Electronic Supplementary Information

An effective fluorescent probe detects glutathione from other sulfhydryl compounds in aqueous solution and its living cell imaging

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1. Materials and Methods

1.1 Materials and Instruments

Unless otherwise stated, all reagents were purchased from Aladdin, J&K or Sinopharm Chemical Reagent Co. and used without further purification. Twice-distilled water was used throughout all experiments. Thin-layer chromatography (TLC) involved silica gel 60F254 plates (Merck KGaA) and column chromatography involved silica gel (mesh 200–300). ^{1}H NMR (300 MHz) and ^{13}C NMR (100 MHz) spectra were carried out on a Bruker Avance 300/400 spectrometer, using $CDCl_3$ or DMSO as solvent and tetramethylsilane (TMS) as internal standard. Melting points were determined on an XD-4 digital micro melting point apparatus. IR spectra were performed with an IR spectrophotometer VERTEX 70 FT-IR (Bruker Optics). HRMS spectra were obtained on a Q-TOF6510 spectrograph (Agilent). UV-vis spectra were measured by use of a Hitachi U-4100 spectrophotometer. Fluorescent measurements were performed on a Hitachi F-7000 luminescence spectrophotometer. Two-photon excitation fluorescence (TPEF) was measured on a SpectroPro300i and the pump laser beam came from a mode-locked Ti: sapphire laser system at the pulse duration of 200 fs, a repetition rate of 76 MHz (Coherent Mira900-D). Quartz cuvettes with a 1 cm path length and 3 mL volume were used for all measurements. The pH was determined with a model PHS-3C pH meter.

1.2 Preparation for the Spectra Measurement

Probe **PPN** was dissolved in acetonitrile to afford the stock solution (1 mM). The amino acids (Cys, Hcy, GSH, arginine, aspartic acid, glutamic acid, glycine, histidine, lysine, proline, threonine, tryptophan, tyrosine), cationic (K^+, Ca^{2+}, Na^+, Mg^{2+}, Zn^{2+},

Fe^{3+}), Na_2S, H_2O_2, glucose and sulfhydryl compounds (2-mercaptoethanol, 4-aminobenzenethiol, 6-mercaptohexan-1-ol,) stocks were all in deionized water with a concentration of 10^{-2} M for fluorescence spectra analysis. Test solutions were prepared by displacing 100 μL of the stock solution and an appropriate aliquot of each testing species solution into a 10 mL volumetric flask, and the solution was diluted to 10 mL in aqueous solution at pH 7.4 (PBS buffer, 1 mM CTAB). The resulting solution was shaken well and incubated for 20 min at room temperature before recording the spectra.

Fluorescence quantum yield was determined in 0.1 N H_2SO_4 aqueous solutions at room temperature with quinine sulfate (Φ_s = 0.54 in 0.1 N H_2SO_4) as standard, and it was calculated by the equation 1.

$$\Phi=\Phi_s(IA_s/I_sA)(\eta^2/\eta_s^2) \qquad (1)$$

in which, A is the absorbance, I is the integrated fluorescence intensity, and η is the refractive index of the solvent [1].

1.3 Calf Serum Cultures

Calf serum (CS, from Hyclone) was divided into 9 groups, each group diluted with different multiples of PBS: no calf serum (PBS); dilution 100 times (0.01 × CS), 50 times (0.02 × CS), 20 times (0.05 × CS), 10 times (0.1 × CS), 5 times (0.2 × CS), 2 times (0.5 × CS), 4/3 times (0.75 × CS) and not diluted (1 × CS). Then CS was incubated with probe **PPN** dissolved in DMSO (stock solution: 0.1 M), and the reaction was blended with the use of an oscillator to a final concentration of **PPN**, 5 μM. After incubation for 60 min at 37 ^oC in the incubator, the reaction liquid

underwent photoluminescence imaging measurement by luminescence microscopy (Nikon TE2000-E). Imaging analysis involved the use of ImageJ.

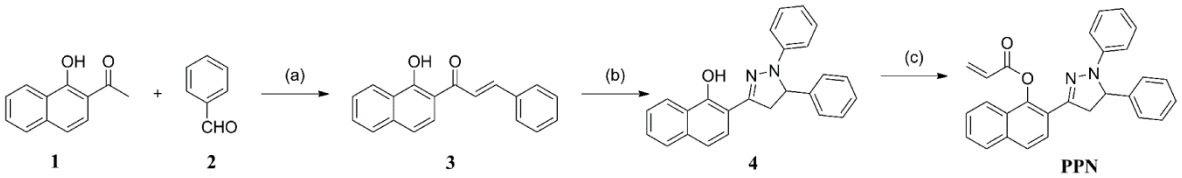
1.4 Cell Cultures and Fluorescence Imaging

A549 cells were cultured in DMEM. The probe was dissolved in DMSO at a storage concentration of 10 mM. Cells were adherent-cultured in 24-well culture plates for 24 h. A549 cells were washed from the RPMI-1640, incubated with 1, 5, 10 μM probe solution for 0.5, 1 h at 37 ^{o}C, respectively. Then washed 3 times with phosphate buffered saline (PBS) and underwent imaging measurement by ultraviolet light with a Nikon TE2000-E fluorescent microscope. Imaging analysis involved the use of ImageJ.

1.5 Cytotoxicity assay

The in vitro cytotoxicity of the probe to A549 cells was measured by a standard sulforhodamine B (SRB) assay. Briefly, A549 cells were loaded in 96-well culture plates at 4×10^4 cell per well. After culture for 12 h, cells were incubated with fresh 1640 containing 10 μM probe for 6 h. Then cells were fixed with 4% TCA for 1 h at 4 ^{o}C, then washed with deionized water 5 times; 50 μL SRB was added to each well, and after sufficient reaction with cells, the remaining SRB was removed by washing each well with 1% acetic acid solution, and 100 μL Tris–HCl was used to dissolve the SRB. Absorbance at 540 nm was measured in a 96-well multiwell-plate reader (TECAN).

2. Synthesis



Reagents and conditions: (a) EtOH, NaOH, r.t. 12 h; (b) EtOH, CH_3COOH, reflux 3 h; (c) CH_2Cl_2, Et_3N, ice-bath 1 h, r.t. 2 h.

Scheme S1. Synthesis of probe **PPN**.

2.1 *Synthesis of 1-(1-hydroxynaphthalen-2-yl)-3-phenylprop-2-en-1-one (**3**)*

Compound **3** was synthesized from 1-(1-hydroxynaphthalen-2-yl)ethanone (**1**) and benzaldehyde (**2**) according to the reported method.[1]

2.2 *Synthesis of 2-(1,5-diphenyl-4,5-dihydro-1H-pyrazol-3-yl)naphthalen-1-ol (**4**)*

Compound **3** (1 mmol) and EtOH (50 mL) were added to a round-bottomed flask, and the mixture was stirred for 10 min at room temperature. Acetic acid (catalyst, 1 mL) followed by phenylhydrazine (3 mmol) were added and the mixture was reflux for 4 h. The progress of the reaction was monitored by TLC. After completion, the reaction mixture was cooled to room temperature; equal volumetric water was added to the reaction mixture. The aqueous layer was separated and extracted with CH_2Cl_2 (3 × 50 mL). The organic phase was combined, dried over $MgSO_4$, filtered and concentrated to give a yellow solid. The crude product was purified by column chromatography on silica gel to afford the desired compound **4**.[2] Yellow solid; m.p. 190 ^oC. IR (KBr, cm^{-1}): 3446 (-OH), 3062 (Ar-H), 2923(-CH_2-). ^1H NMR (300 MHz, DMSO-d_6): δ = 3.39 (dd, 1H, J = 6.9 and 17.7 Hz, pyrazole, 4-H*a*), 4.18 (dd, 1H, J = 12.2 and 17.7 Hz, pyrazole, 4-H*b*), 5.53 (dd, 1H, J = 6.9 and 12.2 Hz, pyrazole, 5-H), 6.78-8.34 (m, 16H, Ar-H), 11.70 (s, 1H, O-H). ^{13}C NMR (100 MHz, DMSO-d_6): 44.22, 62.61, 110.09, 113.43 (2C), 119.55, 119.83, 122.88, 124.34, 125.07, 126.28,

126.50 (2C), 127.93, 128.05, 128.09, 129.55 (2C), 129.65 (2C), 134.50, 142.48, 144.03, 151.52, 153.38. HRMS: m/z $[M+H]^+$ calcd for $C_{25}H_{21}N_2O$: 365.1654, found 365.1702.

2.3 *Synthesis of 2-(1,5-diphenyl-4,5-dihydro-1H-pyrazol-3-yl)naphthalen-1-yl acrylate (Probe **PPN**)*

Compound **4** (1 mmol), CH_2Cl_2 (20 mL) and triethylamine (1.5 mL) were placed in a round-bottomed flask at ice-bath. A solution of acryloyl chloride (3 mmol) in dichloromethane (30 mL) was added over 0.5 h to the mixture. The mixture was stirred at 0 ^oC for 2 h, and then stirred for 1 h at room temperature. H_2O (50 mL) was added to the mixture, and then the aqueous layer was separated and discarded. The organic phase was washed with water (3 × 50 mL), and dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give a yellowish solid. The crude solid was purified by column chromatography on silica gel to obtain product (probe **PPN**) in 38% yield. White solid, m.p. 145-146 ^oC. IR (KBr, cm^{-1}): 3065 (=C-H), 2920 (-CH_2-), 2850 (-CH-), 1740 (C=O). ^1H NMR ($CDCl_3$, 300 MHz): δ ppm 3.25 (dd, 1H, J = 7.0 and 16.8 Hz, pyrazole, 4-H*a*), 3.92 (dd, 1H, J = 12.6 and 16.8 Hz, pyrazole, 4-H*b*), 5.27 (dd, 1H, J = 7.0 and 12.6 Hz, pyrazole, 5-H), 6.15 (dd, 1H, J = 1.5 and 10.5 Hz, -CH=), 6.51-7.89 (m, 18H, -CH=CH_2 and Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$): 45.34, 64.10, 113.74 (2C), 119.39, 121.76, 122.08, 125.19, 125.94 (3C), 126.18, 126.84, 127.12, 127.63, 127.90 (2C), 128.78 (2C), 129.16 (2C), 133.44, 134.23, 142.29, 143.48, 143.63, 144.30, 164.45. HRMS: m/z $[M+H]^+$ calcd for $C_{28}H_{23}N_2O_2$: 419.1760, found 419.1842.

3. Crystal structure of probe PPN by X-ray

Crystal of probe **PPN** suitable for X-ray diffraction was obtained by slow evaporation of a solution of the solid in dichloromethane at room temperature for 4 days. The crystal with approximate dimensions of 0.12 mm x 0.10mm x 0.08 mm for **PPN** was mounted on a Bruker Smart Apex II CCD equipped with a graphite monochromated MoKα radiation (λ = 0.71073 Å) by using φ and ω scan modes and the data were collected at 293 K. The structures of the crystal was solved by direct methods and refined by full-matrix least-squares techniques implemented in the SHELXTL-97 crystallographic software. The non-hydrogen atoms were refined anisotropically. The hydrogen atoms bound to carbon were located by geometrical calculations, with their position and thermal parameters being fixed during the structure refinement. The final refinement converged at R1 = 0.0498, wR2 = 0.1373 for **PPN**.

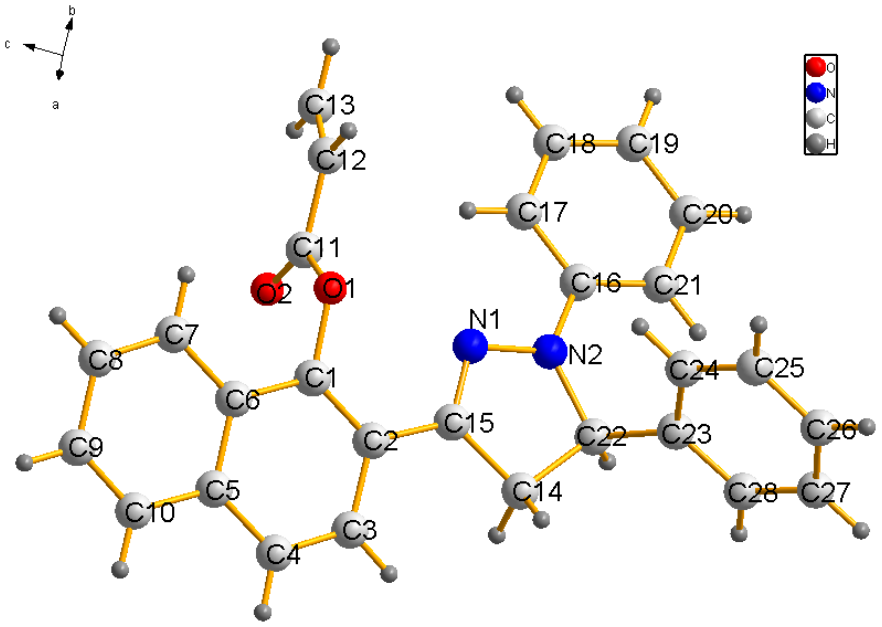
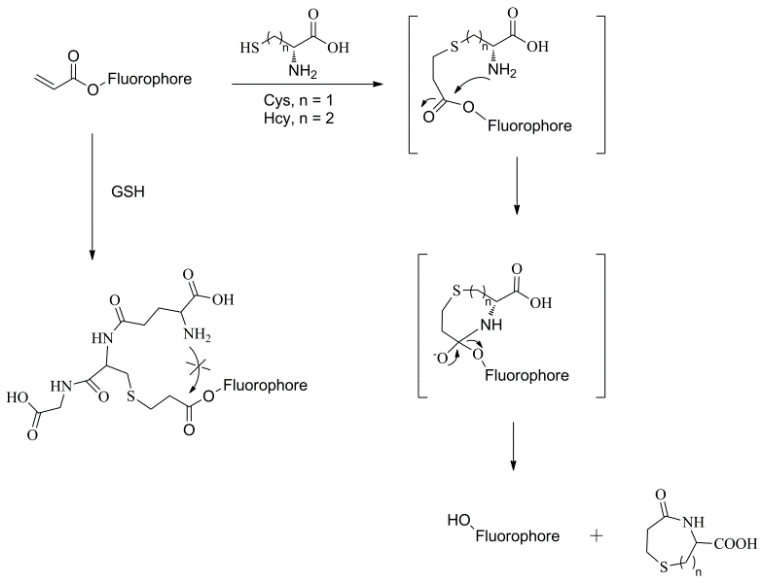


Fig. S1 Crystal structure of probe **PPN**.

4. Supplementary figures



Scheme S2. Proposed mechanism for the sensing of Cys, Hcy and GSH when using acrylate group.

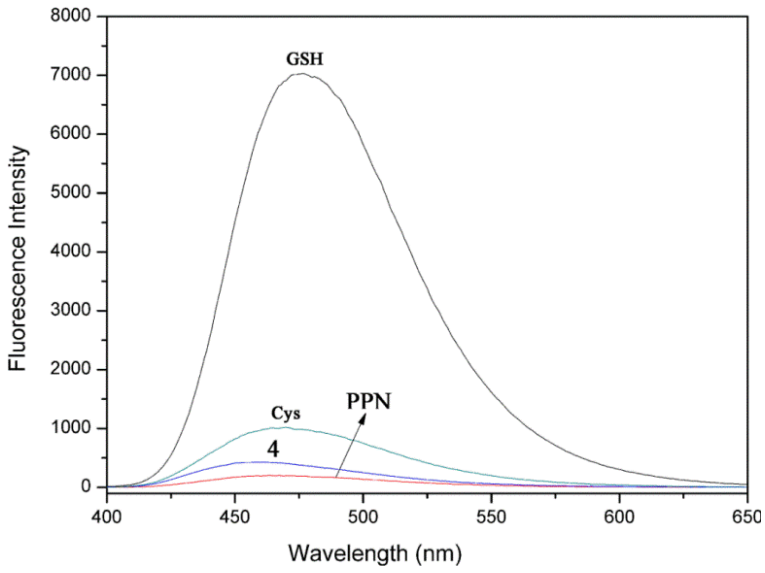


Fig. S2 Fluorescence spectra of compound **4**, **PPN** (2 μM) with or without GSH or Cys (40 μM) in PBS buffer (CTAB 1 mM, pH 7.4, λ_{ex} = 390 nm).

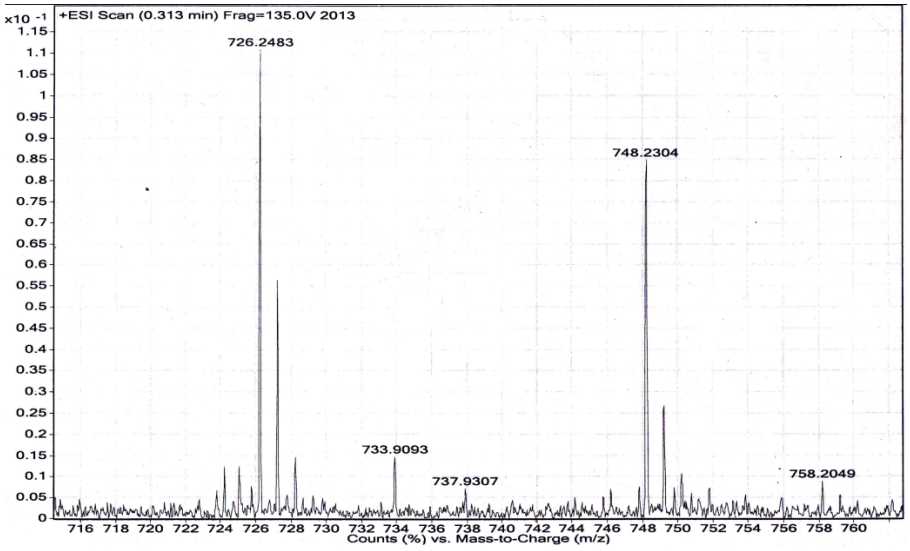


Fig. S3 High resolution mass spectrum of the conjugate addition adduct of **PPN** with GSH.

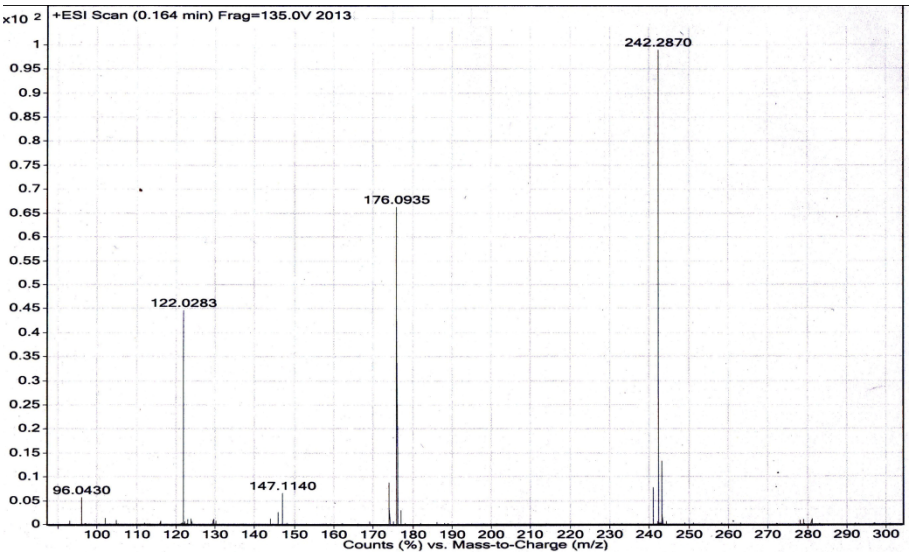


Fig. S4 High resolution mass spectrum of the conjugate addition/cyclization product of **PPN** with Cys.

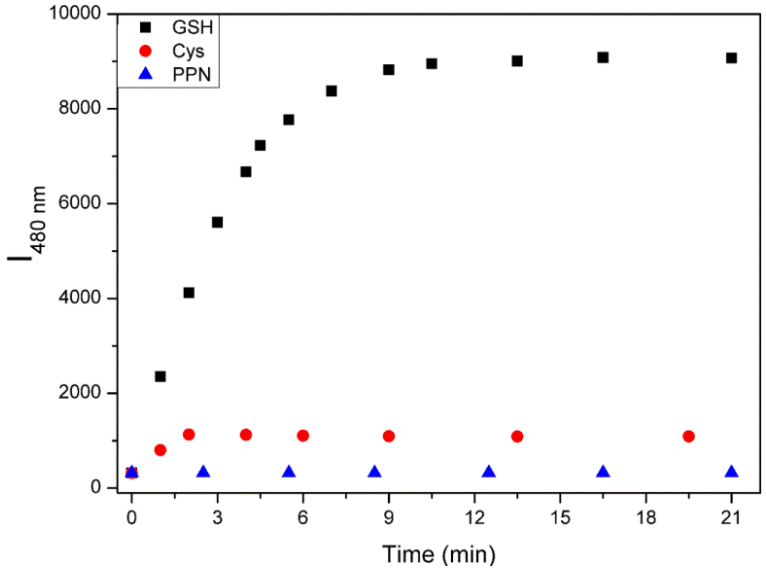


Fig. S5 Time-dependent fluorescent intensity changes of probe **PPN** (2 μM) with or without GSH/Cys (40 μM) in PBS buffer (CTAB 1 mM, pH 7.4, λ_{ex} = 390 nm).

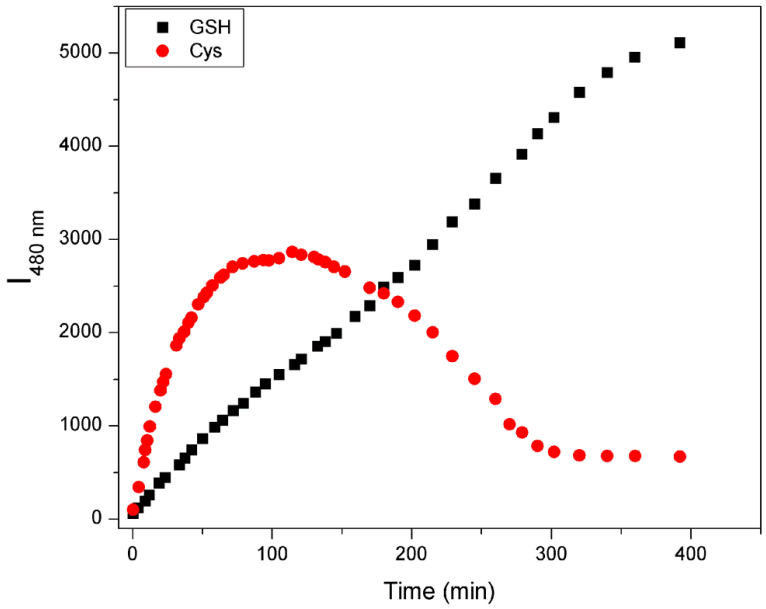


Fig. S6 Time-dependent fluorescent intensity changes of probe **PPN** (2 μM) with GSH or Cys (40 μM) in PBS buffer (PBS/CH_3CN = 1:1, v/v, pH 7.4, λ_{ex} = 390 nm).

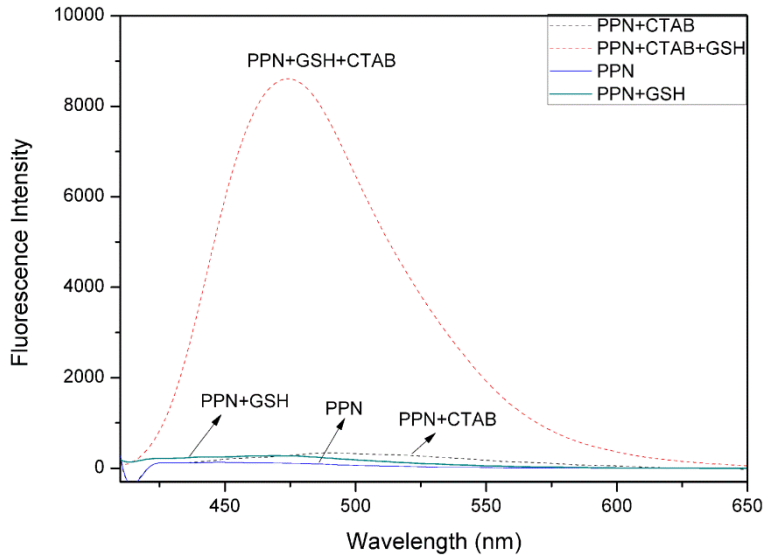


Fig. S7 Fluorescence spectra of **PPN** (2 μM), **PPN** (2 μM) + GSH (40 μM), **PPN** (2 μM) + CTAB (1 mM) and **PPN** (2 μM) + GSH (40 μM) + CTAB (1 mM) in PBS buffer (pH 7.4, λ_{ex} = 390 nm).

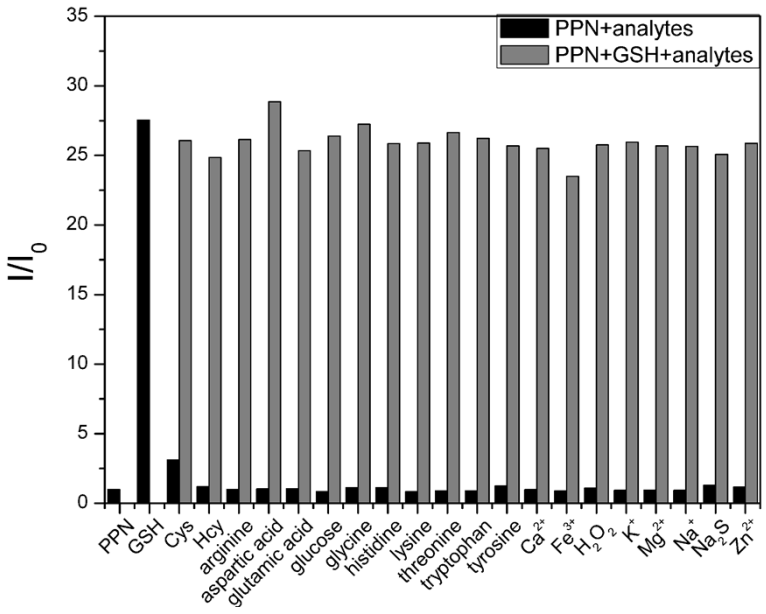


Fig. S8 Fluorescence ratio ($I_{480\ nm}$) of **PPN** (2 μM) without or with GSH (40 μM) in the presence of various analytes (40 μM) in PBS buffer (CTAB 1 mM, pH 7.4, λ_{ex} = 390 nm).

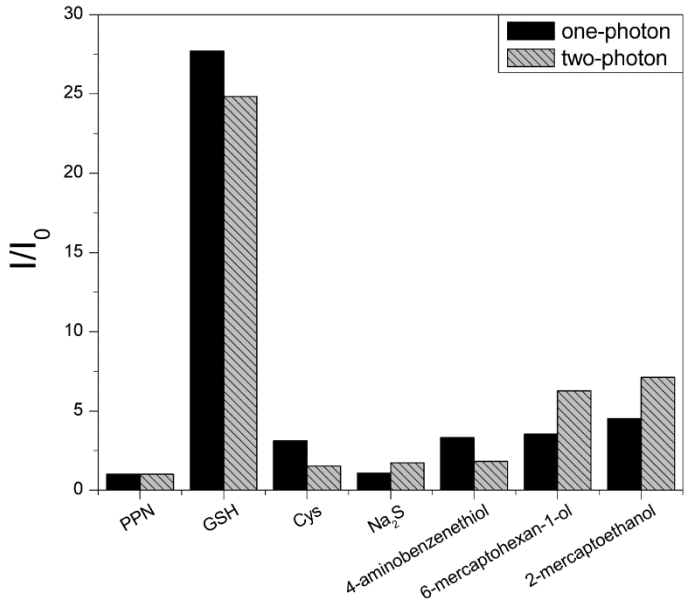


Fig. S9 Fluorescence ratio ($I_{480\ nm}$) of **PPN** (2 μM) with various sulfhydryl analytes (40 μM) in PBS buffer (CTAB 1 mM, pH 7.4, one-photon λ_{ex} = 390 nm, two-photon λ_{ex} = 800 nm).

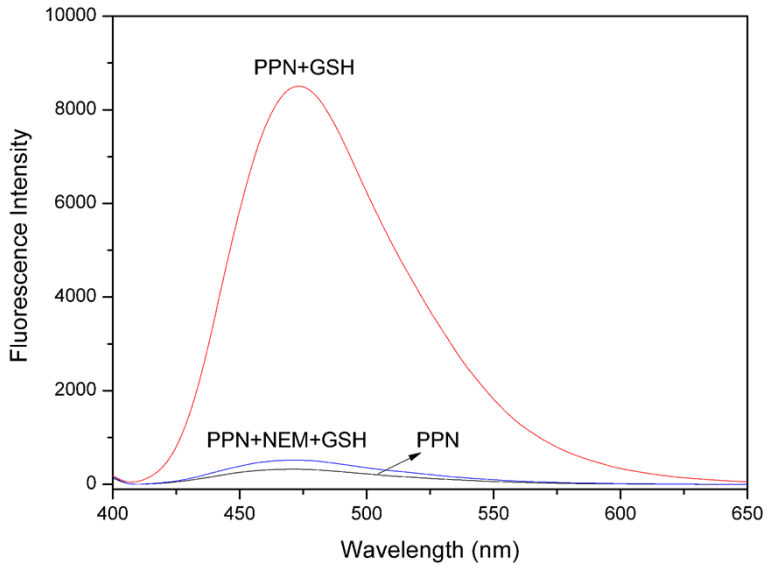


Fig.S10 Fluorescence spectra of **PPN** (2 μM), **PPN** (2 μM) + GSH (40 μM) and **PPN** (2 μM) + GSH (40 μM) + NEM (1 mM) in PBS buffer (CTAB 1 mM, pH 7.4, λ_{ex} = 390 nm).

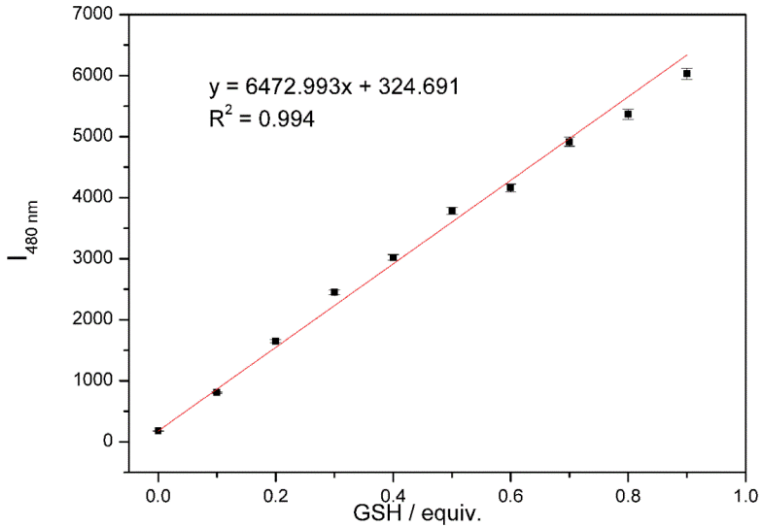


Fig. S11 The plot of fluorescent intensity at 480 nm of probe **PPN** (2 μM) vs equivalents of GSH in PBS buffer (CTAB 1 mM, λ_{ex} = 390 nm, pH 7.4). Data are mean±SE (bars) (n = 3).

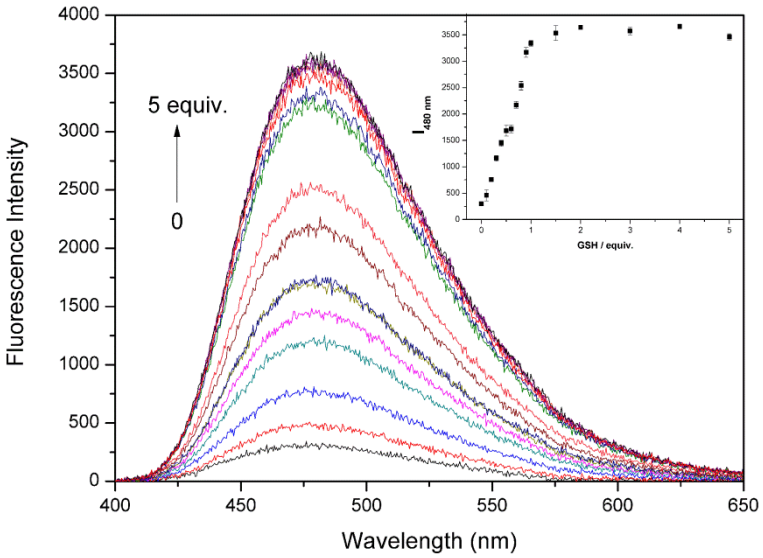


Fig. S12 Two-photon fluorescence spectra of probe **PPN** (2 μM) with GSH (0-5 equiv.) in PBS buffer (CTAB 1 mM, λ_{ex} = 800 nm, pH 7.4). Inset is the plot of two-photon fluorescent intensity of probe **PPN** vs equivalents of GSH. Data are mean±SE (bars) (n = 3).

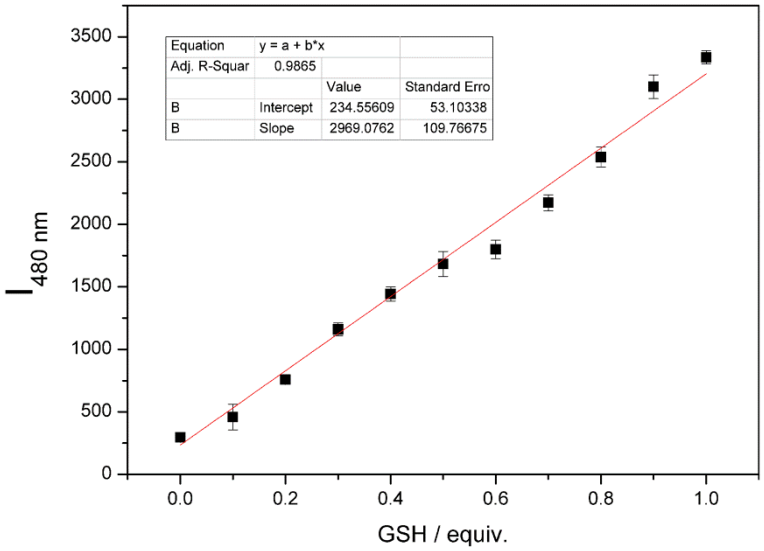


Fig. S13 The plot of two-photon fluorescent intensity at 480 nm of probe **PPN** (2 μM) vs equivalents of GSH in PBS buffer (CTAB 1 mM, λ_{ex} = 800 nm, pH 7.4). Data are mean±SE (bars) (n = 3).

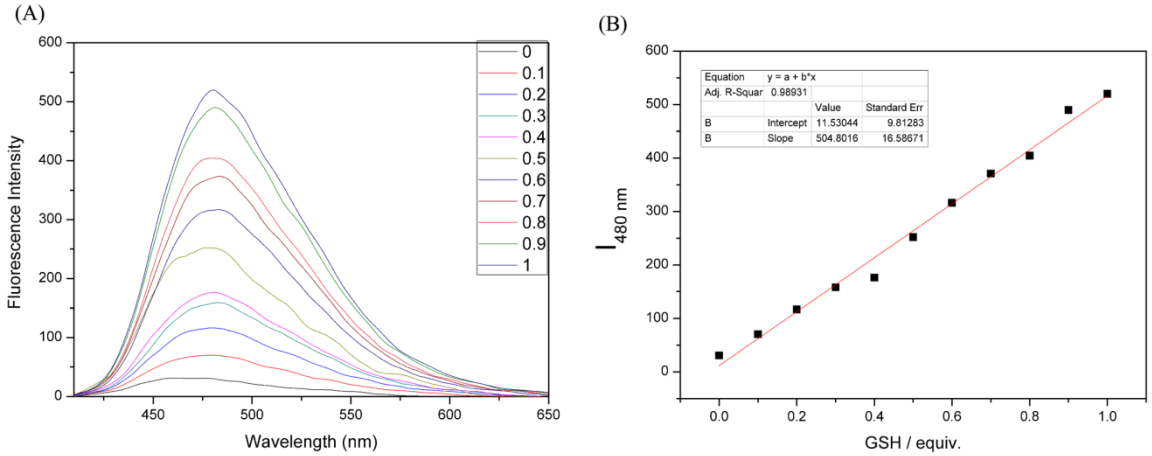


Fig. S14 (A) Fluorescence spectra of probe **PPN** (2 μM) with GSH (0-1.0 equiv.) in PBS buffer; (B) The plot of fluorescent intensity at 480 nm of probe **PPN** (2 μM) vs equivalents of GSH in PBS buffer (PBS/CH_3CN = 1:1, v/v, pH 7.4, λ_{ex} = 390 nm, the samples were measured after incubated 7 h).

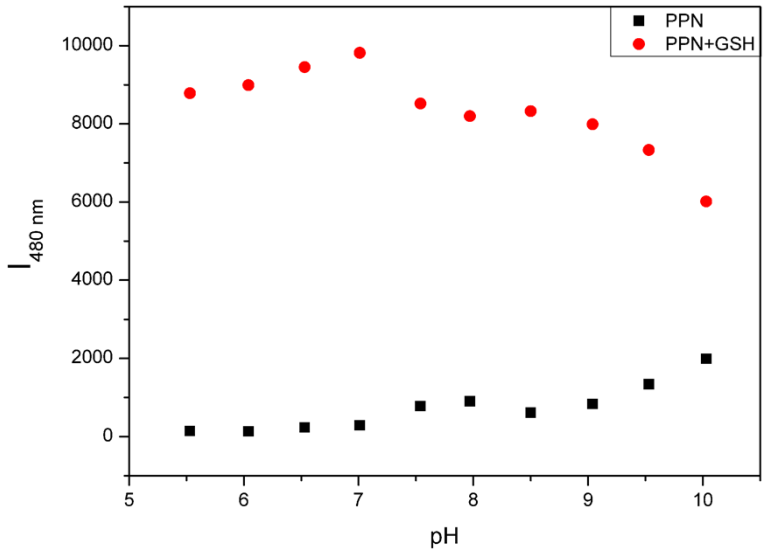


Fig. S15 Fluorescent intensity of **PPN** (2 μM) without and with GSH (40 μM) in PBS buffer (CTAB 1 mM, λ_{ex} = 390 nm, pH 5.53 - 10.00).

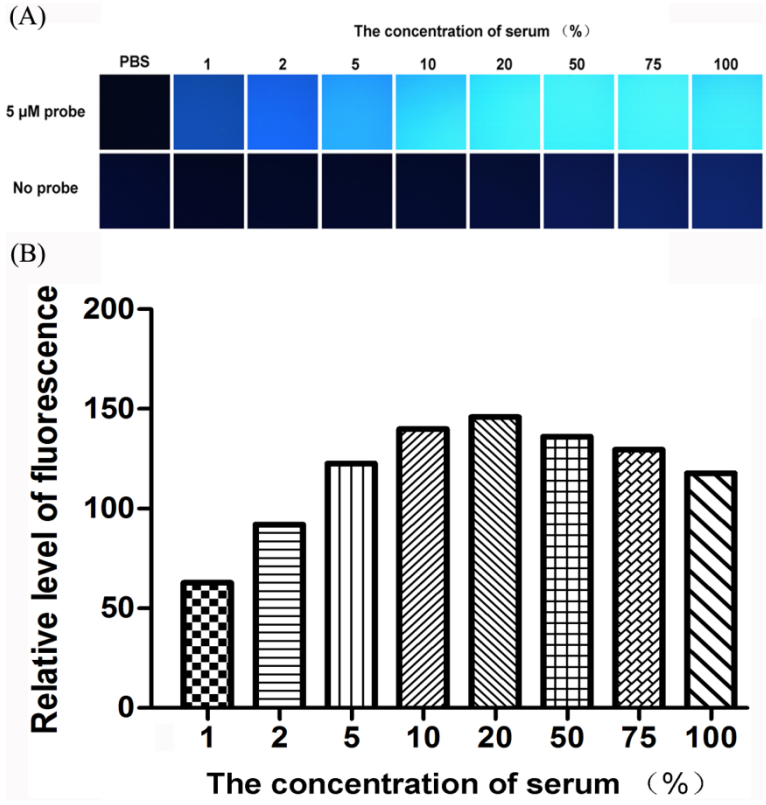


Fig. S16 (A) Fluorescence images of probe **PPN** (5 μM) with different concentrations of calf serum. Calf serum was diluted 100 times (1%), 50 times (2%), 20 times (5%),

10 times (10%), 5 times (20%), 2 times (50%), 4/3 times (75%) with PBS and not diluted (100%). (n > 3) (B) Quantified data of fluorescence images A. The deducted background fluorescence includes two data: **PPN** (5 μM) in PBS and corresponding different concentrations of calf serum.

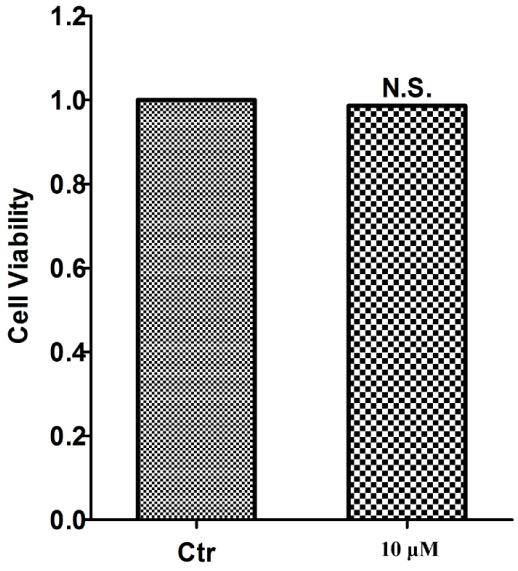


Fig. S17 Viability of A549 cells incubated with probe **PPN** for 6 h.

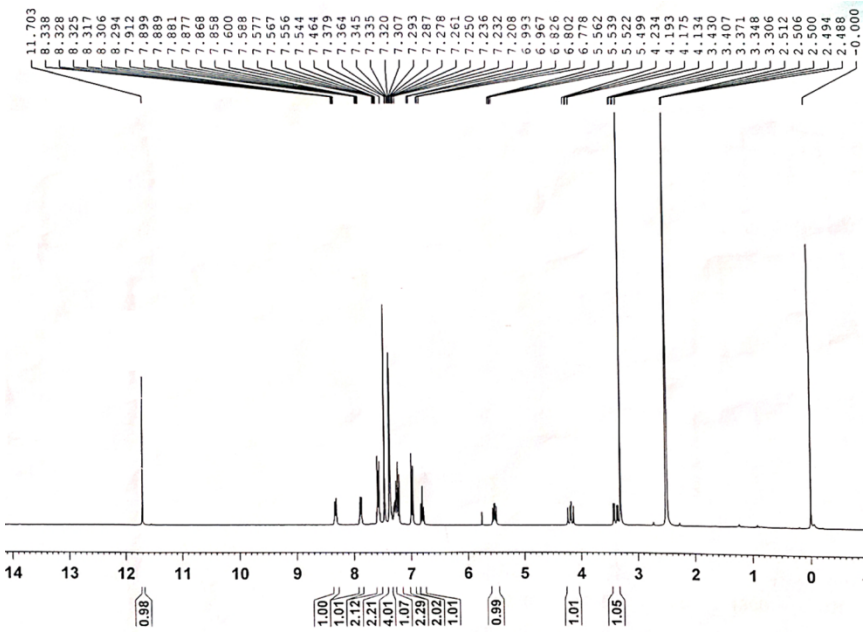


Fig. S18 ^{1}H NMR spectrum of compound **4** (DMSO-d_6).

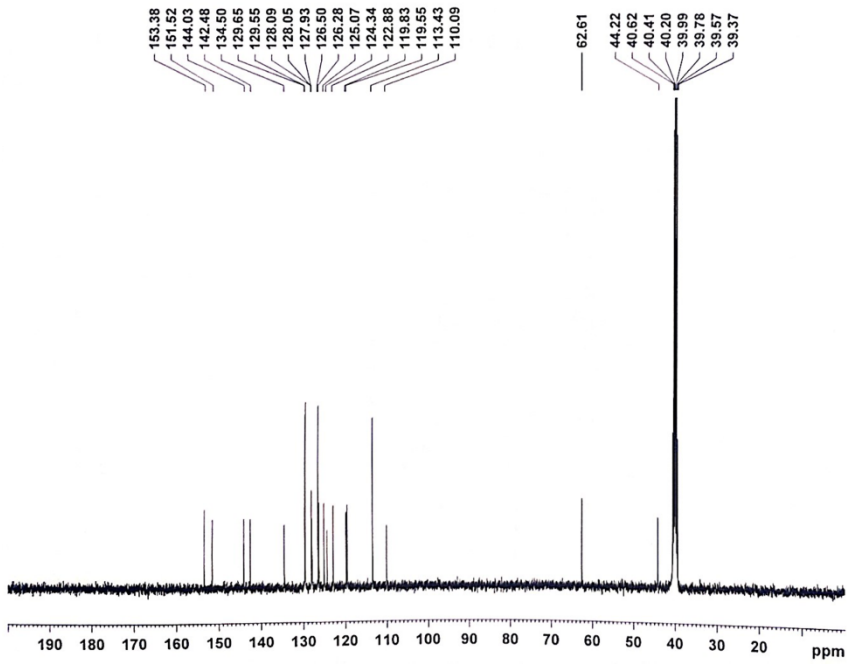


Fig. S19 ^{13}C NMR spectrum of compound **4** (DMSO-d_6).

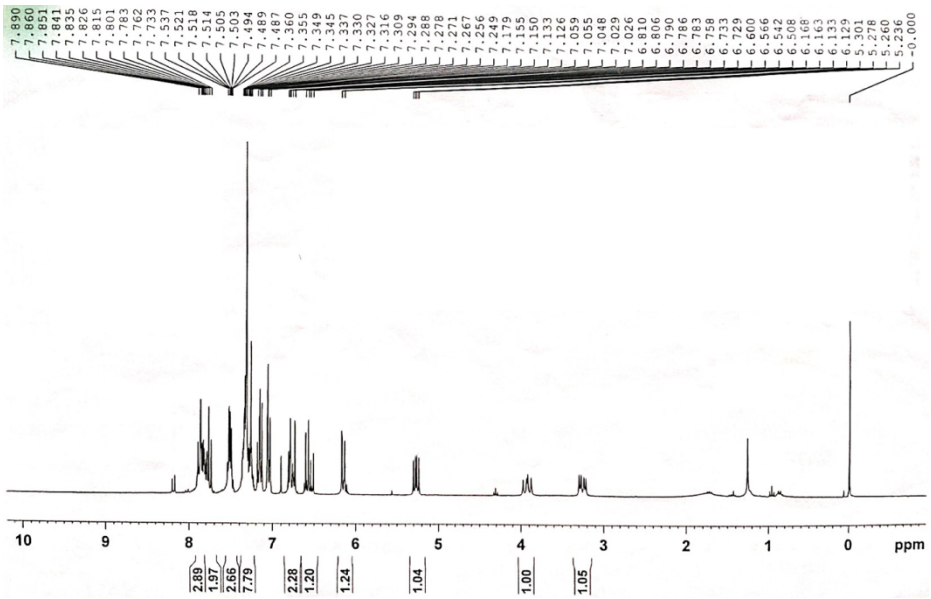


Fig. S20 ^{1}H NMR spectrum of probe **PPN** ($CDCl_3$).

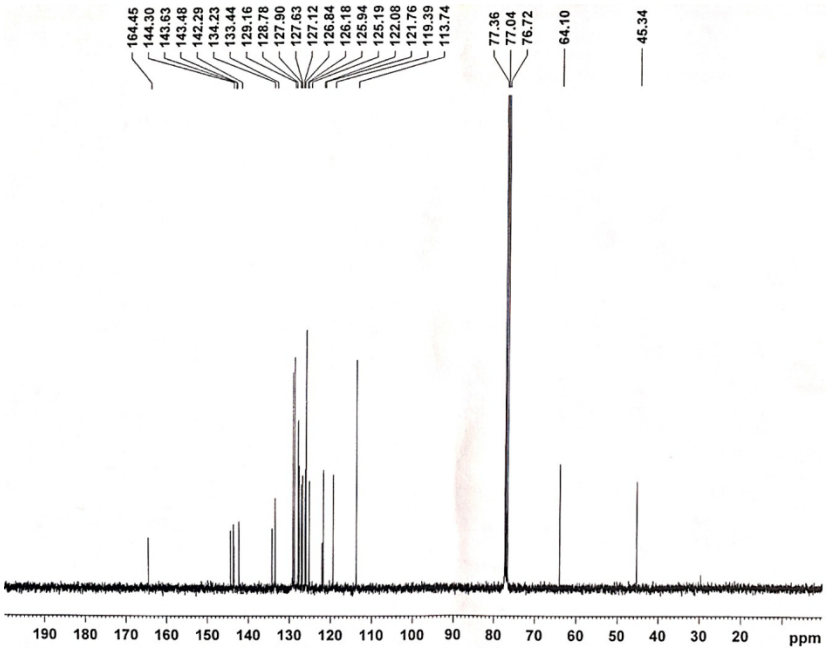


Fig. S21 ^{13}C NMR spectrum of probe **PPN** ($CDCl_3$).

References

1. J. W. Eastman, *Photochem. Photobiol.*, 1967, **6**, 55; A. N. Fletcher, *Photochem. Photobiol.*, 1969, **9**, 439; G. Li, D. Zhu, L. Xue and H. Jiang, *Org. Lett.*, 2013, **15**, 5020.