Electronic Supplementary Information

Aza-BODIPY based near-infrared fluorescent probe for sensitive discrimination of cysteine/homocysteine and glutathione in living cells

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Experimental section

Materials

Alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), cysteine (Cys), N-ethylmaleimide (NEM), glutamic acid (Glu), glutamine (Gln), glutathione (GSH), glycine (Gly), histidine (His), homocysteine (Hcy), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), tryptophan (Trp), and tyrosine (Tyr) were purchased from Sigma-Aldrich. All chemicals were used directly without any further purification. The water used in the experiments was distilled (further treated by ion exchange column from sanda Milli-Q water purification system). Reagents for cell culture, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, and penicillin-streptomycin were purchased from Life Technologies Holding Pte Ltd.

Characterization

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-300 spectrometer, and chemical shifts (δ, ppm) were recorded by using internal reference tetramethyl silane. High-resolution mass spectrometry (HRMS) was performed on a waters Q-tof Premier MS spectrometer. High performance liquid chromatography (HPLC) studies were record by a Shimadzu SPD-20A prominence liquid chromatography. UV-vis absorption spectra were performed on a Shimadzu UV/vis/NIR spectrometer. Fluorescence spectra were recorded with a Shimadzu RF5301PC spectrometer. The absorbance for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed using a Tecan's Infinite M200 microplate reader at wavelength of 490 nm. Confocal laser microscopy (CLSM) images were acquired by a ZEISS LSM 800 Confocal Laser Scanning Microscope.

Preparation of amino acids solutions for fluorescent study

Stock solutions (10 mM) of amino acids including Cys, Hcy, GSH, Asp, Glu, Gln, Ile, Asn, Pro, Trp, Tyr,

His, Cys, Hcy, GSH, Arg, Phe, Ser, Ala, Lys, Gly, Leu and Met in ultra pure water were prepared, and stock solution of probe **1** (0.5 mM) was prepared in DMSO (10 mL). In a typical experiment, test solutions were prepared by using the stock solution and diluting with PBS buffer (10 mM, pH 7.4), and the final test solutions contain 10% DMSO. Fluorescence spectra were measured after the addition of analytes at 25 ^{o}C for 15 min. In all measurements, the excitation wavelength was 470nm and 670 nm for the detection of these biothiols.

Determination of the detection limit

First, the calibration curve was obtained from the plot of fluorescence ratio (F) as a function of the analyte concentration (GSH, Cys or Hcy). The regression curve equation was then obtained for the lower concentration part.

Detection limit = $3 \times$ S.D. / k

Where k is the slope of the curve equation, and S.D. represents the standard deviation for the fluorescence intensity of the assay system in the absence of the analyte.

Kinetic study for the reaction of probe 1 with Cys or Hcy

A solution of Cys or Hcy in phosphate buffer (0.1 M, pH = 7.4) was gradually added to a solution of probe **1** in phosphate buffer (0.1 M, pH = 7.4), and the final concentration of probe **1**, Cys or Hcy was 10 μM. The mixture was stirred at room temperature and monitored by HPLC at different time points. The conversion rate at each time point was calculated and plotted *vs*. reaction time.

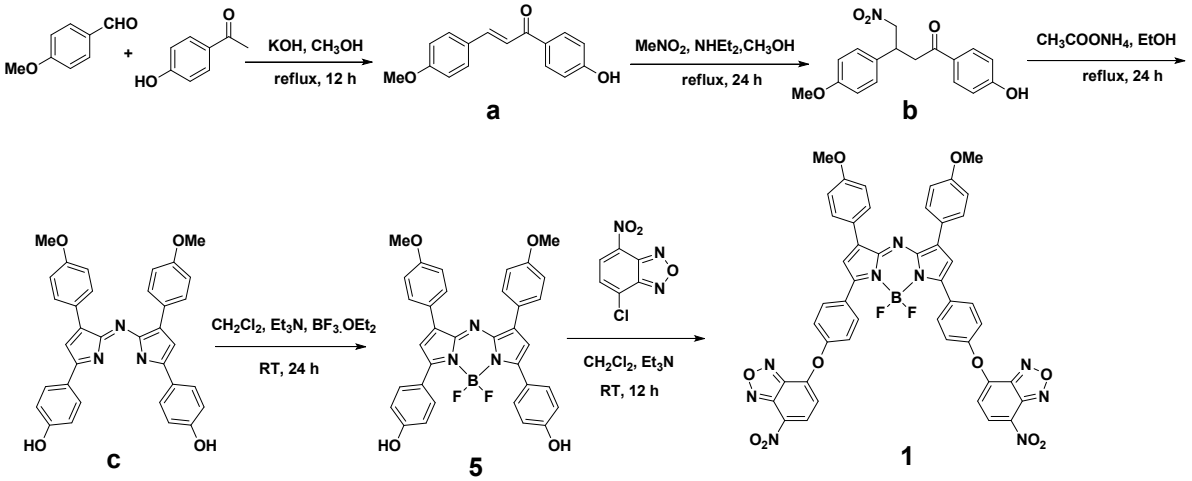
Cell culture, fluorescence imaging and cytotoxicity tests

HeLa cells (human cervical cancer cells) were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin (100 μg/mL), and streptomycin (100 μg/mL). The cell lines were kept in a moist atmosphere containing 5% CO_2 at 37 ^{o}C.

HeLa cells were incubated with probe **1** (5 μM) for 1 h or pretreated with 100 μM GSH, Cys, Hcy or NEM (2.0 mM) for 0.5 h and further incubated with probe **1** at 37 ^{o}C for 1 h, and rinsed with PBS three times to remove free compound before imaging. Confocal fluorescence images of HeLa cells were carried out on ZEISS LSM 800 Confocal Laser Scanning Microscope. The samples were excited at 488 nm and 640 nm. Emission was collected at 510−560 nm (green channel) and 700−750 nm (red channel).

The cytotoxic effect of probe **1** was determined by an MTT assays. HeLa cells were seeded in 96-well assay plates at a density of 10^4 cells per well for 24 h. The as-prepared probe **1** solutions (0.2 μM, 0.5 μM, 1 μM, 2 μM, 5 μM, 10 μM and 20 μM) were added in the serum-free medium and incubated with cells for 12 h or 24 h. The optical absorbance of the cells was detected at 490nm through a Tecan's Infinite M200 microplate reader. The control experiment was conducted by detecting the growth culture medium without probe **1**.

Synthesis and characterization of probe 1



Scheme S1. Synthesis route for probe **1**.

Synthesis of compound a

A solution of 1-(4-hydroxyphenyl)ethanone (1.36 g, 10 mmol) in ethanol (EtOH, 10 mL) was treated with NaOH (10%, 4 ml) under ice-cooling condition, and then anisic aldehyde (1.36 g, 10 mmol) was added dropwise into the solution. The mixture was stirred at room temperature under N_2 atmosphere for 24 h, and then neutralized with dilute HCl. After cooling for 24 h, the crystalline product was filtered, washed with cold ethanol and dried under vacuum. A yellow solid product was obtained. Yield: 2.06 g, 81.1 %. ^{1}H NMR (300 MHz, d_6-DMSO) δ 3.82 (s, 3H), 6.89 (d, J = 8.61 Hz, 2H), 7.01 (d, J = 8.67 Hz, 2H), 7.41 (s, 2H), 7.65 (d, J = 15.51 Hz, 1H), 7.77 (d, J = 15.72 Hz, 1H), 7.82 (d, J = 8.67 Hz, 2H), 8.05 (d, J = 8.64 Hz, 2H), 10.36 (s, 1H); ^{13}C NMR (75.5 MHz, d_6-DMSO) δ 55.34, 114.35, 115.30, 119.60, 127.52, 129.34, 130.51, 131.00, 142.64, 161.10, 161.98, 187.04; HRMS (ESI$^+$): m/z $C_{16}H_{14}O_3$ calcd. 255.1021, found $[M+H]^+$ 255.1020.

Synthesis of compound b

To a stirring solution of compound **a** (1 g, 3.94 mmol) in EtOH (15 mL), diethylamine (4.6 mL, 45 mmol) and nitromethane (4.8 mL, 90 mmol) were added. The mixture was heated under reflux for 24 h. The solution was acidified with dilute HCl after cooling down to room temperature, and then partitioned between EtOAc (50 mL) and H_2O (50 mL). The organic layer was evaporated to dryness, and then purified by column chromatography on silica eluting with CH_2Cl_2 to give the product as a yellow liquid. Yield: 0.93 g, 75.0 %. ^{1}H NMR (300 MHz, d_6-DMSO) δ 3.26-3.45 (m, 2H), 3.70 (s, 3H), 3.90-4.01 (m, 1H), 4.78 (dd, *J* = 9.75, 12.69 Hz, 1H), 4.92 (dd, *J* = 5.73, 12.72 Hz, 1H), 6.80-6.85 (m, 4H), 7.26 (d, *J* = 8.71 Hz, 2H), 7.81 (d, *J* = 8.71 Hz, 2H), 10.36 (s, 1H); ^{13}C NMR (75.5 MHz, d_6-DMSO) δ 40.65, 54.95, 79.94, 113.79, 115.19, 128.09, 128.80, 130.48, 131.88, 158.25, 162.16, 195.46; HRMS (ESI$^+$): m/z $C_{17}H_{17}NO_5$ calcd. 316.1185, found $[M+H]^+$ 316.1189.

Synthesis of compound c

Compound **b** (0.93 g, 2.95 mmol) and ammonium acetate (9.5 g, 123 mmol) were mixed in EtOH (20 mL), and the solution was heated under reflux for 48 h. The reaction was cooled down to room temperature, and partitioned between EtOAc (50 mL) and H_2O (50 mL), The organic layer was

separated, dried over sodium sulfate and evaporated under reduced pressure to give the product as a blue-black solid. Yield: 0.55 g, 68.8 %. ^{1}H NMR (300 MHz, d_6-DMSO) δ 3.84 (s, 6H), 5.32 (s, 1H), 6.98-7.06 (m, 6H), 7.41 (s, 2H), 7.65-7.73 (m, 2H), 7.90 (d, *J* = 8.55 Hz, 4H), 8.06 (d, *J* = 8.61 Hz, 4H), 10.22 (s, 2H); ^{13}C NMR (75.5 MHz, d_6-DMSO) δ 40.65, 54.95, 79.94, 113.79, 115.19, 128.09, 128.80, 130.48, 131.88, 158.25, 162.16, 195.46; HRMS (ESI$^+$): m/z $C_{34}H_{26}N_3O_4$ calcd. 541.2002, found $[M+H]^+$ 541.2010.

Synthesis of compound 5

Under an Ar atmosphere, compound **c** (0.55 g, 1 mmol) was dissolved in dry CH_2Cl_2 (30 mL), and treated with diisopropylethylamine (5.5 mL, 31.6 mmol) and BF_3 diethyletherate (5.5 mL, 43.5 mmol). The solution was stirred at room temperature for 24 h. Then, the reaction mixture was partitioned between CH_2Cl_2 (50 mL) and H_2O (50 mL), and the organic layer was evaporated to dryness. After the evaporation, the resulted residue was subjected to column chromatography for purification using CH_2Cl_2/MeOH (50:1 v/v) as the eluent. The product (compound **5**) was isolated as a red metallic solid. Yield: 0.238 g, 39.7 %. ^{1}H NMR (300 MHz, d_6-DMSO) δ 3.87 (s, 6H), 6.93 (d, *J* = 8.88 Hz, 4H), 7.13 (d, *J* = 8.97 Hz, 4H), 7.43 (s, 2H), 8.05 (d, *J* = 8.88 Hz, 4H), 8.15 (d, *J* = 8.91 Hz, 4H), 10.37 (s, 2H); ^{13}C NMR (75.5 MHz, d_6-DMSO) δ 55.36, 114.32, 115.75, 117.70, 122.00, 124.66, 130.53, 131.83, 141.34, 144.11, 157.00, 160.52, 160.59; HRMS (ESI$^+$): m/z $C_{34}H_{26}BF_2N_3O_4$ calcd. 590.2063, found $[M+H]^+$ 590.2080.

Synthesis of probe 1

A solution of compound **5** (0.238 g, 0.4 mmol) in CH_2Cl_2 (15 mL) was treated with triethylamine (56 µL, 0.4 mmol), and then 4-chloro-7-nitrobenzofurazan (300 mg, 1.51 mmol) was added into the mixture. The mixture was stirred at room temperature for 12 h, and then was partitioned between CH_2Cl_2 (50 mL) and H_2O (50 mL). The organic layer was separated and evaporated under reduced pressure. Purification by column chromatography on silica eluting with CH_2Cl_2/CH_3OH (50:1, v/v) gave the product as a dark blue solid. Yield: 28 mg, 30.6 %. ^{1}H NMR (300 MHz, d_6-DMSO) δ 3.90 (s, 6H), 5.75 (s, 2H), 6.97 (d, *J* = 8.34 Hz, 2H), 7.18 (d, *J* = 8.97 Hz, 4H), 7.60 (s, 2H), 7.63 (d, *J* = 9.00 Hz, 4H), 8.22 (d, *J* = 8.79 Hz, 4H), 8.31 (d, *J* = 8.94 Hz, 4H), 8.17 (d, *J* = 8.34 Hz, 2H); ^{13}C NMR (75.5 MHz, d_6-DMSO) δ 55.50, 111.43, 114.59, 118.69, 120.69, 24.33, 129.22, 130.96, 132.03, 135.30, 143.25, 144.46, 144.86, 145.49, 151.93, 155.28, 157.01, 161.09, 166.9; HRMS (ESI$^+$): m/z $C_{46}H_{28}BF_2N_9O_{10}$ calcd. 916.2099, found $[M+H]^+$ 916.2084.

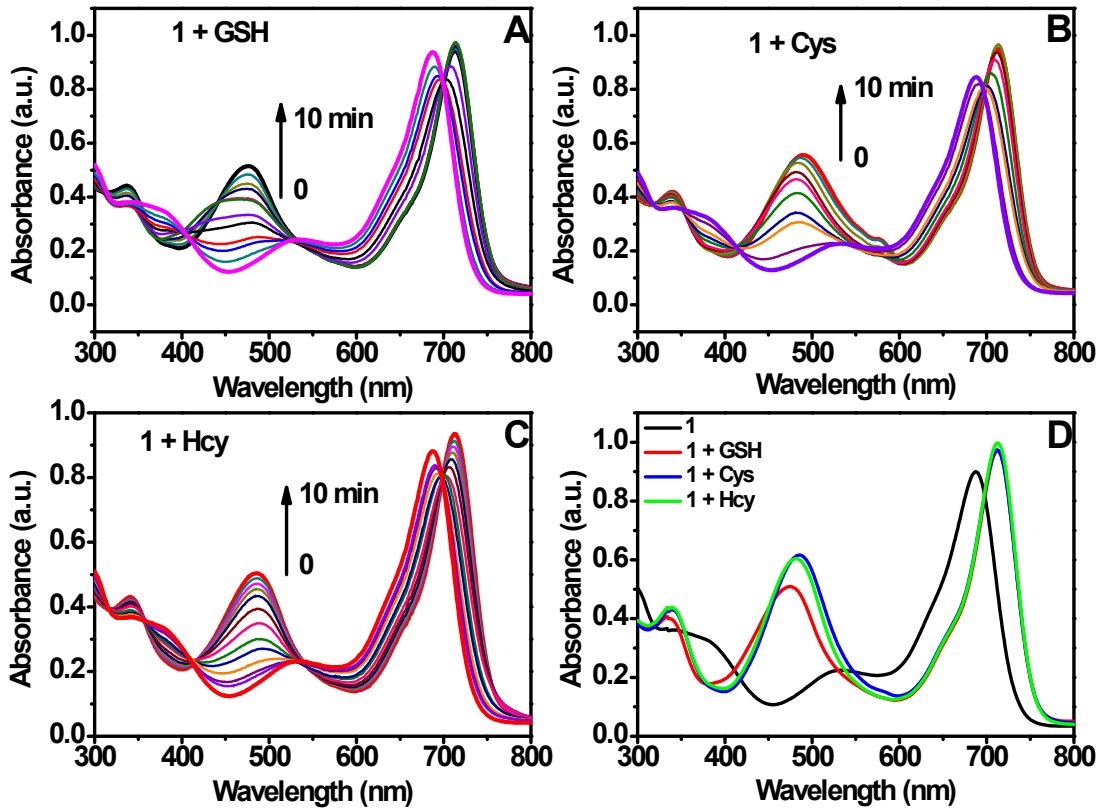


Fig. S1 Time dependent UV spectral changes of probe **1** (5 μM) upon the addition of (A) GSH (100 μM), (B) Cys (100 μM), and (C) Hcy (100 μM) in PBS buffer solution (10mM, pH 7.4, containing 10% DMSO) at 25 ^{o}C. (D) UV-Vis spectral changes of probe **1** (5 μM) upon the addition of GSH (100 μM), Cys (100 μM), and Hcy (100 μM) in PBS buffer solution (10mM, pH 7.4, containing 10% DMSO) at 25 ^{o}C.

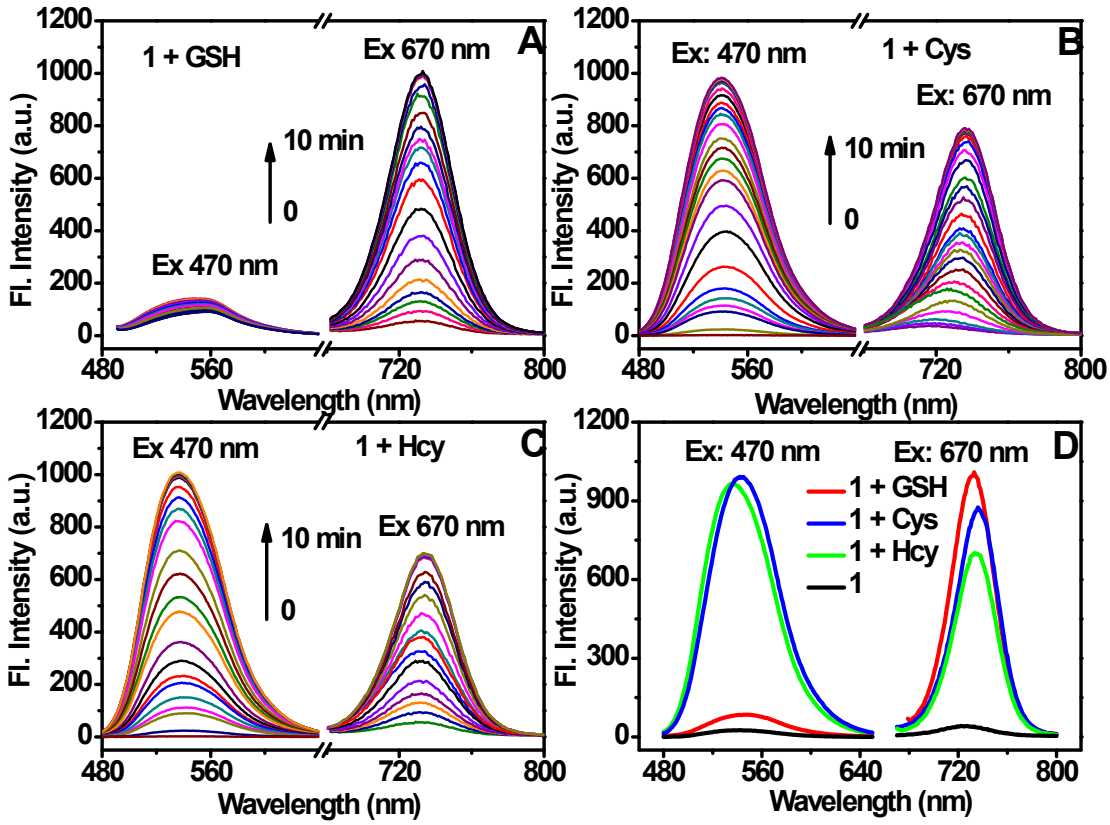


Fig. S2 Time dependent fluorescent spectral changes of probe **1** (5 μM) upon the addition of (A) GSH (100 μM), (B) Cys (100 μM), and (C) Hcy (100 μM) in PBS buffer solution (10 mM, pH 7.4, containing 10% DMSO) at 25 °C. (D) Fluorescent spectral changes of probe **1** (5 μM) upon the addition of GSH (100 μM), Cys (100 μM), and Hcy (100 μM) in PBS buffer solution (10 mM, pH 7.4, containing 10% DMSO) at 25 °C.

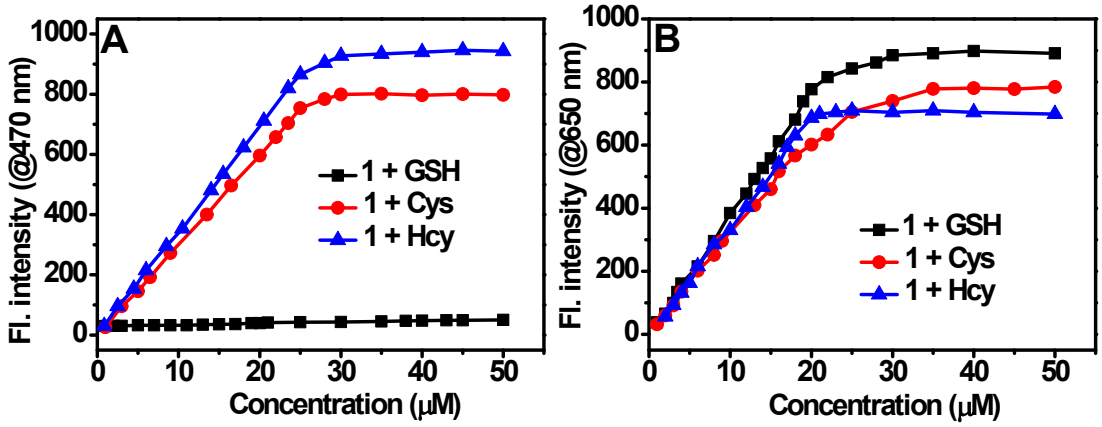


Fig. S3 Concentration dependent fluorescence changes of probe **1** (5 μM) upon the addition of GSH (100 μM), Cys (100 μM), and Hcy (100 μM) in PBS buffer (10 mM, pH 7.4, containing 10% DMSO) at 25 °C. (A) Fluorescence intensity was recorded at 540 nm (λ_{ex} = 470 nm). (B) Fluorescence intensity was recorded at 730 nm (λ_{ex} = 670 nm).

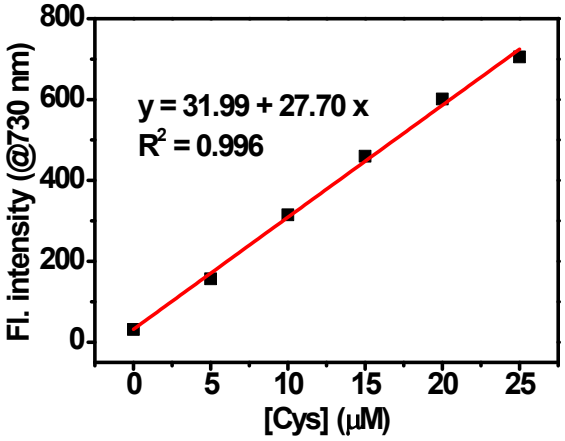


Fig. S4 Fitted calibration curve of the fluorescence intensities at 730 nm as a function of Cys concentrations.

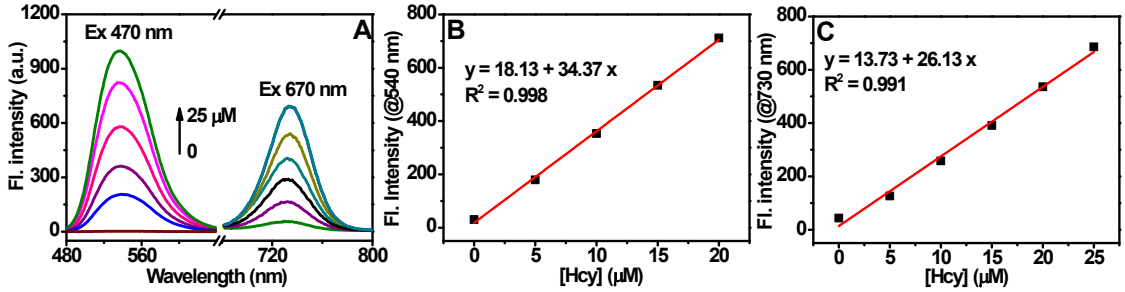


Fig. S5 Fluorescence spectra of probe **1** (5 μM, λ_{ex} = 470 nm and 670 nm) in the presence of various concentrations of Hcy in phosphate buffer (10 mM, pH 7.4, containing 10% DMSO) at 25 °C for 15 min. Fitted calibration curves of the fluorescence intensities at (B) 730 nm and (C) 540 nm as a function of Hcy concentrations.

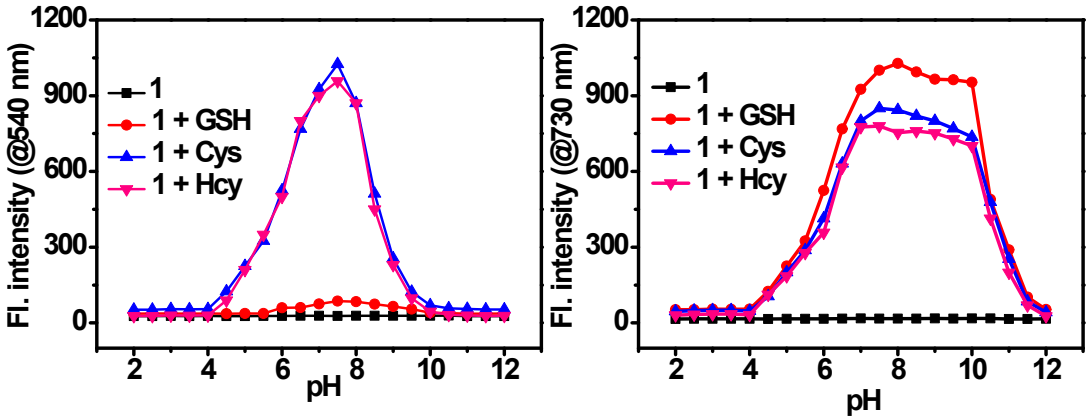


Fig. S6 Changes in fluorescence intensity of probe **1** (5 μM) in PBS buffer at 25 °C measured with and without 20 equiv. of GSH, Cys, and Hcy as a function of pH. (A) λ_{ex}: 470 nm, λ_{em}: 540 nm. (B) λ_{ex}: 580 nm, λ_{em}: 730 nm.

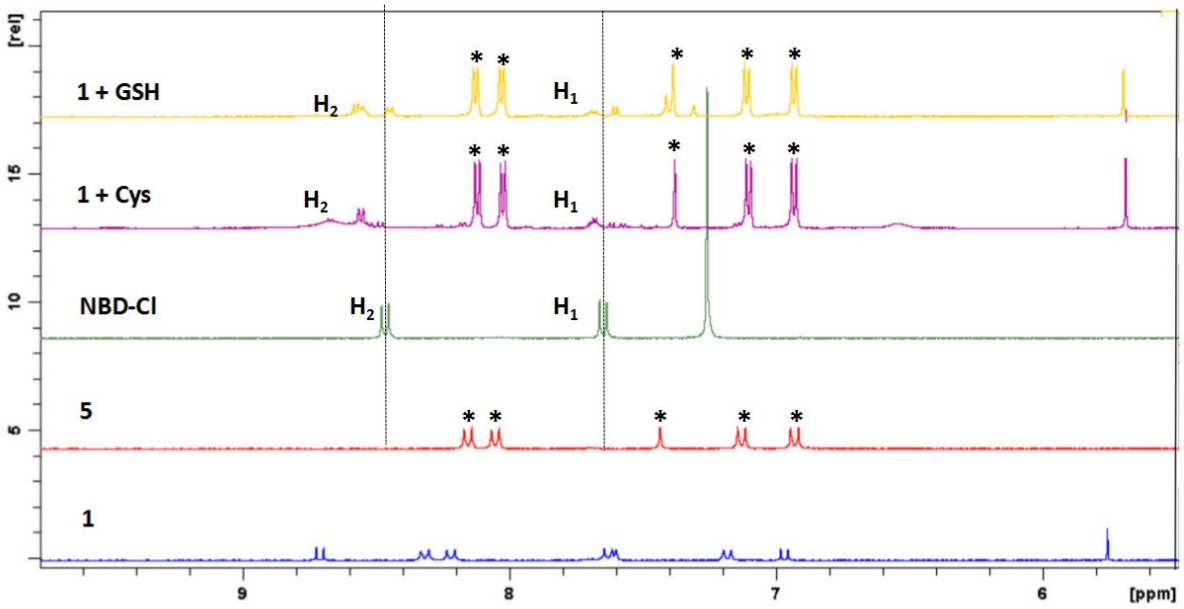


Fig. S7 ^{1}H NMR spectral comparison of compounds **1**, **5**, NBD-Cl (4-chloro-7-nitro-1,2,3-benzoxadiazole), and **1** treated with excessive Cys and GSH.

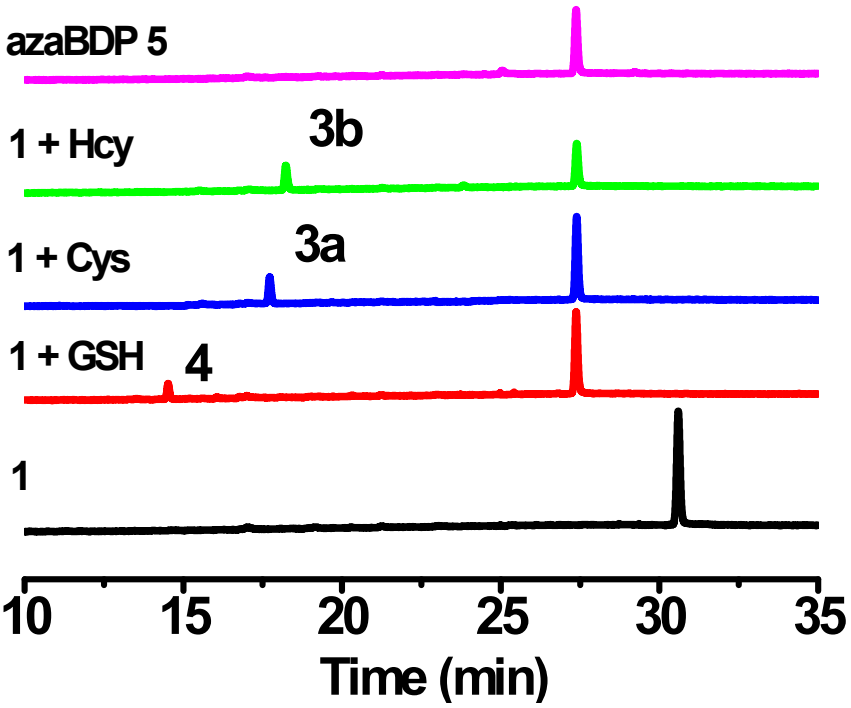


Fig. S8 HPLC chromatographs of probe **1** (5 μM), probe **1** upon the reaction with GSH (20 equiv.), Cys (20 equiv.), and Hcy (20 equiv.), and aza-BDP **5** recorded in a gradient solvent system of CH_3CN and H_2O.

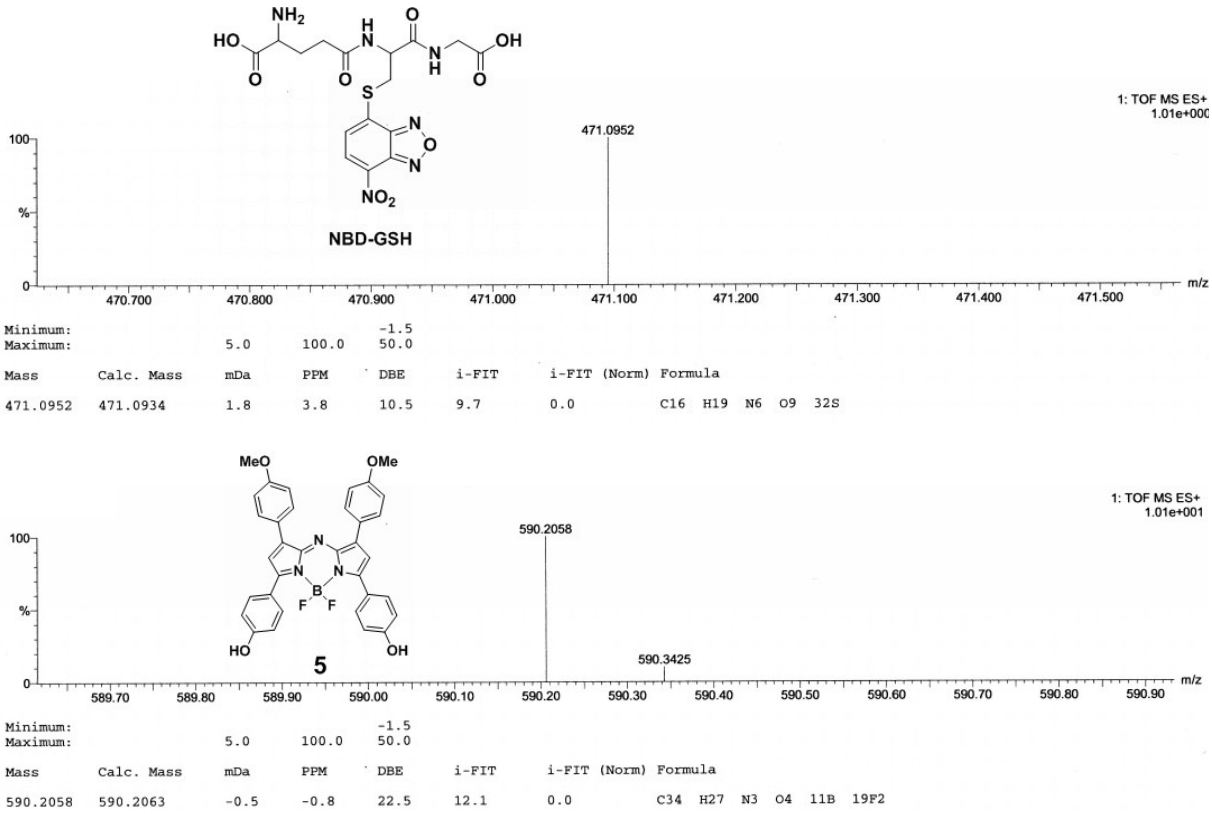


Fig. S9 HRMS spectra of probe **1** (1 μM) treated with excessive GSH (20 μM).

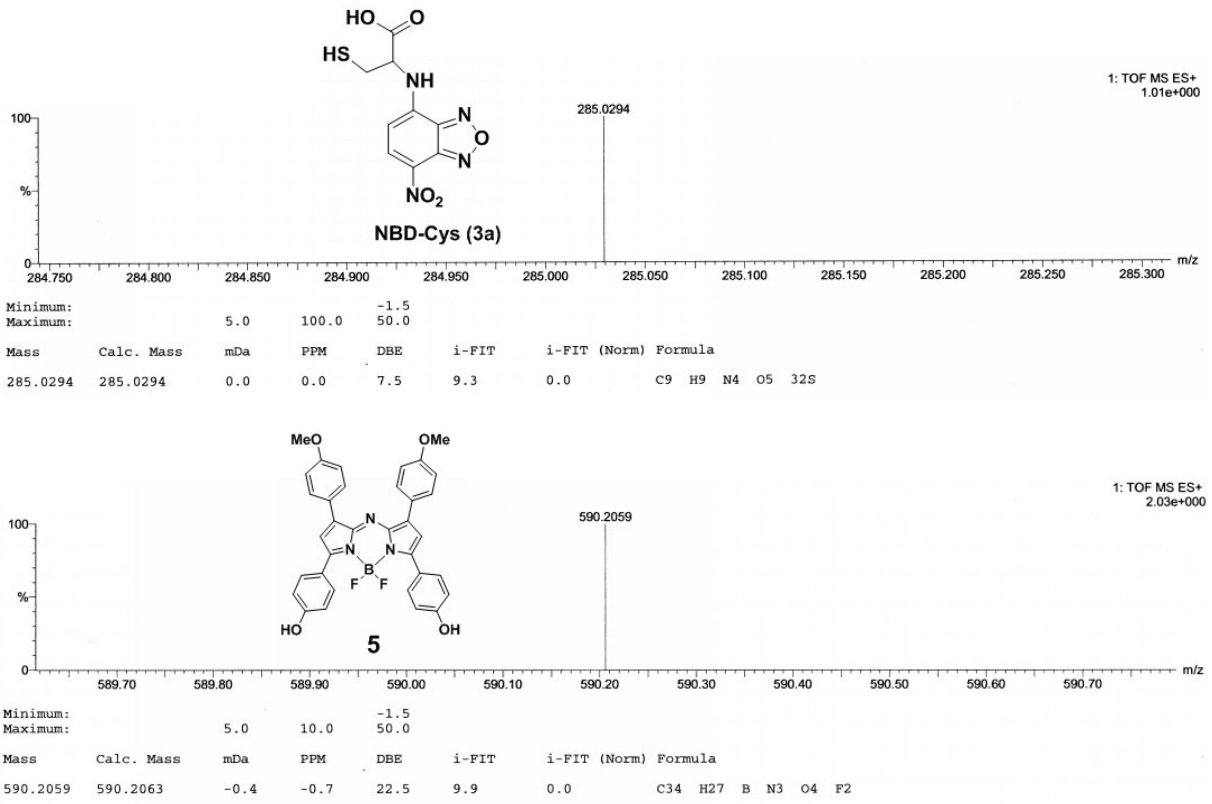


Fig. S10 HRMS spectra of probe **1** (1 μM) treated with excessive Cys (20 μM).

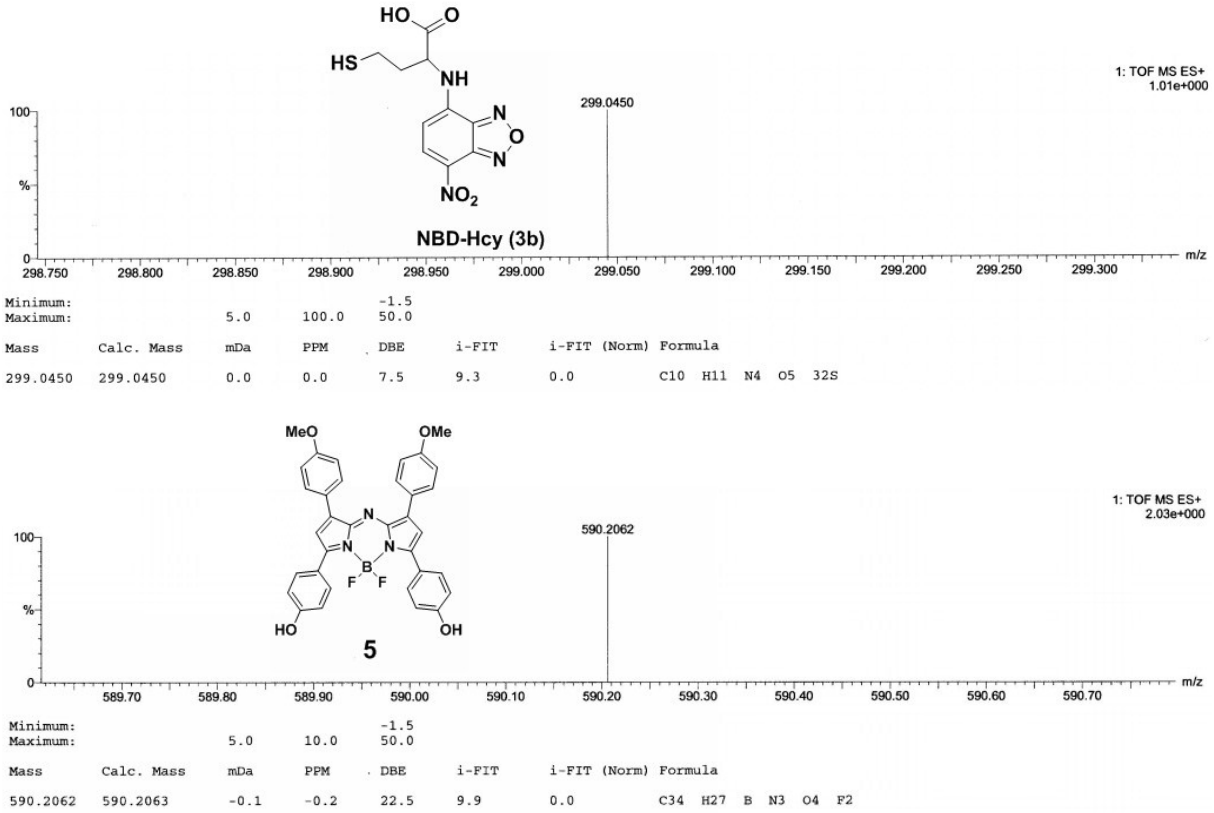


Fig. S11 HRMS spectra of probe **1** (1 μM) treated with excessive Hcy (20 μM).

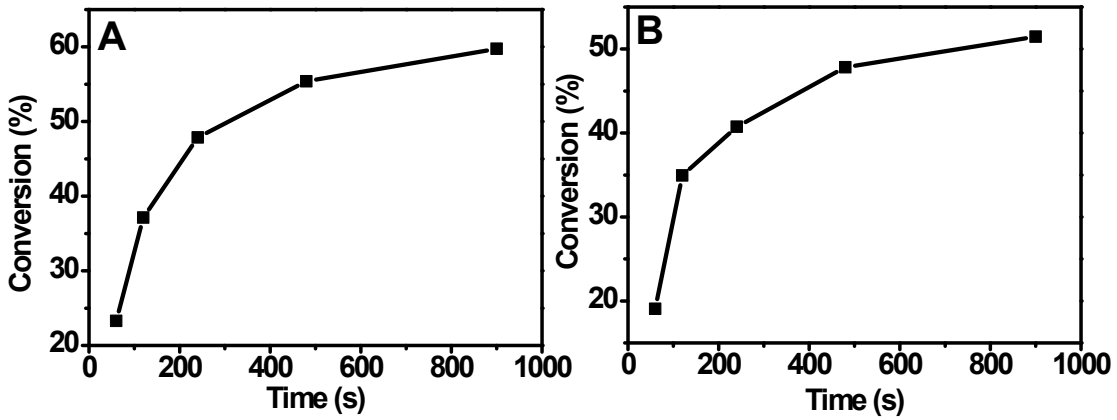


Fig. S12 Conversion rate of the reaction between probe **1** and (A) Cys *vs*. time and (B) Hcy *vs*. time.

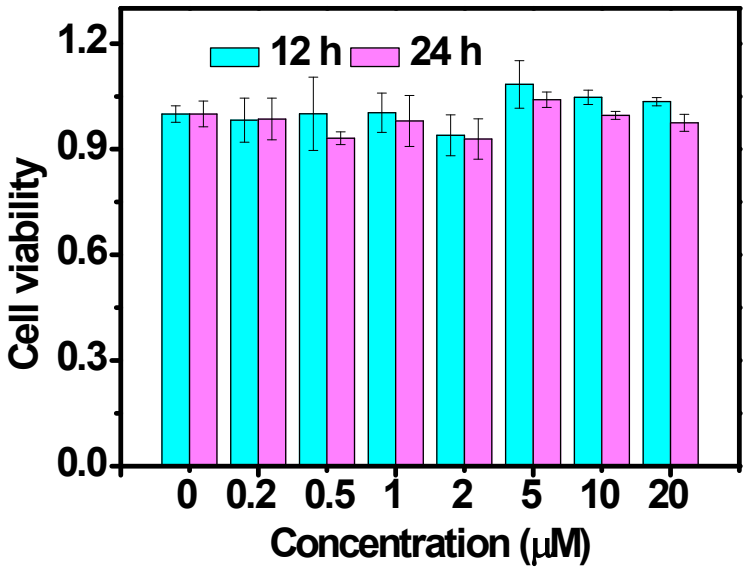


Fig. S13 MTT assay for the viability of Hela cells treated with various concentrations (0.2-20μM) of probe **1** for 12 h and 24 h. Error bars represent the standard deviations of 3 trials.

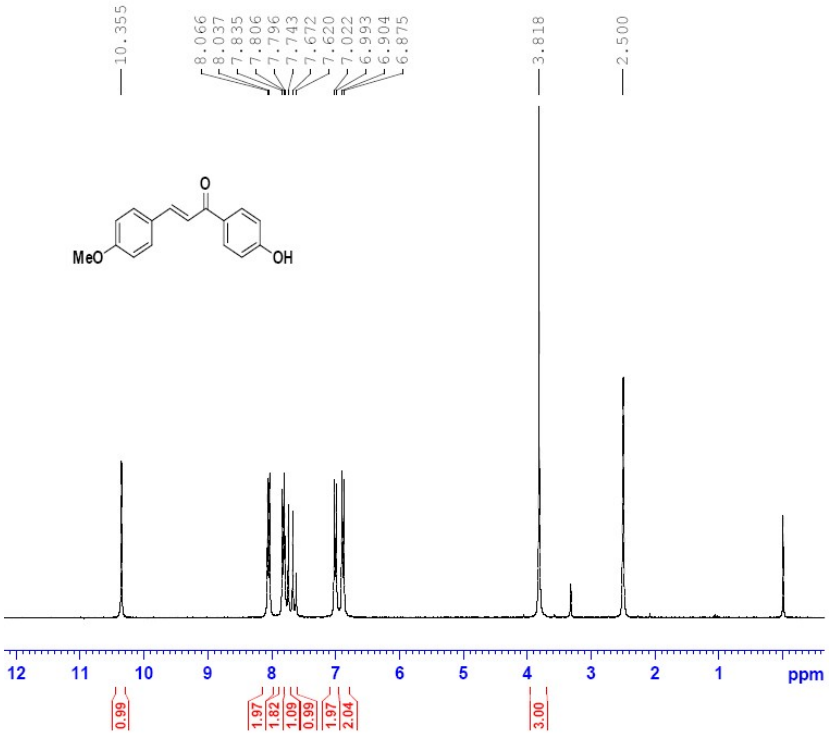


Fig. S14 ^{1}H NMR spectrum of compound **a**.

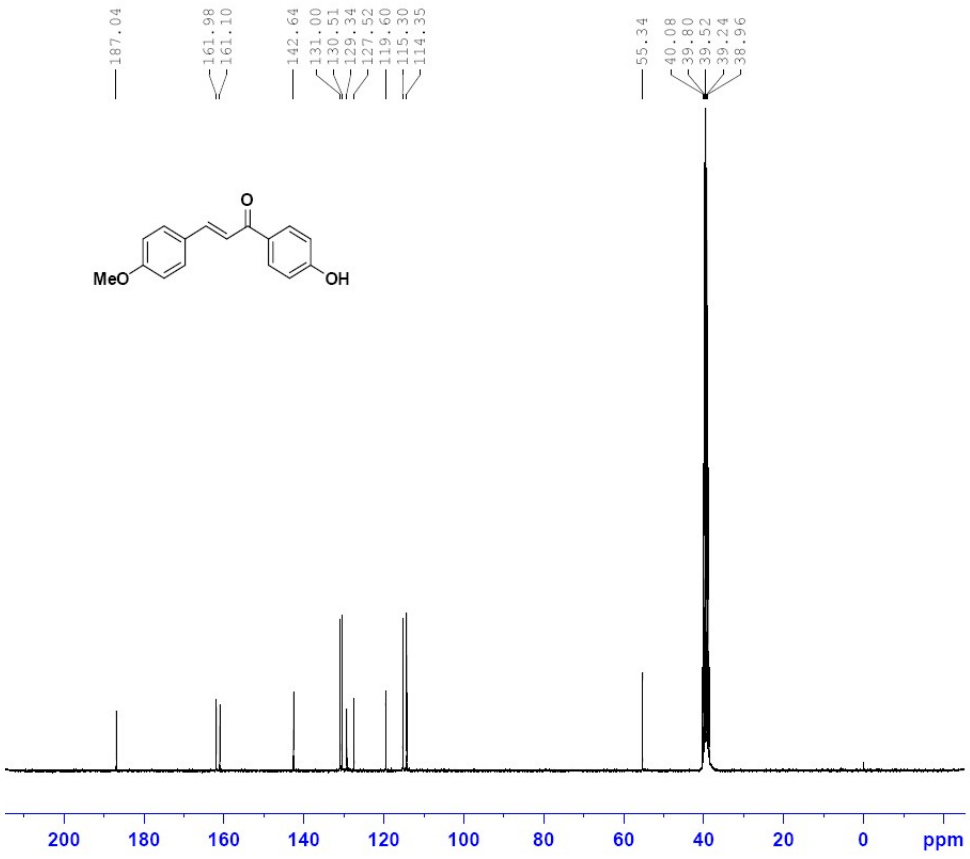
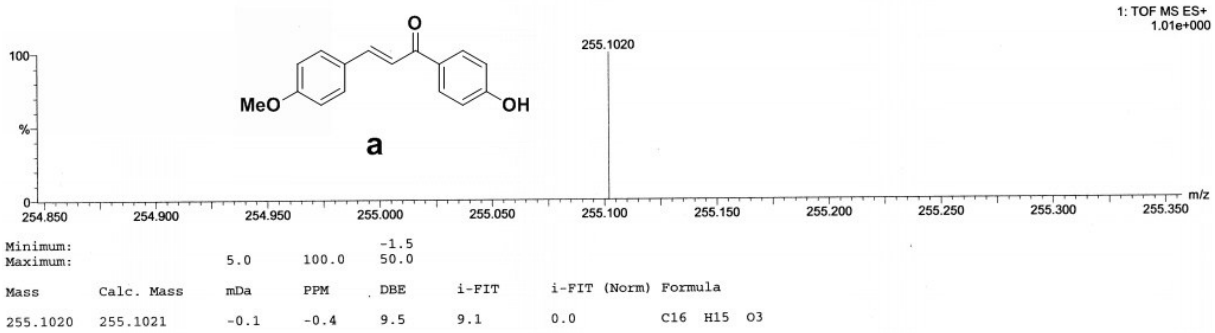


Fig. S15 ^{13}C NMR spectrum of compound **a**.



Minimum:				-1.5			
Maximum:		5.0	100.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
255.1020	255.1021	-0.1	-0.4	9.5	9.1	0.0	C16 H15 O3

Fig. S16 HRMS of compound **a**.

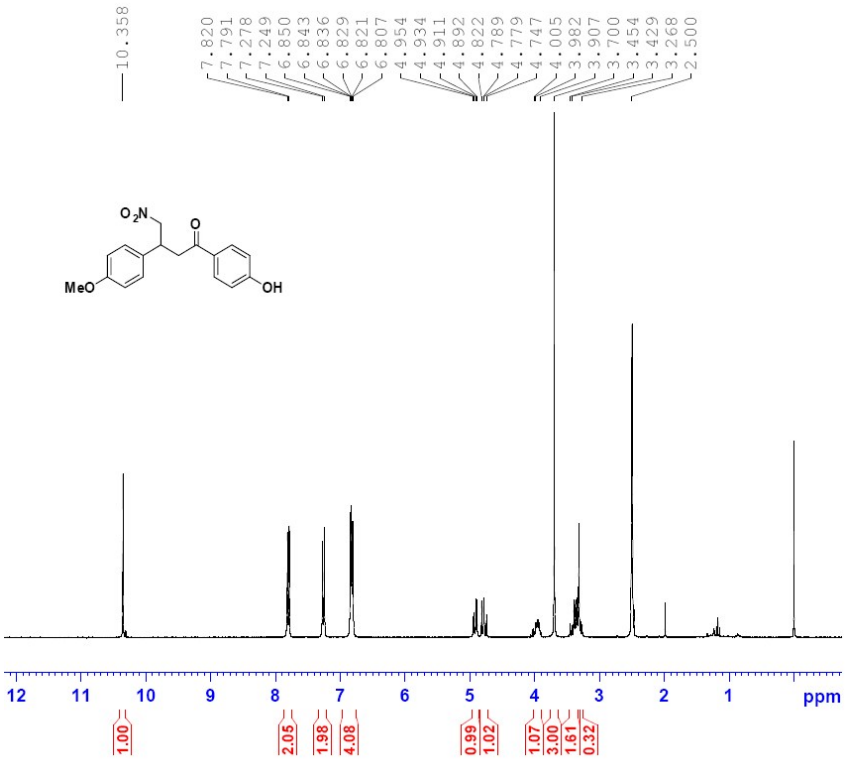


Fig. S17 ^{1}H NMR spectrum of compound **b**.

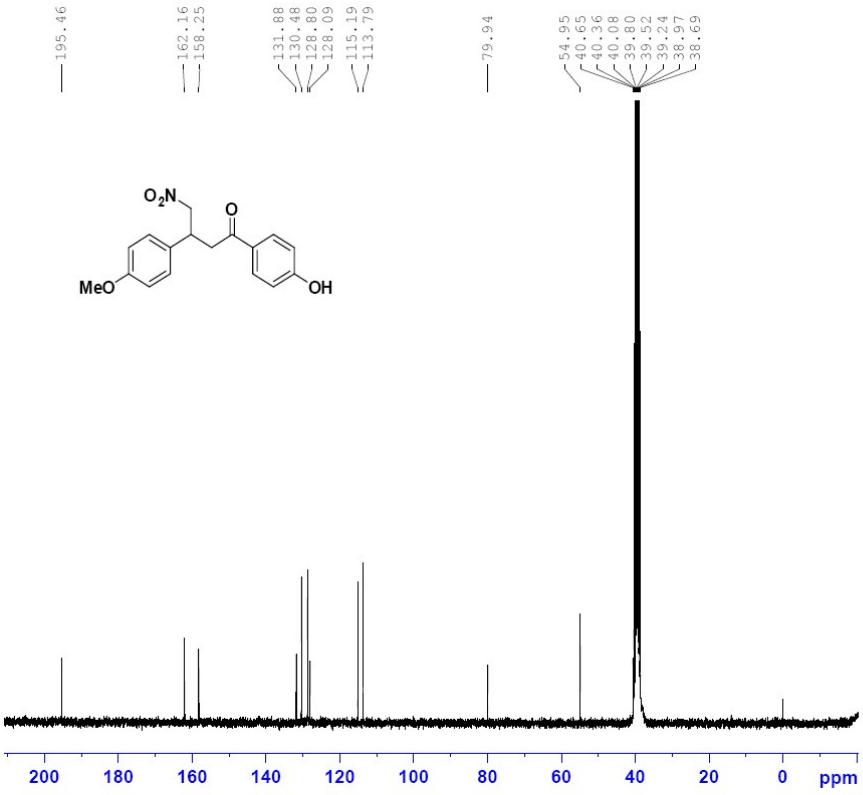
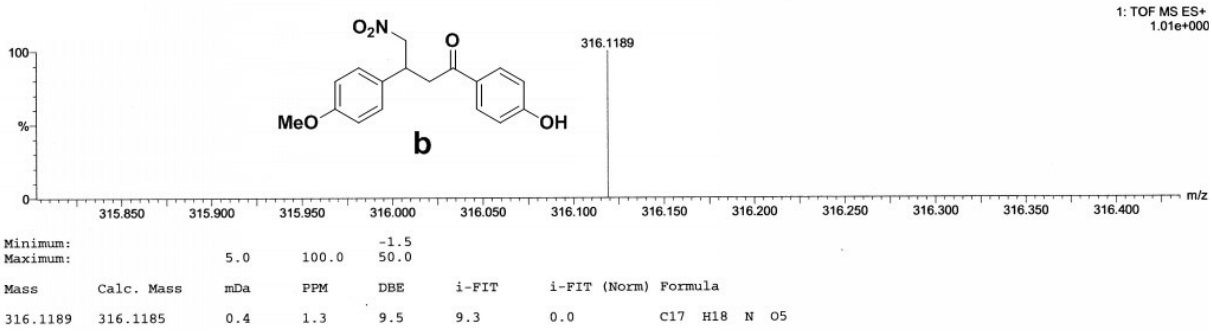


Fig. S18 ^{13}C NMR spectrum of compound **b**.



Minimum:				-1.5			
Maximum:		5.0	100.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
316.1189	316.1185	0.4	1.3	9.5	9.3	0.0	C17 H18 N O5

Fig. S19 HRMS of compound **b**.

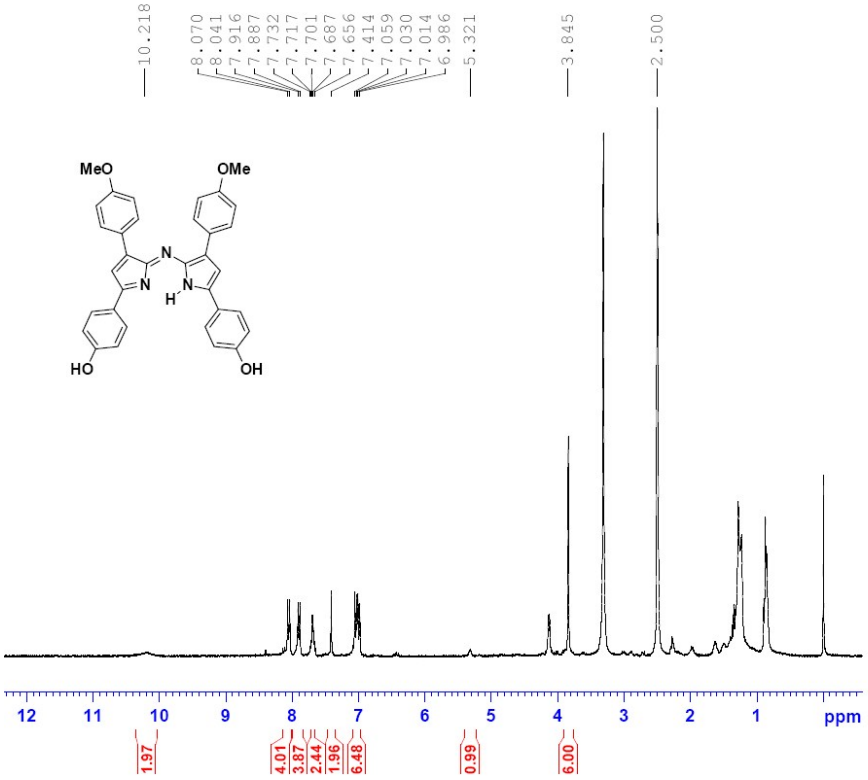


Fig. S20 ^{1}H NMR spectrum of compound **c**.

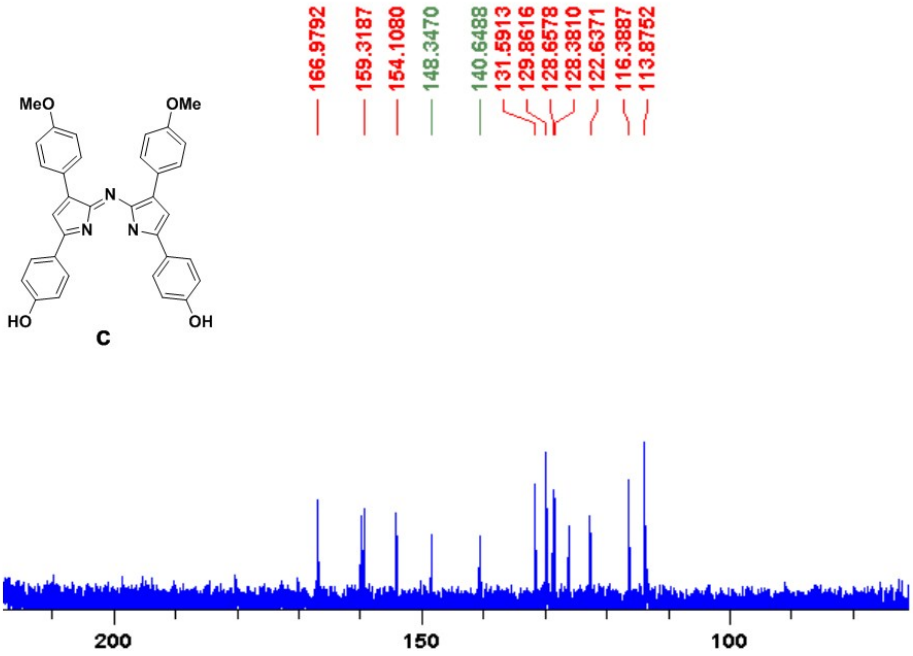


Fig. S21 ^{13}C NMR spectrum of compound **c**.

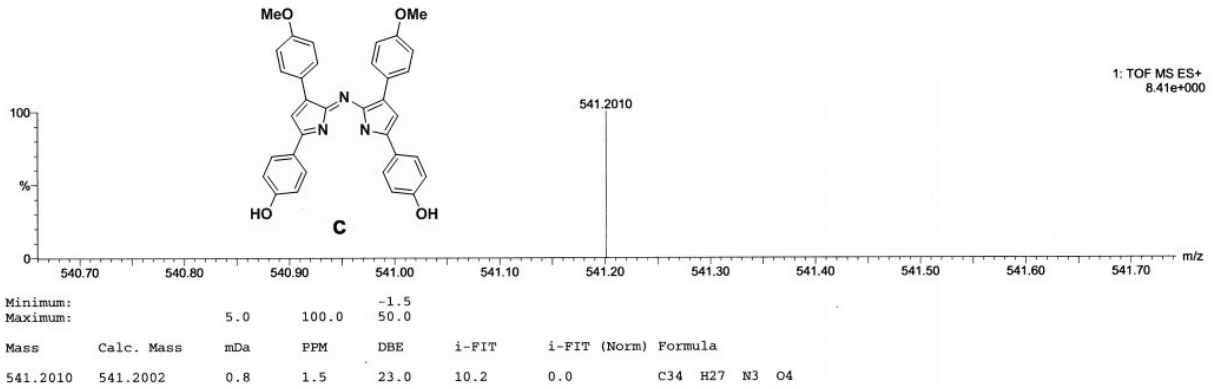


Fig. S22 HRMS of compound **c**.

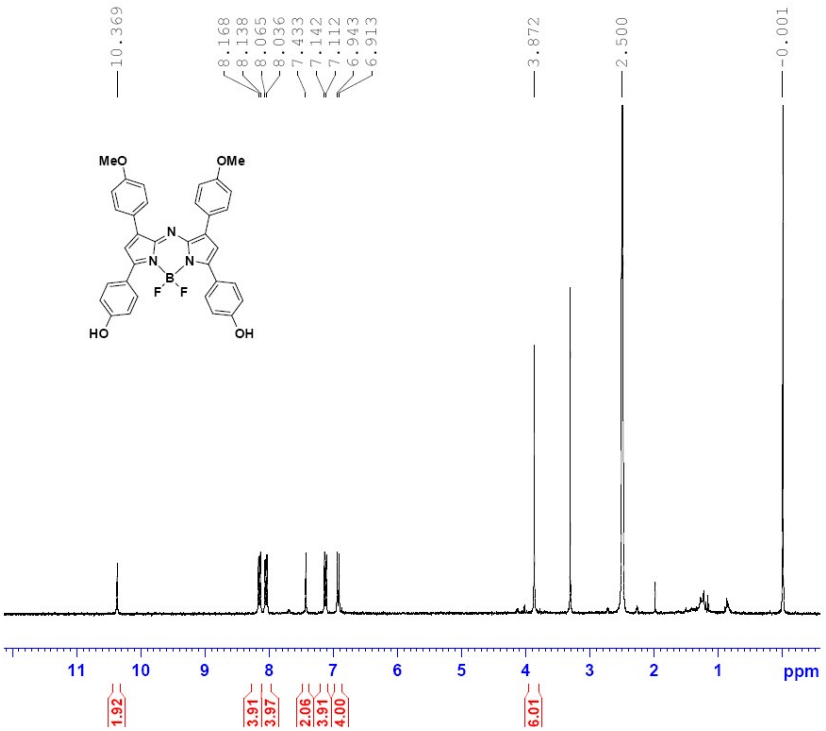


Fig. S23 ^{1}H NMR spectrum of compound **5**.

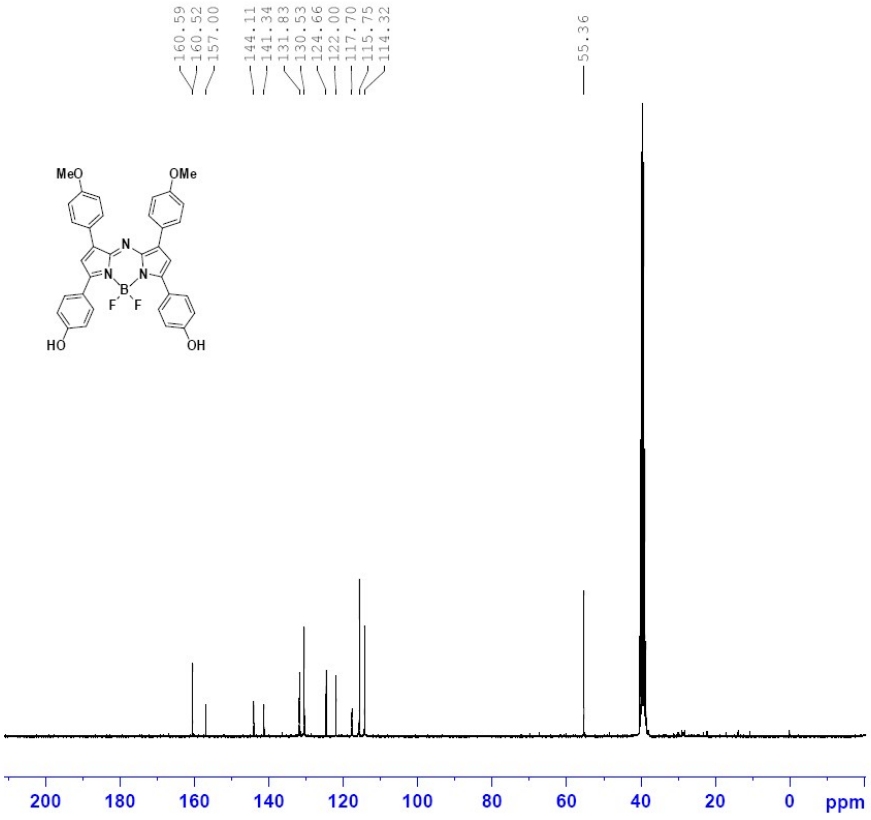
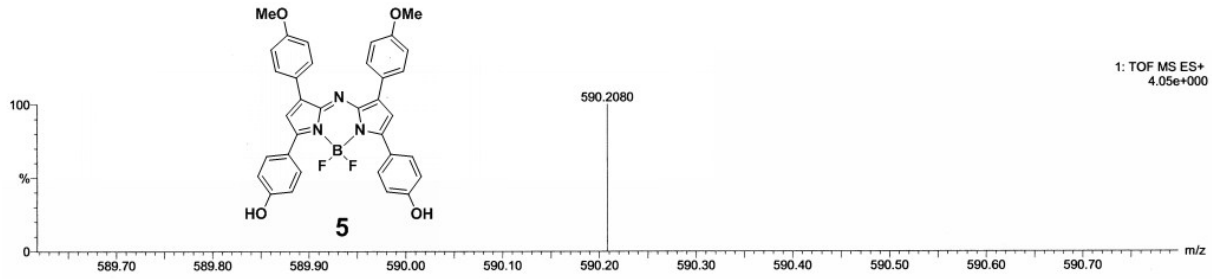


Fig. S24 ^{13}C NMR spectrum of compound **5**.



Minimum:				-1.5			
Maximum:		5.0	100.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
590.2080	590.2063	1.7	2.9	22.5	9.6	0.0	C34 H27 N3 O4 F2 11B

Fig. S25 HRMS of compund **5**.

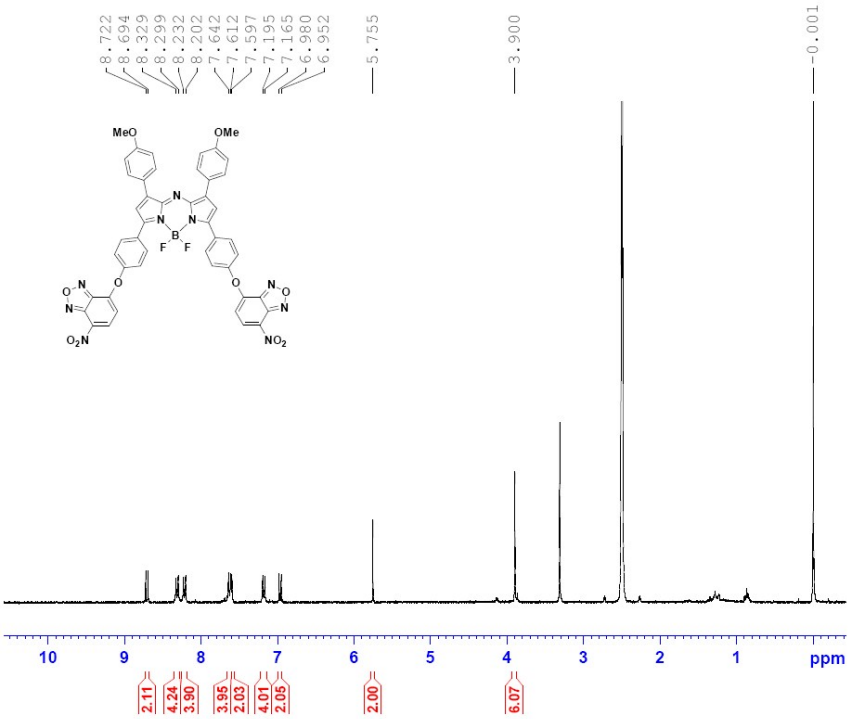


Fig. S26 ^{1}H NMR spectrum of compound **1**.

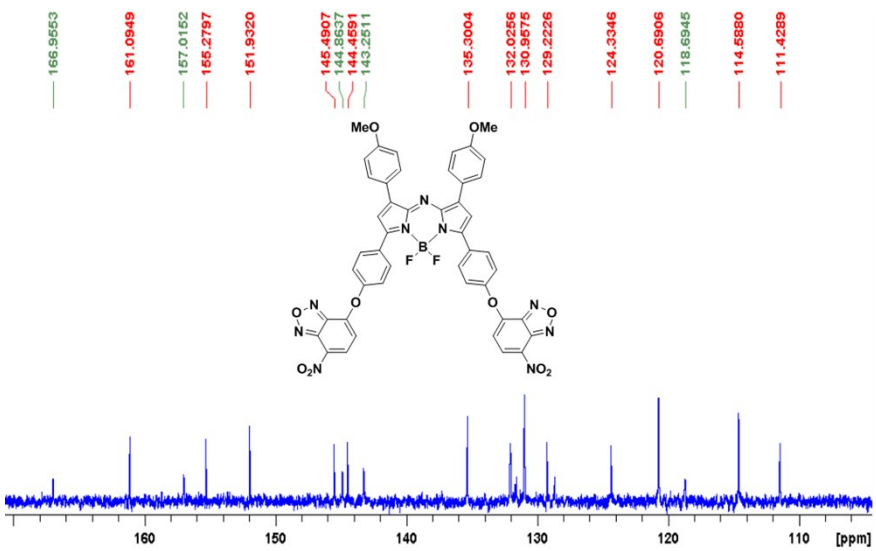
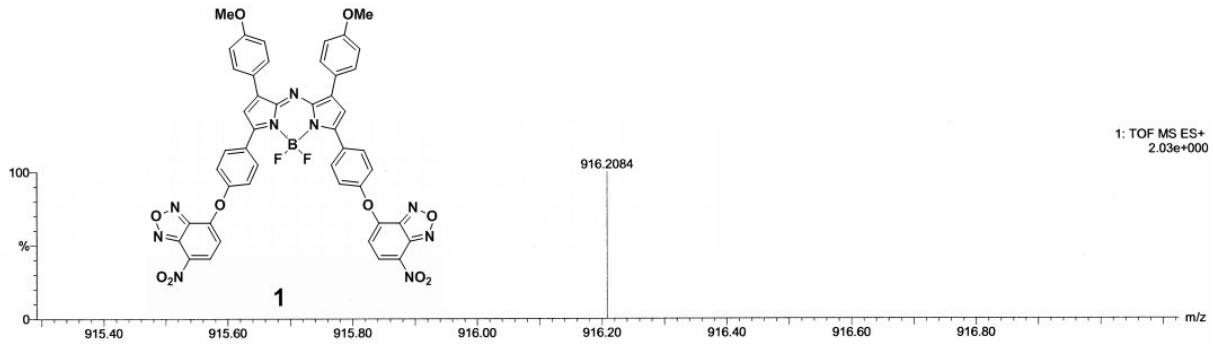


Fig. S27 ^{13}C NMR spectrum of compound **1**.



Minimum:				-1.5			
Maximum:		5.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
916.2084	916.2099	-1.5	-1.6	36.5	10.4	0.0	C46 H29 B N9 O10 F2

Fig. S28 HRMS of compound **1**.