Supporting Information

Ligand-displacement based two-photon fluorogenic probe for visualizing mercapto biomolecules in live cells, Drosophila brains and Zebrafishes

Yanfei Zhao[a], Yun Ni[a], Liulin Wang[a], Chenchen Xu[a], Chenqi Xin[a], Chengwu Zhang[a], Gaobin Zhang[a], Xiaoji Xie[a], Lin Li*[a] and Wei Huang*[ab]

[a.]Key Laboratory of Flexible Electronics (KLOFE) & Institute of Advanced Materials (IAM), Jiangsu National Synergetic Innovation Center for Advanced Materials (SICAM), Nanjing Tech University (NanjingTech), 30 South Puzhu Road, Nanjing, 211816, P. R. China.

[b.]Shaanxi Institute of Flexible Electronics (SIFE), Northwestern Polytechnical University (NPU), 127 West Youyi Road, Xi'an 710072, China

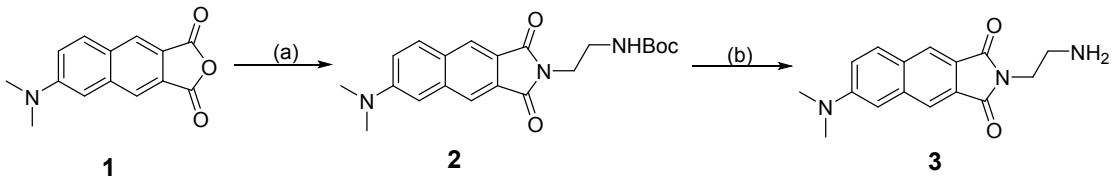
** Corresponding author E-mail: iamlli@njtech.edu.cn, iamwhuang@njtech.edu.cn*

1. General procedures:

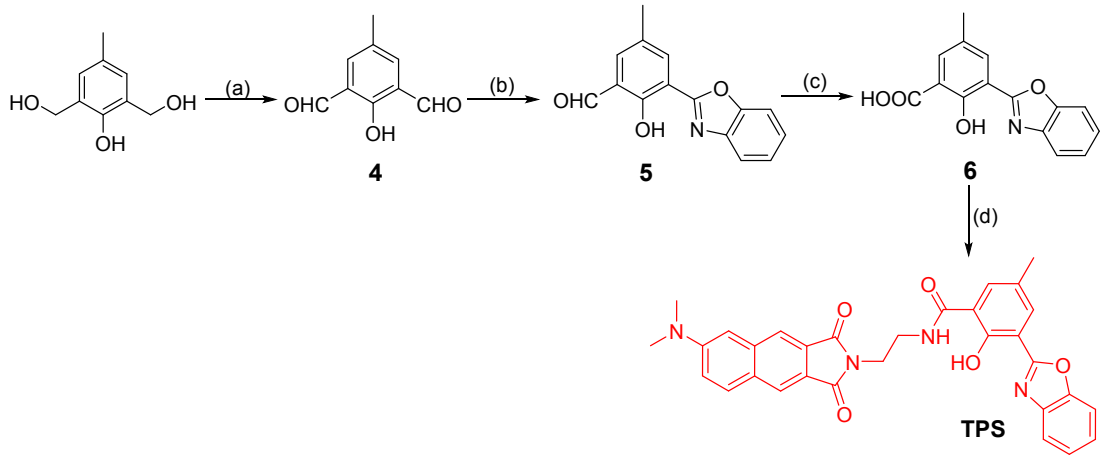
All images were acquired on Zeiss LSM880 NLO (2+1 with BIG) Confocal Microscope System equipped with objective LD C-Apochromat 63x/1.15 W Corr M27, cell incubator with temperature control resolution ± 0.1 ^{o}C, 405 nm Diode laser, Argon ion laser (458, 488 and 514 nm), HeNe laser (543 and 594 nm), Rack LSM 880 incl. 633 nm laser, and a Spectra Physics femtosecond Ti: sapphire laser (~4 W at 800 nm) which corresponded to approximately 1% (~40 mW at 800 nm, the output laser pulses have a tunable center wavelength from 690 nm to 1040 nm with pulse duration of 150 < fs and a repetition rate of 80 MHz) average power in the focal plane as the

excitation source, with main beam splitter wheel VIS equipped for ROGB lasers/Axio imager beam coupling optics for NLO and 405 nm laser and 8 channels AOTF for simultaneous control of 8 laser lines. A PMT detector ranging from 420 nm to 700 nm for steady-state fluorescence and non-descanned detectors (BiG.2) for the two-photon excited fluorescence, were used. Internal photomultiplier tubes were used to collect the signals in 8-bit unsigned 1024×1024 pixels at a scan speed of 200 Hz. Images were processed with Zeiss User PC Advanced for LSM system (BLUE).

2. Synthesis and characterization



Scheme S1. Synthesis scheme of two-photon fluorophore: (a) tert-butyl(2-aminoethyl)carbamate, Ethanol, reflux; (b) 20% TFA/DCM, 0 °C ~ room temperature.



Scheme S2. Synthesis scheme of two-photon fluorescent ligand (**TPS**). (a) MnO_2, $CHCl_3$ reflux; (b) i. 2-aminophenol, toluene, N_2, reflux, 3 h; ii. DDQ, DCM/THF = 3/1, N_2, reflux, 5 h; (c) $NaClO_2$, NaH_2PO_4, DMSO, room temperature, 0.5 h; (d) DMF, EDCl, room temperature.

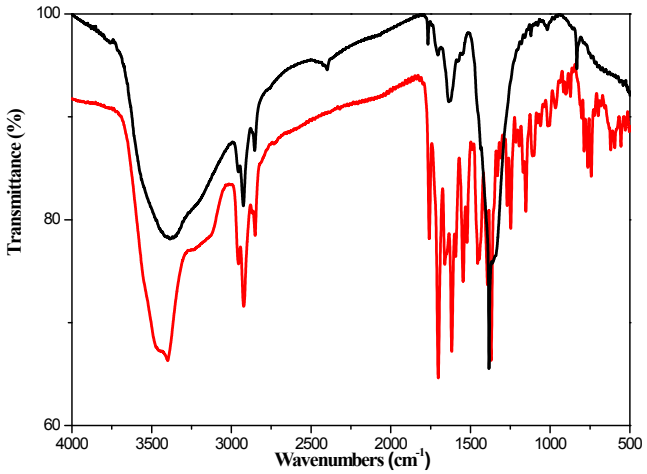


Fig. S1 IR spectrum of **TPS** (red line) and **TPFeS** (black line).

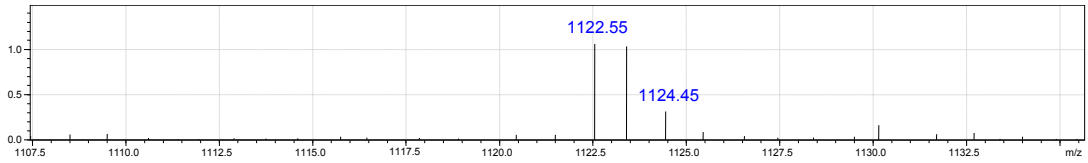


Fig. S2 Mass spectrum of **TPFeS**.

3. Photophysical properties of **TPS** in deionized water with 0.05% Triton X-100.

Table S1 Photophysical properties of **TPS**

Compound	λ^a	Φ^b	$\delta\Phi^c$
TPS	380/550	0.50	103
Flu1	352/498	—	128
Fluorescein	470/520	0.85	—

—: Not determined.

[a]Peak position of the longest absorption/emission band.

[b]Quantum yields determined by using fluorescein aqueous NaOH (0.1M) as standard.

[c]The maxima two-photon action cross section values upon excitation wavelength of fluorophore from 750 to 840 nm in GM (1 GM = 10^{-50} cm^4 s $photon^{-1}$) by using **Flu1** as standard.[5]

4. Spectroscopic Materials and Methods

All spectroscopic measurements were performed in deionized water with 0.05% Triton X-100, pH 7.35, 37 °C. The detection limit of **TPFeS** was determined based on a reported method (Fig. 3).[6]

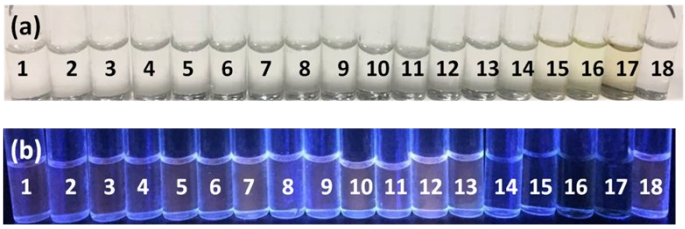


Fig. S3 Color changes of **TPS** (10 μM) in presence of different cations: Ni(II) (1), K(I) (2), Na(I) (3), Cd(II) (4), Ba(II) (5), Mn(II) (6), Mg(II) (7), Co(II) (8), Zn(II) (9), Ca(II) (10), Cr(III) (11), Al(III) (12), Ag(I) (13), Fe(II) (14), Pd(II) (15), Fe(III) (16), Cu(II) (17) and **TPS** (18). (a) In visible light naked eye and (b) under a hand-hold UV lamp.

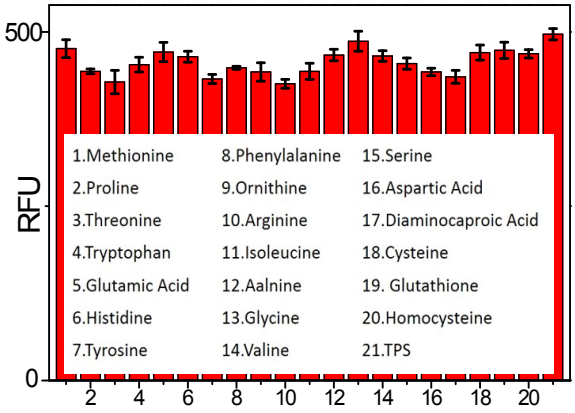


Fig. S4 Effect of the 20 amino acids (10 eq.) toward **TPS** (10 μM) in deionized water with 0.05% Triton X-100 at 37°C. RFU = relative fluorescence units.

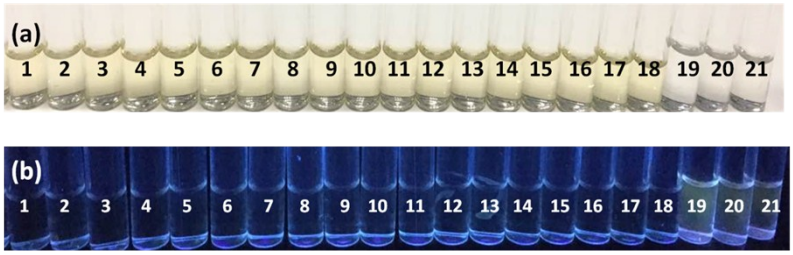


Fig. S5 Color changes of **TPFeS** (10 μM) in presence of different amino acids: Met (1), Pro (2), Thr (3), Try (4), Glu (5), His (6), Tyr (7), Phe (8), Orn (9), Arg (10), Dia (11), Ala (12), Gly(13), Val (14), Ser (15), Asp (16), Ile (17), **TPFeS** (18), Cys (19), GSH (20) and Hcy (21). (a) In visible light naked eye and (b) under a hand held UV lamp.

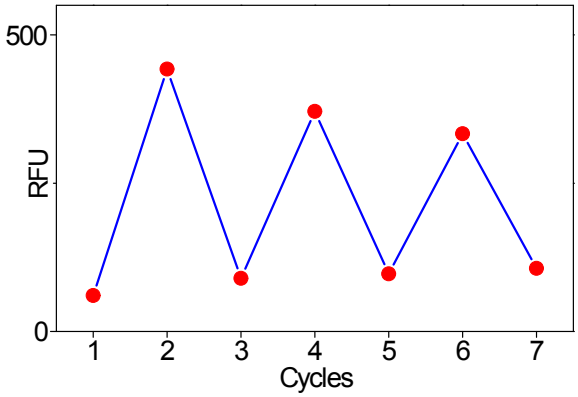


Fig. S6 Fluorescence intensity of **TPFeS** (10 μM) upon the alternate addition of GSH/Fe(III) with concentration of 2 equiv. /1 equiv. (Cycle 1: 0 μM, 2: GSH 20 μM, 3: Fe(III) 10 μM, 4: GSH 20 μM, 5: Fe(III) 10 μM, 6: GSH 20 μM and 7: Fe(III) 10 μM) in assay buffer.

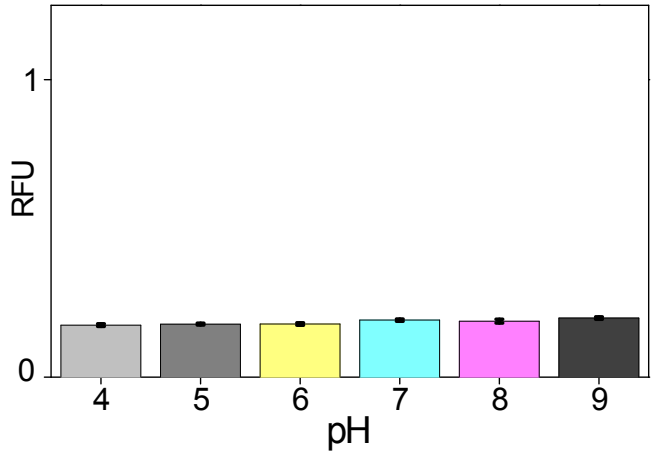


Fig. S7 Fluorescence intensities at 530 nm of **TPFeS** (5 μM) normalized the value of **TPS** at various pH values.

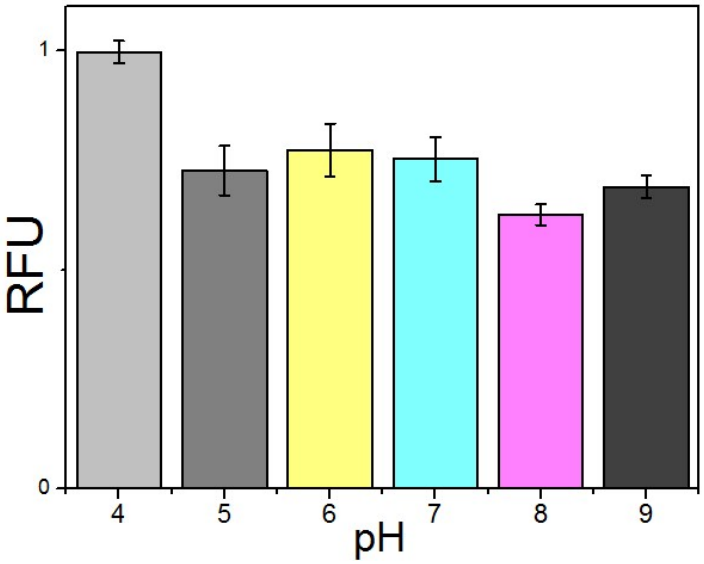


Fig. S8 Fluorescence intensities at 530 nm of **TPS** (5 μM) (**TPFeS** after addition of GSH) normalized the value of the maximum fluorescence of **TPS** at various pH values.

5. Cell\Drosophila\Zebrafish culture and fluorescence imaging

The human hepatocellular carcinoma (HepG2) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing supplemented with 10% fetal bovine serum (FBS), 100.0 mg/L streptomycin, and 100 IU/mL penicillin. HepG2 cells were seeded in glass-bottom dishes (Mattek) and grown till 70 ~ 80% confluency.

The cytotoxicity activity of probe was determined by using the XTT colorimetric cell proliferation kit (Roche) following manufacturer's guidelines. Briefly, different cells were grown to 20 ~ 30% confluency (since they will reach 80 ~ 90% confluency within 48 to 72 hrs in the absence of compounds) in 96-well plates under the conditions described on above. The medium was aspirated, and then washed with PBS, and then treated, in duplicate, with 0.1 mL of the medium containing different concentrations of **TPS**, **TPFeS**, **TPS**+Cu(II), **TPS**+Pd(II)(1~50 μM). Probe was applied from DMSO stocks whereby DMSO never exceeded 1% in the final solution. The same volume of DMSO was used as a negative control; and the same volume of Staurosporine (STS, 200 nM) was used as a positive control. After a total treatment time of 12 hrs, proliferation was assayed using the XTT colorimetric cell proliferation kit (Roche) following manufacturer reference. DMSO was used as a negative control.

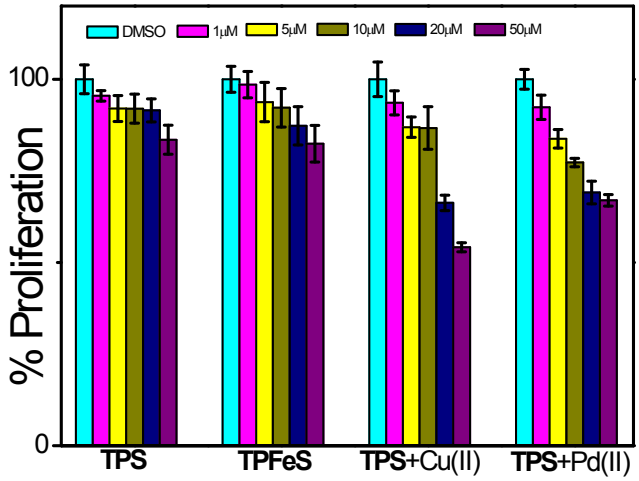


Fig. S9 Viability of HepG2 cells in the presence of **TPS**, **TPFeS**, **TPS**+Cu(II), **TPS**+Pd(II) (0~50 μM) as measured by using XTT assays.

For imaging of Drosophila brains, the fresh brains are infiltrated with PBS buffer in 0.5 mL tubes and about 5 brains in each tube, which were incubated with 10 mM NEM(±) for 30 mins at 37 °C beforehand in PBS (10 μM), followed by 10 μM of **TPS**/**TPFeS** for 2 hr at 37 °C. For imaging of Zebrafishes, all Zebrafish embryos were passed through three successive washes of buffer solution before observation. Before imaging, the fish was anesthetized because we use 0.01%-0.02% tricaine in egg water to imprison it. Zebrafishes were cultured for 5-day-old for two-photon bioimaging in vivo. An then, 5-day-old Zebrafishes were raised with **TPS** (10 μM) and **TPFeS** (10 μM) at 28 ^{o}C for 2 hrs. Two-photon fluorescence images of Drosophila brains and Zebrafishes were obtained by exciting samples with laser source set at wavelength 800 nm, respectively. A staining free group was used as control. All images were taken at the same way.

Fig. S10 Two-photon fluorescence imaging of 5-day-old Zebrafish without any staining at the same condition as used in Fig. 5b.

5. References

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6. M. Shortreed, R. Kopelman, M. Kuhn, B. Hoyland, *Anal. Chem.*, 1996, **68**, 1414.

6. ^{1}H NMR and ^{13}C NMR spectra.

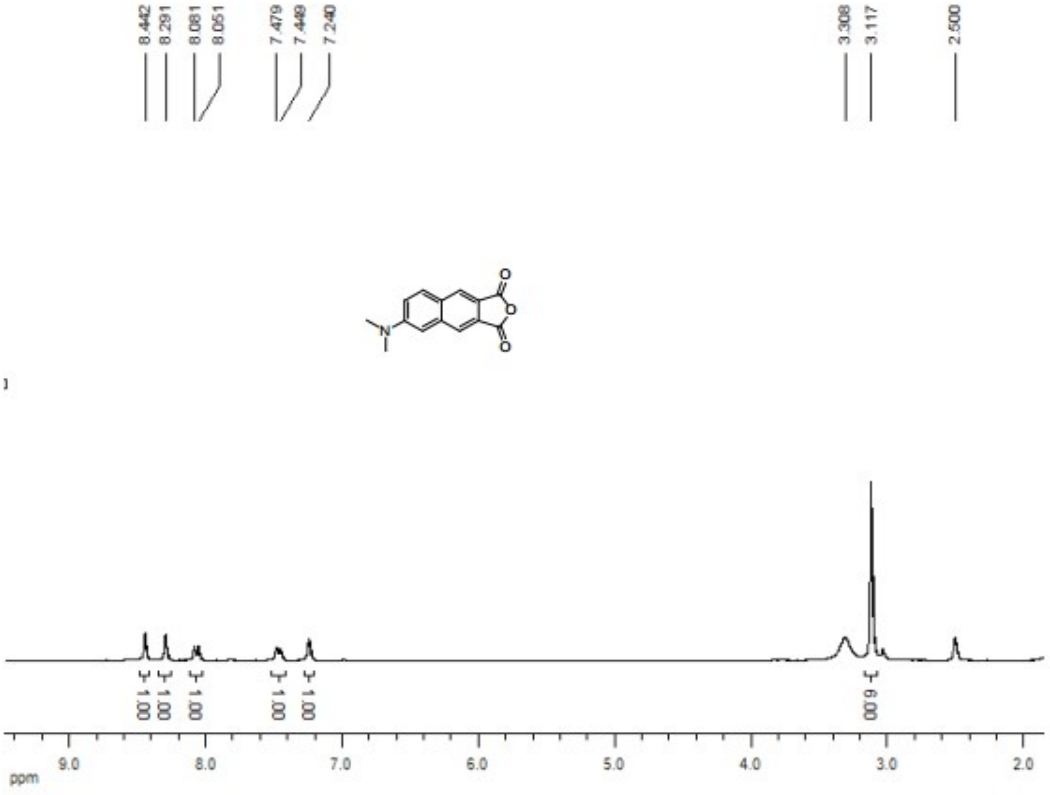

Fig. S9 ^{1}H NMR spectrum of **1**.

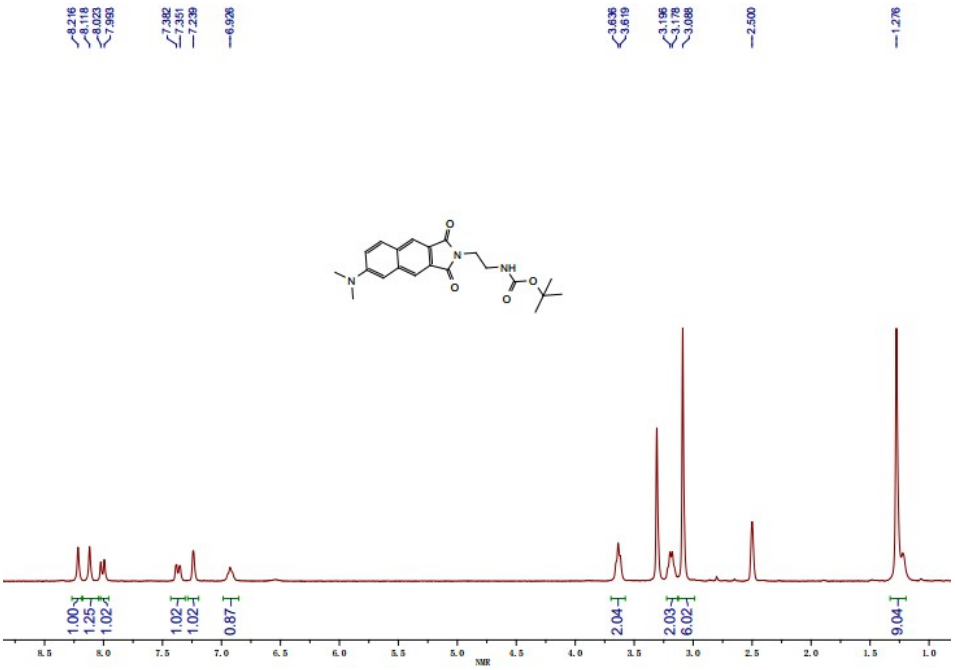

Fig. S10 ^{1}H NMR spectrum of 2.

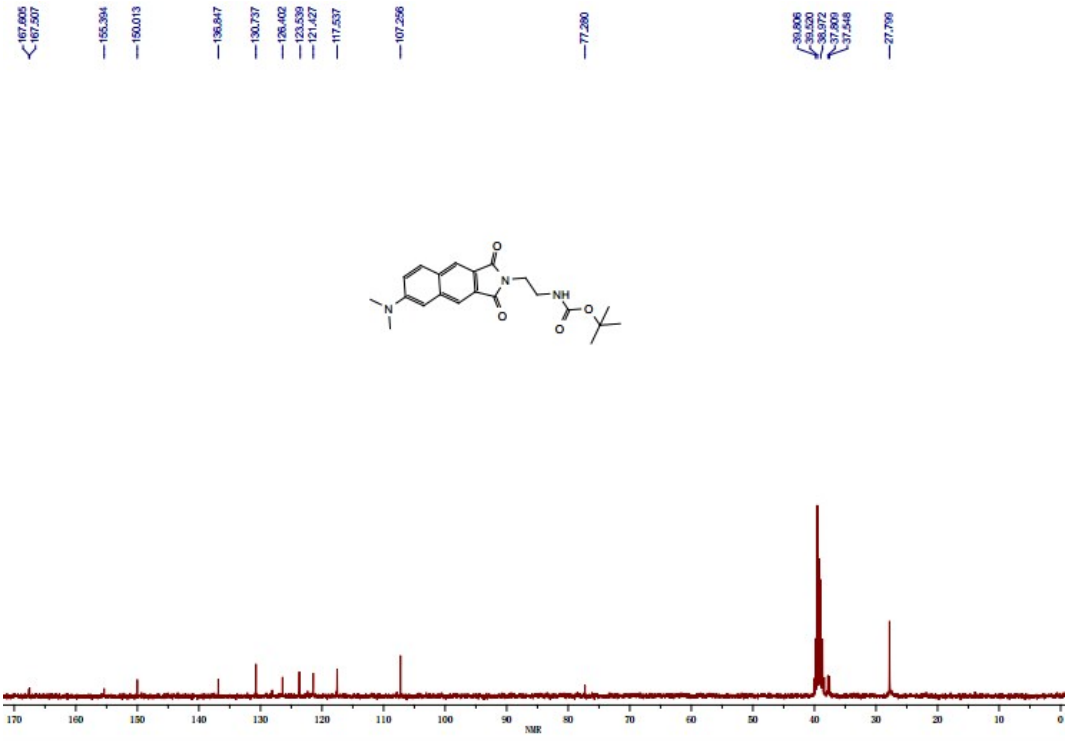

Fig. S11 ^{13}C NMR spectrum of 2.

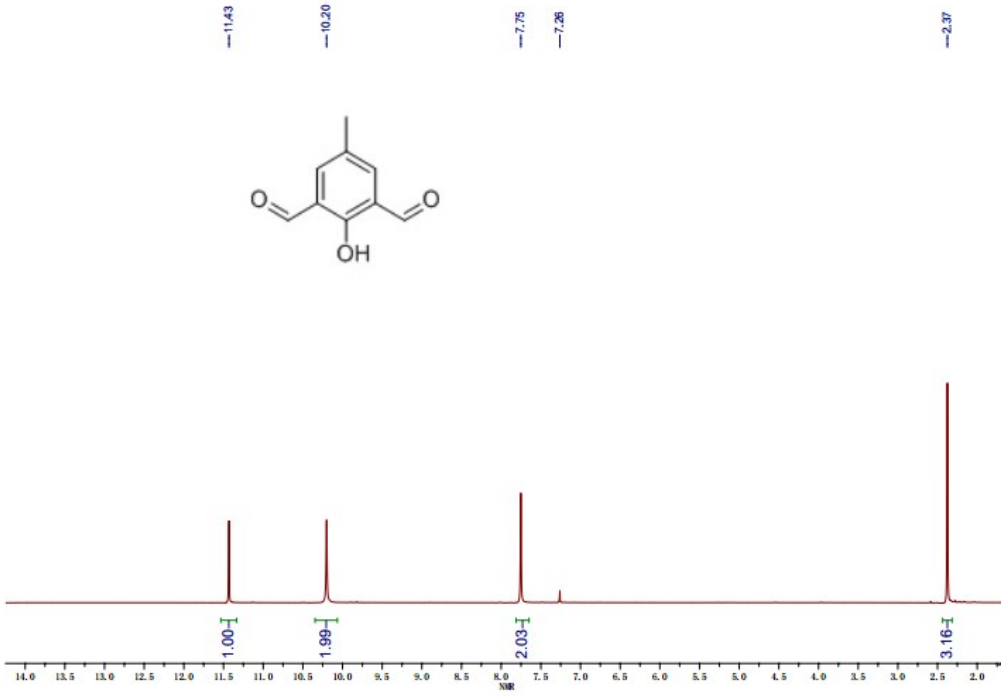

Fig. S12 ^{1}H NMR spectrum of 4.

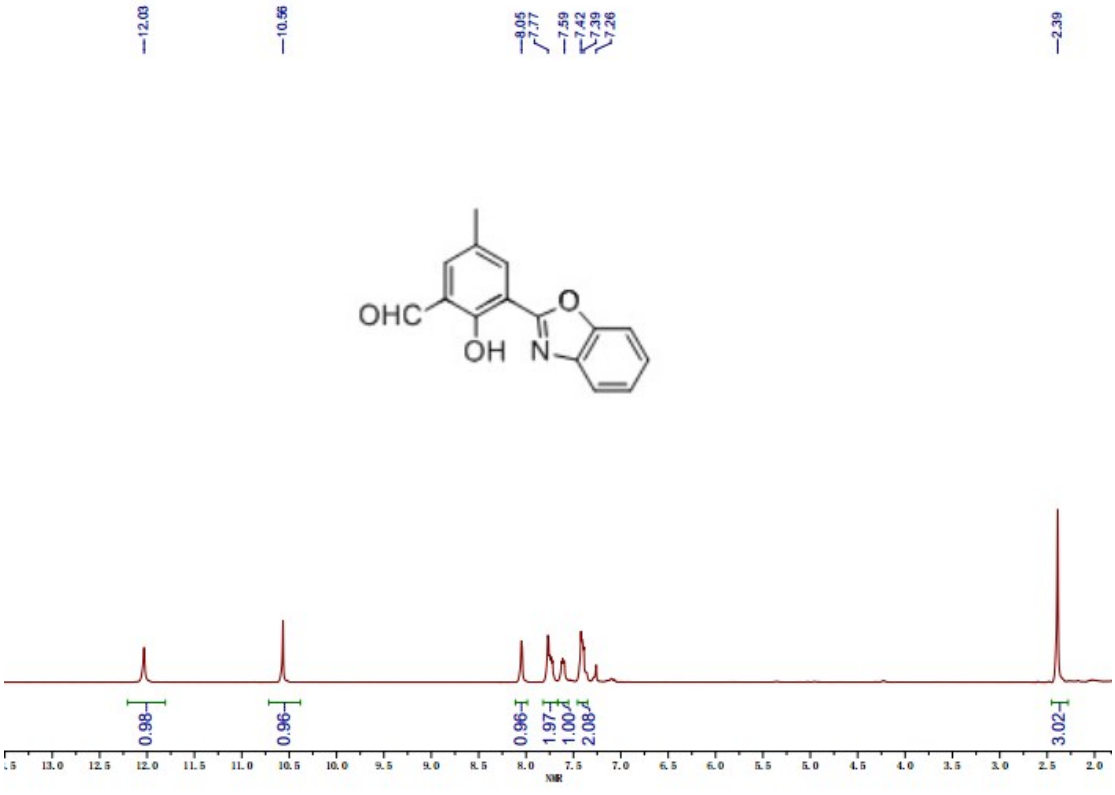

Fig. S13 [1]H NMR spectrum of 5.

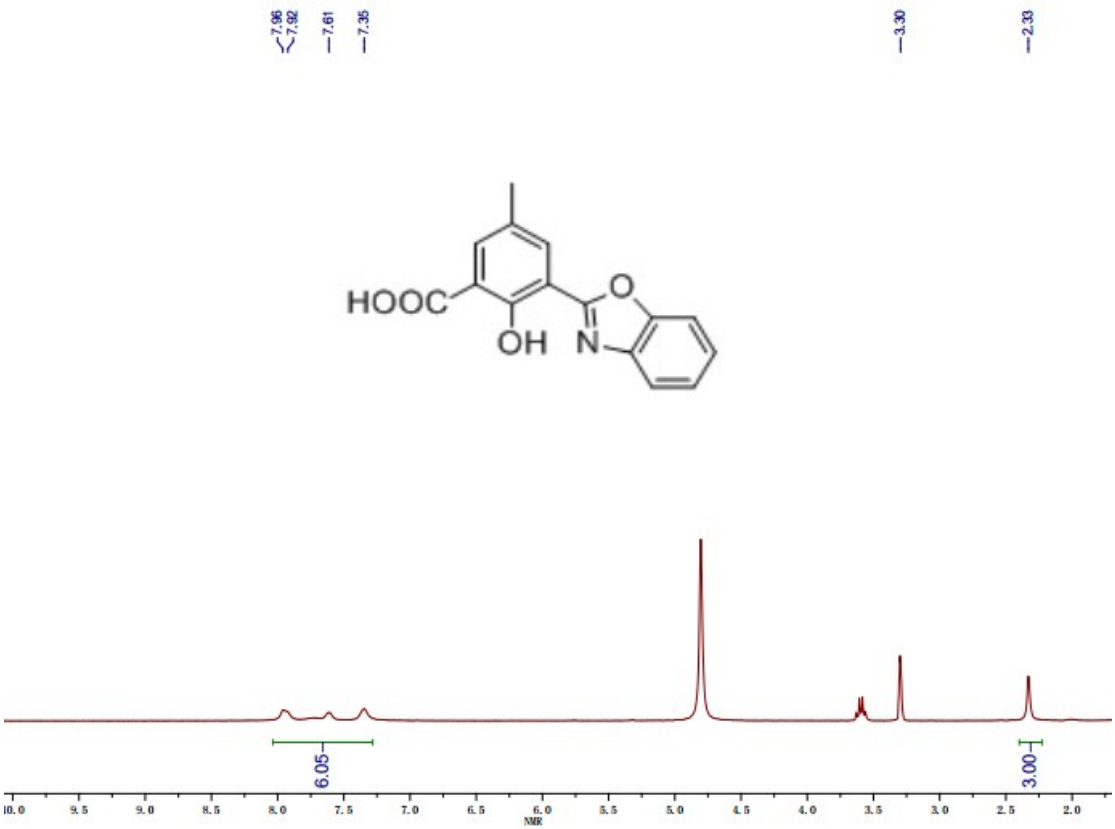

Fig. S14 [1]H NMR spectrum of 6.

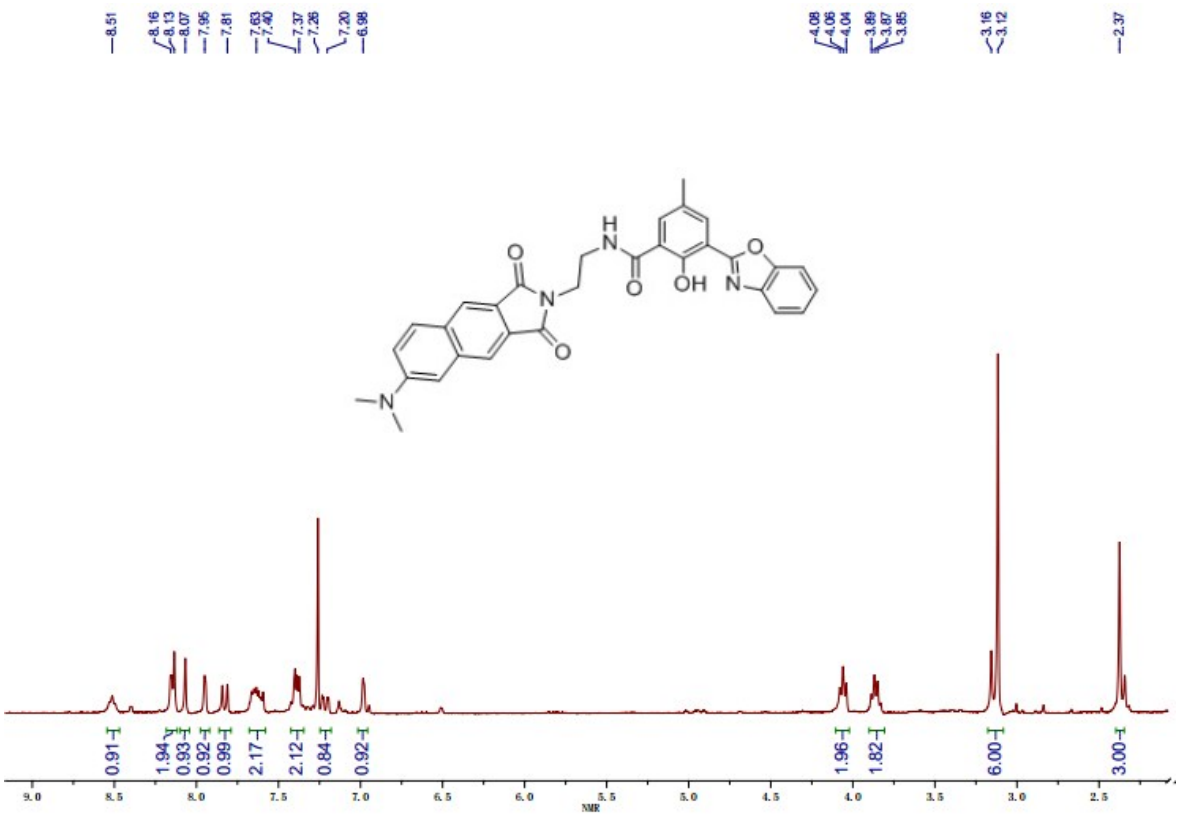

Fig. S15 ^{1}H NMR spectrum of TPS.

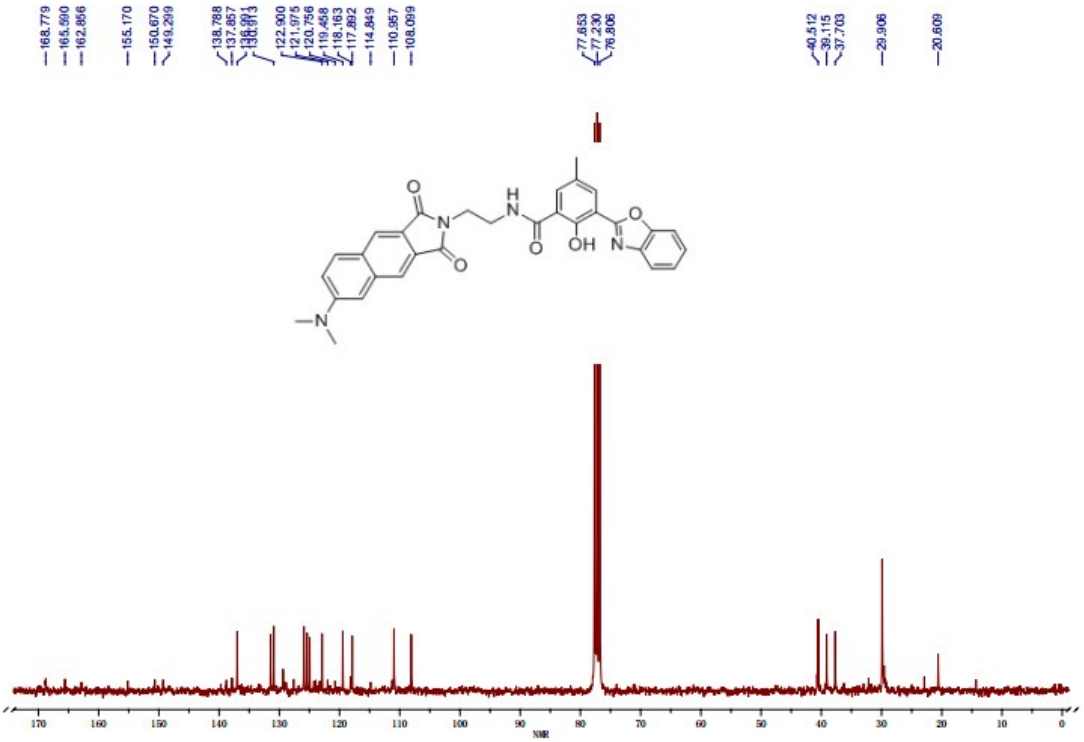

Fig.S16 ^{13}C NMR spectrum of TPS.