Supporting Information

Rational Design of Ratiometric Near-Infrared Aza-BODIPY-Based Fluorescent Probe for *in Vivo* Imaging of Endogenous Hydrogen Peroxide

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1. Cell Experiment

Cell Lines: The human epithelioid cervical carcinoma cell line A549 were purchased from the Institute of Cell Biology (Shanghai, China). Cells were all propagated in T-75 flasks cultured at 37 ºC under a humidified 5% CO_2 atmosphere in RPMI-1640 medium or DMEM medium (GIBCO/Invitrogen, Camarillo, CA, USA), which were supplemented with 10 % fetal bovine serum (FBS, Biological Industry, Kibbutz Beit Haemek, Israel) and 1 % penicillin-streptomycin (10,000 U mL^{-1} penicillin and 10 mg/ml streptomycin, Solarbio life science, Beijing, China)

***In Vitro* Cellular Imaging:** The A549 cells at 1×10^5 cells/well were seeded onto glass-bottom petri dishes with complete medium (1.5 mL) for 12 h. Then the cells pre-incubated with and without H_2O_2 were exposed to desired concentrations of **BP_5-NB-OB** (10 μM) for 40 min. PBS buffers (pH 7.4) was used to washed cells for three times to clean the background. The images were then photographed by using a confocal laser scanning microscope with 633 nm as the excitation wavelength and 690-710 nm, 715-735 nm as the emission wavelength.

The cell cytotoxicity of **BP_5-NB-OB** to A549 cells were measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cytotoxicity was evaluated by Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the factory's instruction. Cells were plated in 96-well plates in 0.1 mL volume of DMEM or RPMI-1640 medium with 10 % FBS, at a density of 1×10^4 cells/well and added with desired concentrations of **BP_5-NB-OB**. After incubation for 24 h, absorbance was measured at 410 nm with a Tecan GENios Pro multifunction reader (Tecan Group Ltd., Maennedorf, Switzerland). Each concentration was measured in triplicate and used in three independent experiments. The relative cell viability was calculated by the equation: cell viability (%) = $(OD_{treated}/OD_{control}) \times 100\%$

Animals: All animal studies were approved by the Animal Care and Use Committee of Shanghai Bioray Biotechnology in accordance with the guidelines for the care and use of laboratory animals. The 3-4-week-old female BALB/cA nude mice were producted from Shanghai Bioray Biotechnology and maintained under standard conditions. The animals were housed in sterile cages within laminar airflow hoods in a specific pathogen-free room with a 12-h light/12-h dark schedule and fed autoclaved chow and water *ad libitum*. Number of qualitative qualifications: Production Permit No.: SYXK(Shanghai) 2015-0011

***In vivo* imaging in tumor-bearing mice:** The nude mice were inoculated with A549 cell on their right flanks by injecting 10^6 cells subcutaneously. When the tumors grew up to 10 mm in diameter, **BP_5-NB-OB** (administered at a dose of 0.2 $mg\cdot Kg^{-1}$) in PBS was in-situ injected into the A549 cell tumor-bearing nude mice. *In vivo* imaging was recorded at different time internals after

injection **BP_5-NB-OB** using PerkinElmer IVIS Lumina Kinetic Series III imaging system. The concentration of the injected solution is 30 μM (PBS, pH = 7.4). In the *in vivo* imaging, λ_{ex} = 630-650 nm, λ_{em} = 710-730 nm.

2. Time Dependence and ESI-MS Spectra Characterization of NB-B Reaction with H_2O_2

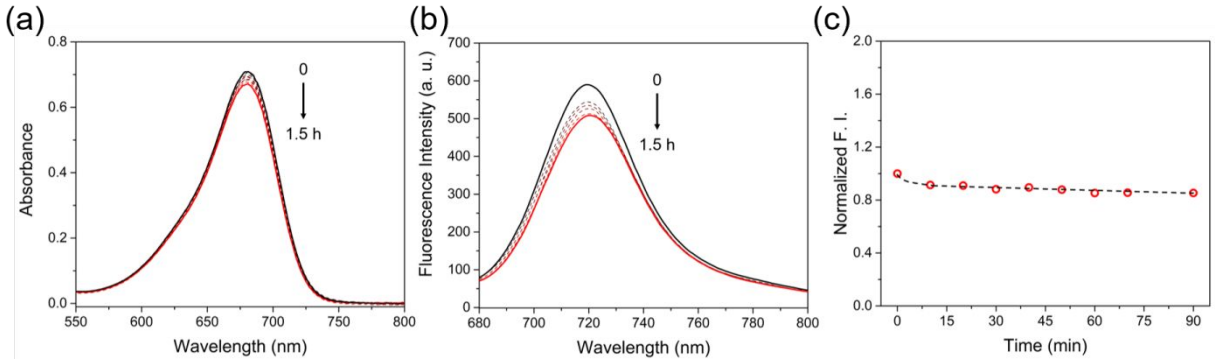


Figure S1 Time dependence of absorption (a) and fluorescence (b) spectra of NB-B in the presence of 1 mM H_2O_2. (c) Time dependence of normalized fluorescence intensity at 720 nm for NB-B in the presence of 1 mM H_2O_2. Solvent: a mixed PBS buffer solution (PBS/MeCN, v/v = 1/1, pH = 6.0, 0.01mM).

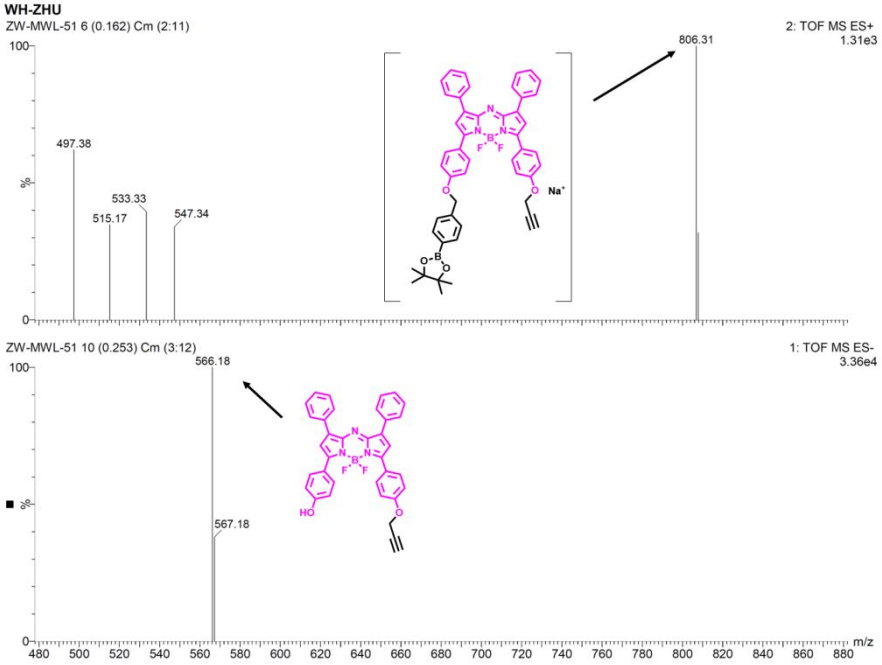


Figure S2 HRMS spectrum of the products from the reaction of NB-B with 10 equiv of H_2O_2.

3. Time Dependence and ESI-MS Spectra Characterization of NB-OB Reaction with H_2O_2

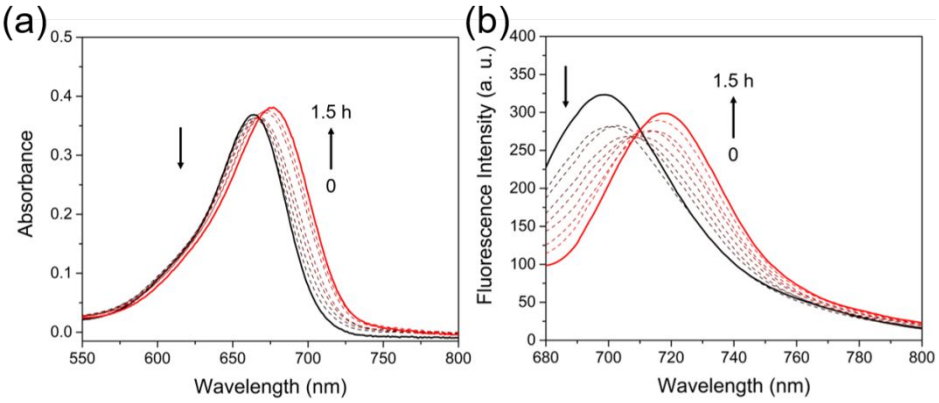


Figure S3 Time dependence of absorption (a) and fluorescence (b) spectra of NB-OB in the presence of 1 mM H_2O_2. Solvent: a mixed PBS buffer solution (PBS/MeCN, v/v = 1/1, pH = 6.0, 0.01 mM).

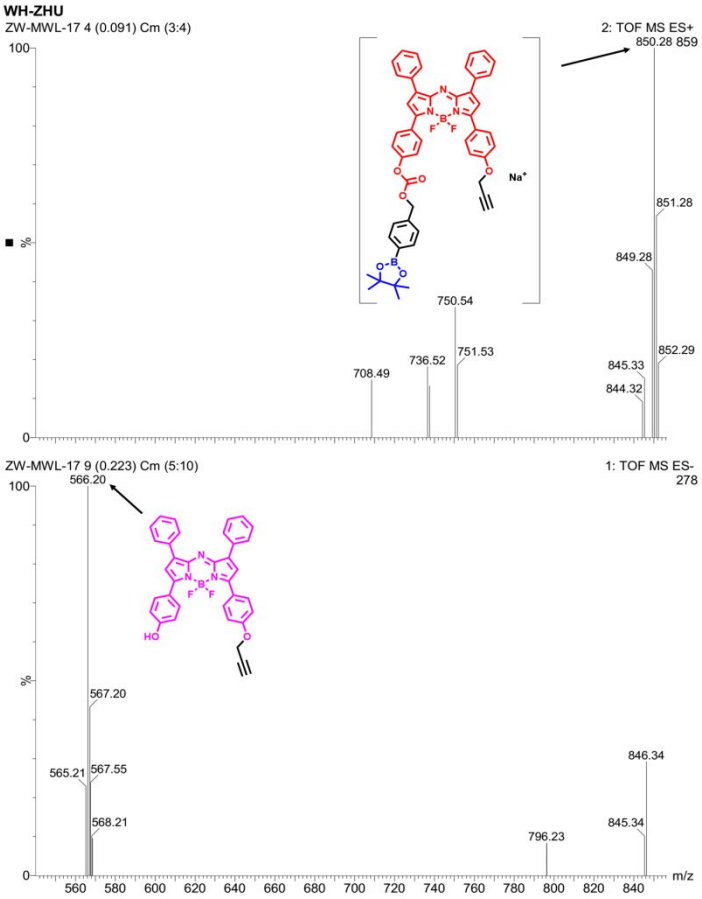


Figure S4 HRMS spectrum of the products from the reaction of NB-OB with 10 equiv of H_2O_2.

4. Properties of BP_5-NB-OB and BP_5-NB-OH

Table S1 Fluorescence quantum yield of BP_5-NB-OB.

	BP_5-NB-OB (700 nm)	
Solvent	MeCN	PBS/MeCN (v/v = 1/1, pH = 6.0)
Φ_F	0.325	0.404

The relative fluorescence quantum yield Φ_F value was determined using indocyanine green dye ICG as a reference.

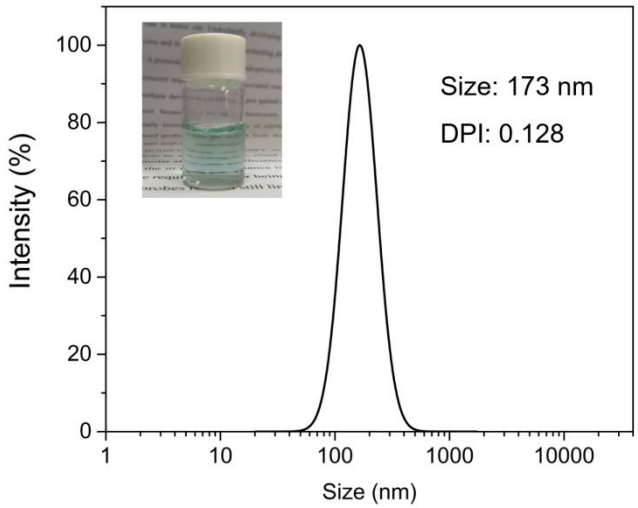


Figure S5 Size distribution of BP_5-NB-OB in aqueous solution.

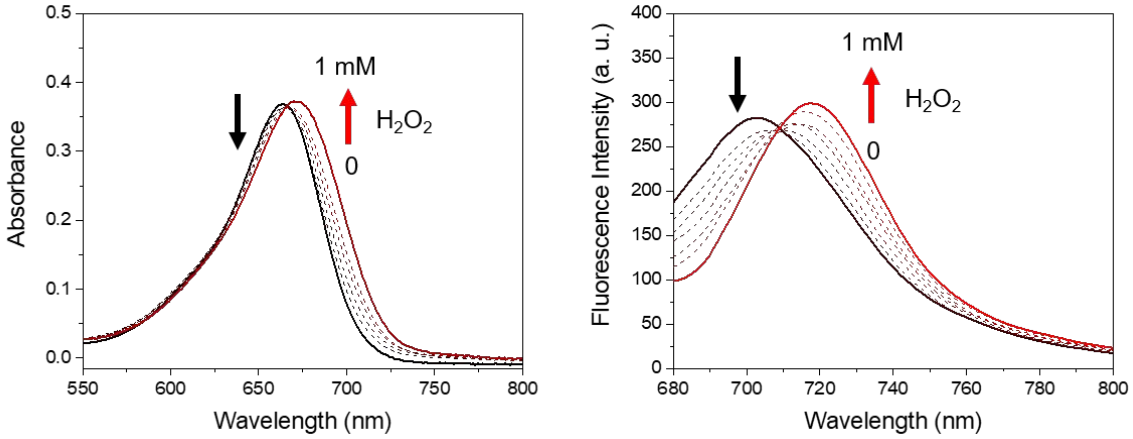


Figure S6 The absorption spectra and fluorescence spectra of BP_5-NB-OB in the presence of H_2O_2. Solvent: a mixed PBS buffer solution (PBS/MeCN, v/v = 1/1, pH = 6.0, 0.01 mM).

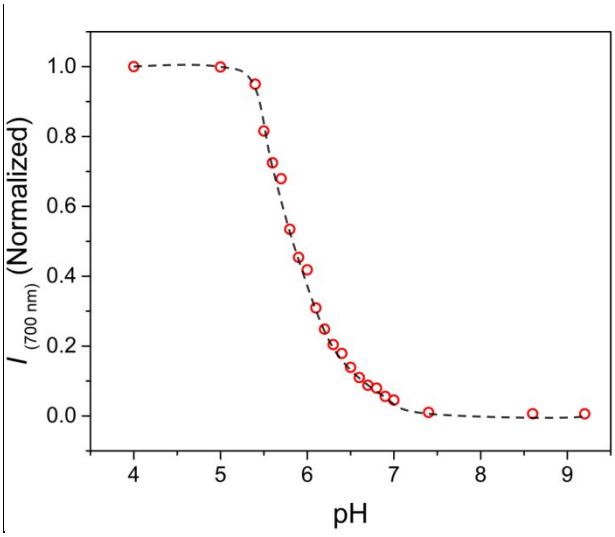


Figure S7 Fluorescence intensity of BP_5-NB-OH at 700 nm with pH changing from 4.0 to 9.2, λ_{ex} = 675 nm.

5. Characterization of Intermediate Compounds and BP_5-NB-OB

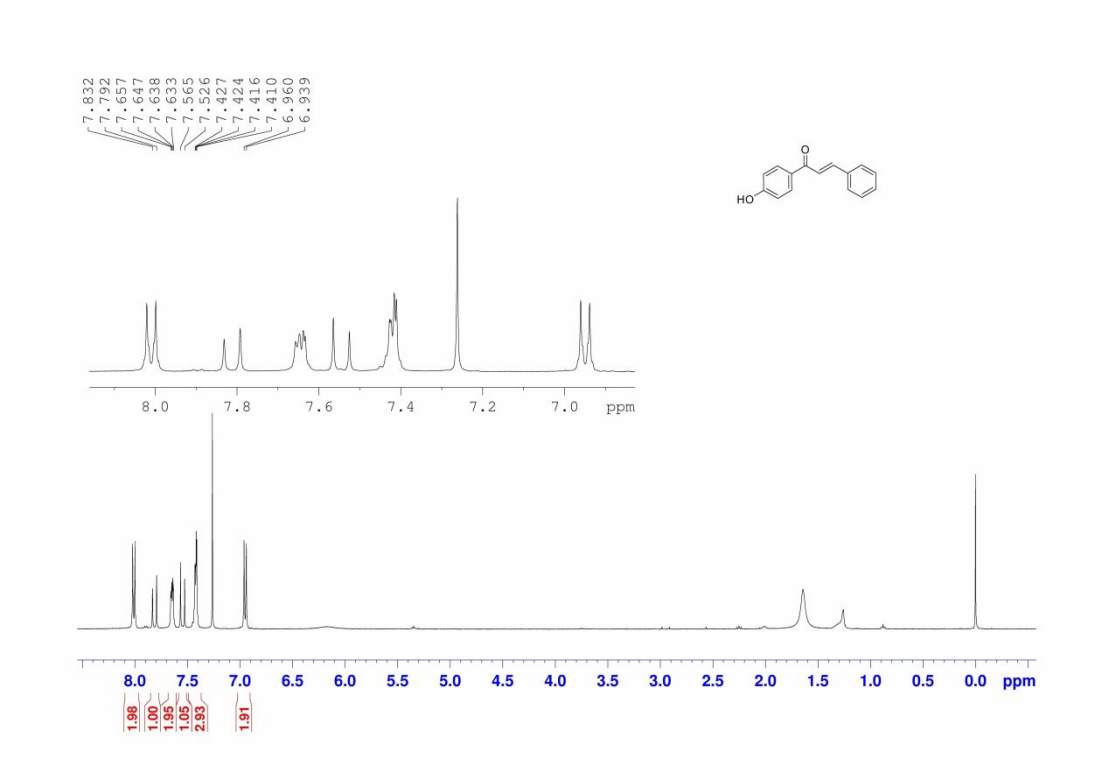


Figure S8 ^{1}H NMR spectrum of chalcone in $CDCl_3$

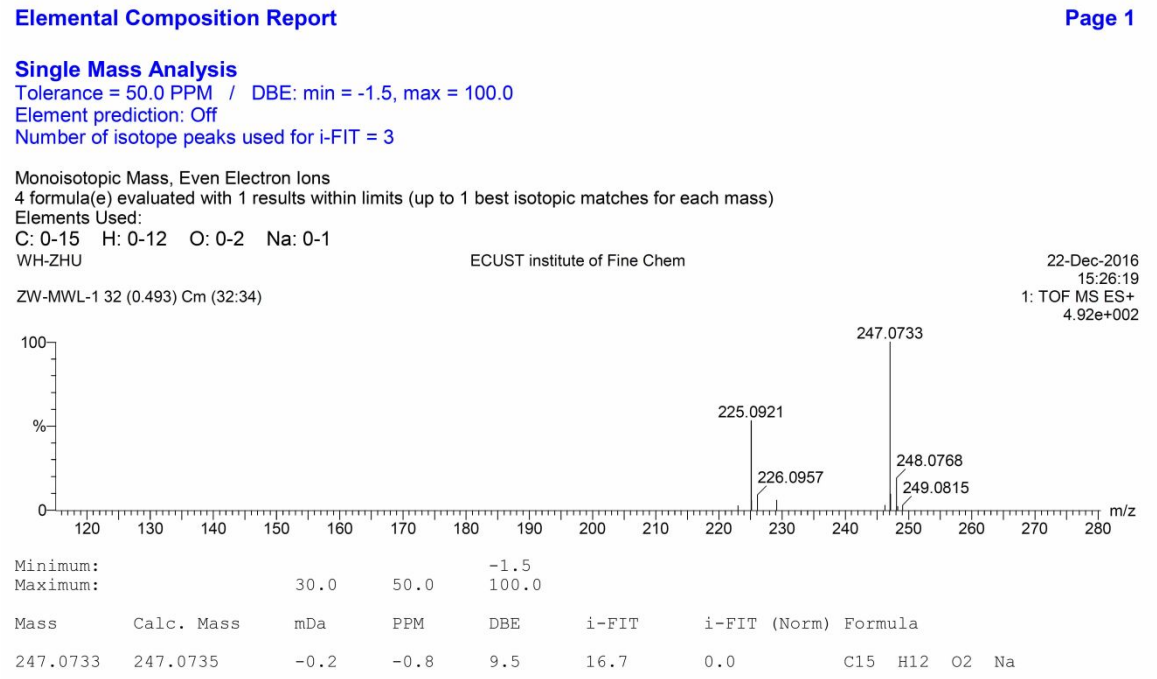


Figure S9 HRMS spectrum of chalcone

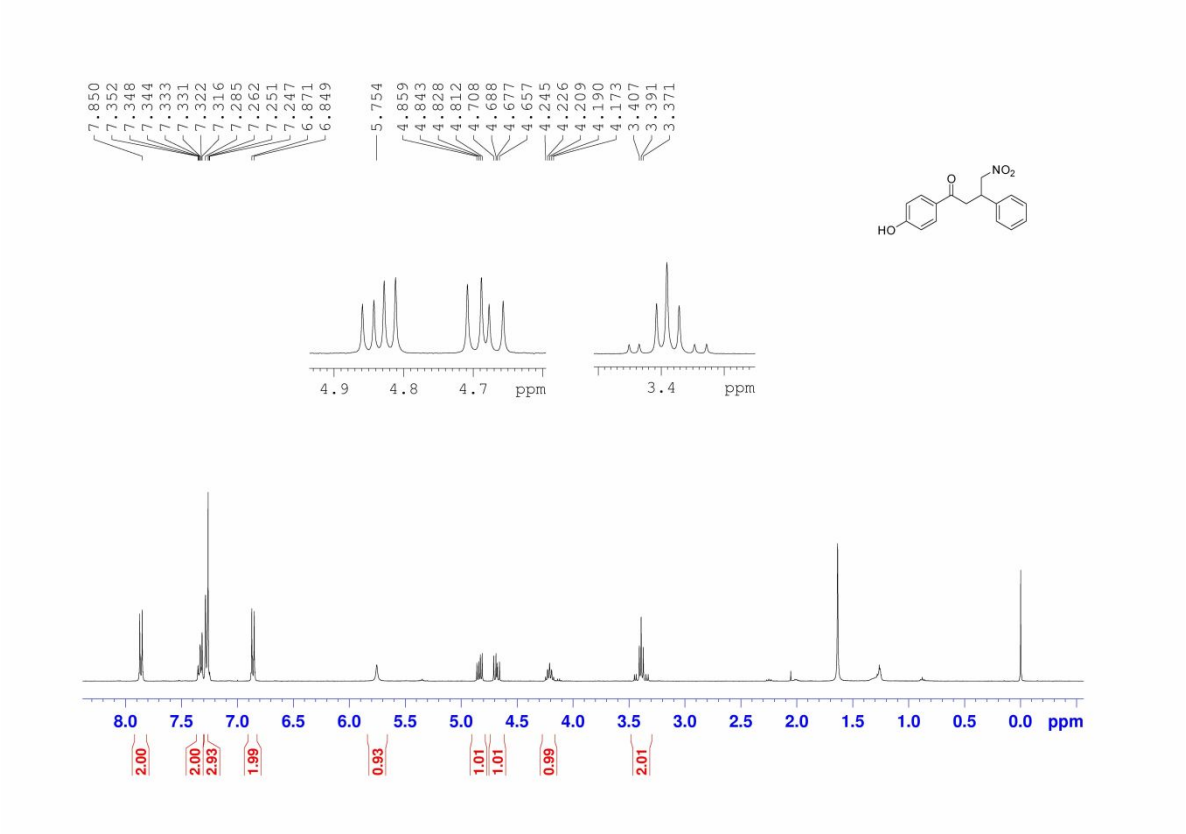


Figure S10 ^{1}H NMR spectrum of chalcone-NO_2 in $CDCl_3$

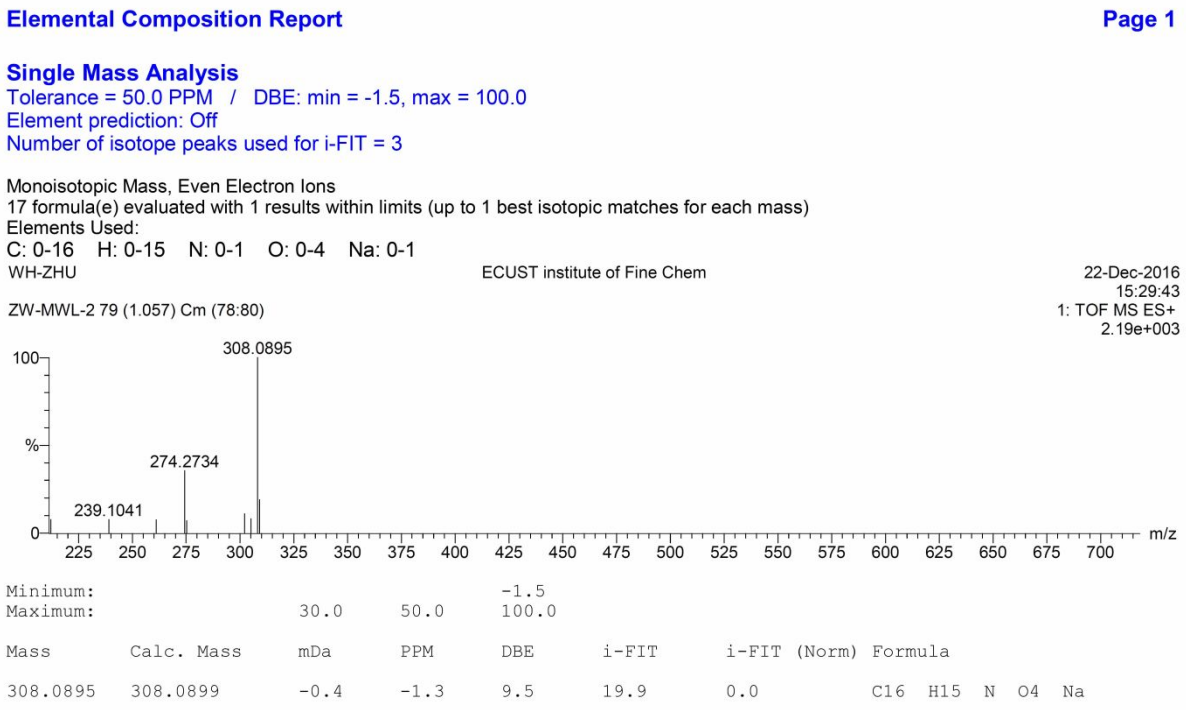


Figure S11 HRMS spectrum of chalcone-NO_2

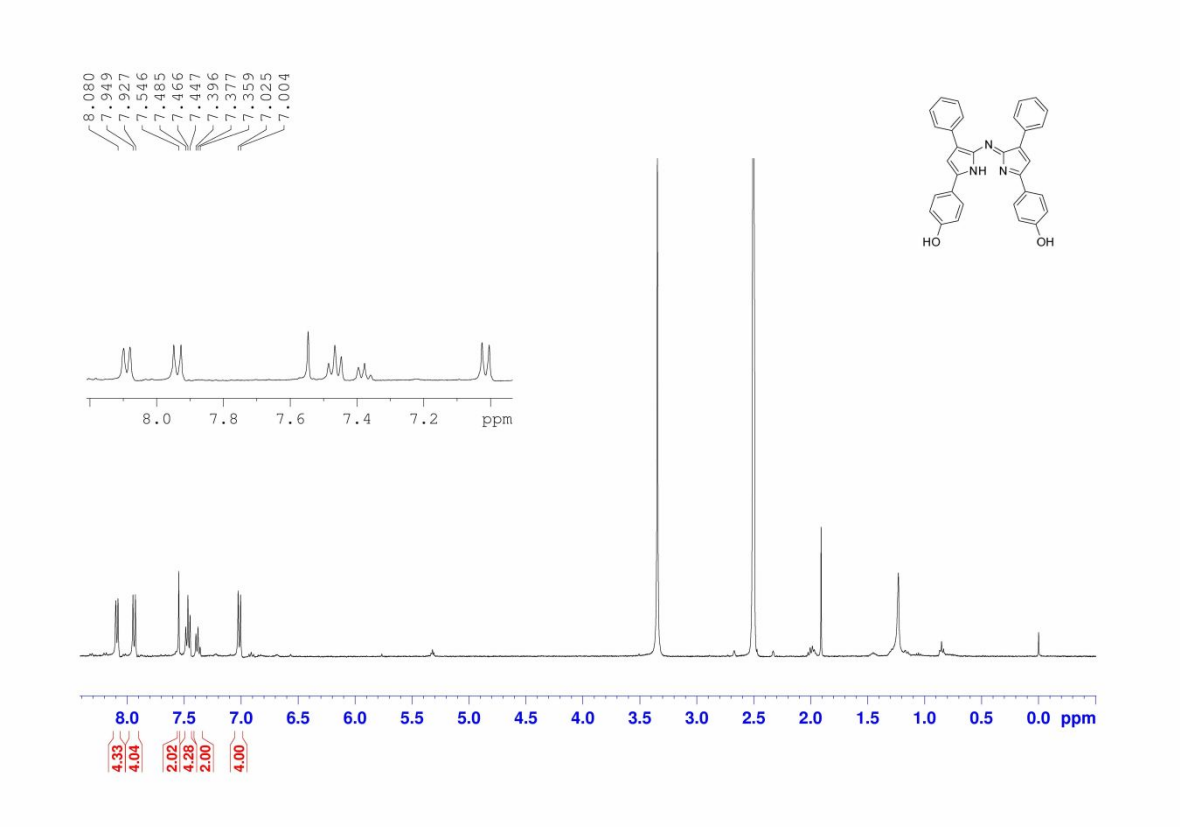


Figure S12 ^{1}H NMR spectrum of N-OH in DMSO-d_6

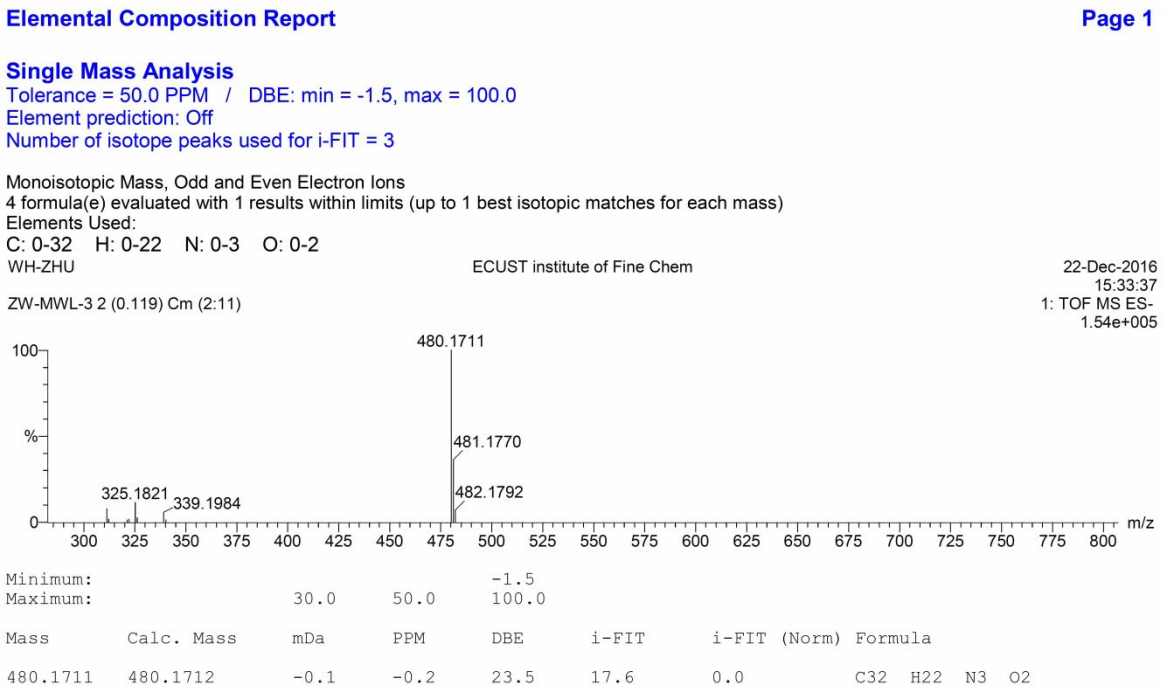


Figure S13 HRMS spectrum of N-OH

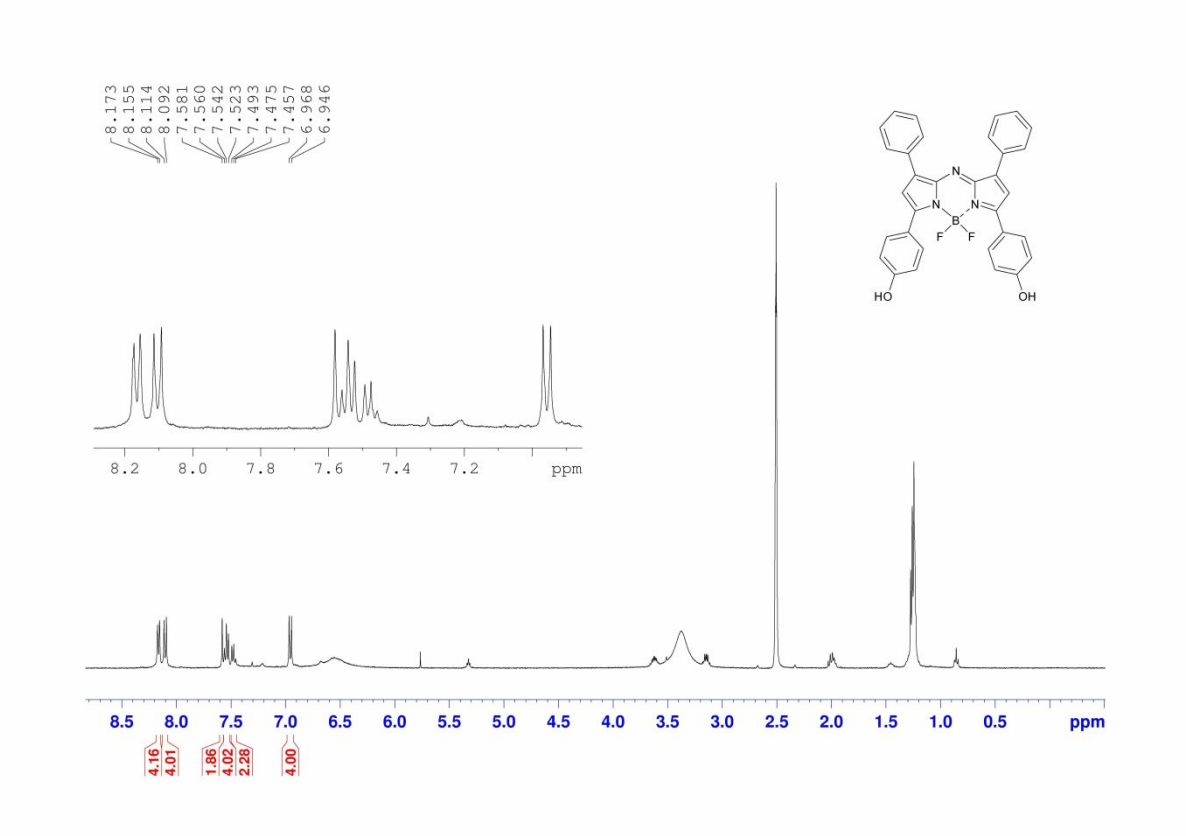


Figure S14 ^{1}H NMR spectrum of NB-diOH in DMSO-d_6

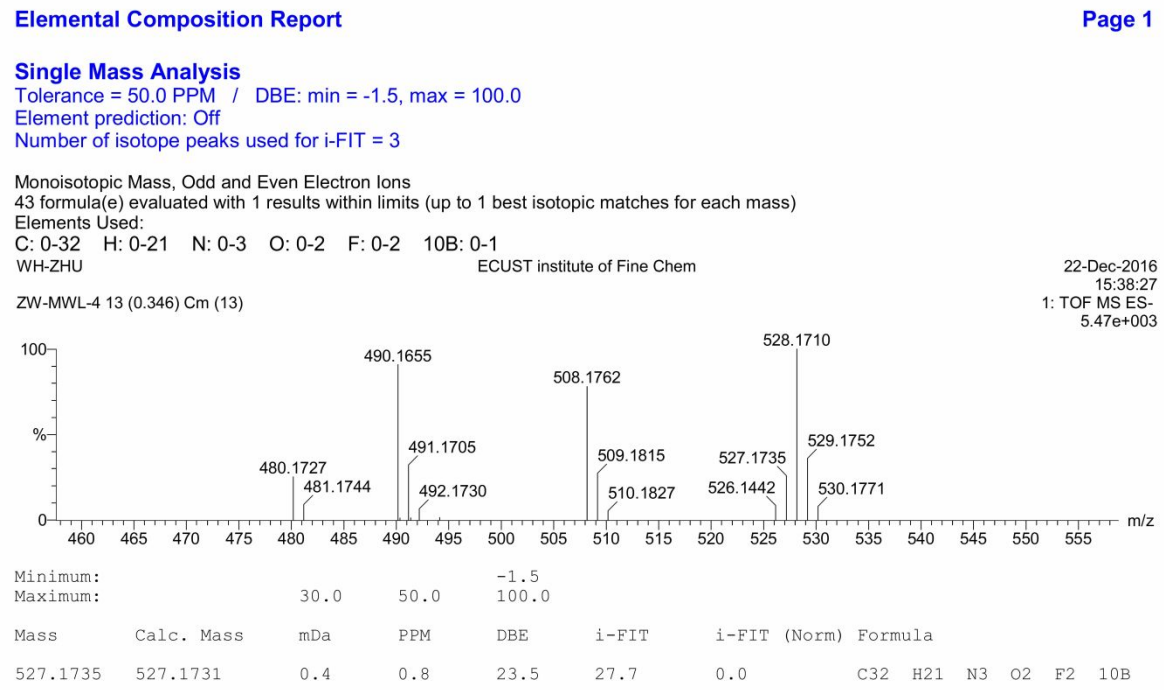


Figure S15 HRMS spectrum of NB-diOH

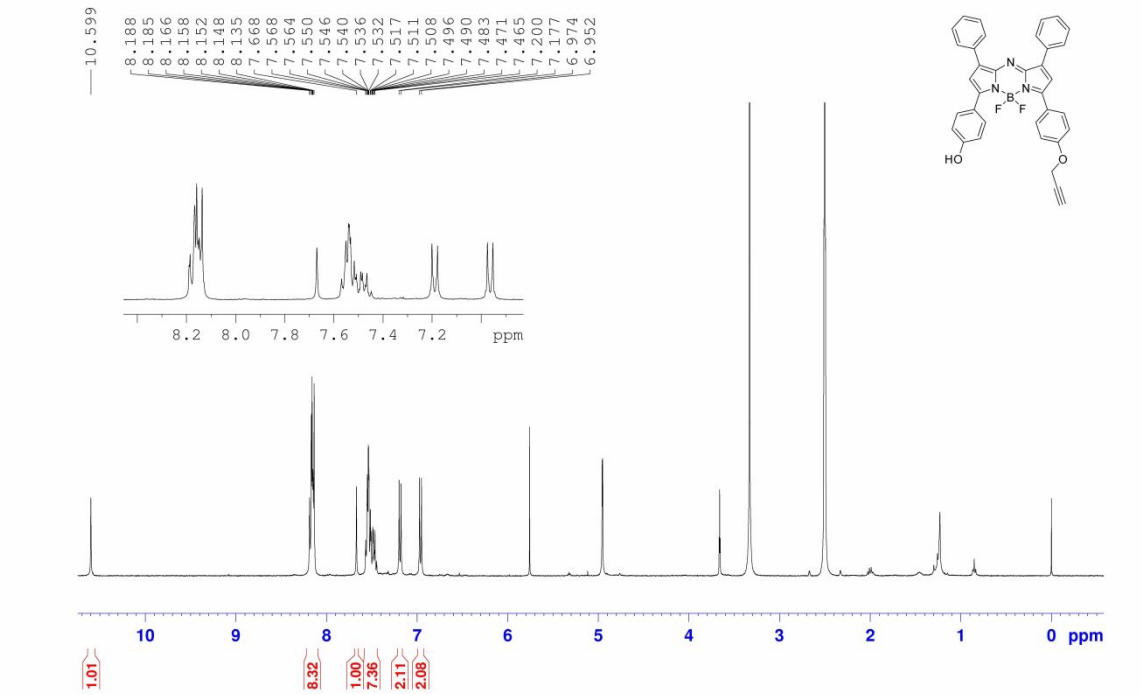


Figure S16 ^{1}H NMR spectrum of NB-OH in DMSO-d_6

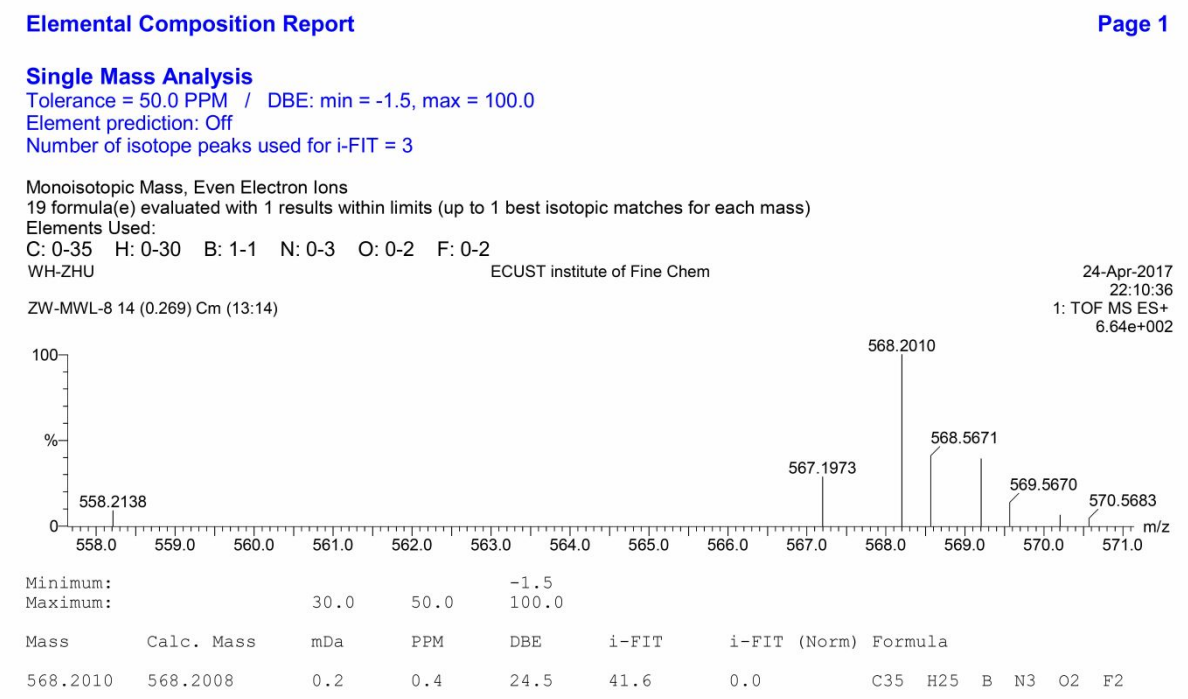


Figure S17 HRMS spectrum of NB-OH

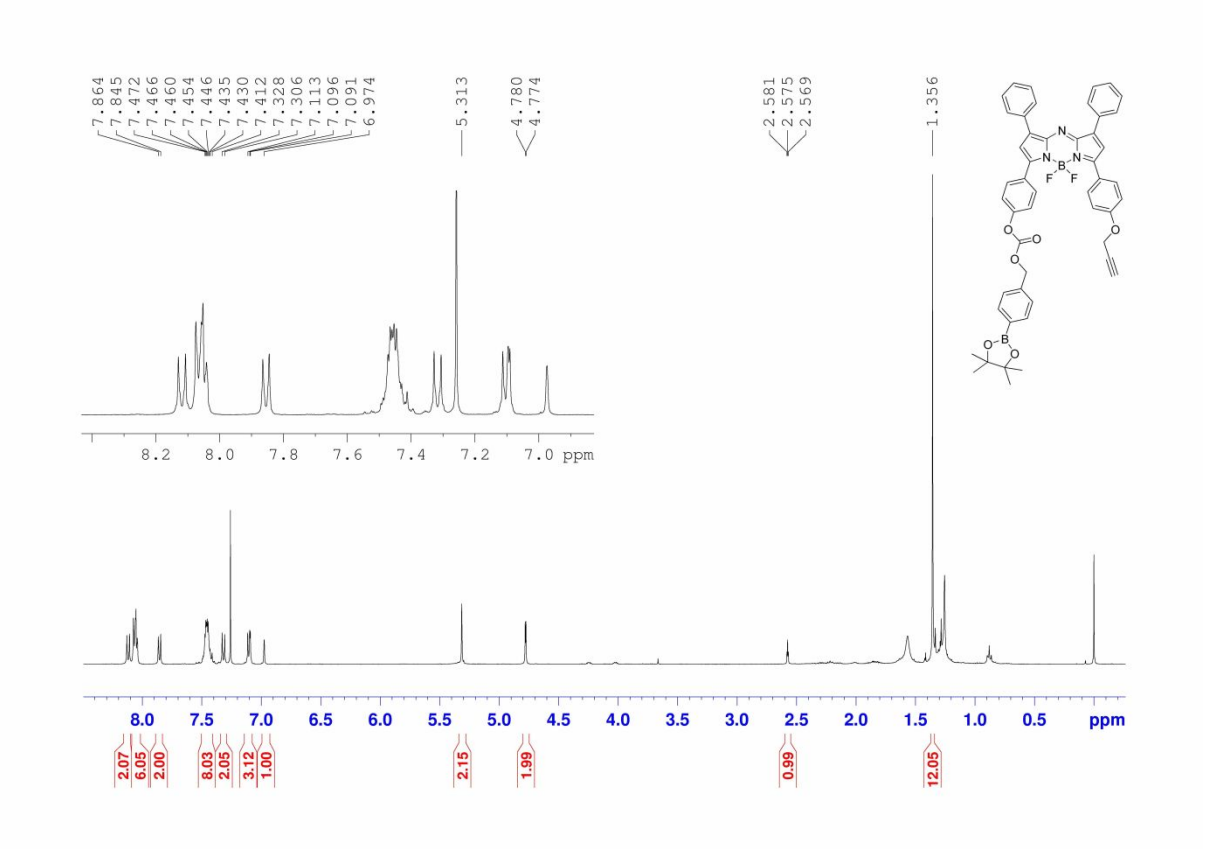


Figure S18 ^{1}H NMR spectrum of NB-OB in $CDCl_3$

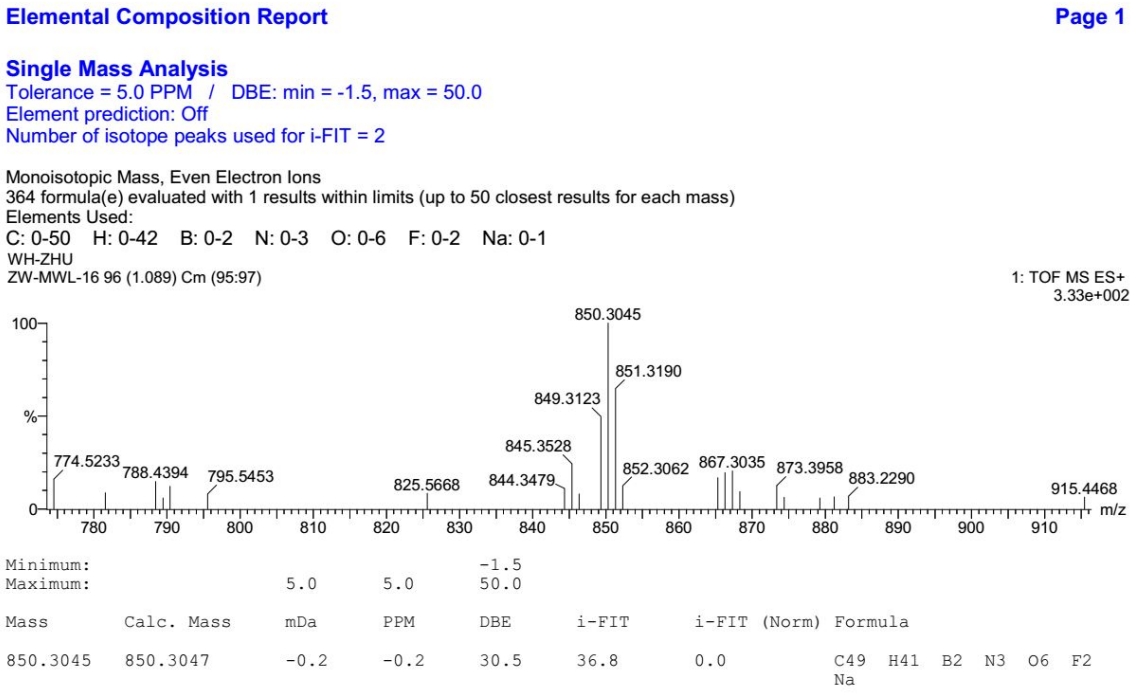


Figure S19 HRMS spectrum of NB-OB

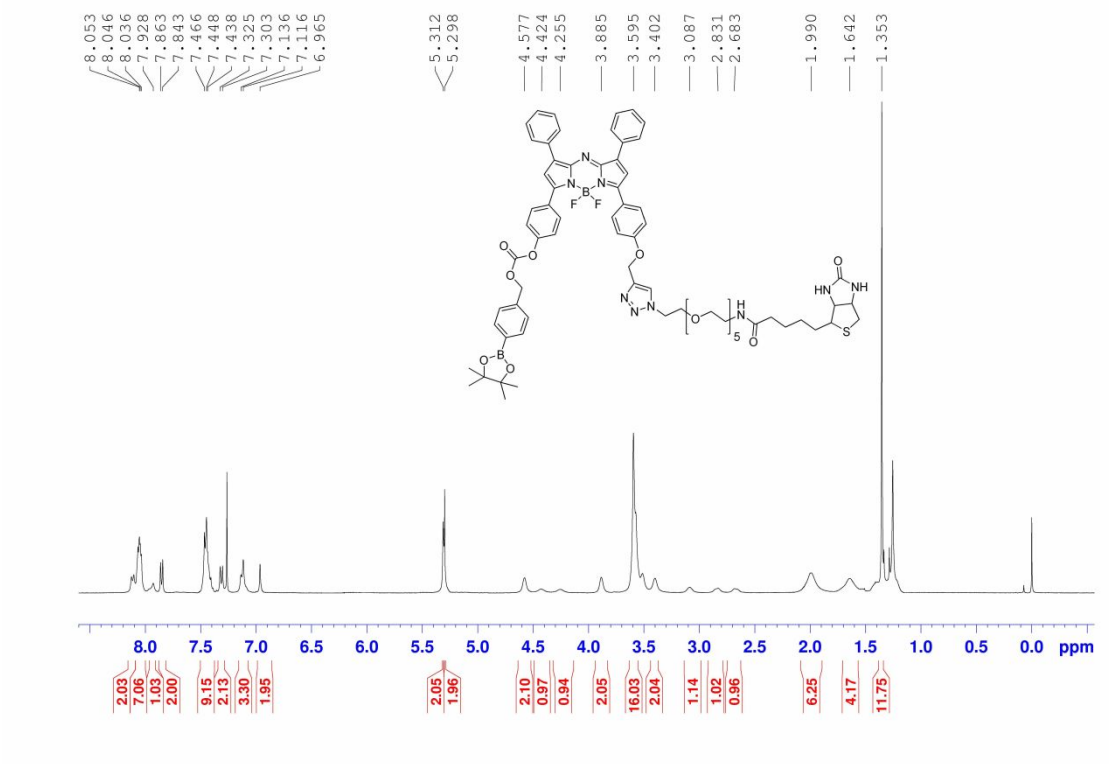


Figure S20 ^{1}H NMR spectrum of BP_5-NB-OB in $CDCl_3$

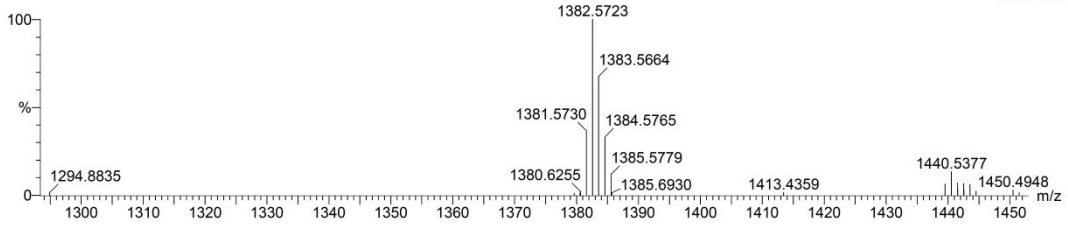
Elemental Composition Report **Page 1**

Single Mass Analysis
Tolerance = 5.0 PPM / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 2

Monoisotopic Mass, Even Electron Ions
4871 formula(e) evaluated with 1 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 0-71 H: 0-81 N: 0-9 O: 0-13 S: 0-1 B: 0-2 F: 0-2 Na: 0-1

WH-ZHU
ZW-MWL-50 52 (0.581) Cm (51:53)

1: TOF MS ES+
1.14e+003



Minimum:				-1.5			
Maximum:		5.0	5.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
1382.5723	1382.5726	-0.3	-0.2	35.5	41.8	0.0	C71 H81 N9 O13 S B2 F2 Na

Figure S21 HRMS spectrum of BP_5-NB-OB

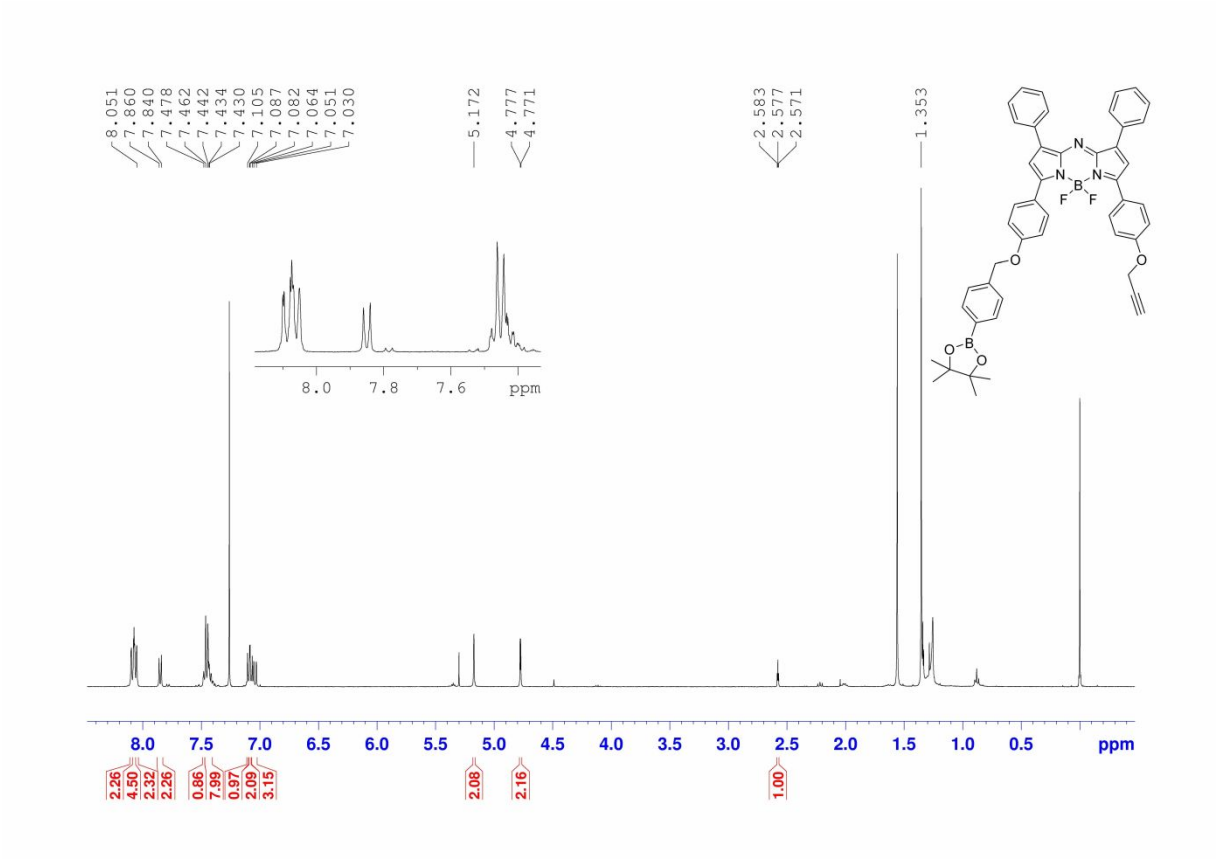


Figure S22 ^{1}H NMR spectrum of NB-B in $CDCl_3$