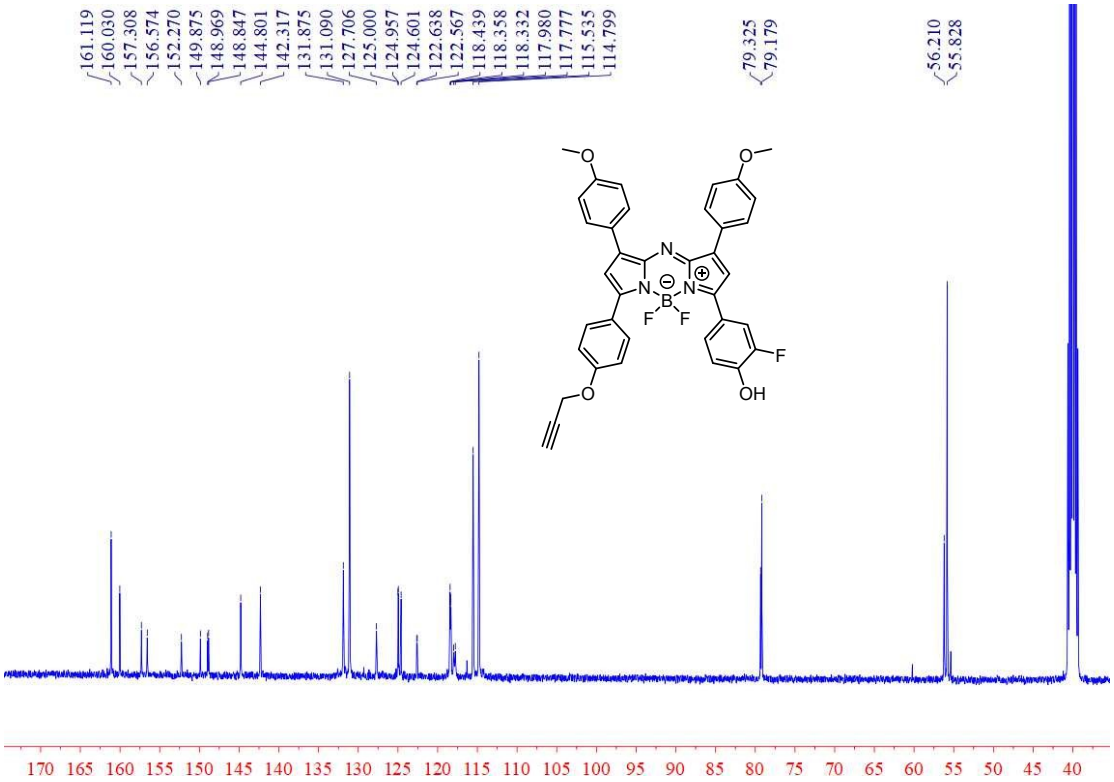
1.13
1.15
2.10
7.06
2.00
5.83
1.06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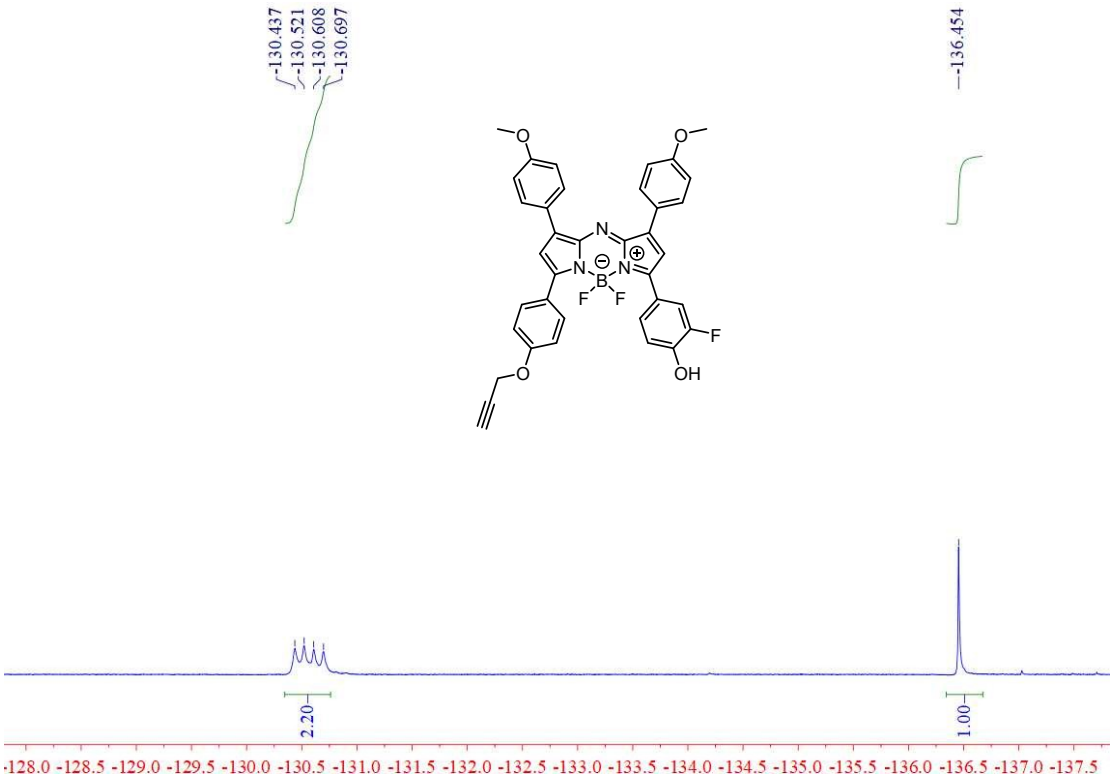
156.574
152.270
149.875
148.969
148.847
144.801
142.317
131.875
131.090
127.706
125.000
124.957
124.601
122.638
122.567
118.439
118.358
118.332
117.980
117.777
115.535
114.799
79.325
79.179
56.210
55.828
O
O
N
N
B
F
F
F
OH
O
170 165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40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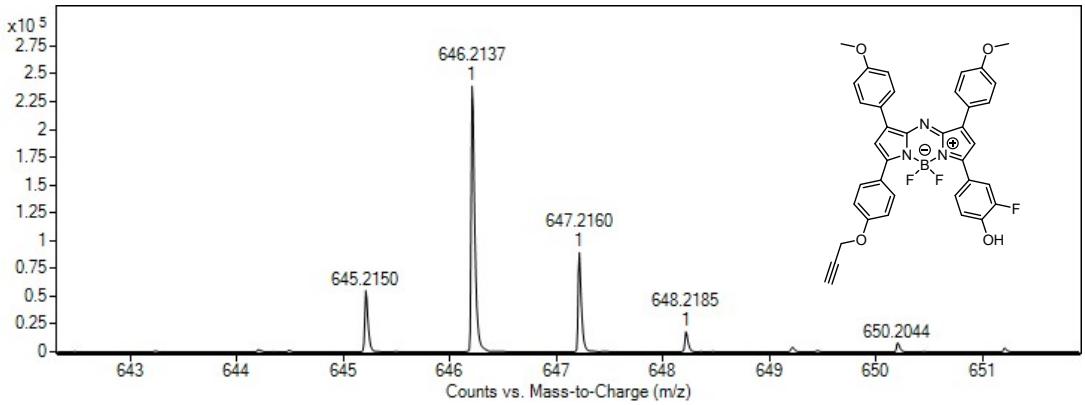
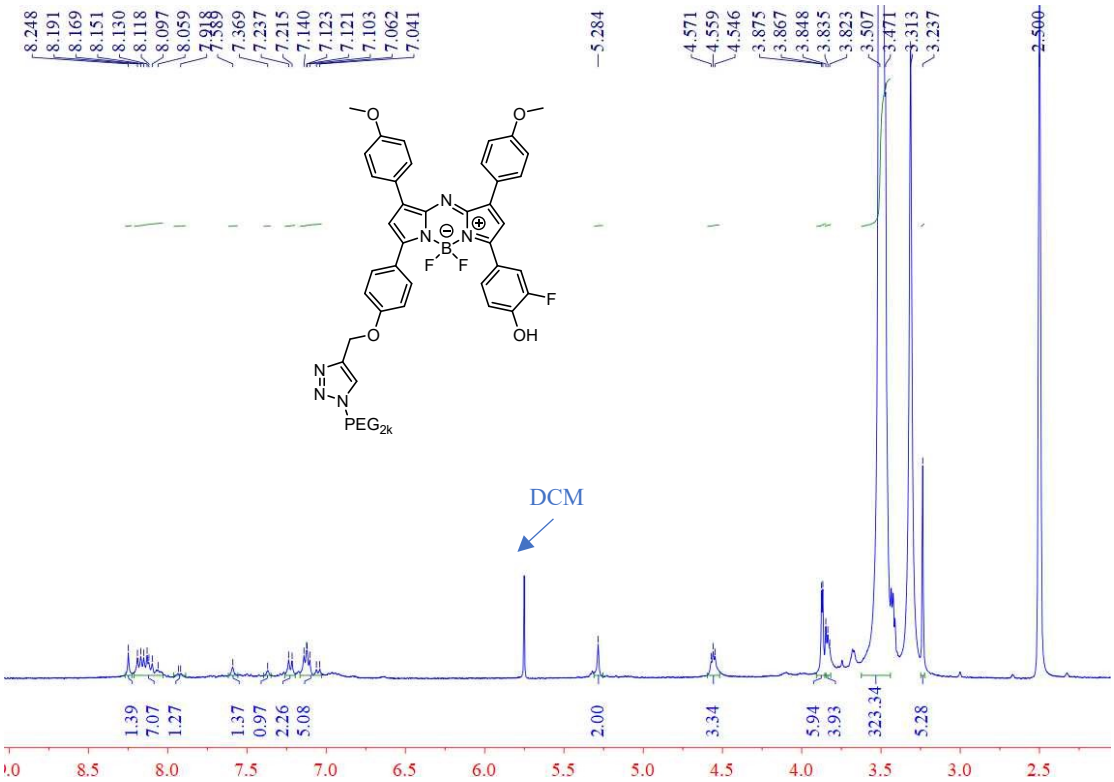
-130.521
-130.608
-130.697
-136.454
O
O
N
N
B
F
F
F
OH
O
2.20
1.00
-128.0 -128.5 -129.0 -129.5 -130.0 -130.5 -131.0 -131.5 -132.0 -132.5 -133.0 -133.5 -134.0 -134.5 -135.0 -135.5 -136.0 -136.5 -137.0 -137.5


x10 5
2.75
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
646.2137
1
647.2160
1
645.2150
648.2185
1
650.2044
643
644
645
646
647
648
649
650
651
Counts vs. Mass-to-Charge (m/z)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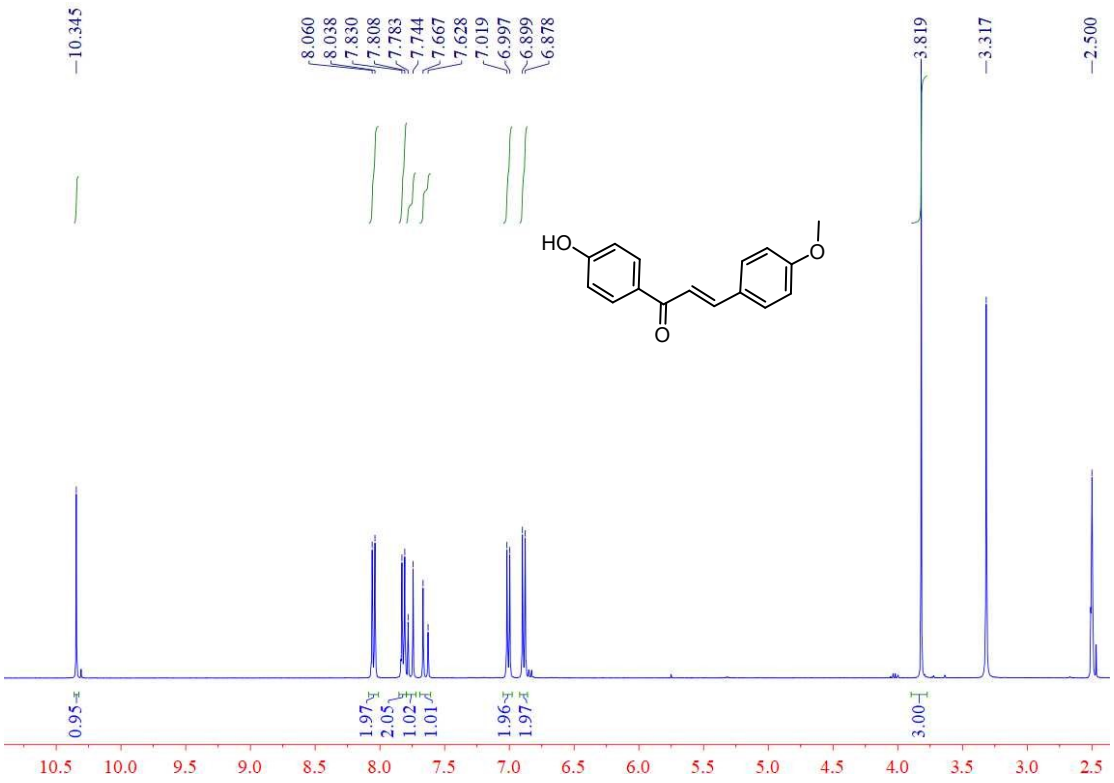
8.191
8.169
8.151
8.130
8.118
8.097
8.059
7.589
7.369
7.237
7.215
7.140
7.123
7.121
7.103
7.062
7.041
5.284
4.571
4.559
4.546
3.875
3.867
3.848
3.835
3.823
3.507
3.471
3.313
3.237
2.500
PEG2k
DCM
1.39
7.07
1.27
1.37
0.97
2.26
5.08
2.00
3.34
5.94
3.93
323.34
5.28
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5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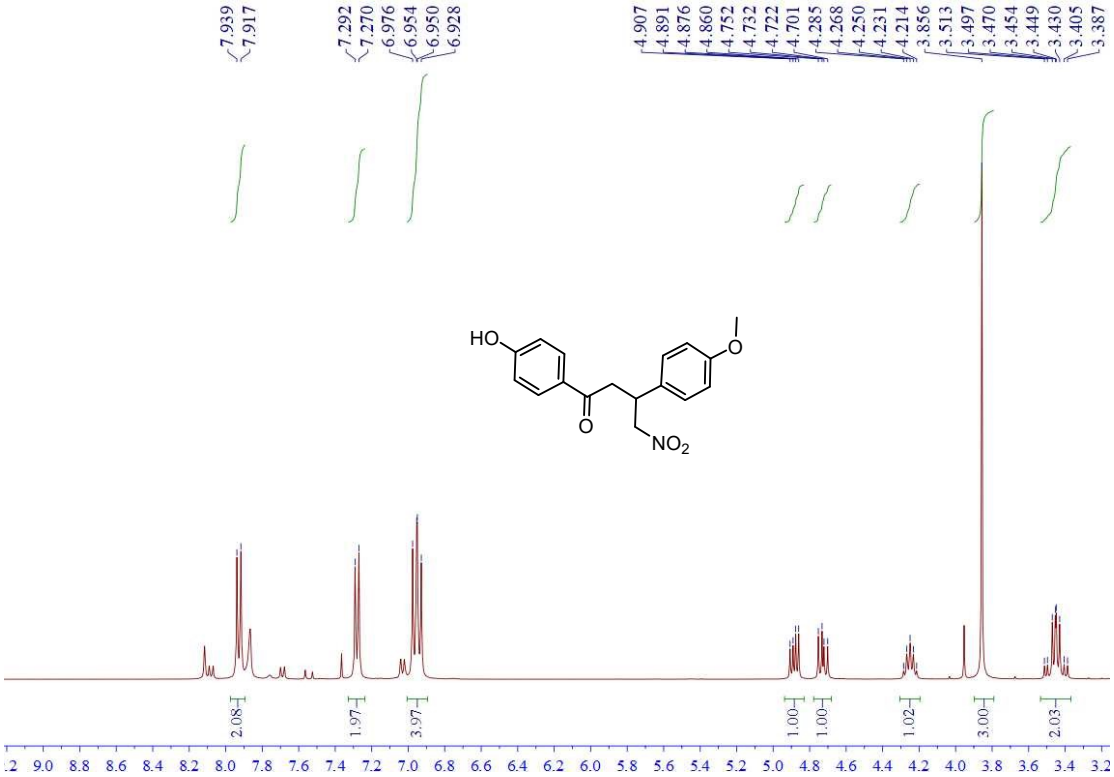
8.060
8.038
7.830
7.808
7.783
7.744
7.667
7.628
7.019
6.997
6.899
6.878
3.819
3.317
2.500
HO
O
O
0.95
1.97
2.05
1.02
1.01
1.96
1.97
3.00
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5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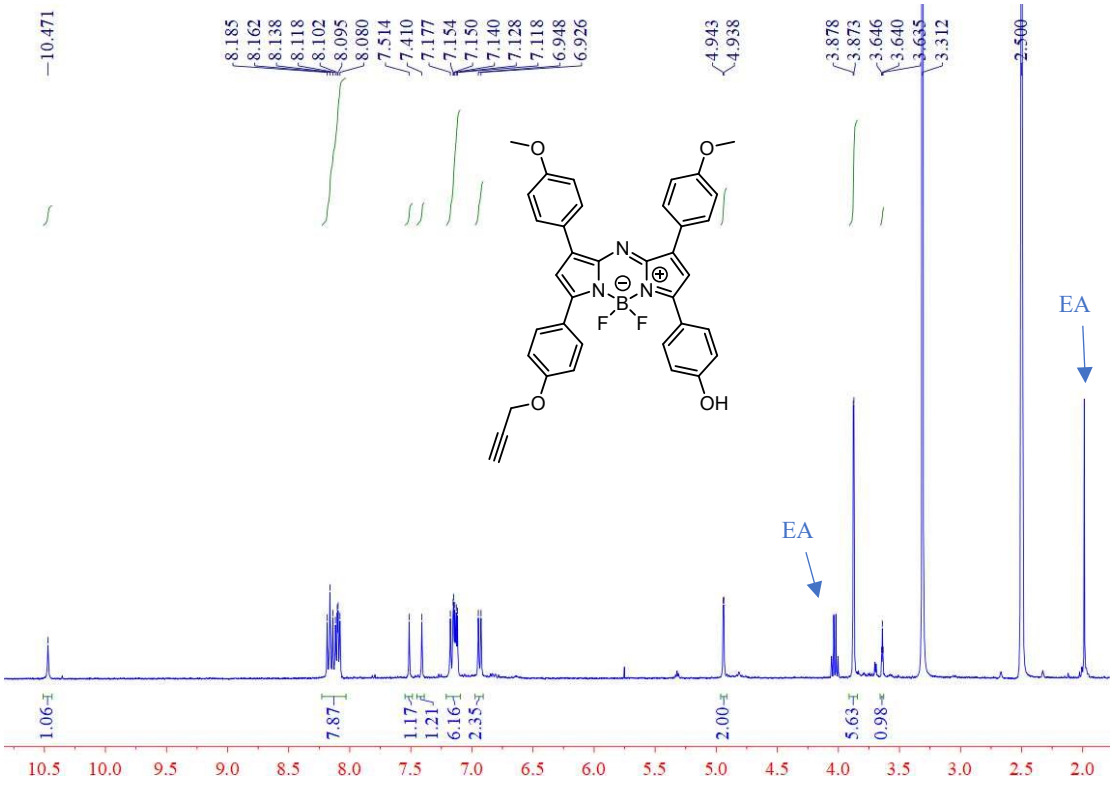
7.270
6.976
6.954
6.950
6.928
4.907
4.891
4.876
4.860
4.752
4.732
4.722
4.701
4.285
4.268
4.250
4.231
4.214
3.856
3.513
3.497
3.470
3.454
3.449
3.430
3.405
3.387
HO
O
O
NO2
2.08
1.97
3.97
1.00
1.00
1.02
3.00
2.03
.2 9.0 8.8 8.6 8.4 8.2 8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2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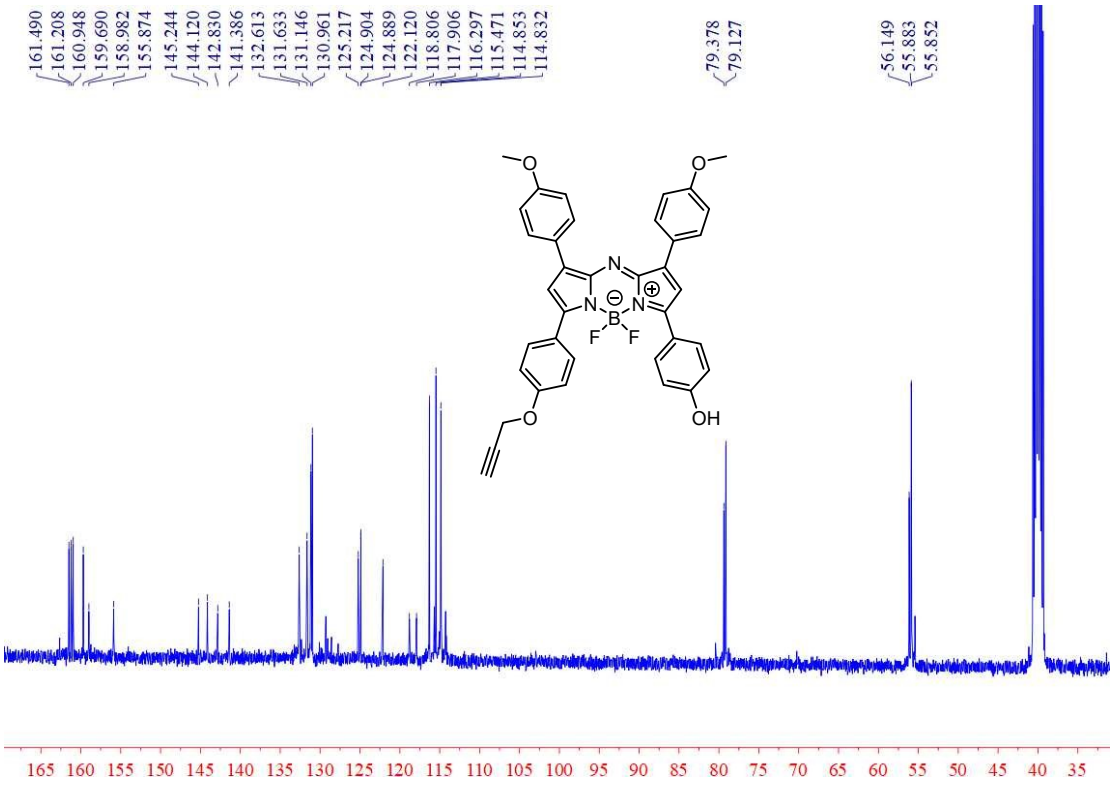
8.185
8.162
8.138
8.118
8.102
8.095
8.080
7.514
7.410
7.177
7.154
7.150
7.140
7.128
7.118
6.948
6.926
4.943
4.938
3.878
3.873
3.646
3.640
3.635
3.312
2.500
EA
EA
1.06
7.87
1.17
1.21
6.16
2.35
2.00
5.63
0.98
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0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161.208
160.948
159.690
158.982
155.874
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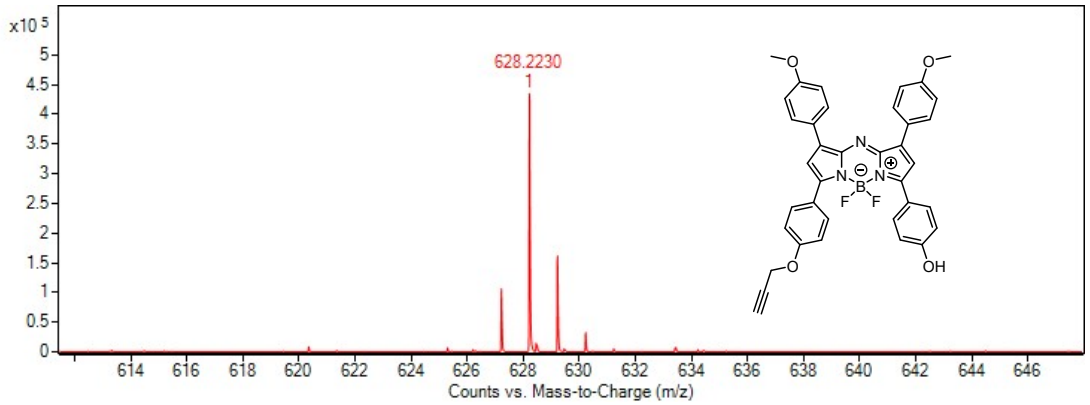
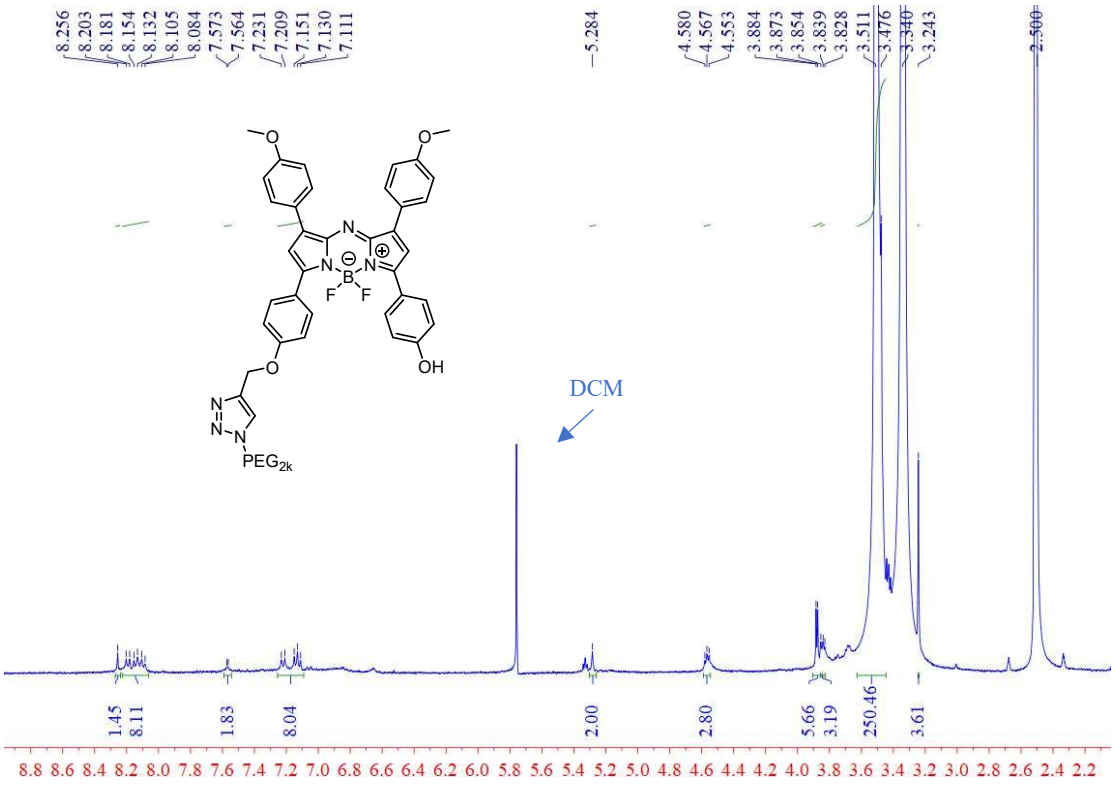
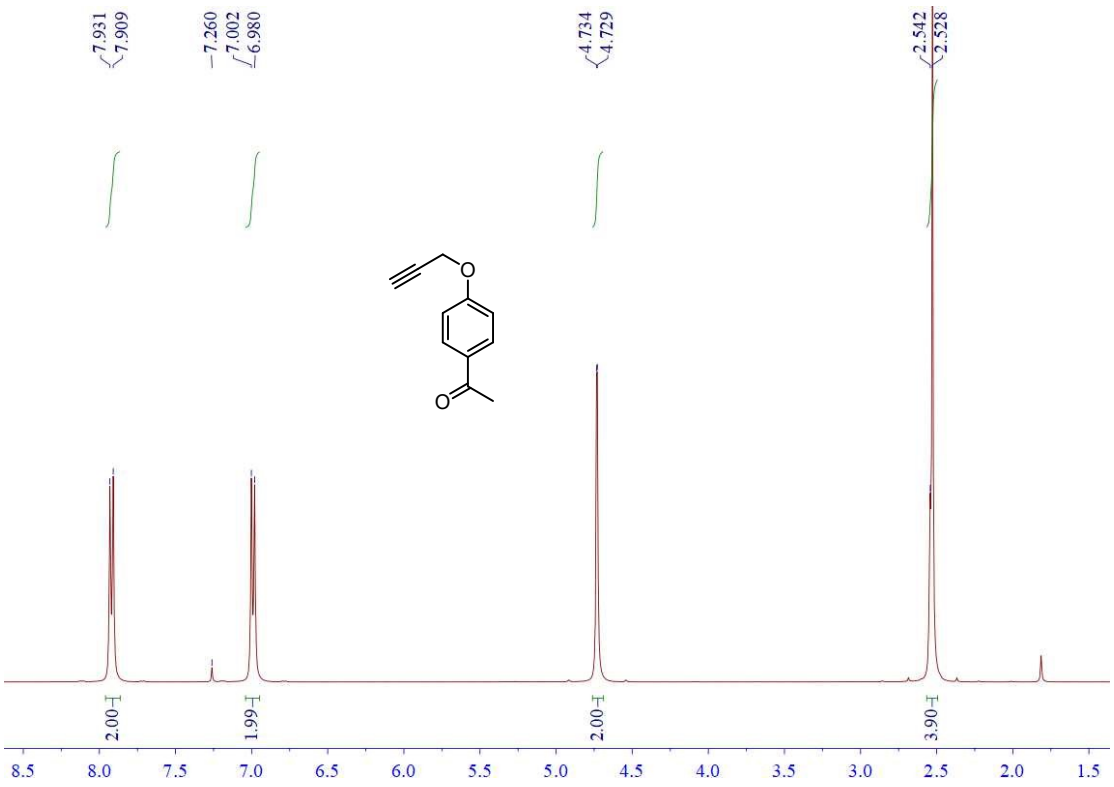
144.120
142.830
141.386
132.613
131.633
131.146
130.961
125.217
124.904
124.889
122.120
118.806
117.906
116.297
115.471
114.853
114.832
79.378
79.127
56.149
55.883
55.852
165 160 155 150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35


x10 5
628.2230
1
Counts vs. Mass-to-Charge (m/z)


DCM
PEG2k


7.931
7.909
7.260
7.002
6.980
4.734
4.729
2.542
2.528
O
O
2.00
1.99
2.00
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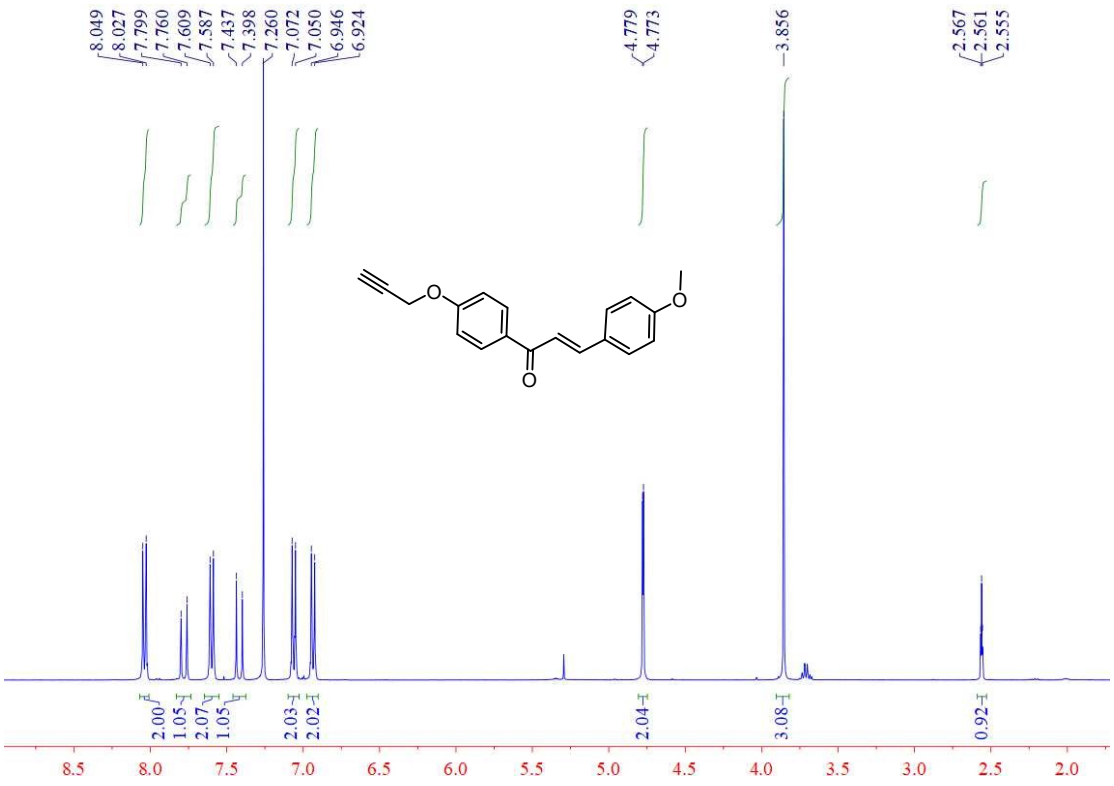
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O
O
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1.05
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2.02
2.04
3.08
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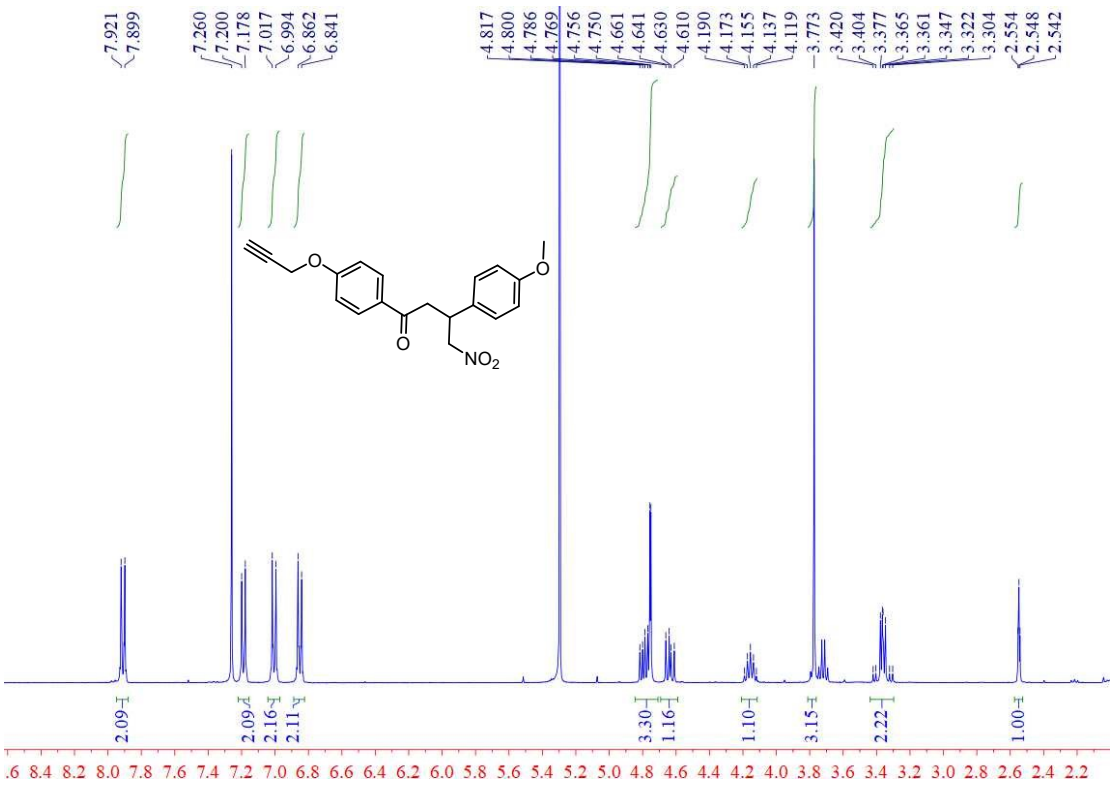
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