Electronic Supplementary Information (ESI)

In Vivo Real-time Tracking of the Tumor-Specific Bio-Catalysis of Cascade Nanotheranostics Enables Synergistic Cancer Treatment

Ruofei Wang,‡ Chenxu Yan,‡ Hehe Zhang, Zhiqian Guo,* Wei-Hong Zhu

Key Laboratory for Advanced Materials and Joint International Research Laboratory of Precision Chemistry and Molecular Engineering, Feringa Nobel Prize Scientist Joint Research Center, Institute of Fine Chemicals, School of Chemistry and Molecular Engineering, East China University of Science and Technology, Shanghai 200237, China.

‡These authors contributed equally to this work.

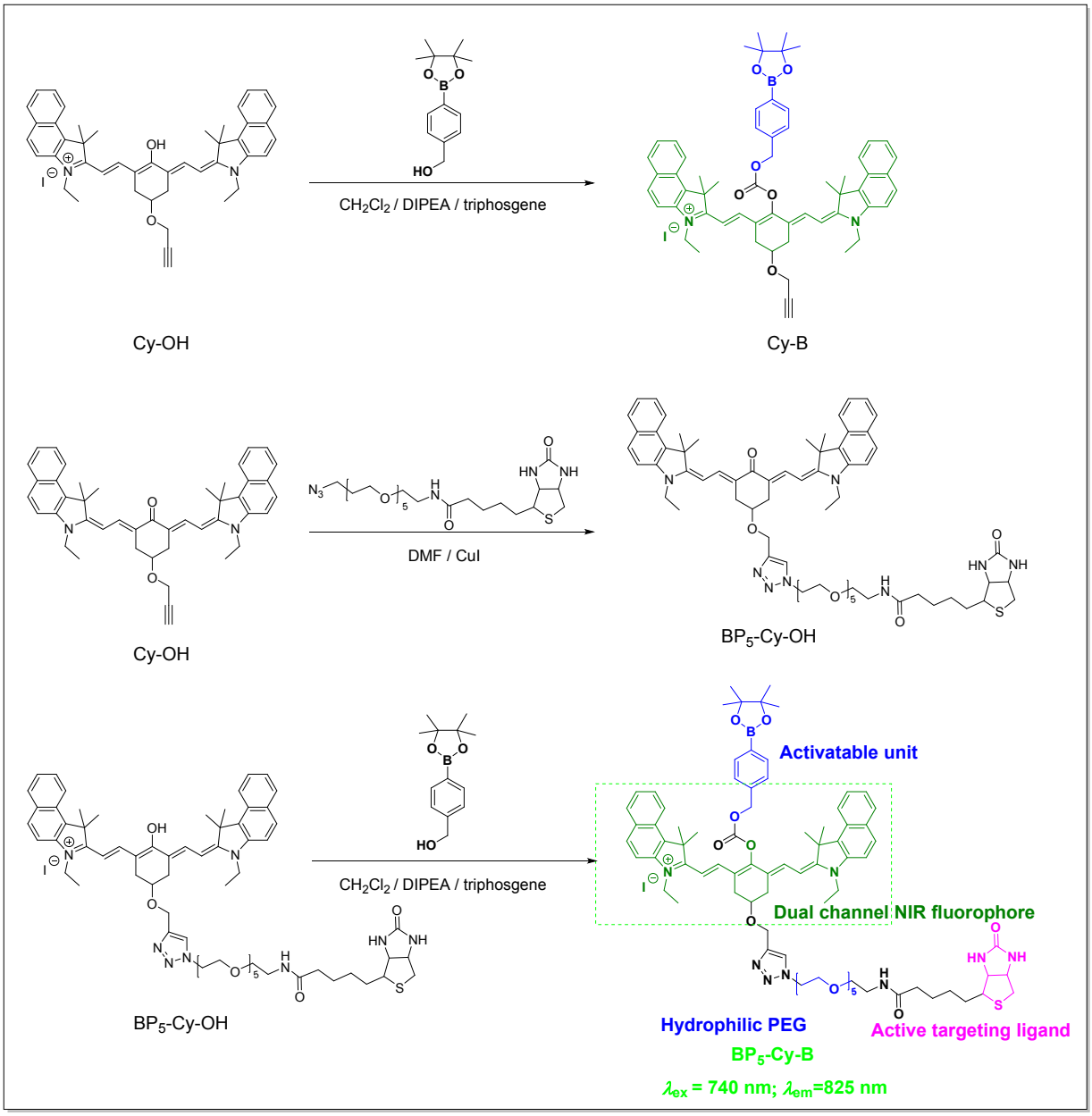
E-mail: guozq@ecust.edu.cn

Contents

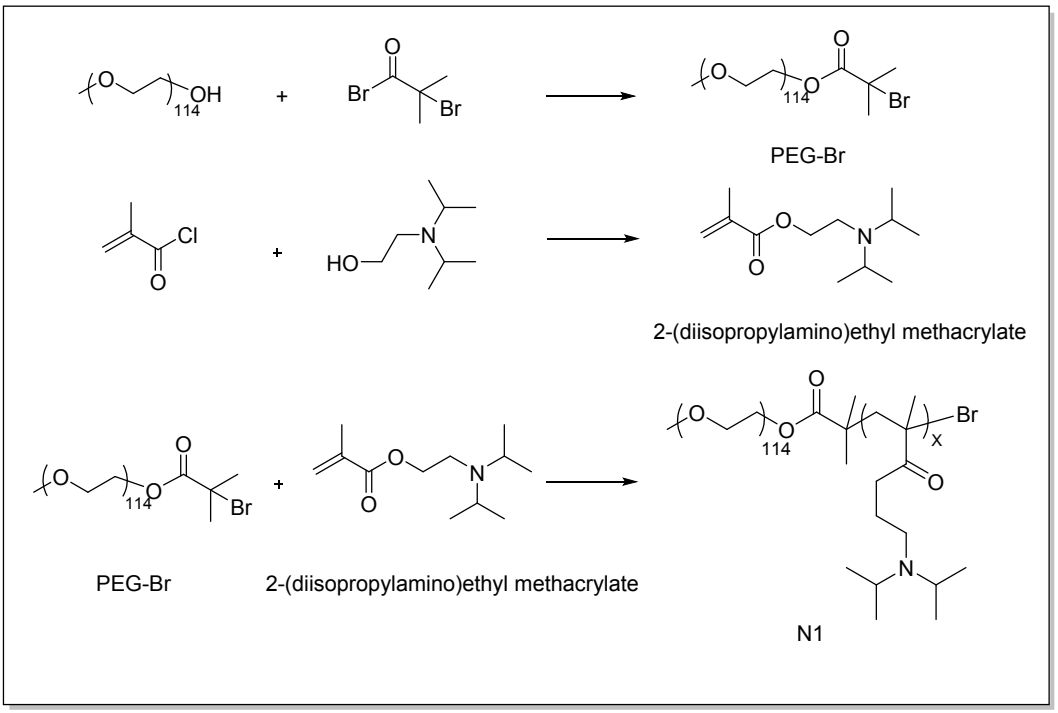
1. Experimental Section

Synthesis of BP_5-Cy-B, Cy-B and N1

The intermediate compound Cy-OH[1] and N1[2] was synthesized by the established procedures from our group.



Scheme S1. Synthetic route of Cy-B and BP_5-Cy-B



Scheme S2. Synthetic route of N1

Materials and General Methods

Unless special stated, all solvents and chemicals were purchased from commercial suppliers in analytical grade and used without further purification. Biotin-PEG_5-N_3 was supplied by Biomatrik Inc. (Jiaxing, China). The ^1H and ^{13}C NMR spectra were recorded on a Bruker AM 400 spectrometer, using TMS as an internal standard. High resolution mass spectrometry data were obtained with a Waters LCT Premier XE spectrometer. Absorption spectra were collected on a Varian Cary 500 spectrophotometer, and fluorescence spectra measurements were performed on a Varian Cary Eclipse fluorescence spectrophotometer. Particle size was measured by dynamic light scattering (DLS) with a Malvern Zetasizer Nano S90. Confocal fluorescence images were taken on confocal laser scanning microscope (LEICA TCS SP8). *In vivo* fluorescence images were measured with a PerkinElmer IVIS Lumina Kinetic Series III imaging system.

Synthesis of pH-Sensitive Copolymer

pH-sensitive copolymer were synthesized by atom transfer radical polymerization (ATRP) method. First, 2-(diisopropylamino)ethyl methacrylate (1.71 g, 8 mmol), PMDETA (17.3 mg, 0.1 mmol), and PEG-Br (0.5 g, 0.1 mmol) were charged into a polymerization tube. Then a mixture of anhydrous DMF (2 mL) was added to dissolve the monomer and initiator. After three cycles of freeze-pump-thaw to remove oxygen, CuBr (14 mg, 0.1 mmol) was added into the reaction tube under nitrogen atmosphere, and the tube was sealed in vacuo. The polymerization was carried out at 40 ^oC for 8 hours. After polymerization, the reaction mixture was diluted with 10 mL THF, and passed through an Al_2O_3 column to remove the catalyst. The THF solvent was removed by rotovap. The residue was dialyzed in distilled water and lyophilized to obtain a white powder.

Synthesis of Cy-B

To a mixture of 4-(hydroxymethyl) phenylboronic acid pinacol ester (183 mg, 0.78 mmol), triphosgene (77 mg, 0.26 mmol) and dry CH_2Cl_2 (30 mL) was added N,N-Diisopropylethylamine (168 mg, 1.3 mmol) dropwise at room temperature. The resulting solution was stirred overnight at room temperature. After removal of unreacted phosgene gas by flushing argon gas, a solution of Cy-OH (200 mg, 0.26 mmol) in $CHCl_3$ (10 mL) was added to the mixture and the reaction mixture was stirred overnight at room temperature. After removing the solvent under reduced pressure, the crude product was purified by silica gel chromatography using ethyl acetate/petroleum ether (v/v, 1:1) as the eluent to afford Cy-B as a green solid (88 mg): Yield 10% ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ: 1.09 (t, 12H, J = 8 Hz, -CH_3), 1.15 (s, 12H, -CH_3), 1.28 (t, 6H, J = 6 Hz, -CH_3), 2.68 (m, 2H, -CH_2-), 3.09 (t, 2H, J = 8 Hz, -CH_2-), 3.51 (s, 1H, -CH-), 4.08 (s, 1H, -CH-), 4.38 (t, 2H, J = 4 Hz, -CH_2-), 5.53 (s, 2H, -CH-), 6.32 (d, 2H, J = 6 Hz, ph-H), 7.54 (t, 2H, J = 8 Hz, ph-H), 7.62 (d, 2H, J = 8 Hz, ph-H), 7.70 (t, 2H, J = 8 Hz, ph-H), 7.77 (d, 4H, J = 12 Hz, ph-H), 7.83 (d, 2H, J = 4 Hz, ph-H), 8.10 (m,6H, ph-H). Mass spectrometry (ESI-MS, m/z): $[M-I]^+$ calcd for $C_{59}H_{64}BN_2O_6^+$, 907.4857; found, 907.4860.

Synthesis of BP_5-Cy-OH

Cy-OH (200 mg, 0.26 mmol) and Biotin-PEG_5-N_3 (276 mg, 0.52 mmol) were dissolved in dry DMF (10 mL) with CuI (99 mg, 0.52 mmol). Then the reaction mixture was stirred overnight at room temperature under an argon atmosphere. The solution was added with CH_2Cl_2 (50 mL) and washed with water (50 mL × 3), dried over Na_2SO_4, filtered and evaporated to BP_5-Cy-OH, and then the crude product was purified by silica gel chromatography using dichloromethane/methyl alcohol (v/v, 50:1) as the eluent to afford BP_5-Cy-OH as a red solid (350 mg): Yield 73%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.34 (t, 6H, J = 8 Hz, -CH_3), 1.43 (m, 4H, -CH_2-), 2.00 (s, 12H, -CH_3), 2.22 (t, 3H, J = 6 Hz), 2.59 (d, 2H, J = 12 Hz, -CH_2-), 2.71 (s, 1H, -CH-), 2.88 (m, 1H, -CH-), 3.14 (m, 2H, -CH_2-), 3.18 (d, 1H, J = 4 Hz, -CH-), 3.53 (t, 3H, J = 6 Hz), 3.63 (m, 24H, -CH_2-), 4.30 (m, 1H, -CH-), 4.48 (t, 1H, J = 6 Hz, -CH-), 4.56 (t, 2H, J = 4 Hz, -CH_2-), 4.85 (s, 2H, -CH_2-), 5.12 (s, 1H, -CH-), 5.50 (d, 2H, J = 16 Hz, -CH-), 5.95 (s, 1H, -CH-), 7.29 (d, 2H, J = 8 Hz, ph-H), 7.49 (t, 2H, J = 6 Hz, ph-H), 7.81 (m, 6H, ph-H), 8.05 (d, 2H, J = 12 Hz, ph-H), 8.36 (d, 2H, J = 16 Hz, -CH-). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ 173.41, 164.62, 140.92, 134.58, 130.03, 129.77, 129.51, 128.97, 126.87, 122.64, 122.42, 121.83, 109.13, 91.62, 74.45, 70.48, 70.02, 69.45, 62.07, 61.70, 50.37, 48.72, 39.13, 37.26, 35.86, 31.72, 29.71, 28.01, 27.92, 25.62, 11.65. Mass spectrometry (ESI-MS, m/z): $[M-I]^+$ calcd for $C_{67}H_{87}N_8O_9S^+$, 1179.6317; found, 1179.6322.

Synthesis of BP_5-Cy-B

To a mixture of 4-(hydroxymethyl) phenylboronic acid pinacol ester (35 mg, 0.45 mmol), triphosgene (44 mg, 0.15 mmol) and dry CH_2Cl_2 (30 mL) was added N,N-diisopropylethylamine (161 mg, 1.25 mmol) dropwise at room temperature. The resulting solution was stirred overnight at room temperature. After removal of unreacted phosgene gas by flushing argon gas, a solution of BP_5-Cy-OH (200 mg, 0.15 mmol) in $CHCl_3$ (10 mL) was added to the mixture and the reaction mixture was stirred overnight at room temperature. After removing the solvent under reduced pressure, the crude product was purified by silica gel chromatography using ethyl acetate/petroleum ether (v/v, 1:1) as the eluent to afford BP_5-Cy-B as a green solid (80 mg): Yield 8%. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.22 (s, 12H, -CH_3), 1.32 (s, 2H, -CH_2-), 1.49 (m, 16H, -CH-), 1.77 (s, 6H, -CH_3), 2.25 (t, 2H, J = 8 Hz, -CH_2-), 2.73 (m, 3H, -CH_3), 2.90 (m, 1H, -CH-), 3.12 (m, 2H, -CH_2-), 3.28 (d, 2H, J = 12 Hz, -CH_2-), 3.43 (d, 2H, J = 4 Hz, -CH_2-), 3.62 (m, 24H, -CH_2-), 3.95 (s, 2H, -CH_2-), 4.08 (s, 1H, -CH-), 4.63 (d, 2H, J = 4 Hz, -CH_2-), 4.99 (s, 2H, -CH_2-), 5.42 (s, 2H, -CH_2-), 6.31 (d, 2H, J = 12 Hz, -CH-), 7.43 (d, 2H, J

= 8 Hz, ph-H), 7.56 (d, 2H, *J* = 8 Hz, ph-H), 7.66 (t, 2H, *J* = 8 Hz, ph-H), 7.85 (d, 2H, *J* = 12 Hz, -CH-),7.96 (m, 10H, ph-H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm): δ173.02, 139.49, 139.19, 137.23, 135.28, 134.06, 131.93, 130.82, 130.18, 128.09, 127.85, 125.05, 122.06, 119.20, 110.66, 100.64, 84.03, 77.24, 71.19, 70.87, 70.57, 70.18, 69.79, 69.52, 61.77, 60.09, 55.38, 53.61, 53.45, 50.87, 50.12, 41.89, 40.64, 40.08, 39.11, 35.75, 30.26, 29.71, 28.07, 27.47, 25.48, 24.76, 18.66, 17.40, 12.94, 12.03. Mass spectrometry (ESI-MS, m/z): $[M-I]^+$ calcd for $C_{81}H_{104}BN_8O_{13}S^+$, 1439.7533; found, 1439.7537.

Preparation of BN

In a typical procedure for the preparation of BN nanoparticles: 2 mg BP_5-Cy-B and 20 mg N1 was dissolved in 1.0 mL of DMSO and stirred at room temperature (25 ^{o}C) for 10 min. Then the mixture was slowly added into 9.0 mL of deionized water and stirred at room temperature (25 ^{o}C) for 10 min. Subsequently, the solution was dialyzed against deionized water for 24 h (molecular weight cutoff = 8,000 g mol^{-1}) and the deionized water was exchanged for 4 times.

Preparation of BNG

In a typical procedure for the preparation of BNG nanoparticles: 2 mg BP_5-Cy-B and 20 mg N1 was dissolved in 1.0 mL of DMSO and stirred at room temperature (25 ^{o}C) for 10 min (mixture 1). 2 mg GOD was dissolved in 9.0 mL of deionized water and stirred at room temperature (25 ^{o}C) for 10 min (mixture 2). Then the mixture 1 was slowly added into mixture 2 and stirred slightly for another 10 min. Subsequently, the solution was dialyzed against deionized water for 24 h (molecular weight cutoff = 8,000 g mol^{-1}) and the deionized water was exchanged for 4 times.

Cell Experiment

Cell Lines

The A549 cell line 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^{-1} streptomycin, Solarbio life science, Beijing, China).

In Vitro Cytotoxicity Assay

The cell cytotoxicity of BN, N1 & GOD, BNG and BNG & GSH to A549 cells, 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 (μM) of BN, N1 & GOD, BNG and BNG & GSH. After incubation for 48 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 (%) = $(OD_{treated}/OD_{control}) \times 100\%$

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. To explore the effect of H_2O_2 in vitro, A549 cells were incubated with BP_5-Cy-B (10 μM) for 30 min, then the probe treated cells were incubated with H_2O_2 (10 μM) for another 10 min. For the endogenous H_2O_2 detection, cells were successively incubated with TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl, ROS scavenger) (10 μM) at 37 °C for 30 min, BP_5-Cy-B (10 μM) for another 30 min and PMA (phorbol myristate acetate, trigger production of ROS in mitochondria via activating of protein kinase C) for 30 min. For the effect of PEG segment, cells were successively incubated with TEMPO (10 μM) at 37 °C for 30 min, BP_5-Cy-B (10 μM) and Cy-B (10 μM) for another 30 min, respectively. After washing the culture dishes three times with PBS, the images were then photographed by using a Confocal laser scanning microscope Leica TCS SP8 (63 × oil lens) with 540 nm, 740 nm as the excitation wavelength and 650 nm and 825 nm as the emission wavelength.

Colocalization

For the co-staining experiment, A549 cells were incubated with the mixture of probe BP_5-Cy-B (10 μM) and Mito-Tracker Green FM (200 nM) for 2 h. Fluorescence images were acquired with a LEICA TCS SP8 laser confocal fluorescence microscope.

Animals

The 3-4-week-old female BALB/cA nude mice were produced from East China Normal University, 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.

Real-time *in vivo* imaging in tumor-bearing mice

The nude mice were inoculated with A549 cell on their right flanks by injecting 106 cells subcutaneously. When the tumors grew up to 10 mm in diameter, BN and BNG (administered at a BP_5-Cy-B-equivalent dose of 0.1 mg kg^{-1}) in PBS were intravenously injected via tail vein into the A549 cell tumor-bearing nude mice. The real-time *in vivo* imaging was recorded at different time internals after BN and BNG injection. *In vivo* fluorescence images were measured with IVIS Spectrum CT imaging system, respectively. After injection, the mice were sacrificed at 12 h. The grafted tumor tissues and major organs, including kidney, lung, spleen, liver, heart, were excised and washed with 0.9% saline. The optical images of the organs and tissues were taken using a PE *in vivo* Professional Imaging System as described above.

2. ACQ of BP_5-Cy-B

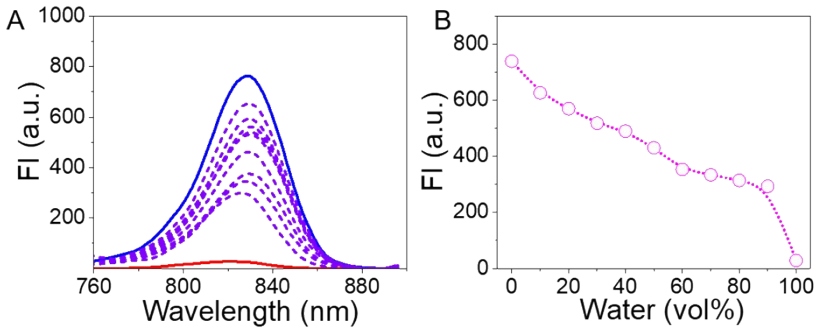


Fig. S1. (A) The fluorescence spectra of BP_5-Cy-B (λ_{ex} = 740 nm) in THF/water mixtures with different fractions of water. (B) Fluorescence intensity at 825 nm, showing typical ACQ (Aggregation-Caused Quenching) effects at 25 °C.

3. Selectivity of BP_5-Cy-B and Real-time Targeting GOD Bio-catalysis

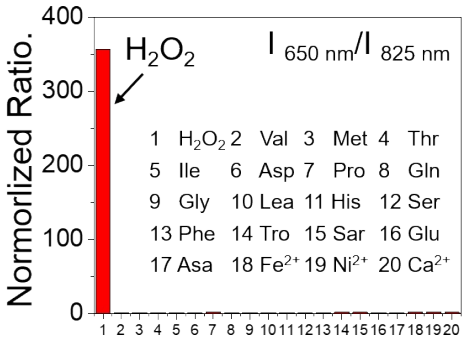


Fig. S2. Selectivity of BP_5-Cy-B toward various potential reactive species.

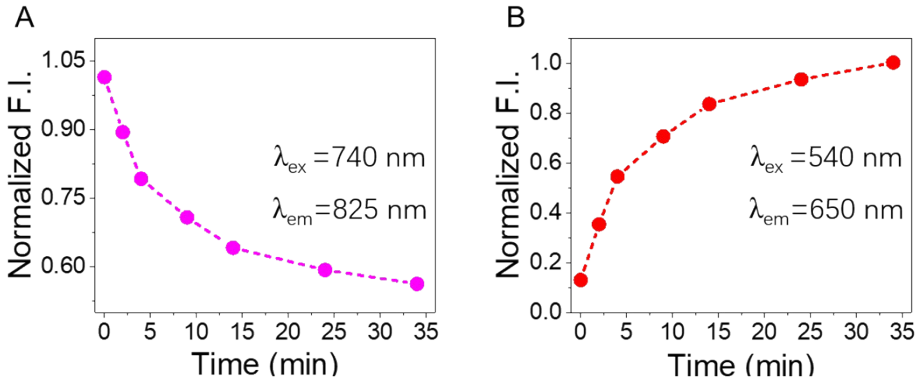
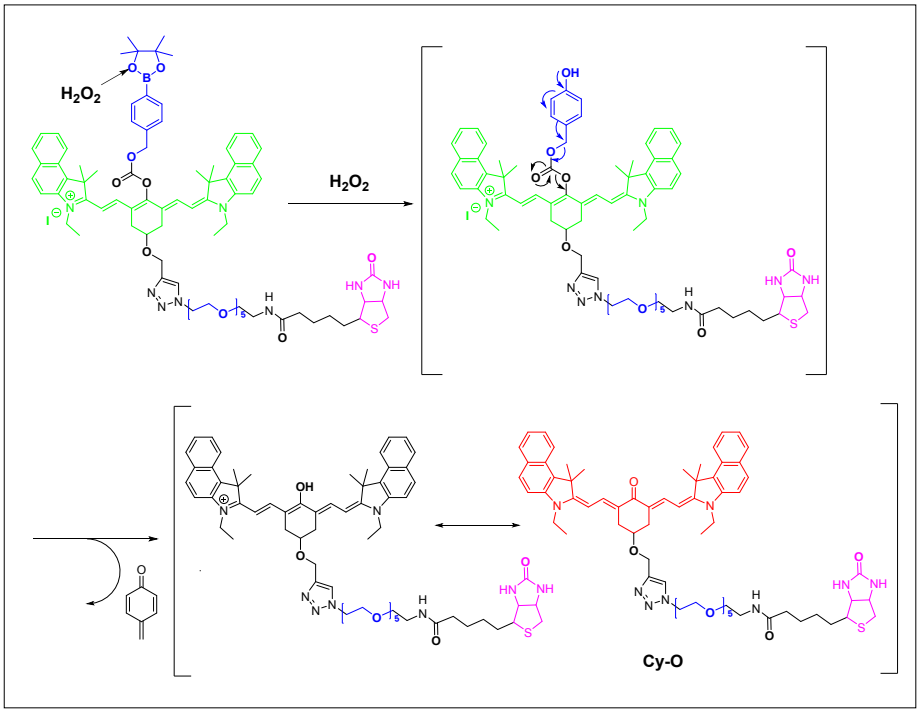


Fig. S3. Normalized fluorescence intensity with λ_{em} = 825 nm (A) and λ_{em} = 650 nm (B) of BP_5-Cy-B in the presence of glucose (5 mg mL^{-1}) and GOD (20 U mL^{-1}) in mixtured solution (DMSO/H_2O, v/v= 1/3, pH = 7.4, 37 °C).

4. Proposed Mechanism of BP_5-Cy-B Reaction with H_2O_2



Scheme S3. Proposed Mechanism of BP_5-Cy-B Reaction with H_2O_2

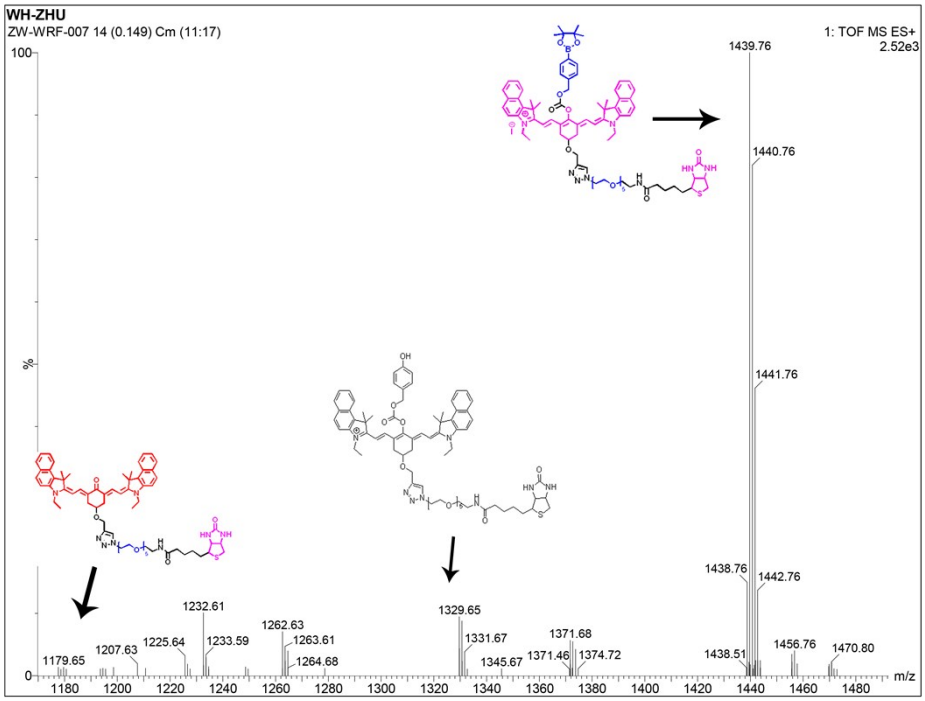


Fig. S4. Sensing mechanism were further confirmed by ESI-MS analyses

5. The Stability of BNG

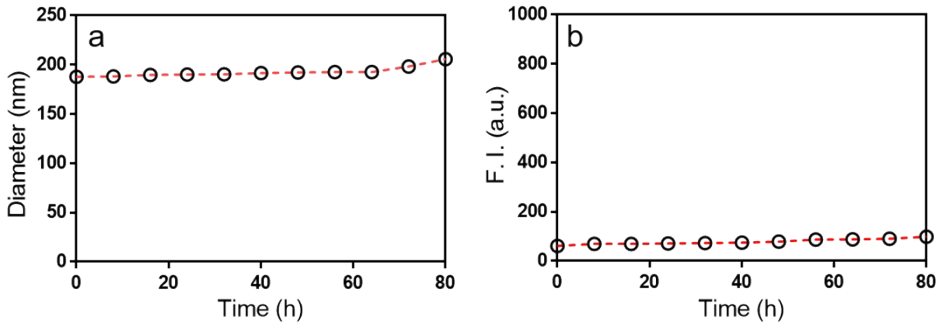


Fig. S5 Time dependent diameter (a) and fluorescence intensity (b) at 825 nm of BNG in PBS buffer solution at 37 °C.

Note: The diameter and fluorescence intensity at 825 nm remained stable within 80 h, which suggested BNG did not dissociate with time. Those results confirmed that BNG exhibited excellent stability for long storage.

6. Targeting Mitochondria Ability

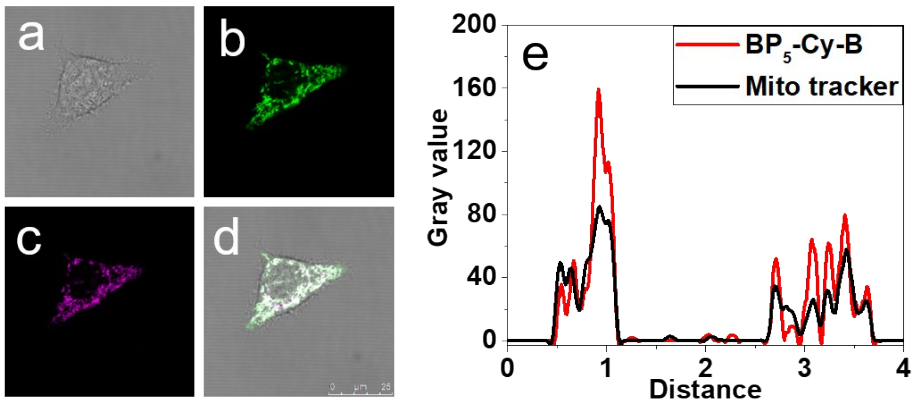


Fig. S6. Fluorescence images of mitochondria co-localized experiment in HeLa cells. The cells were incubated with the mixture of BP_5-Cy-B and Mito-Tracker Green for 2 h. (b) Mito-Tracker Green (200 nM, λ_{ex} = 488 nm, λ_{em} = 500 - 550 nm). (c) BP_5-Cy-B (10 μM, λ_{ex} = 740 nm, λ_{em} = 750 - 800 nm). (d) Overlay of (b) and (c). (e) Intensity correlation plot. Note: BP_5-Cy-B display site-specifically internalized in mitochondria in living cells.

7. Cancer Cells Targeting of BP_5-Cy-B

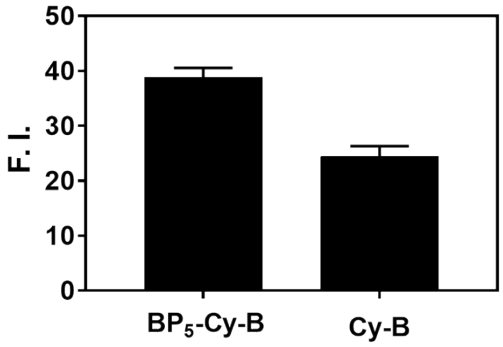


Fig. S7. Normalized intensity from Fig. $4D_3$ and Fig. $4E_3$

8. Cancer Cells Imaging of GSH Depletion

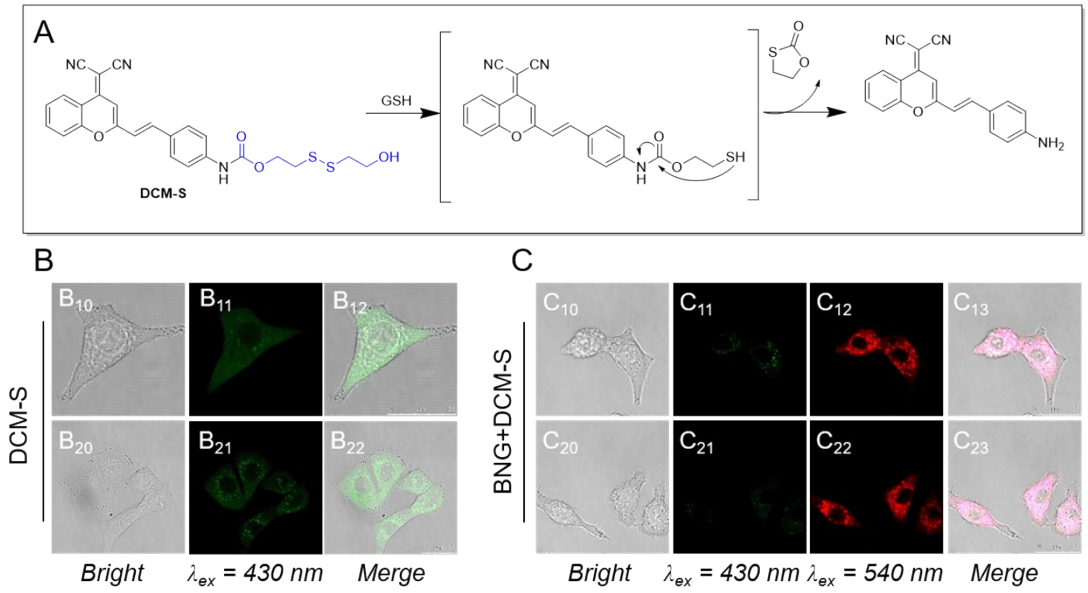


Fig. S8 (A) The reaction mechanism of DCM-S with GSH. (B) Confocal laser scanning microscopy images of A549 cells only co-cultured with DCM-S (20 μM). Images were taken from 600-620 nm (λ_{ex} = 430 nm; two parallel experiments). (C) Confocal laser scanning microscopy images of A549 cells incubation firstly with BNG (2 hours) and then DCM-S (20 μM, 40 min). Images were taken from 600 - 620 nm (λ_{ex} = 430 nm) and 640 - 690 nm (λ_{ex} = 540 nm).

DCM-S could display a fluorescence light-up signal (λ_{ex} =430 nm) after reaction with GSH in cancer cells.[3] (Note: BNG did not display fluorescence signal when excited in 430 nm. So there is no fluorescence interference between DCM-S and BNG.) As expected, cells pretreated with DCM-S and BNG displayed much weaker fluorescence signal (λ_{ex} = 430 nm). These cell imaging results strongly supported the released quinone methide could deplete cellular GSH.

9. Characterization of Intermediate Compounds and BP_5-Cy-B

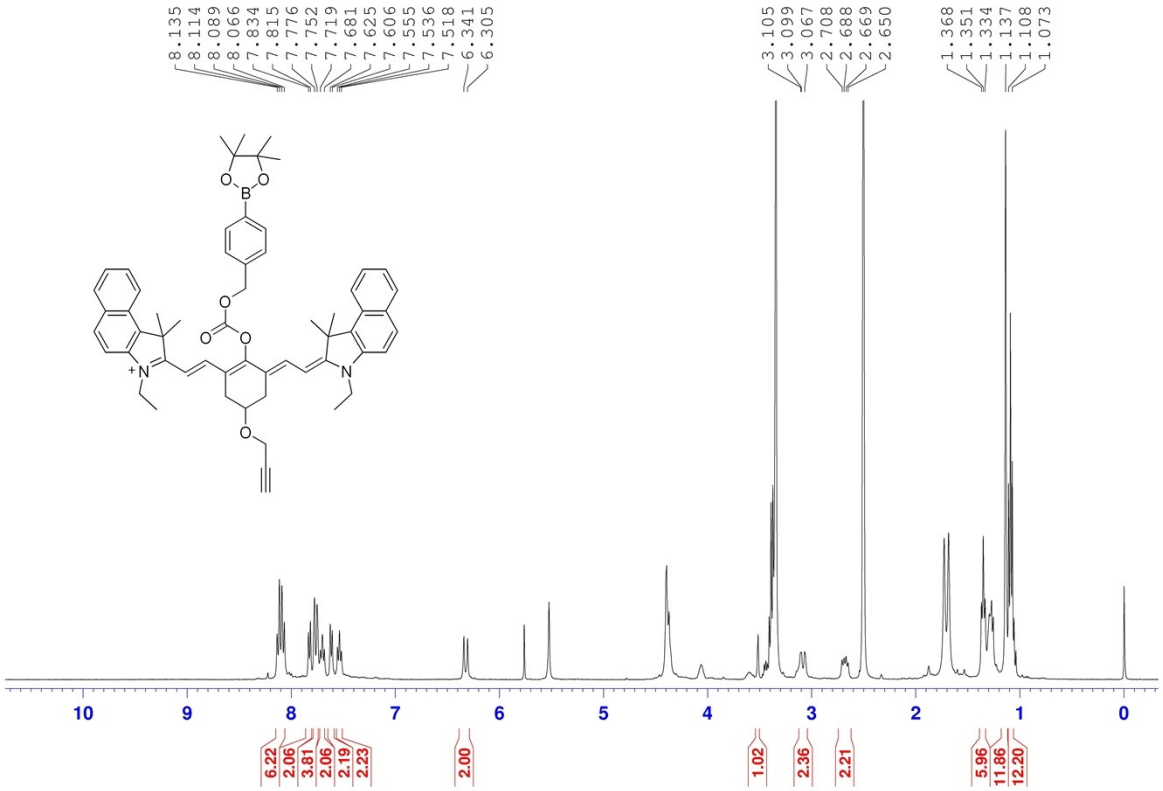


Fig. S9. ^{1}H NMR spectrum of Cy-B in DMSO

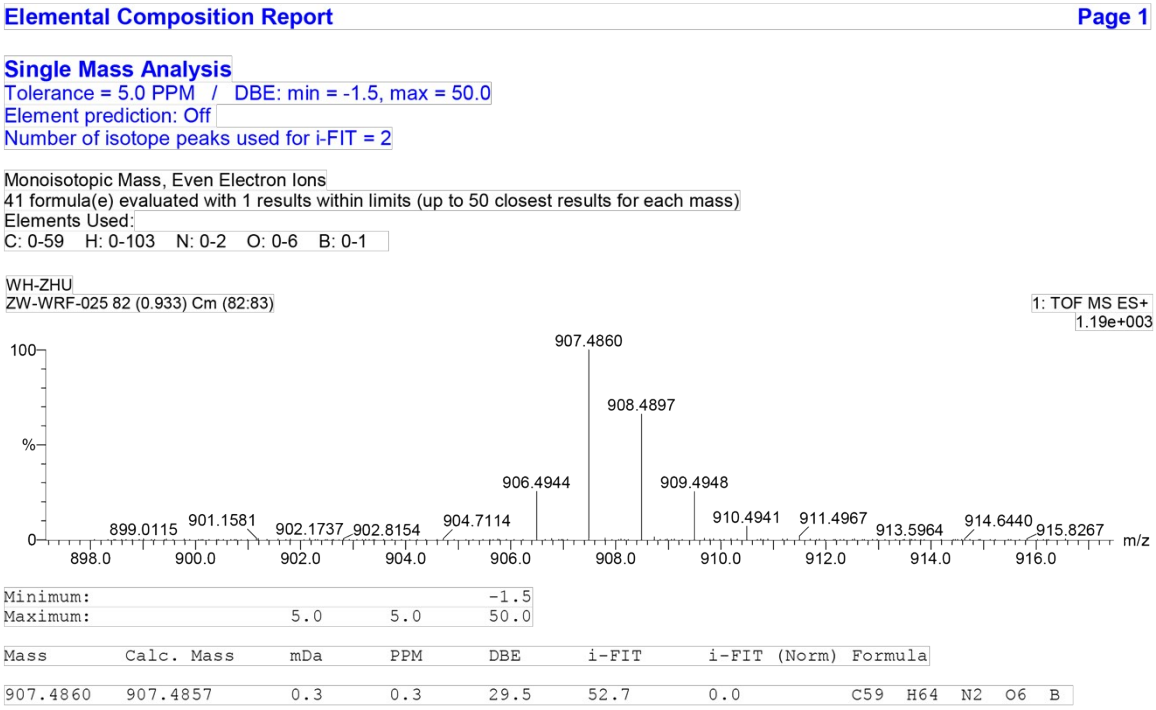


Fig. S10. HRMS spectrum of Cy-B

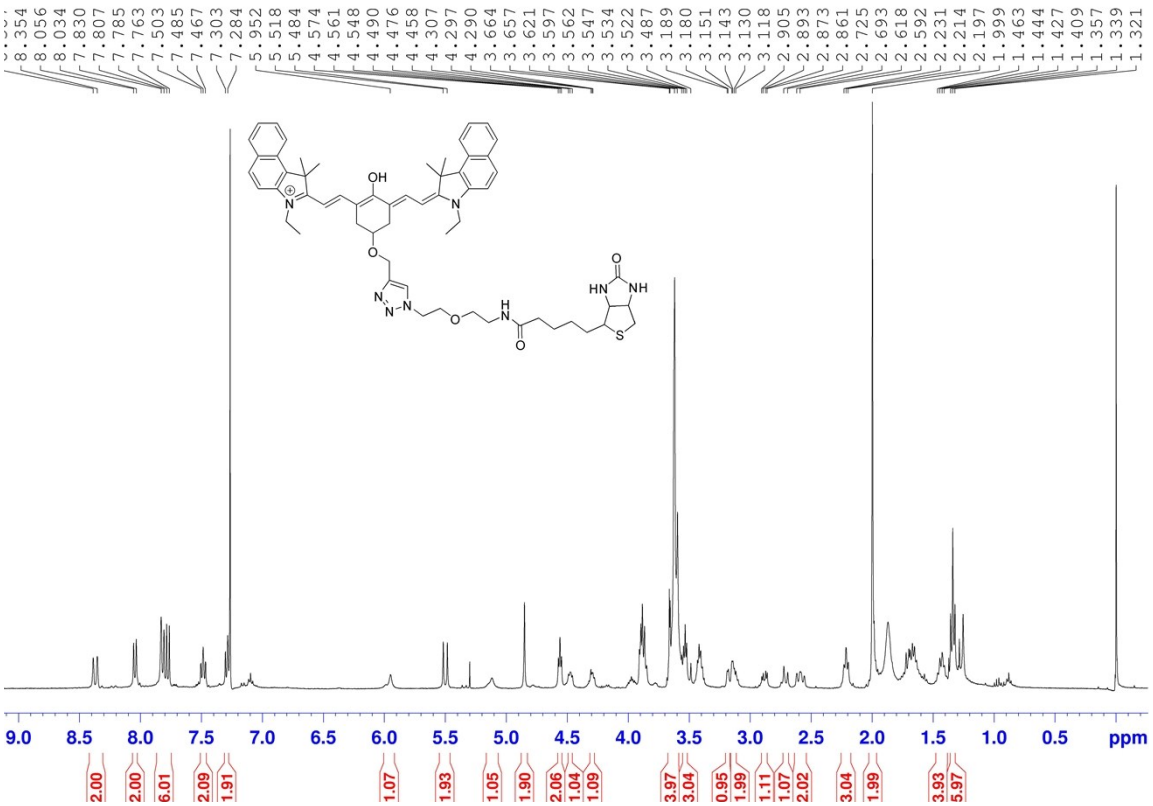


Fig. S11. ^{1}H NMR spectrum of BP_5-Cy-OH in $CDCl_3$

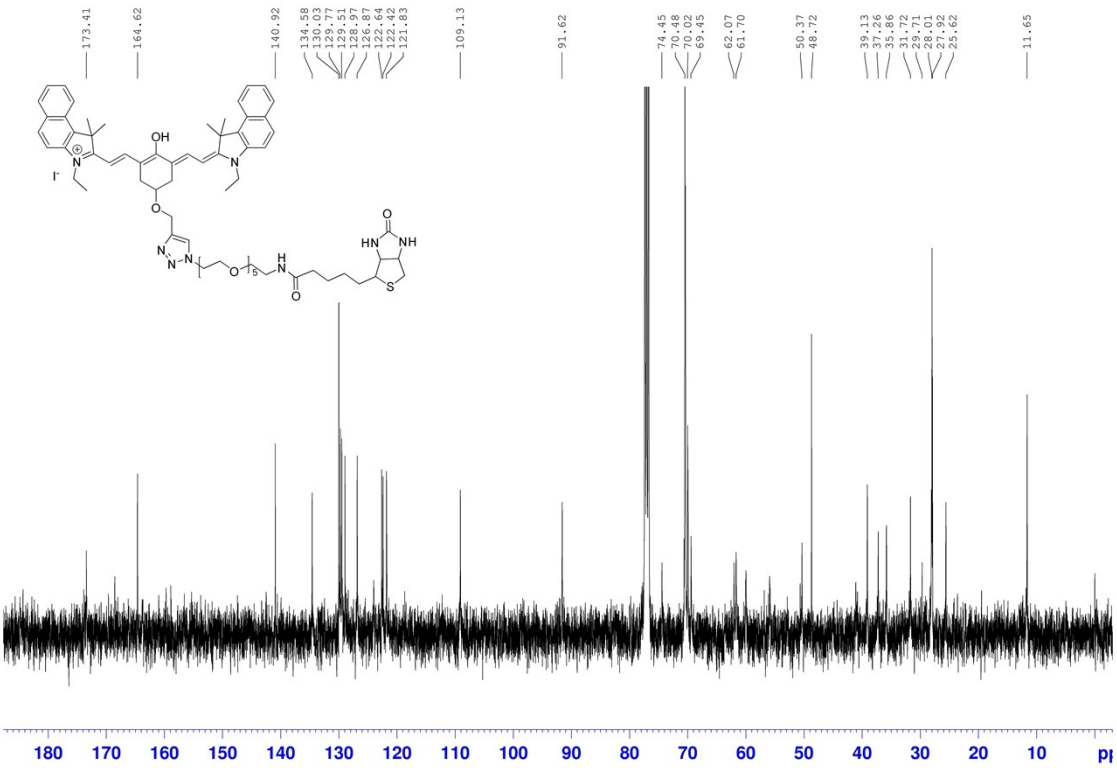


Fig. S12. ^{13}C NMR spectrum of BP_5-Cy-OH in $CDCl_3$

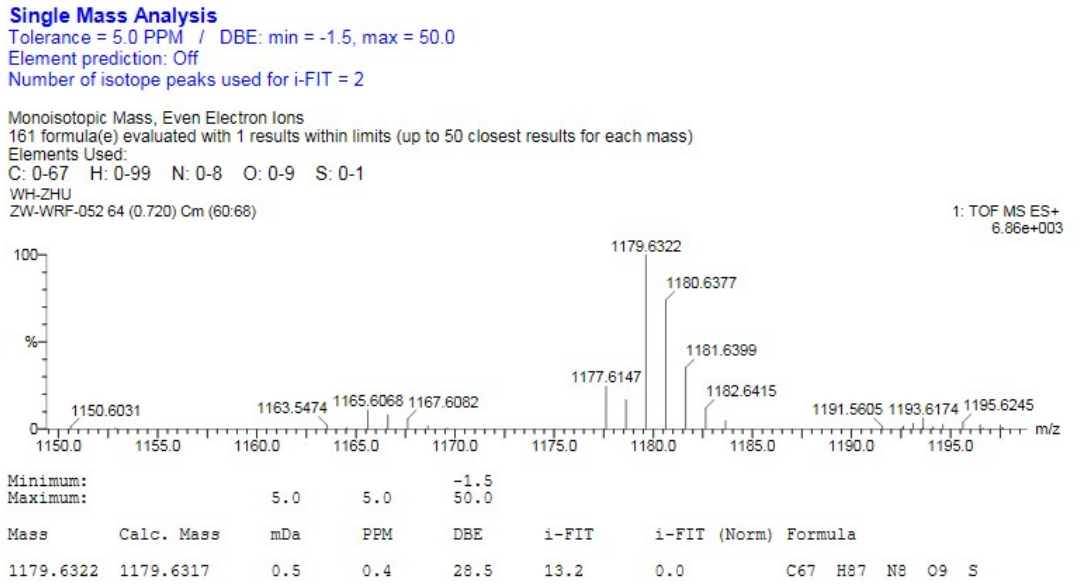


Fig. S13. HRMS spectrum of BP_5-Cy-OH

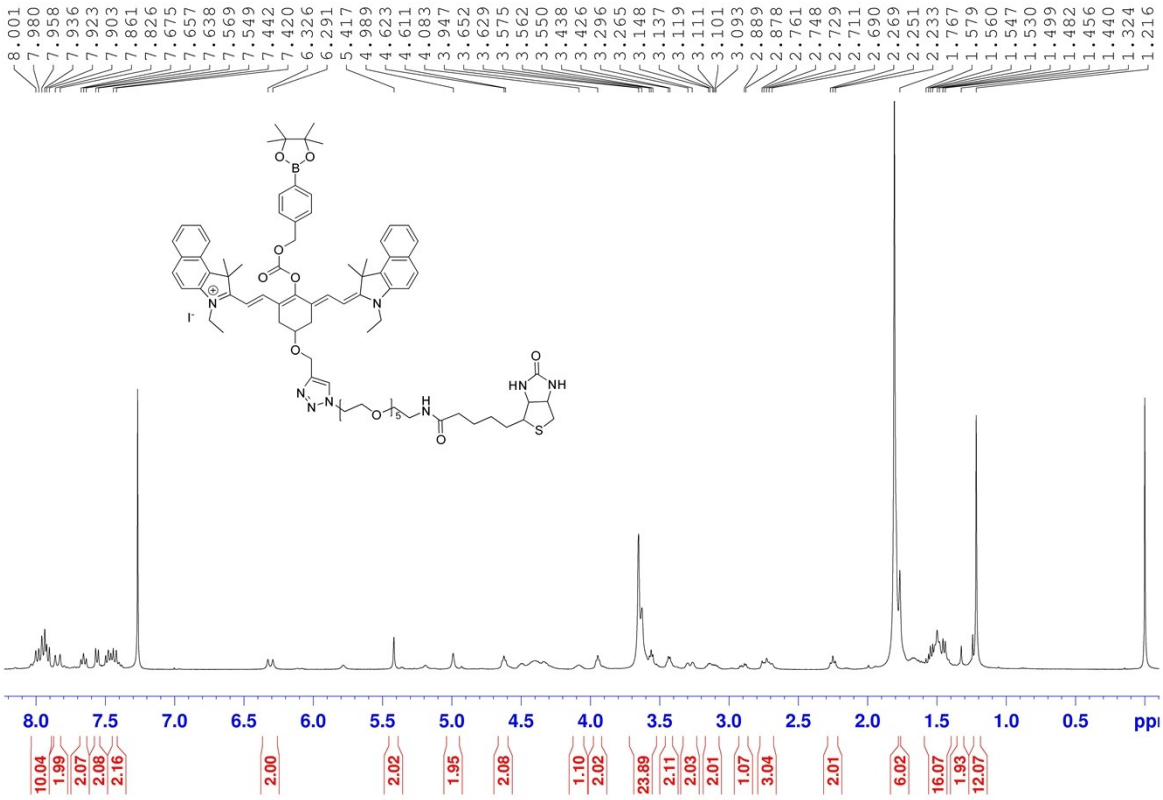


Fig. S14. ^{1}H NMR spectrum of BP_5-Cy-B in $CDCl_3$

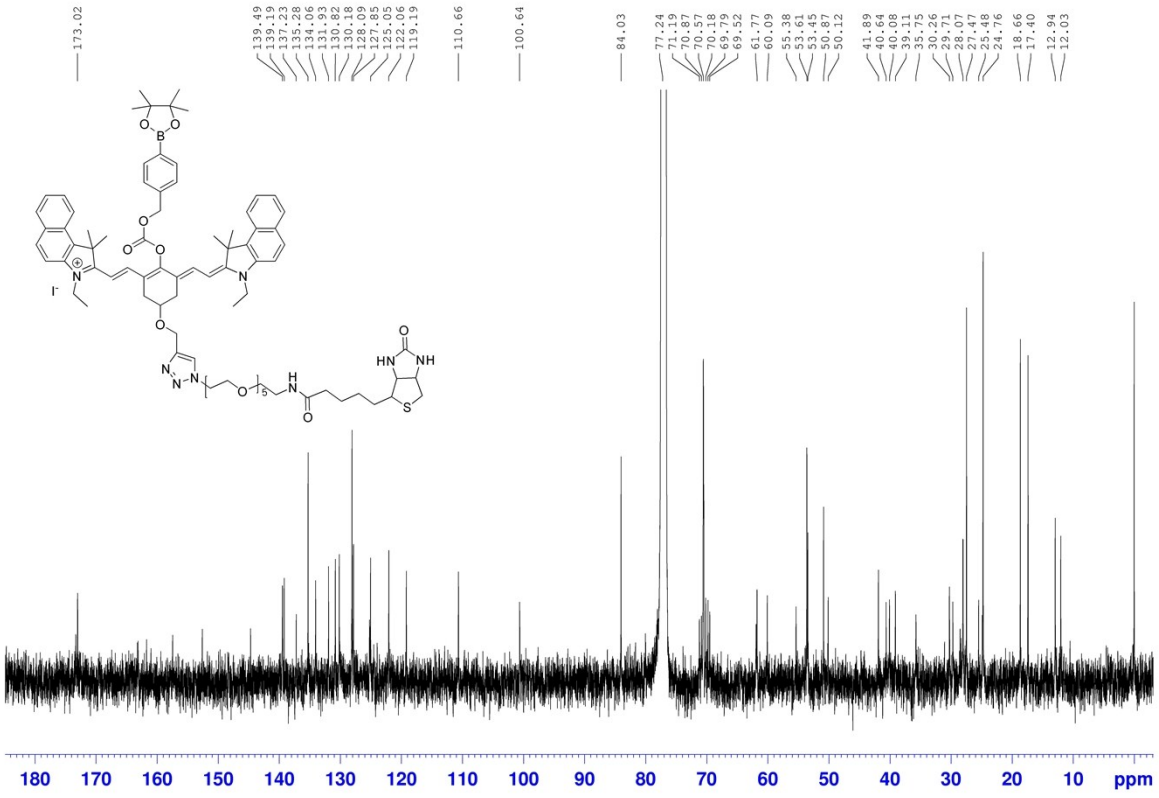


Fig. S15. ^{13}C NMR spectrum of BP_5-Cy-B in $CDCl_3$

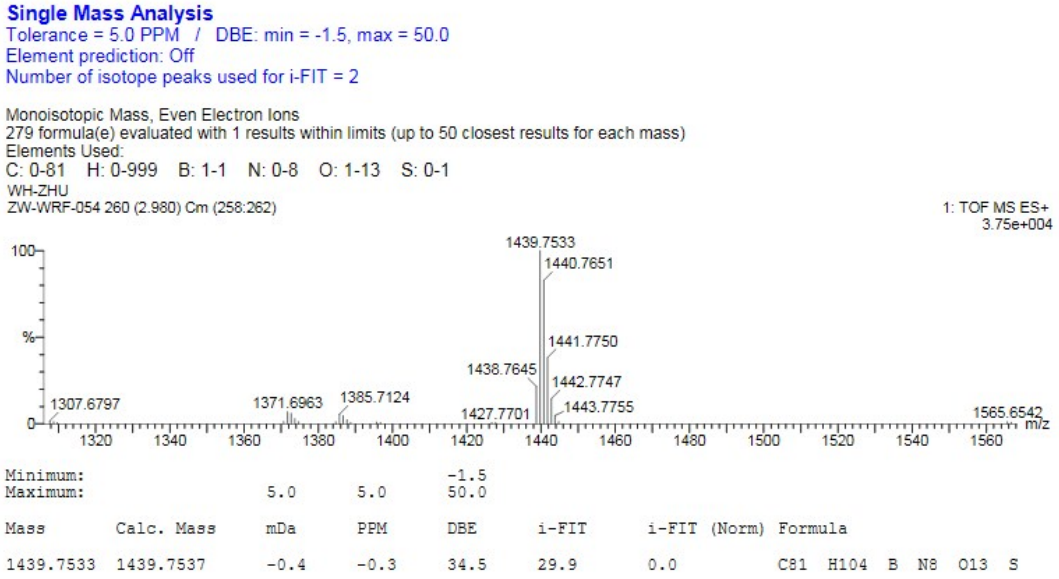


Fig. S16. HRMS spectrum of BP_5-Cy-B

References

1. M. Z. Ye, X. H. Wang, J. B. Tang, Z. Q. Guo, Y. Q. Shen, H. Tian and W. H. Zhu, *Chem. Sci.*, 2016, **7**, 4958-4965.

2. C. X. Yan, Z. Q. Guo, Y. J. Liu, P. Shi, H. Tian and W. H. Zhu, *Chem. Sci.*, 2018, **9**, 6176-6182.
3. X. M. Wu, X. R. Sun, Z. Q. Guo, J. B. Tang, Y. Q. Shen, T. D. James, H. Tian, W. H. Zhu, *J. Am. Chem. Soc.,* 2014, **136**, 3579-3588.