A self-assembled nanoprobe for the long term cancer cell nucleus-specific staining and two-photon breast cancer imaging

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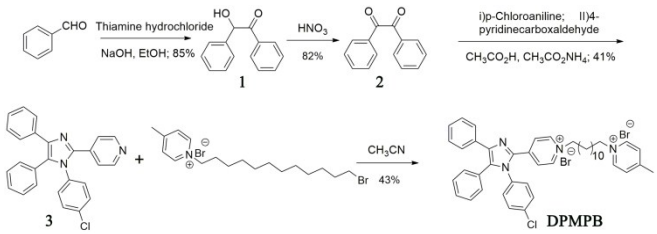
1. Materials and instruments

All chemicals and reagents were used as received useless otherwise specified. Vitamin B1, benzaldehyde, and *p*-chloroaniline (99.5%) were purchased from Energy Chemical Co., Ltd (China). 4-Pyridine carboxaldehyde (98%) and pyridine were purchased from Sinopharm Chemical Reagent Co., Ltd (China). 4',6-diamidino-2-phenylindole (DAPI) and calf thymus DNA were purchased from Sigma-Aldrich. Roswell Park Memorial Institute (RPMI)-1640 medium, phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Invitrogen. The cell counting kit-8 (CCK-8) cytotoxicity assay kit was a commercial product of Beyotime Biotechnology (China). Milli-Q water was supplied by Milli-Q Plus System (Millipore Corporation, United States).

^{1}H and ^{13}C NMR spectra were measured on a Bruker ARX 400 NMR spectrometer using $CDCl_3$ or DMSO-d_6 as the solvent and tetramethylsilane (TMS; $\delta = 0$) as the internal reference. UV-vis Absorption spectra were performed on a UV-2450 scanning spectrophotometer (Shimadzu, Japan). Fluorescent spectra were recorded on a Shimadzu RF-5301 equipped with a 1 cm quartz cell. Dynamic light scattering measurements were performed at 25 ^{o}C on Zestier Nano ZS (Malvern Instruments Ltd, UK). The morphology of **DPMPB-FONs** was characterized by a double beam electron microscope (Helios Nanolab 600i). Fluorescent images were collected on Leica DMI4000B fluorescence microscope. Two-photon confocal images were collected on a Leica confocal laser scanning microscope(TCS SP8 MP, Germany).

2. Synthesis



Scheme S1. Synthetic route for **DPMPB**

2-hydroxy-1,2-diphenylethanone (compound 1): In a 50 mL round bottom flask, 1.75 g vitamin VB_1, 3.5 mL water and 15 mL ethanol were added and the flask was allowed to cool in an ice bath. At the same time, 5mL of 10% sodium hydroxide solution in a test tube was also placed in the ice bath to cool. Then with cooling in ice bath, the sodium hydroxide solution was added to the reaction solution, and continued to shake. During the process, pH of the solution was adjusted to 9-10 until the solution was yellow, then the ice bath was removed. This solution was next added with the newly steamed benzaldehyde and installed with reflux condenser in a water bath with the temperature of 65-75^{o}C for 1.5 h, without heating the mixture to boiling the whole time. The pH of the solution was maintained in the range of 8 to 9 during the reaction. At the end, the reaction mixture became an orange uniform solution. The reaction mixture was cooled to room temperature before precipitating pale yellow crystals 1.80 g (85% yields). ^{1}H NMR (400 MHz, CDCl3): δ 4.56 (s, 1H), 5.94 (s, 1H), 7.27–7.44 (m, 7H), 7.50 (t, 1H), 7.94 (d, 2H).

Benzil (compound 2): Compound **1** (2.0 g, 0.188 mol) and 10 mL conc. HNO_3 was taken in a 100 mL round bottom flask and heated on a boiling water bath with occasional shaking until the evolution of oxides of nitrogen seized. The reaction mixture was then poured onto crushed ice, and stirred well till the yellow solid separated out. It was then filtered and recrystallized in ethanol to yield benzil 1.685 g (82% yields). ^{1}HNMR (400 MHz, $CDCl_3$, δ): 7.96-7.99 (m, 4H), 7.63-7.67 (m, 2H), 7.49–7.53 (m, 4H). ^{13}CNMR (100 MHz, $CDCl_3$, δ): 194.61, 134.93, 132.97, 129.90, 129.05.

4-(1-(4-chlorophenyl)-4, 5-diphenyl-1H-imidazol-2-yl) pyridine (compound 3): 4-chloroaniline (1.27g, 10.00 mmol) and 4-pyridine carboxaldehyde (1.06 g, 10.00 mmol) were dissolved in acetic acid (100 mL) and stirred for 1 h at room temperature. Benzil (2.10 g, 10.00 mmol) and ammonium acetate (3.55 g, 46.00 mmol) were added subsequently. The mixture was heated at 120 °C overnight. After quenching the reaction, the dark solution was poured into copious amounts of water. After neutralization, the mixture was filtered and washed with water. Purification by silica gel column chromatography gave the product as a white solid powder (1.67 g, yield = 41%). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.51-8.52 (d, 2H), 7.56 - 7.58 (d, 2H), 7.31-7.33 (m, 3H), 7.25-7.30 (m, 6H), 7.20-7.23 (m, 1H), 7,12-7.13 (d, 2H), 7.02-7.03 (d, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 149.69, 143.74, 139.37, 137.55, 135.07, 134.98, 133.64, 132.25, 130.96, 129.74, 129.63, 129.40, 128.63, 128.55, 128.25, 127.23, 127.06, 122.33.

Compound DPMPB: Compound **3** (407 mg, 1.00 mmol) was reflux with Compound **4** (419 mg, 1.00 mmol) in 15 mL acetonitrile for 12 h. After the mixture cooled to room temperature, it was evaporated under reduced pressure to remove the solvent. The residue was purified by a silica gel column chromatography using a DCM and methanol mixture (30:1 v/v) as the elution solvent to give compound **DPMPB** as a green powder (355 mg, yield = 43%). ^{1}H NMR (400 MHz, DMSO-d_6, δ): 9.04-9.06 (t, 4H), 8.00-8.01 (d, 2H), 7.83-7.84 (d, 2H), 7.52-7.56 (m, 6H), 7.39-7.40 (m, 3H), 7.27-7.33 (m, 5H), 4.53-4.60 (m, 4H), 2.61 (s, 3H), 1.86-1.89 (m, 4H), 1.20-1.24 (m, 16H). ^{13}C NMR (100 MHz, DMSO-d_6, δ): 159.16, 155.15, 144.21, 140.96, 140.72, 140.19, 136.20, 135.11, 134.82, 133.43, 131.38, 130.88, 130.41, 129.83, 129.24, 129.16, 128.92, 128.77, 126.93, 124.29, 70.21, 63.25, 60.42, 60.25, 31.08, 30.88, 29.46, 29.33, 29.25, 28.85, 28.84, 25.83, 21.81.

3. Absorbance spectra of DPMPB in different solvents.

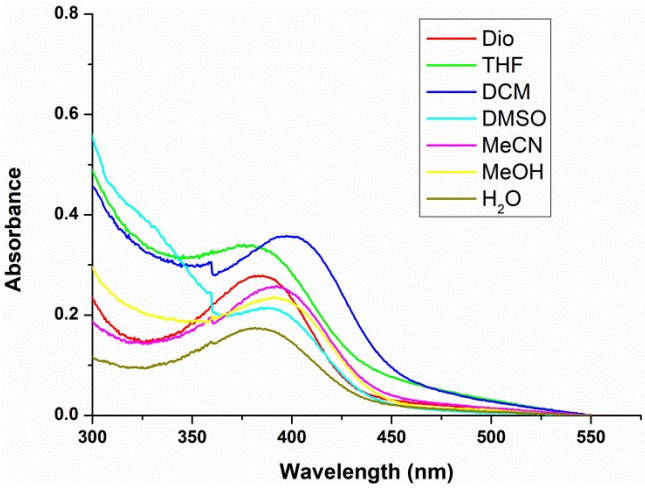


Figure S1. Absorbance spectra of DPMPB (10 μM) in different solvents.

4. Fluorescent spectra spectra of DPMPB in different solvents and computational calculations.

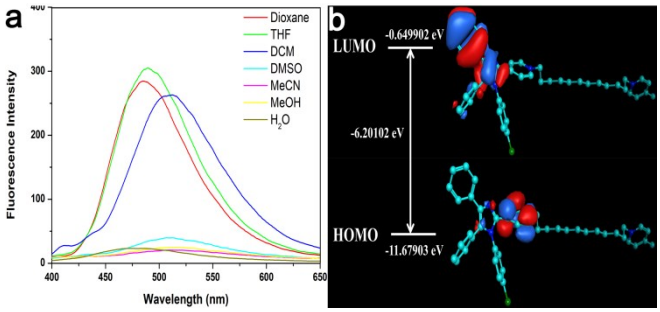


Figure S2. (a) Emission spectra of **DPMPB** in different solvents. Concentration:10 μM; λex = 385 nm. (b) Calculated HOMO and LUMO electron cloud distribution of **DPMPB**.

5. Fluorescent spectra of DPMPB in different concentrations.

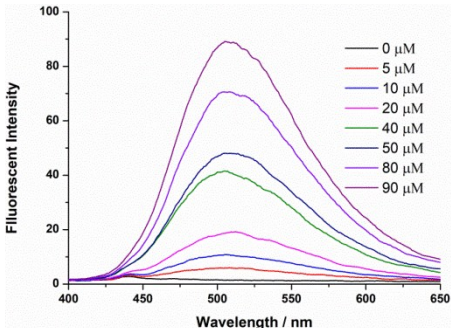


Figure S3. Fluorescent spectra of different concentration of **DPMPB** in aqueous.

6. Preparation of DPMPB-FONs and SEM analysis

The stock solution of **DPMPB** (1×10^{-3} M) was prepared by dissolving in dimethyl sulfoxide. A volume of 10 μL of this solution was injected into phosphate buffer (2 mL) maintained at pH 7.4 with vigorously shaken, and the solution (5 μM) was kept under room temperature. Nanoparticle formation was confirmed by DLS and SEM analyses by drop casting on carbon-coated copper grid (400 mesh), after drying in vacuum.

7. Zeta potential of DPMPB-FONs in aqueous.

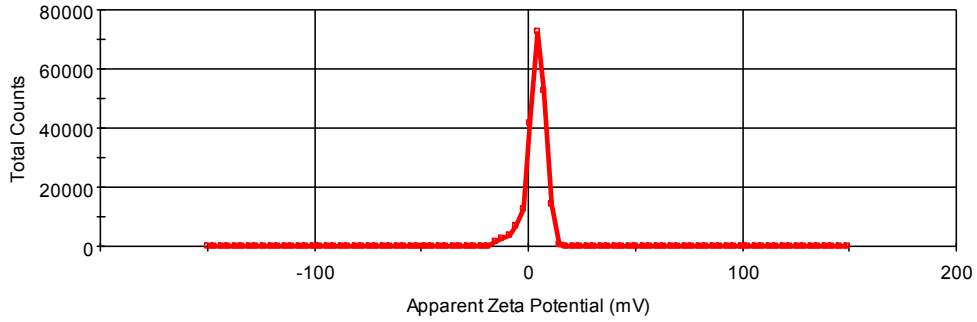


Figure S4. Zeta potential of **DPMPB-FONs (**5 μM**)** in aqueous.

8. Cell culture

C666-1 cells, CNE2 cells, AGS cells and GES-1 cells were cultured in RPMI-1640 media, respectively. All the cells were cultured in media supplemented with 10% heat-inactivated FBS, 100 units per mL penicillin and 100 mg mL^{-1} streptomycin, in a humidity incubator with 5% CO_2 at 37°C. Before the experiment, the cells were pre-cultured until confluence was reached.

9. Cell imaging

C666-1 cells, CNE2 cells, AGS cells and GES-1 cells were seeded on a 35 mm Petri dish with a glass cover slide. C666-1 cells, CNE2 cells, AGS cells and GES-1 cells were cultured on coverslips in a 6-well plate, respectively.） After overnight cell culture, the cells were incubated in an aqueous solution of **DPMPB-FONs** (5 μM) for 30 min, respectively. Then the cells were washed with fresh phosphate buffered saline (PBS; pH=7.4) three times before fluorescence imaging. Fluorescent images of the stained cells were taken on a fluorescence microscope (Leica DMI4000B Germany); excitation: 330–380 nm, exposure time: 200 ms.

10. Fluorescent images of DPMPB-FONs in cancer cells and normal cells

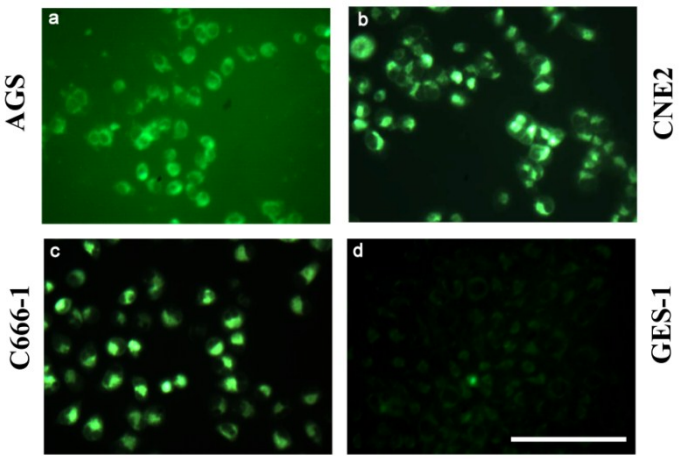


Figure S5. Differentiation of cancer cells from normal cells by **DPMPB-FONs**. (a-d) Fluorescent images of different (a-c) cancer cells and (d) normal cells stained with **DPMPB-FONs**. (5 μM) for 30 min; Scale bar 25 μm.

11. Fluorescent images of cancer cells stained with DPMPB-FONs for 22 h and 29 h

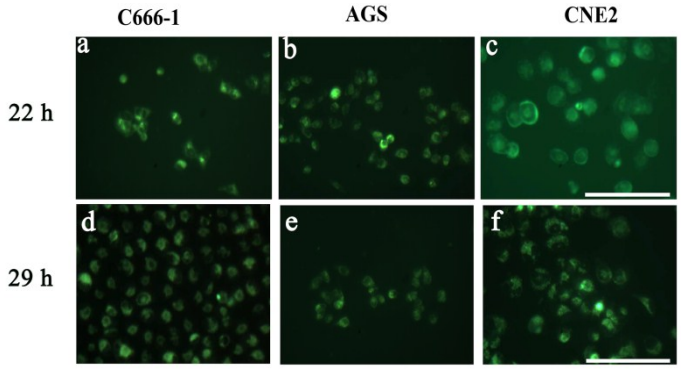


Figure S6. Fluorescent images of different cancer cells stained with **DPMPB-FONs** (5 μM) for 22 h and 29 h; Scale bar 25 μm.

12. Confocal laser fluorescence microscopic images of GES-1 cells treated with DPMPB-FONs and DAPI

Place a coverslip in each well of a 6-well plate. The C666-1 cells, CNE2 cells and AGS cells were digested with trypsin, respectively, to prepare a single cell suspension and then transferred to a 15 ml centrifuge tube for centrifugation (1000 rpm, 5 min). After removal of the supernatant, add the appropriate medium to prepare the single cell suspension. A drop of suspension was added to the middle of the coverslip in the 6-well plate and incubated for about 3-4 hours under 37 ° C in a 5% CO_2. After the cells were adhered, 1 ml of culture medium was added to each well and incubated for about 24 hours. The culture medium in 6-well plates was aspirated and 1 ml of culture solution containing 5 μM of **DPMPB-FONs** was added to each well and incubated for about 4 hours. Remove the coverslip and wash with PBS 3 times. Thereafter, add appropriate amount of DAPI staining 5-10min, and then washed several times with PBS. A drop of 50% glycerol (glycerol: PBS = 1: 1) was added to the slide and observed by confocal fluorescence microscopy.

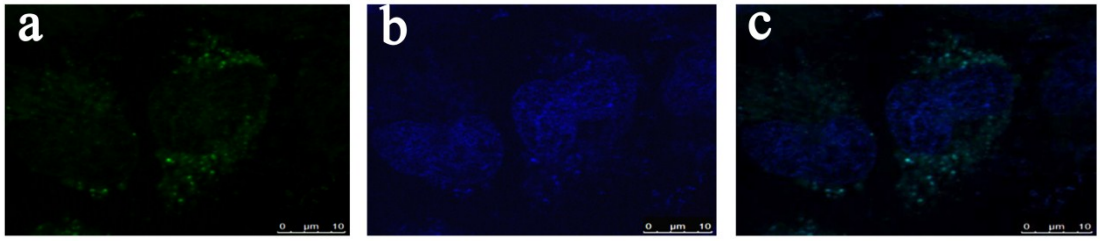


Figure S7. Confocal laser fluorescence microscopic images of GES-1cells treated with 5 μM **DPMPB-FONs** and DAPI (1.0 μM), respectively. a) Fluorescence imaging of **DPMPB-FONs** in GES-1 cells collected at 480-530 nm and excited at 400 nm. b), Fluorescence image of DAPI in GES-1 cells, collected by a 450-470 nm band path filter with excitation at 358 nm. c), a) and b) Merged image of GES-1 cells, Scale bar: 10 μm.

13. Confocal laser fluorescence microscopic images of C666-1, CNE2 and AGS cells treated with DPMPB-FONs and DAPI for 112 hours.

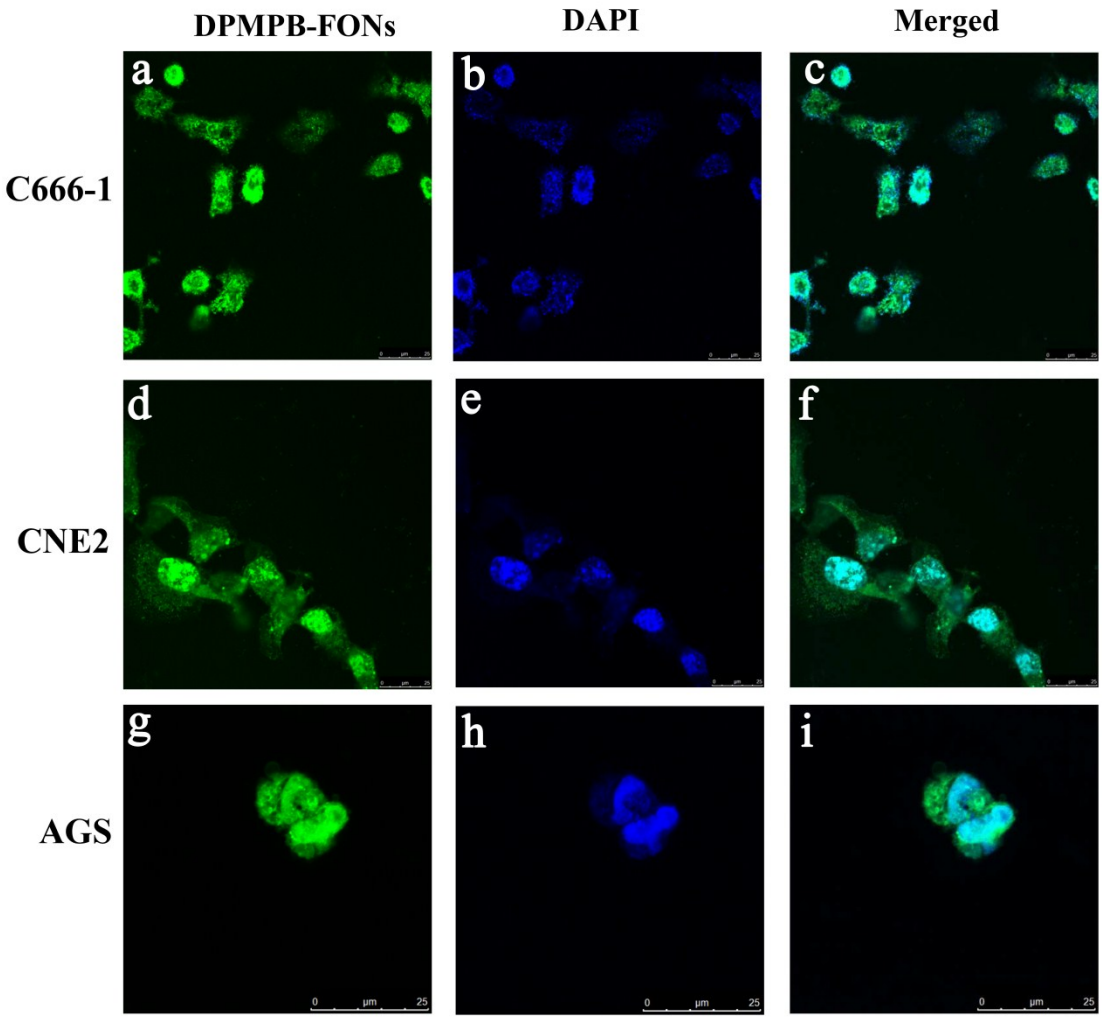


Figure S8. Confocal laser fluorescence microscopic images of C666-1, CNE2 and AGS cells treated with 5 μM **DPMPB-FONs** and DAPI (1.0 μM) for 112 hours, respectively. (a), (d) and (g) Fluorescence imaging of **DPMPB-FONs** in C666-1, CNE2 and AGS cells, respectively collected at 480-530 nm and excited at 400 nm. (b), (e) and (h) Fluorescence image of DAPI in C666-1, CNE2 and AGS cells, respectively collected by a 450-470 nm band path filter with excitation at 358 nm. (c), (f) and (i) Merged image of C666-1, CNE2 and AGS cells, respectively. Scale bar: 25 μm.

14. Titration of DPMPB-FONs against DNA

DPMPB DMSO stock solution was diluted with PBS buffer in EP tube. In each tube, varying amount of double stranded DNA (dsDNA), were added into the solution. The final concentration of **DPMPB** was maintained as 5 μM. The fluorescence of the solution was recorded upon excitation at 385 nm and the emission was collected at 500 nm.

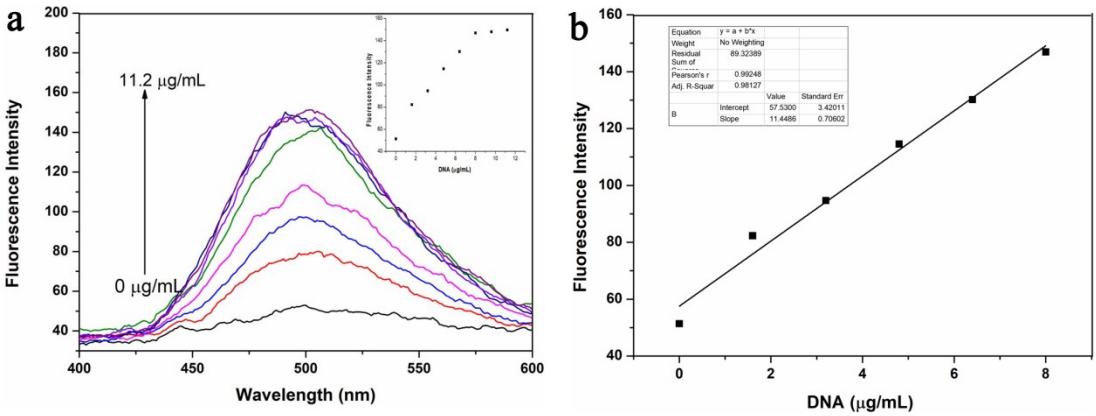


Figure S9. (a) Fluorescence responses of **DPMPB-FONs** (5μM) toward different concentrations of calf thymus DNA sodium salt (0–11.2 μg/mL) in PBS buffer (pH = 7.4). Inset: fluorescence intensity at 500 nm as a function of DNA concentration. (b) Fluorescence intensity at 500 nm of **DPMPB-FONs** versus DNA concentration.

15. Cell Viability Assay

The cell viability effects of **DPMPB-FONs** were investigated by CCK-8 assay using a cell counting kit-8 according to the manufacture's protocols. Briefly, GES-1 cells were seeded in a 96-well plate (1×10^4 cells/well) and treated with different concentrations of **DPMPB-FONs** for 48 h under cell culture conditions (37 ^oC and 5% CO_2). Then 10 μL of CKK-8 was added to the each well for 1 h and the absorbance at 450 nm was examined with a microplate reader (Synergy HT, Biotech). The optical density (OD) values were determined to reflect the viable cell population from each well.

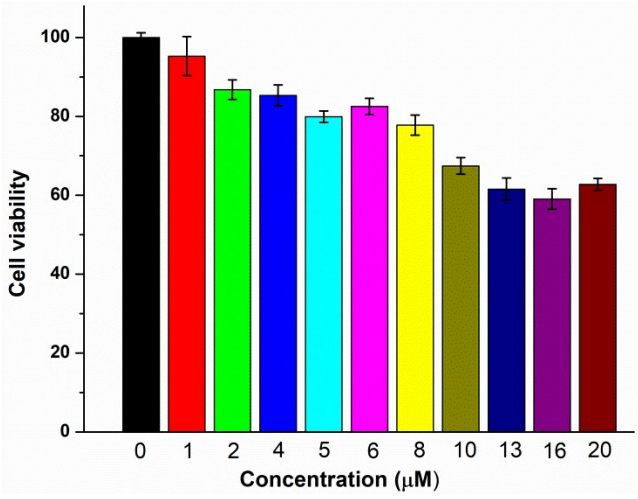


Figure S10. Viability of GES-1cells in the presence of different concentrations of **DPMPB-FONs** for 48 h.

16. Fluorescence microscopy imaging of breast cancer and para-carcinoma tissue.

The breast cancer tissue and para-carcinoma tissue were removed from a 44-year-old breast cancer patient. The two tissues were imprint to the glass slide and immobilized with 95% ethanol about 3-5min. After that, removed the ethanol and dropped DPMPB-FONs (5 μM) onto the glass slide. 30 minute later, the fluorescence was observed by fluorescence microscopy.

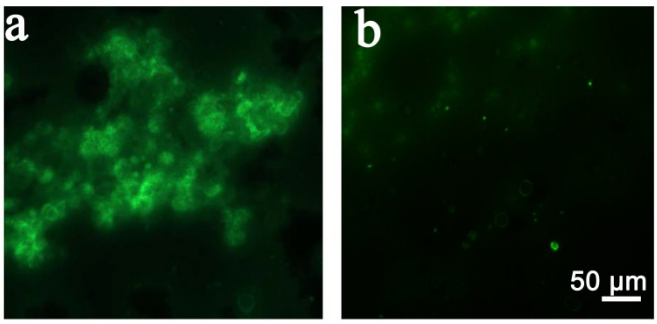


Figure S11. (a) Fluorescent imaging of breast cancer tissue stained with 5 μM **DPMPB-FONs** for 5 min. (b) Fluorescent imaging of para-carcinoma tissue stained with 5 μM **DPMPB-FONs** for 5 min.

17. Two photon fluorescence images of para-carcinoma tissue

The breast cancer tissue and para-carcinoma tissue were removed from a 44-year-old breast cancer patient. The breast cancer tissues or para-carcinoma tissue were cut into 100-200 μm thick slices using a freezing microtome. Slices were attached to the slide and treated with 10 μM **DPMPB-FONs** nanoprobe for 7.5 hour at 37°C and washed three times with 10 mM PBS. Then, the slices were incubated with 5 μM DAPI for 20 min and washed three times with 10 mM PBS. The slices were transferred to slice boxes for two-photon fluorescence imaging observation.

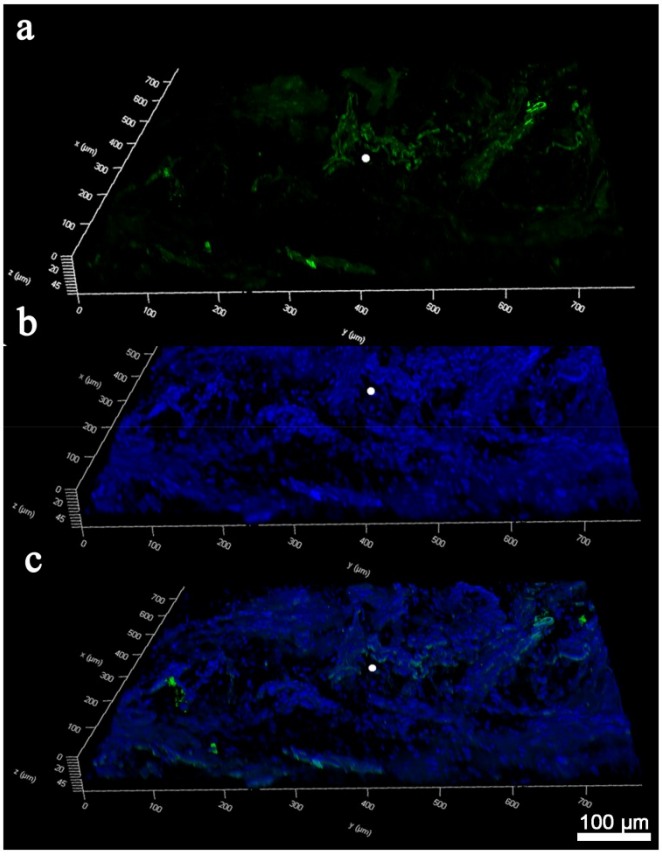


Figure S12. (a) 3D reconstruction fluorescence images of para-carcinoma tissue stained with 5 μM **DPMPB-FONs** in two-photon mode by collecting the emissions at 480-530 nm upon excitation at 760 nm. (b) 3D projection fluorescence images of para-carcinoma tissue stained with 1 μM DAPI in two-photon mode by collecting the emissions at 450-470 nm upon excitation at 358 nm. (c) Merged image of (a) and (b). Scale bar: 100 μm.

18. Characterization of compound

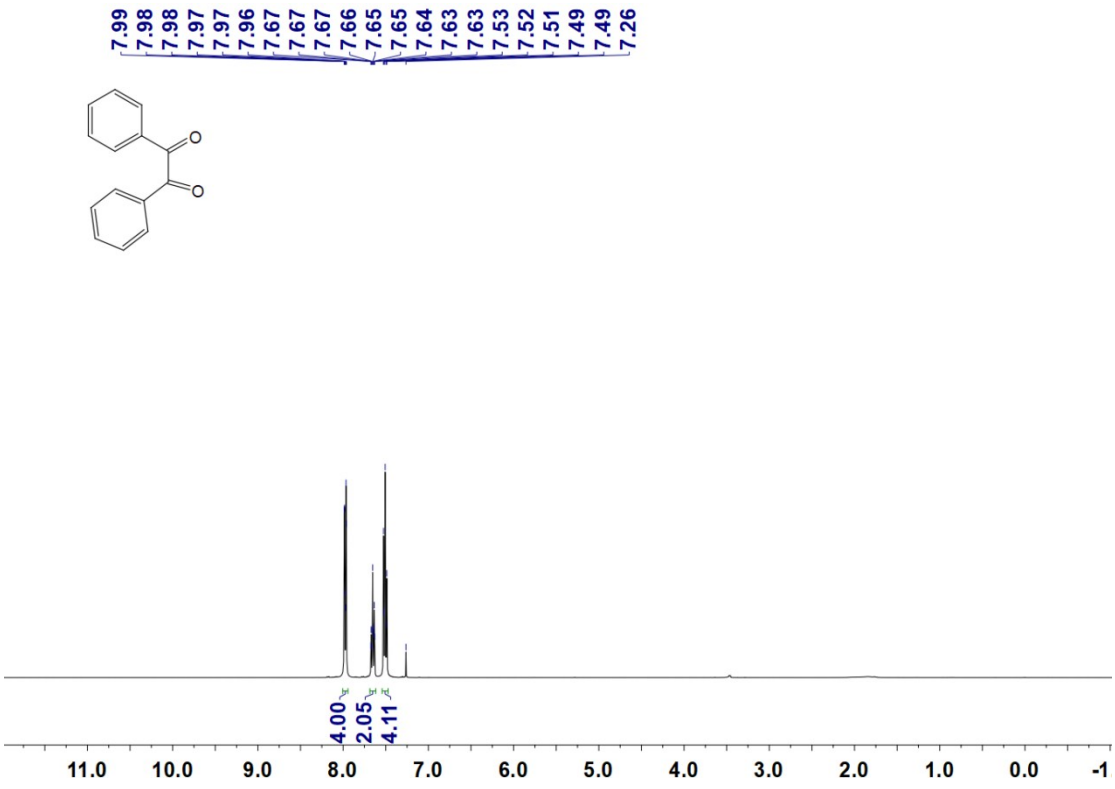


Figure S13. 400MHz spectra of compound **2** in $CDCl_3$.

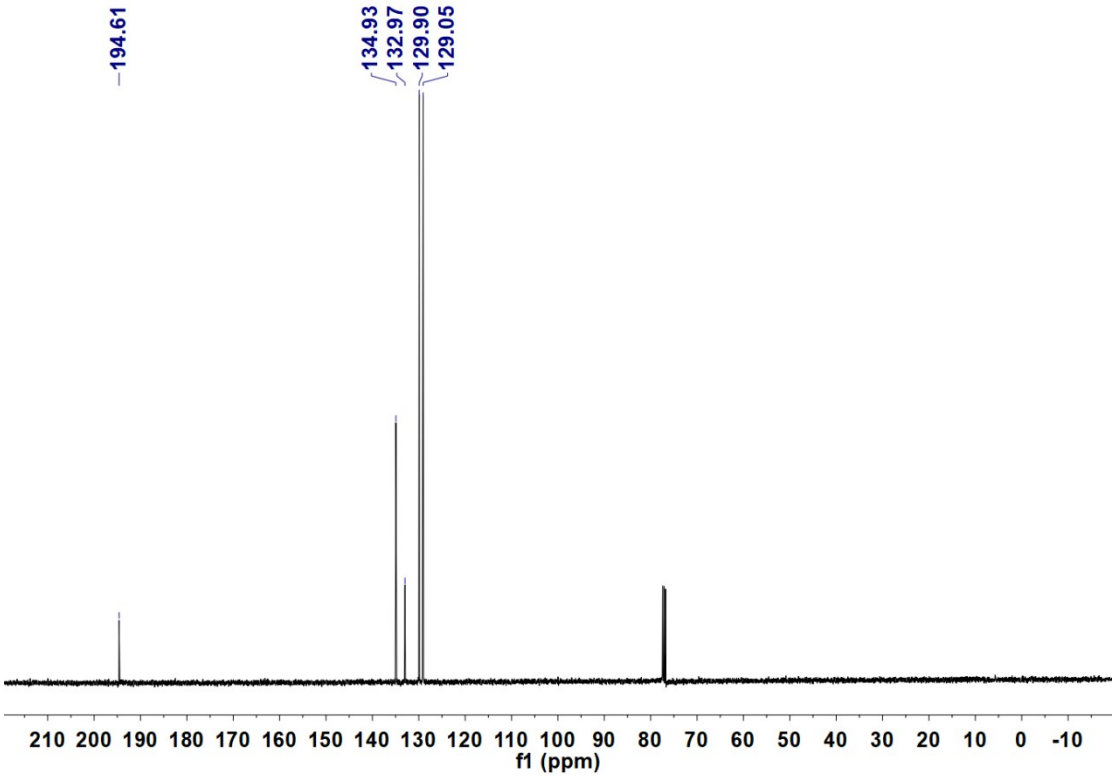


Figure S14. 100MHz ^{13}C-NMR spectra of compound **2** in $CDCl_3$.

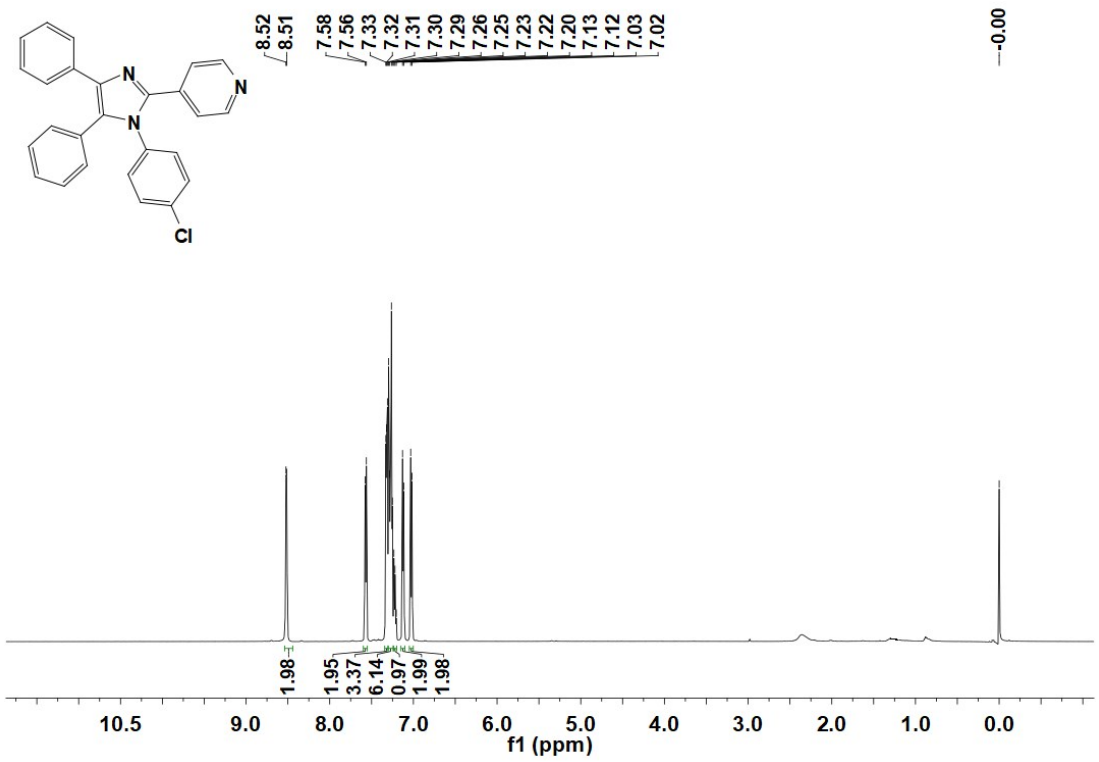


Figure S15. 400MHz spectra of compound **3** in $CDCl_3$.

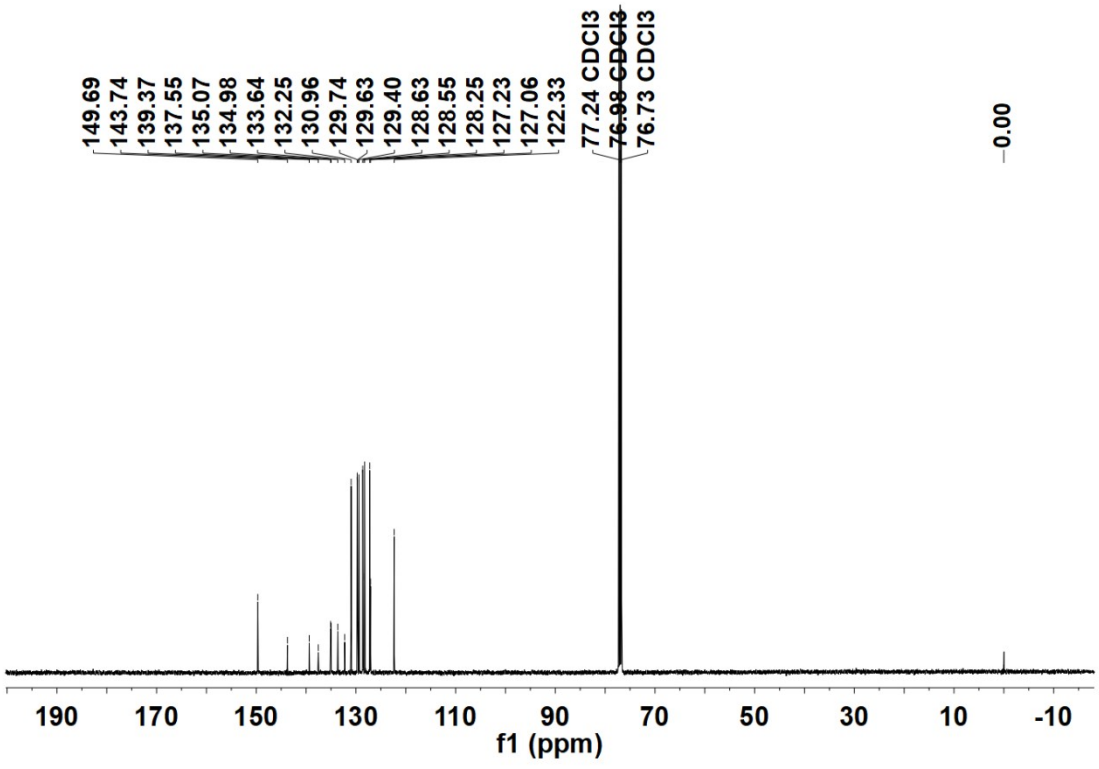


Figure S16. 100MHz ^{13}C-NMR spectra of compound **3** in $CDCl_3$.

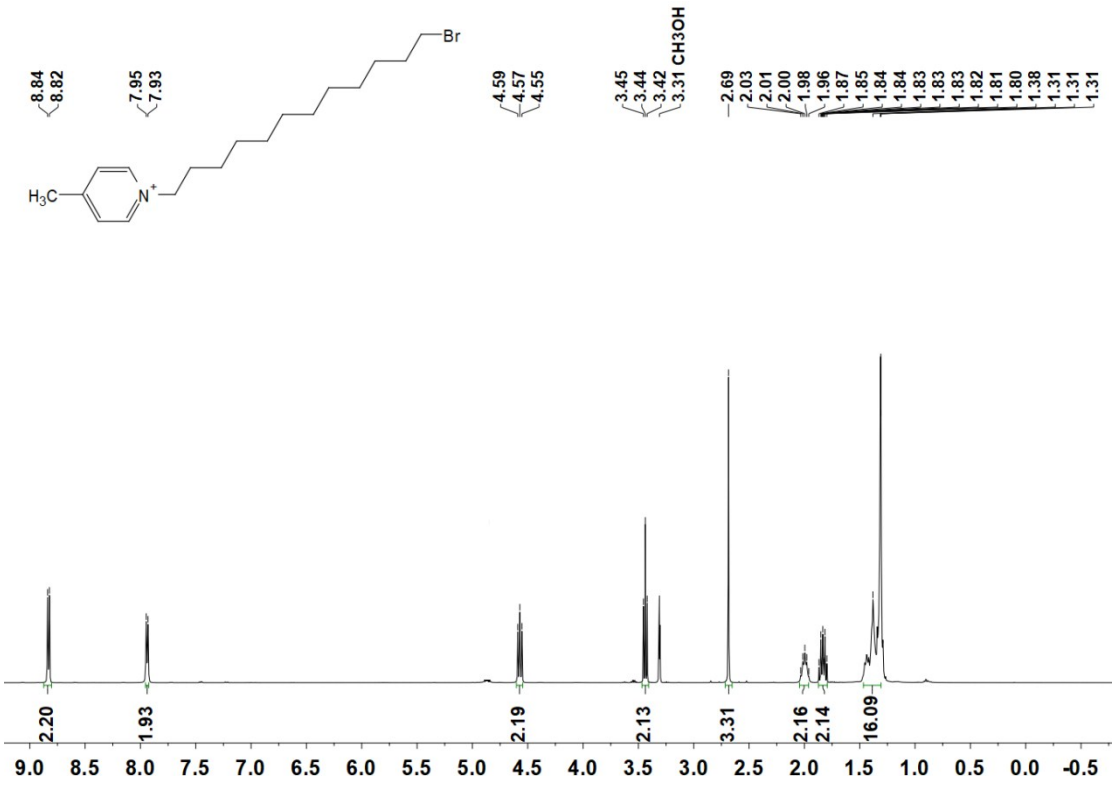


Figure S17. 400MHz spectra of compound **4** in CD_3OD

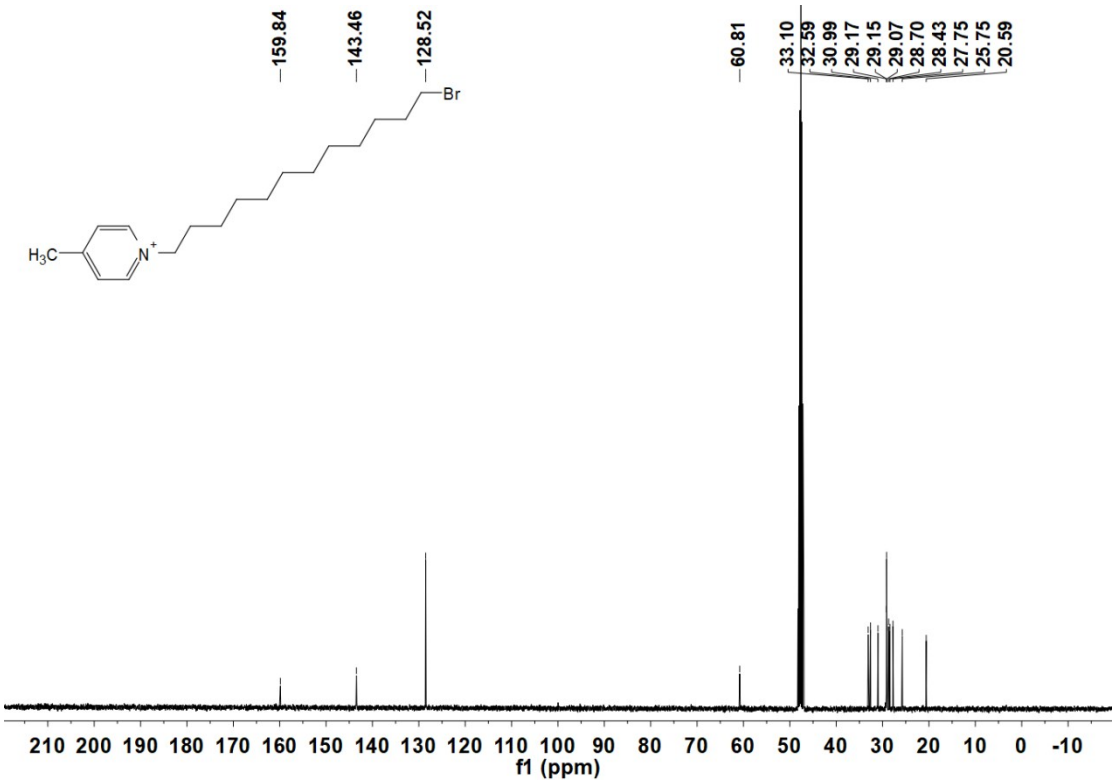


Figure S18. 100MHz ^{13}C-NMR spectra of compound **4** in CD_3OD

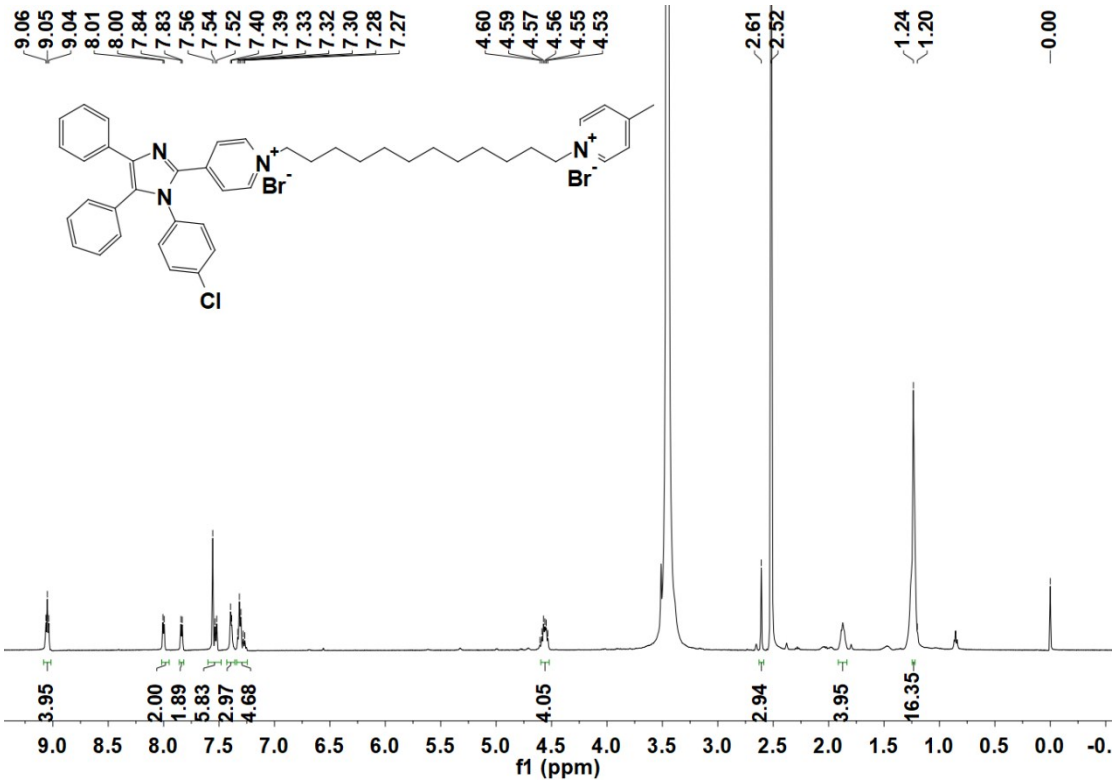


Figure S19. 400MHz NMR spectra of compound **DPMPB** in DMSO-d_6

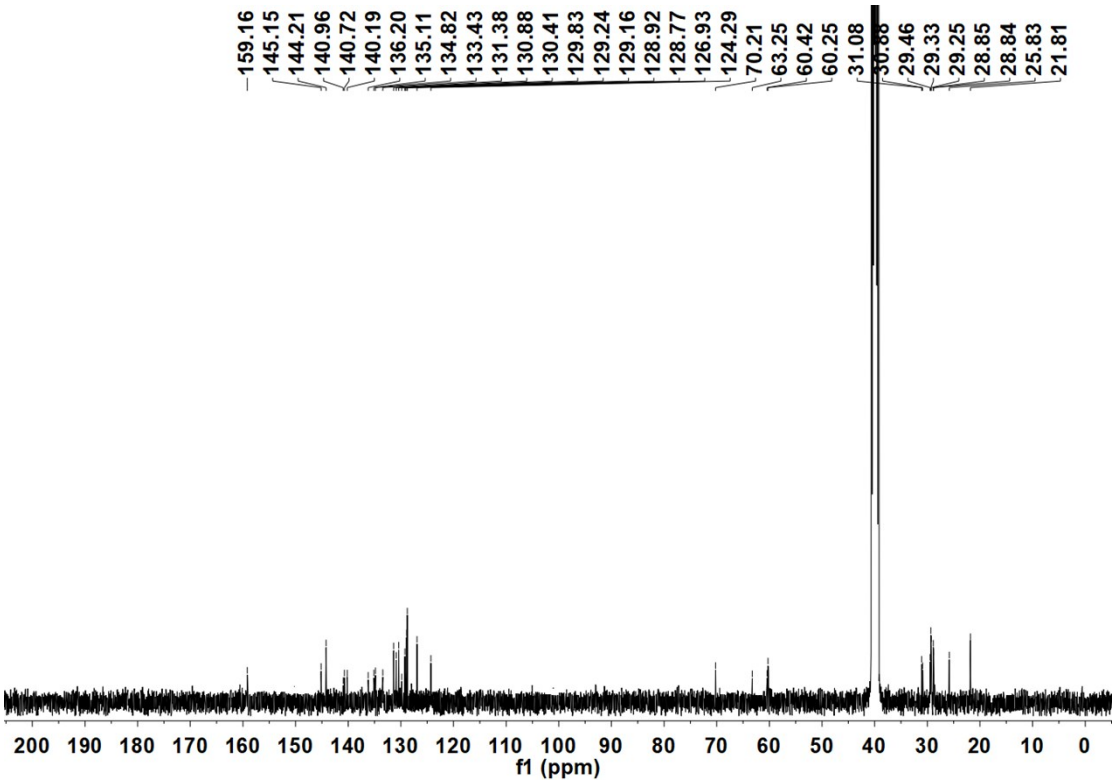


Figure S20. 100MHz ^{13}C-NMR spectra of compound **DPMPB** in DMSO-d_6