Supporting Information

A Novel NIR-II Fluorescent Probe for Hydrogen Peroxide Detection in Drug-Induced Liver Injury

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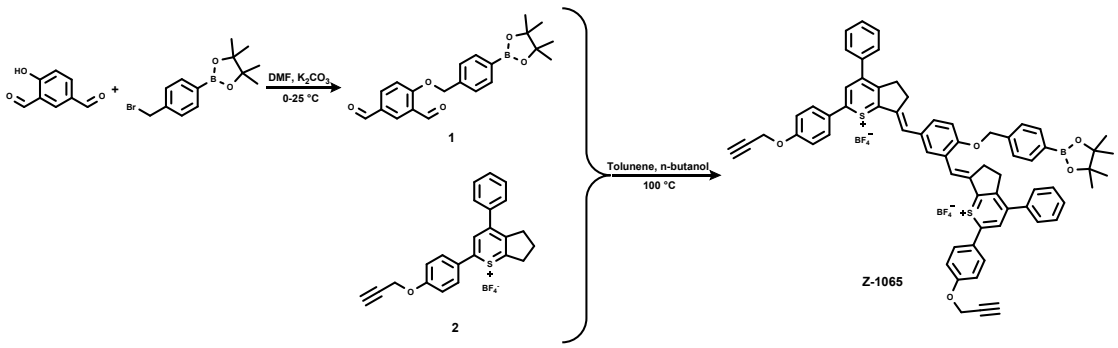
1. Experimental procedures

1.1 Reagents and Instruments

4-hydroxy-isophthalaldehyde, 4-(bromomethyl)benzeneboronic acid pinacol ester, p-hydroxybenzaldehyde, benzaldehyde, propargyl bromide, cyclopentanone, tetrahydropyrrole, thioacetic acid and boron (tri) fluoride etherate were purchased from Aladdin Reagents. N, N-dimethyl-formamide (DMF), dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were analytical grade reagents. L-02 cells were gifted from Nanjing University. H_2O_2, isoniazid (INH) and N-Acetyl-L-cysteine (NAC) were purchased from Sigma Aldrich.

NMR spectra were recorded on a Bruker Ultra Shield Plus 400 MHz spectrometer (^{1}HNMR 400 MHz and ^{13}C NMR 100 MHz) and referenced to tetramethylsilane (TMS) as the internal standard.The chemical shifts are reported as ppm and solvent residual peaks were shown as following: DMSO-*d*6 δ H (2.50 ppm) and δ C (39.52 ppm). Absorption data was measured by Shimadzu UV-3600 ultraviolet-visible-near-infrared (UV-Vis-NIR) spectrophotometer. The NIR-II fluorescence spectra were monitored on a commercial NIR-II spectrophotometer (Fluorolog 3, Horiba) equipped with an 808 nm diode laser and an InGaAs NIR detector. The 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay was performed by a BioTek Synergy HTX microplate reader. Confocal fluorescence imaging was conducted on ZEISS LSM880 laser scanning confocal microscope. The NIR-II fluorescence images were taken with NIR-II in Vivo Imaging System (Series II 808/900-1700, Wuhan Grand-imaging Technologies Co., Ltd. (excitation: 808 nm laser with 20 mW·cm^{-2}, emission filter: 900-1700 nm)

1.2 Synthesis of Z-1065



Scheme S1. Synthetic route.

Compound 1: 4-hydroxy-isophthalaldehyde (0.15 g, 1.0 mmol) were dissolved anhydrous DMF

(3.0 mL) under ice bath. K_2CO_3 (0.28 g, 2.0 mmol) was added to the cooled solution and stirred for 10 min. Then, 4-Bromomethylphenylboronic acid pinacol ester (0.36 g, 1.2 mmol) was added into the above solution and the reaction mixture was stirred at room temperature for 12 h. The solution was dissolved in EtOAc (100.0 mL), followed by washing with saturated NH_4Cl solution for three times and drying over Na2SO4. After the removal of solvent under reduced pressure, the crude product was subjected to silica gel chromatography with EtOAc/petroleum ether (1/2, v/v) as eluent to afford a buff solid product. Yield: 0.33 g (90.0 %). *Characterization of Compound 1*: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (s, 1H), 9.96 (s, 1H), 8.26 (d, J = 2.1 Hz, 1H), 8.17 (dd, J = 8.7, 2.1 Hz, 1H), 7.72 (d, J = 7.9 Hz, 2H), 7.55 (d, J = 7.9 Hz, 2H), 7.51 (d, J = 8.7 Hz, 1H), 5.46 (s, 2H), 1.30 (s, 12H).

Compound 2: The synthesis of compound 2 was according to previous reports.[1-4]

Z-1065: Compound 1 (0.183 g, 0.5 mmol) and compound 2 (0.473 g, 1.1 mmol) were dissolved in toluene/n-butanol (5.0 mL/5.0 mL). Under a nitrogen atmosphere, the reaction mixture was stirred overnight at 100 °C. After the removal of solvent under reduced pressure, the crude product was silica gel chromatography with CH_3OH/CH_2Cl_2 (1/10, v/v) as eluent to afford a deep brown solid product. Yield: 0.30 g (46.0%). *Characterization of Z-1065*: ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.20 (s, 2H), 7.87 (s, 4H), 7.68 (d, 4H), 7.58 – 7.52 (m, 3H), 7.46 – 7.39 (m, 6H), 7.36 – 7.30 (m, 9H), 6.99 (d, J = 9.0 Hz, 1H), 5.01 (s, 2H), 4.86-4.79 (d, J = 2.6 Hz, 4H), 3.73-3.70 (m, J = 7.8, 4.5, 3.6, 0.9 Hz, 4H), 3.67 – 3.57 (m, 4H), 2.54 (d, J = 2.6 Hz, 2H), 1.23 (s, 12H). Calcd for: $C_{67}H_{57}BO_5S_2^{2+}$ [M-BF_4]: 508.1865, Measured M.W. 508.2844 [M-BF_4+H].

1.3 Photostability and responsiveness test

Photophysical property：Z-1065 was dissolved in 10 – 100 mM solution in dichloromethane. The solutions were kept at 25 ℃ for 60 min and then measure the absorption spectrum and fluorescence spectra of different concentration solutions. For photostability experiments, Z-1065 solution and ICG solution (0.3 mM for both) were subjected to 808 nm laser irradiation at 0.5 W/cm-2 for different time before fluorescence spectra measurements.

Responsiveness test: Z-1065 was dissolved in DMSO. H2O2 with varied doses from 0 – 240 μM was added into Z-1065 solution so that the final concentration is 30 mM in DMSO solution, and fluorescence spectra of each of the solutions were measured. For pH anti-interference

experiments, HCl/ NaOH with buffer solution was added in to obtain different pH buffer solutions. For selectivity experiments, H_2O_2 (240 μM) and other substances were added into Z-1065 (30 mM) in DMSO solution. Afterwards the fluorescence spectra and NIR-II fluorescence imaging of each solution were measured.

1.4 Cell experiments

Cell culture: L-02 cells were incubated in Roswell Park Memorial Institute (RPMI) 1640, containing 10% fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO2 and 95% air.

Cell viability: Cells were seeded into 96-well microplates and cultured in RPMI 1640 medium for 24 hours. Subsequently, the cells were incubated with different concentrations of Z-0165 (0, 10, 20, 40, 80, and 160 μM) for another 24 hours. After this incubation period, the medium was replaced with fresh medium containing 10 μL of MTT (prepared in advance at a final concentration of 5 mg/mL), and the cells were further incubated for 4 hours in a constant temperature incubator. Following this, the medium was carefully removed, and 100 μL of dimethyl sulfoxide (DMSO) was added to each well of the 96-well plate. The plate was then gently shaken to dissolve the formazan crystals. Finally, the absorbance was measured using a microplate reader at 490 nm.

Mitochondrial analysis： When the L-02 cells reached approximately 50% confluence, they were washed with PBS to remove any remaining RPMI 1640 medium. A 1 mM stock solution of Z-1064 was added to the RPMI 1640 medium to create a staining solution with a final concentration of 50 μM. The cells were incubated with this staining solution for 6 hours at 37°C, followed by a PBS wash. Subsequently, a 0.5 mM solution of Mito-Tracker Green was added to the RPMI 1640 medium to create a staining solution with a final concentration of 500 nM for Mito-Tracker Green. The cells were incubated with this solution for 30 minutes at 37°C, followed by two PBS washes, and then used for bio-imaging. Under CLSM, Z-1064 was excited at 514 nm, and the emission was collected at 560–635 nm. Mito-Tracker Green was excited at 458 nm, and the emission was collected at 488–516 nm. No background fluorescence was detected under these conditions.

ROS level detection： For H_2O_2 imaging experiments, the commercial probe DCFH-DA was first used to verify the level of isoniazid-induced H_2O_2 production in cells and the inhibition effect of NAC on H_2O_2 levels. L-02 cells were divided into three groups: group 1, the control, where cells were incubated normally; group 2, cells treated with 30 μM INH for 12 hours; and group 3, cells pretreated with different concentrations of NAC (30 μM) for 2 hours, followed by treatment with

30 μM INH for 12 hours. Finally, all groups were incubated with 10 μM DCFH-DA for 20 minutes before confocal imaging. The excitation wavelength for the green channel was 488 nm, and the emission wavelength ranged from 510 to 627 nm.

Cell imaging with CLSM: L-02 cells were divided into three groups: group 1, the control, where cells were incubated normally; group 2, cells treated with 30 μM INH for 12 hours; and group 3, cells pretreated with 30 μM NAC for 2 hours followed by treatment with 30 μM INH for 12 hours. Finally, all groups were incubated with 50 μM Z-1065 for 4 hours prior to confocal imaging. The excitation wavelength for the red channel was 531 nm, and the emission wavelength ranged from 560 to 635 nm.

Cell imaging with NIR-II Fluorescenc imaging system: For the INH-induced cell model of liver injury in the NIR-II window, L-02 cells were divided into four groups: group 1, cells incubated normally; group 2, cells treated with 30 μM INH for 12 hours; group 3, cells pretreated with 30 μM NAC for 2 hours followed by treatment with 30 μM INH for 12 hours. Finally, all groups were incubated with 50 μM Z-1065 for 4 hours prior to imaging. NIR-II fluorescence imaging was performed using an NIR-II fluorescence imaging system under an 808 nm laser and a 980 nm long-pass filter with an exposure time of 2000 ms.

1.5 Animal experiments

Statement of Animal Experiments: Female BALB/c mice (6-8 weeks old, 19-21 g) were purchased from Jiangsu KeyGEN Biotechnology Co., Ltd. The animal experiments were approved by and conducted in compliance with the regulations of the Laboratory Animal Center of Jiangsu KeyGEN Biotechnology Co., Ltd. Mice were randomly divided to establish animal models and support subsequent experimental investigations. Before the imaging experiments, the hair of the mice was removed using depilatory cream.

INH-Induced Liver Injury Mouse Models: Female BALB/c mice (6-8 weeks old, 19-21 g) were randomly divided into three groups. For the isoniazid (INH)-induced liver injury model groups, the mice received intraperitoneal injections of 160 mg/kg isoniazid. For the liver protection group, the mice received intraperitoneal injections of 160 mg/kg isoniazid and 100 mg/kg N-acetylcysteine. For the control group, the mice received intraperitoneal injections of an isovolumetric amount of PBS.

NIR-II Fluorescence Imaging: The model mice were administered 100 μg of Z-1065 via tail

vein injection. All mice were anesthetized with 2.5% isoflurane in oxygen, and whole-body NIR-II fluorescence images were obtained using an NIR-II in vivo imaging system with an 808 nm laser and a 980 nm long-pass filter with an exposure time of 500 ms. Imaging was performed at the following time points: 0 min, 15 min, 30 min, 45 min, 60 min, and 75 min. For ex vivo NIR-II fluorescence imaging, 75 minutes after probe injection, the mice were sacrificed and major organs such as the heart, liver, spleen, lungs, kidneys, and intestines were harvested for imaging. The NIR fluorescent signal of each organ was recorded.

2. Supplemental figures

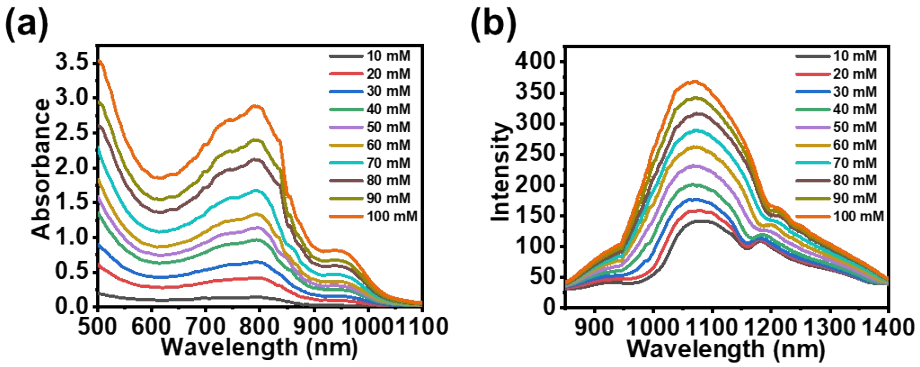


Fig. S1 Optical properties of Z-1065 measured in DCM with different concentrations.

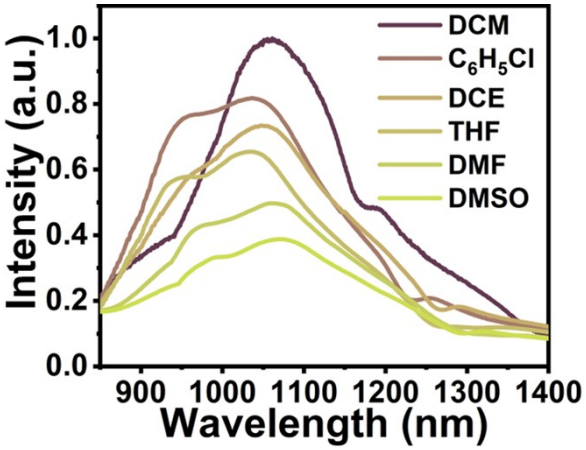


Fig. S2. Emission of Z-1065 (30 mM) measured in different solvents including dichloromethane (DCM), chlorobenzene, dichloroethane (DCE), tetrahydrofuran (THF), dimethylformamide (DMF) and dimethyl sulfoxide (DMSO).

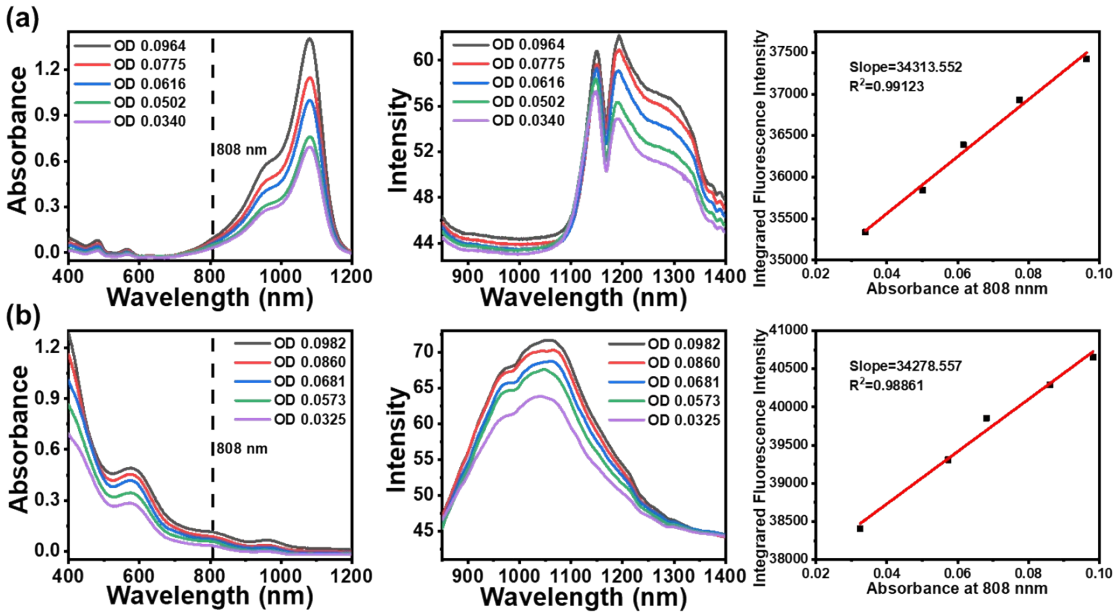


Fig. S3. Quantum yield measurements of (a) IR-26 in DCE and (b) Z-1065 in DCM. A reference IR-26 (0.05%) in DCE was chosen.

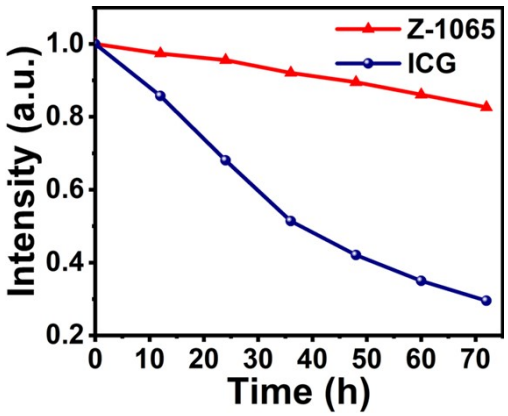


Fig. S4. Stability of Z-1065 measured by fluorescent intensity in medium containing 10% FBS compared with ICG under similar experimental conditions.

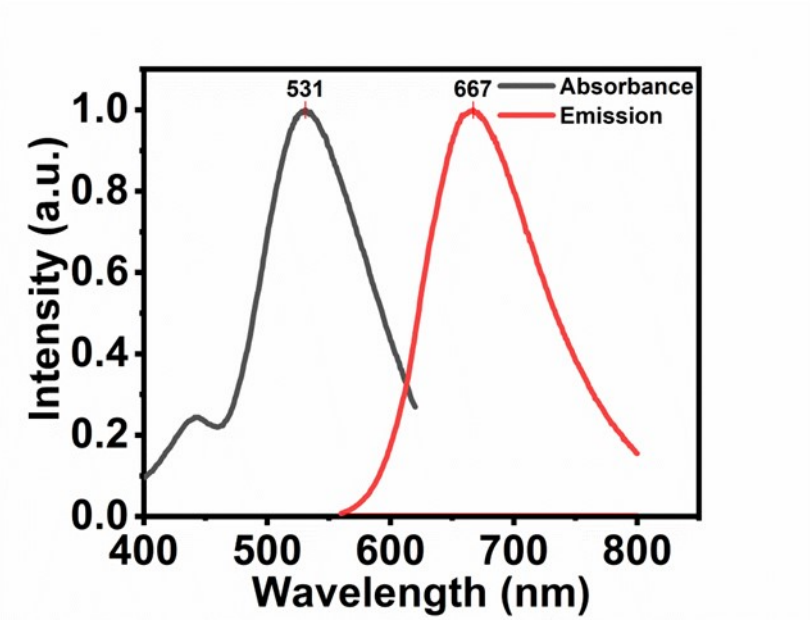


Fig. S5. Absorption and emission of Z-1065 in the NIR-I window.

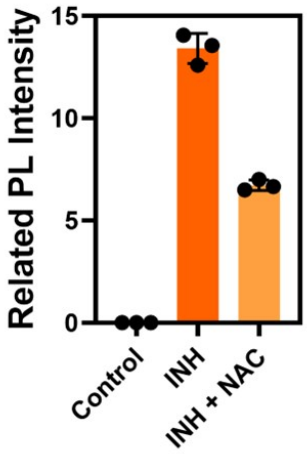


Fig. S6. Quantitative analysis of ROS levels in L-02 cells with different treatments.

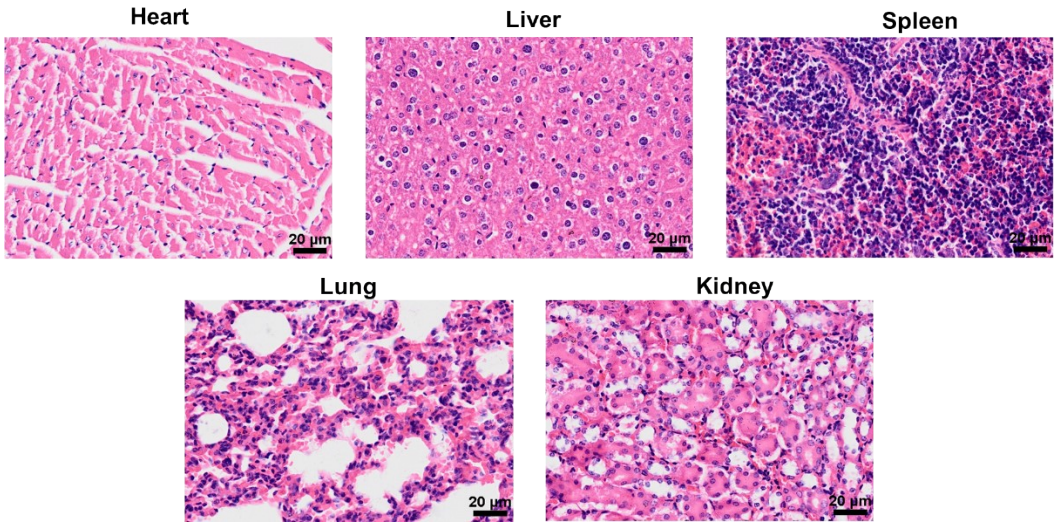


Fig. S7. Major organs (Heart, Liver, Spleen, Lung, and Kidney) in normal mice using H&E staining injected with the probe Z-1065.

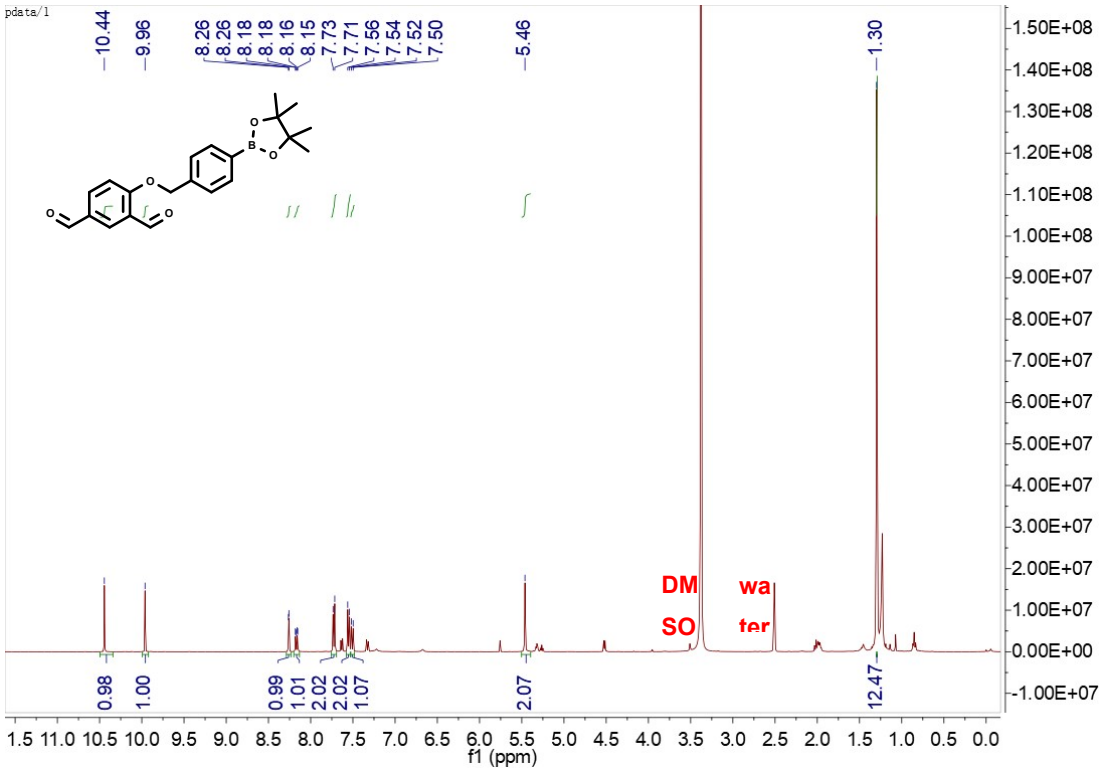


Fig. S8. ^{1}H NMR spectra of compound 1 in DMSO-d_6.

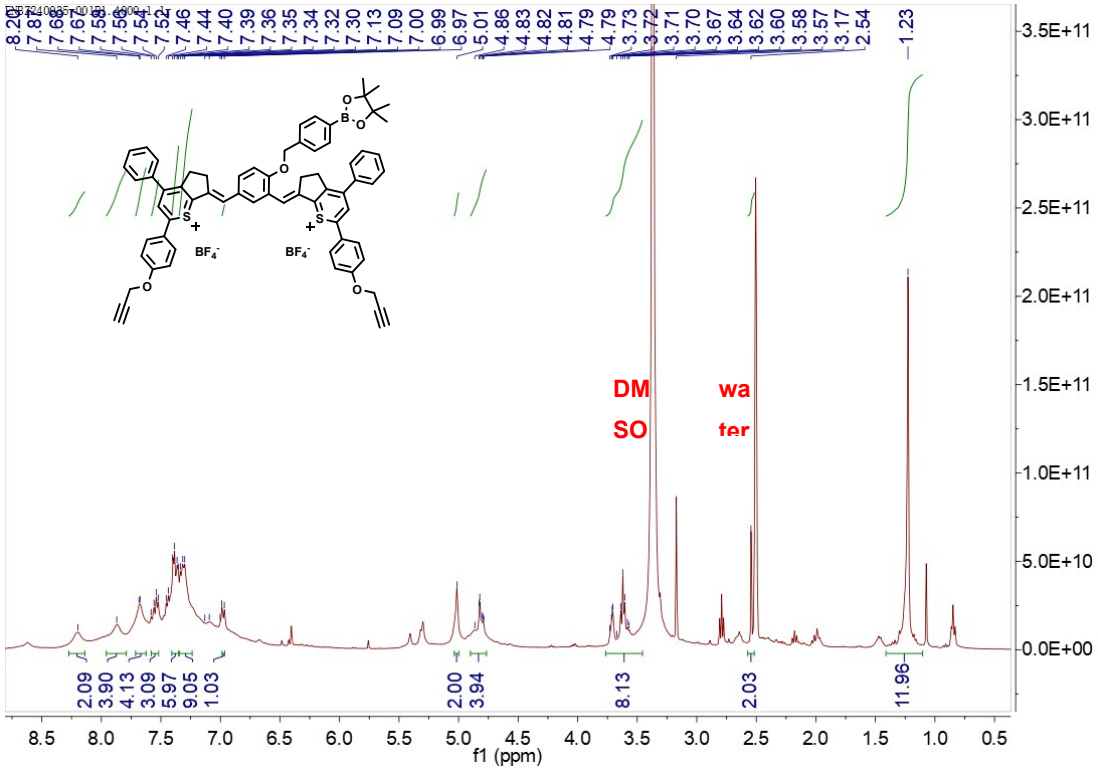


Fig.S9. ^{1}H NMR spectra of the probe Z-1065 in DMSO-d_6.

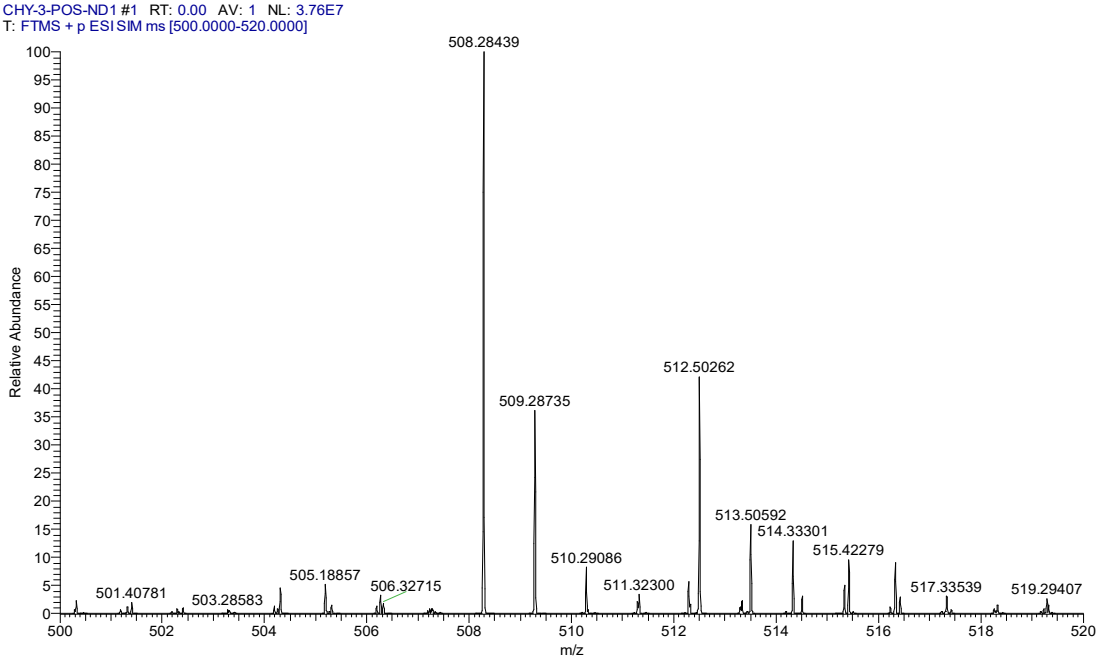


Fig.S10. MS of Z-1065.

References:

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4. B. Wang, H. Zhou, L. Chen, Y. Ding, X. Zhang, H. Chen, H. Liu, P. Li, Y. Chen, C. Yin and Q. Fan, *Angew. Chem. Int. Ed.*, 2024, DOI: 10.1002/anie.202408874, e202408874.