Supporting Information

A Near Infrared Fluorescence Probe for Detection and Imaging Prolyl Aminopeptidase Activity in Living Cells

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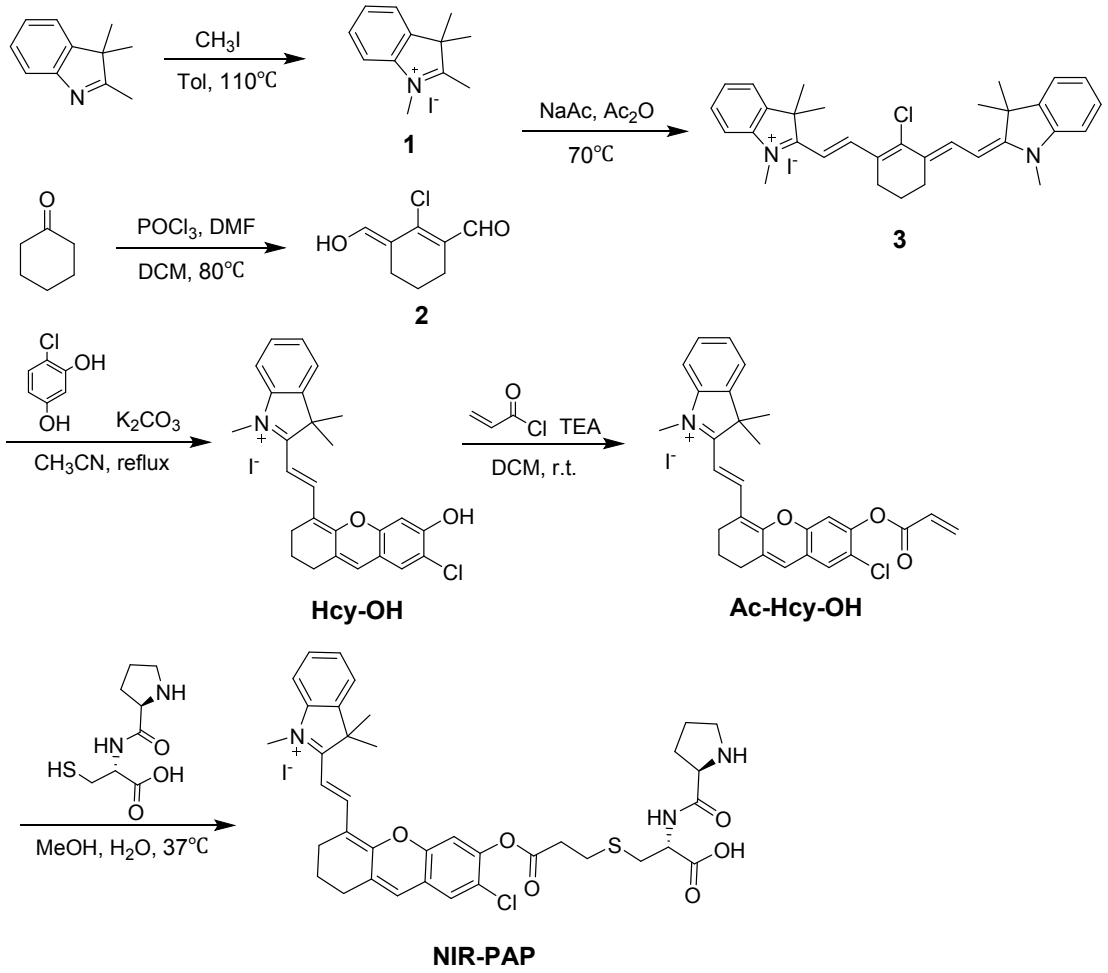
Scheme S1. Synthesis routes.



Synthesis of 1,2,3,3-tetramethyl-3H-indol-1-ium iodide (1). To a solution of iodomethane (3.4 g, 24 mmol) in toluene (30 mL), 2,3,3-trimethyl-3H-indole (2.5 g, 12.0 mmol) was added. After stirring at 110 °C overnight, the solid product was collected by filtration, washed with diethy ether (150 mL) and EtOAc (150 mL). Then it was dried under vacuum to afford the product with a yield of 81%. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.91-7.93 (1H, m, Ar-H), 7.83-7.85 (1H, m, Ar-H), 7.59-7.65 (2H, m, Ar-H), 3.99 (3H, s, CH_3), 2.79 (3H, s, CH_3), 1.54 (3H, s, 2 × CH_3). ^{13}C NMR (100MHz, DMSO-d_6) δ (ppm): 196.47, 142.58, 142.08, 129.78, 129.28, 123.78, 115.61, 54.42, 35.28, 22.20, 14.74. MS (ESI) m/z: $[M-I]^+$ calcd 174.13, found 174.20.

Synthesis of 2-chloro-3-(hydroxymethylene) cyclohex enecarbaldehyde (2). A solution of $POCl_3$ (4.5 mL, 48 mmol) in dichloromethane (10.0 mL) was slowly added to an ice-cooled solution of DMF (4.8 mL, 63 mmol) in DCM (10 mL). Cyclohexanone (1.3 mL, 12 mmol) was then added in via a syringe. The reaction mixture was refluxed for 3 h at 80 °C and then cooled in ice. Ice water (200 mL) was added slowly while stirring. The precipitate was then filtered, washed and dried to obtain compound 2 as a pale yellow solid with a yield of 79%.

Synthesis of 2-((E)-2-((E)-2-chloro-3-(2-((E)-1,3,3-trimethylindolin-2-ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-1,3,3-trimethyl-3H-indol-1-ium iodide (3). Sodium acetate (0.6 g, 6.6 mmol) was added to a solution of compound **1** (2.3 g, 6.6 mmol) and compound **2** (0.5 g, 3.3 mmol) in acetic anhydride (15 mL). Then the mixture was heated to 70 °C for 2 h, resulting in a green solution. The mixture was washed with saturated aqueous sodium bicarbonate. The solution was then extracted with DCM and concentrated in vacuum. The crude product was purified by silica gel column chromatography with DCM/MeOH (30/1, v/v) as the eluent to give compound **3** as a green solid with a yield of 56%. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm): 8.35 (2H, d, J =14.0 Hz, 2 × CH=), 7.38-7.43 (4H, m, Ar-H), 7.20-7.29 (4H, m, Ar-H), 6.22 (2H, d, J

=14.0 Hz, 2 × CH=), 3.76 (6H, s, 2 × CH_3), 2.75 (4H, t, J =6.0 Hz, 2 × CH_2), 1.95-2.01 (2H, m, CH_2), 1.73 (12H, s, 4 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm): 172.88, 150.64, 144.36, 142.76, 140.90, 128.83, 127.72, 125.35, 122.13, 110.85, 101.64, 49.25, 32.61, 28.08, 26.75, 20.69. MS (ESI) m/z: $[M-I]^+$ calcd 483.26, found 483.18.

Synthesis of (E)-7-chloro-4-(2-(1,3,3-trimethyl-3H-1l4-indol-2-yl)vinyl)-2,3-dihydro-1H-xanthen-6-ol (Hcy-OH). 4-chlorobenzene-1,3-diol (0.13 g, 0.9 mmol) and potassium carbonate (0.12 g, 0.9 mmol) were mixed in acetonitrile (5.0 mL), and the mixture was stirred for 10 min at room temperature under an argon atmosphere. Then compound **3** (0.18 g, 0.3 mmol) in acetonitrile (10.0 mL) was added to the mixture, followed by heating at 80 °C for 5 h. After removal of the solvent under reduced pressure, the crude product was purified by silica gel chromatography with DCM/MeOH (40/1, v/v) as the eluent to give Hcy-OH as a green solid with a yield of 32%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.11 (1H, d, J =13.6 Hz, Ar-H), 7.44 (1H, s, Ar-H), 7.29-7.33 (3H, m, Ar-H), 7.09 (1H, t, J =7.6 Hz, Ar-H), 6.86 (1H, d, J =8.0 Hz, Ar-H), 6.72 (1H, s, Ar-H), 5.61 (1H, d, J =13.6 Hz, CH=), 3.38 (3H, s, CH_3), 2.63-2.73 (4H, m, 2 × CH_2), 1.88-1.97 (2H, m, CH_2), 1.69 (6H, s, 2 × CH_3). ^{13}C NMR (100MHz, $CDCl_3$) δ (ppm): 174.84, 166.49, 160.23, 157.85, 143.77, 139.59, 133.01, 132.25, 128.14, 127.25, 122.46, 122.11, 116.68, 116.16, 115.59, 107.82, 103.82, 94.45, 47.49, 31.51, 29.70, 28.61, 24.48, 21.29. MS (ESI) m/z: $[M-I]^+$ calcd 418.16, found 418.17.

MTT assay. The cytotoxicity of probe against HepG-2 cells, HeLa cells, MCF-7 cells and L-02 cells was studied using a standard methyl thiazolyltetrazolium (MTT) assay. The cells were seeded into a 96-well plate at 5 × 10^4 cells /well in DMEM including 10% fetal bovine serum (FBS), and the cells were incubated at 37 °C under 5 % CO_2 for 24 h. Then the medium was removed and washed with cold PBS for three times. Cells were then incubated with fresh DMEM medium containing various concentrations of NIR-PAP (5-50 μM) for 12 h. 20

μL of MTT solution (5.0 mg/mL) was then added to each well and the cells were incubated for another 4 h. Afterwards, 100 μL of the supernatant was removed and 150 μL DMSO was added to each well to dissolve the formazan crystals. The cell viability was determined by measuring the absorbance at 450 nm with a microplate reader.

Cell lysate experiment. First, cell lysate with a volume of 100 μL was extracted from 2 × 10^6 cells. P-chloromercuribenzoic acid (200 μM) was mixed with cell lysates (10 μL) and incubated at 37 °C for 3 h. For comparison, cell lysates (10 μL) were also mixed with Tris-HCl buffer and incubated at 37 °C for 1 h. Then, NIR-PAP (10 μM) was added and the solution was incubated at 37 °C for 2 h. All the fluorescence spectra were recorded in the range from 700 nm to 850 nm with an excitation wavelength of 680 nm, using slit widths of 10 nm for both excitation and emission.

HPLC analysis of PAP mediated reactions. The HPLC chromatogram of Hcy-OH, NIR-PAP and its product upon reaction with PAP were performed on a system with a C18 column (150 × 4.6 mm, 5 μm). The conditions were as follows: methanol/H_2O (0.5% CH_3COOH) = 70/30 (v/v); flow rate: 1 mL/min; detection wavelength: 650 nm.

Determination of fluorescence quantum yield. The quantum yields of NIR-PAP and Hcy-OH were determined according to Equation 1, [S1] using ICG as a reference.

$$\Phi_F = \frac{I\,A_R}{I_R\,A}\left(\frac{n}{n_R}\right)\Phi_R \qquad (1)$$

where ΦF is the quantum yield, I is the integrated area under the fluorescence spectra, A is the absorbance, n is the refractive index of the solvent, and R refers to the reference fluorophore.

Reference

S1 D. Magde, R. Wong and P. G. Seybold, *Photochem. Photobiol.*, 2002, **75**, 327-334.

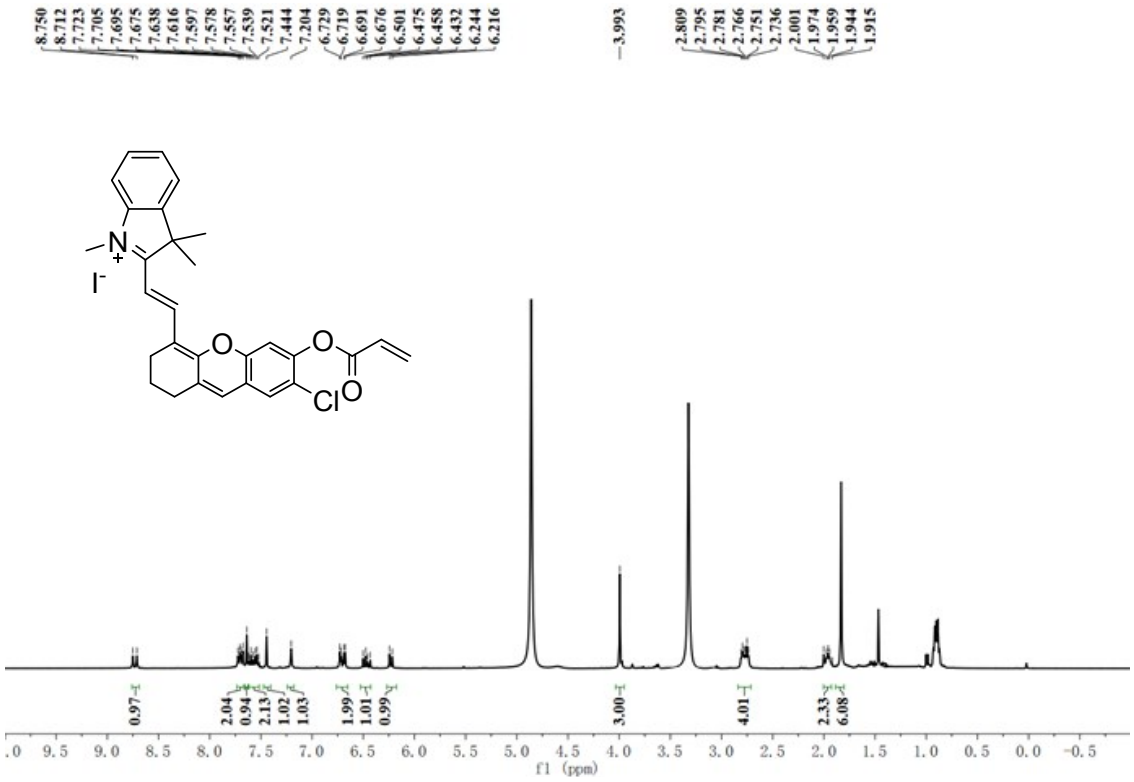


Fig. S1 ^{1}H NMR spectrum of Ac-Hcy-OH in CD_3OD.

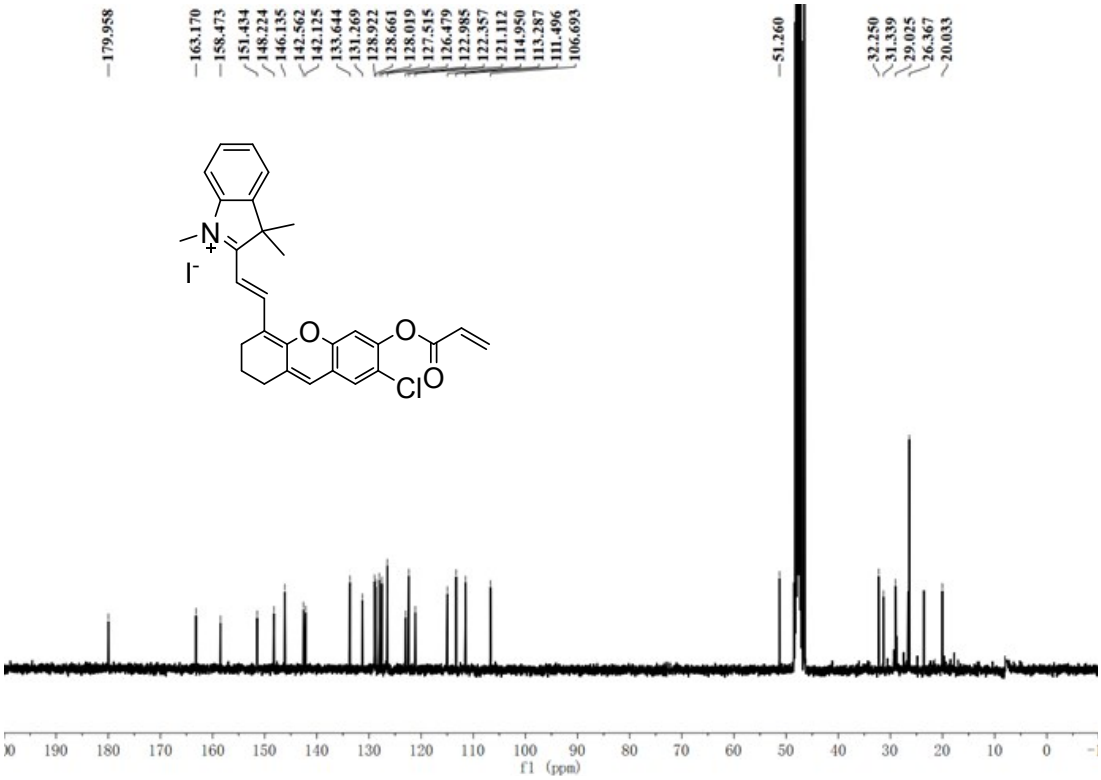


Fig. S2 ^{13}C NMR spectrum of Ac-Hcy-OH in CD_3OD.

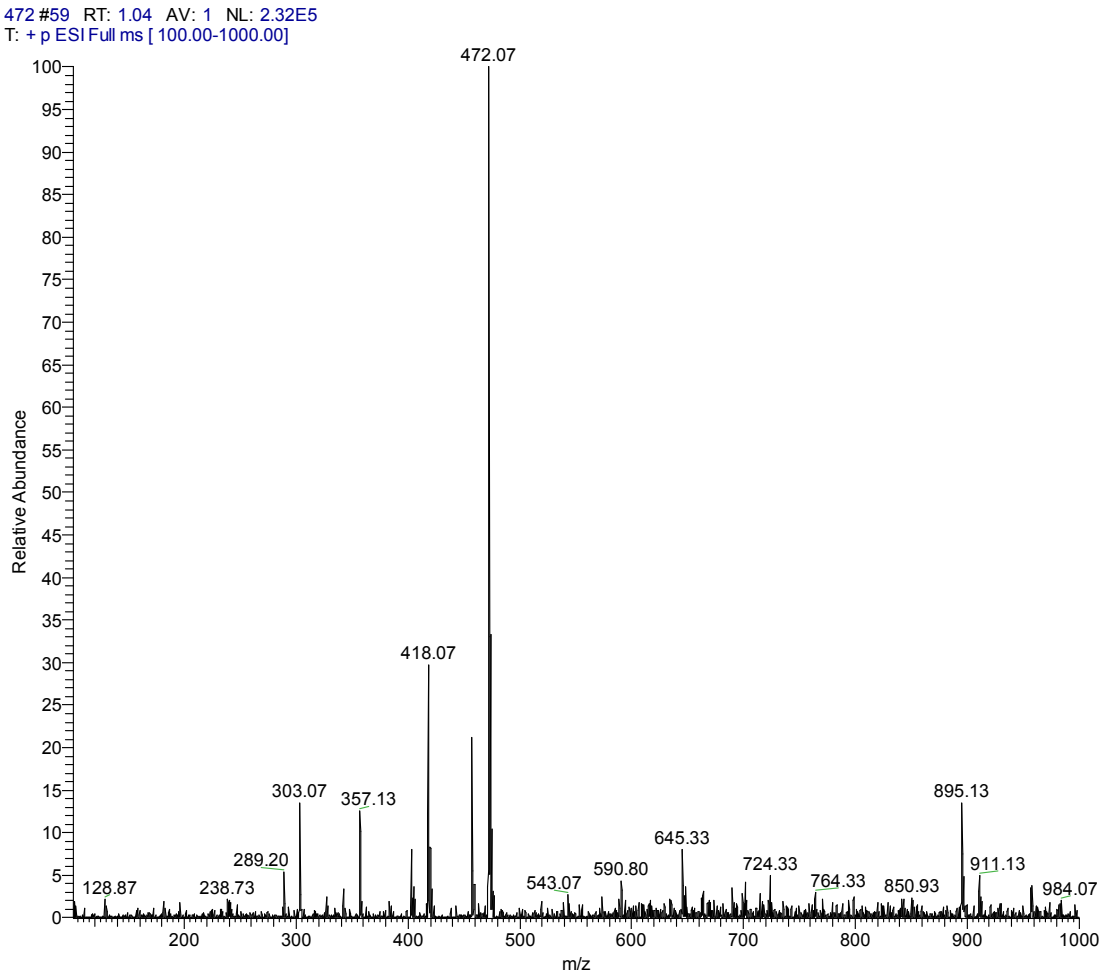


Fig. S3 ESI-MS spectrum of Ac-Hcy-OH.

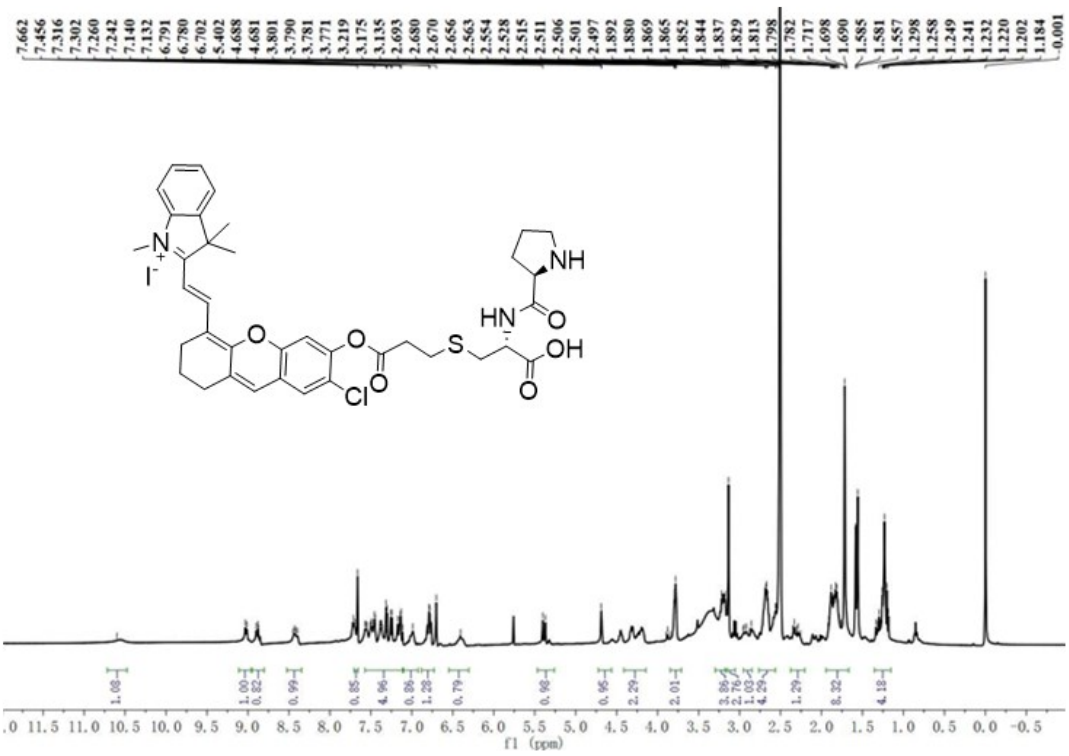


Fig. S4 ^{1}H NMR spectrum of NIR-PAP in DMSO-d_6.

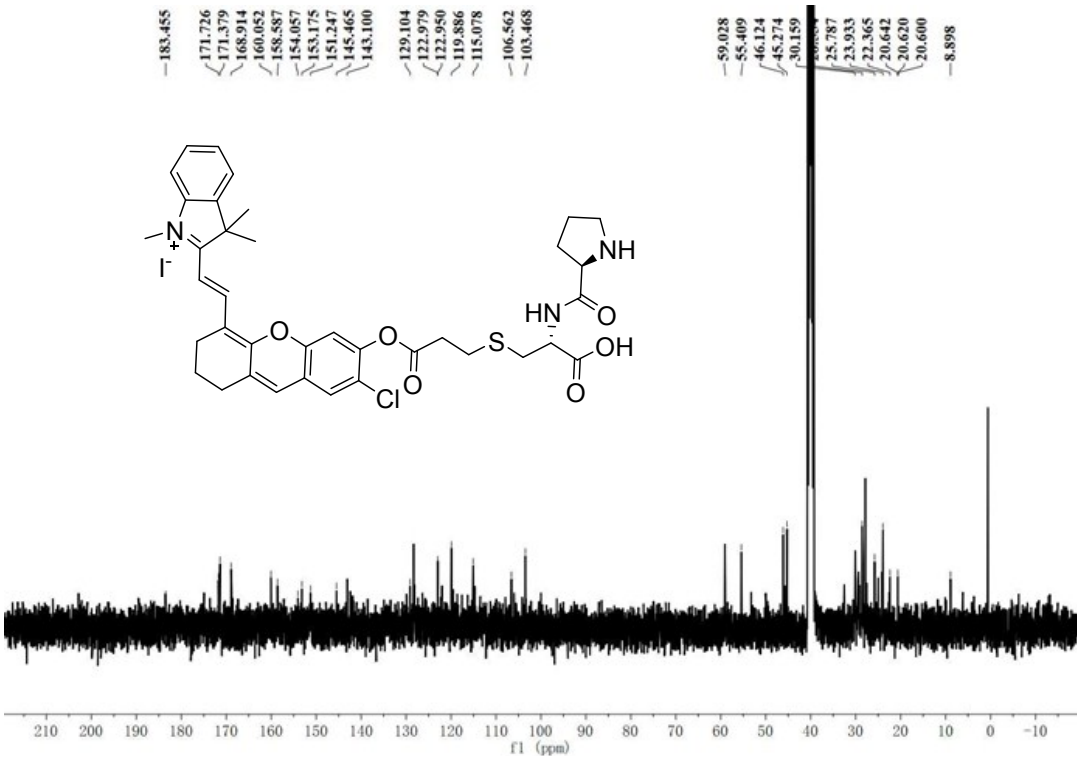


Fig. S5 ^{13}C NMR spectrum of NIR-PAP in DMSO-d_6.

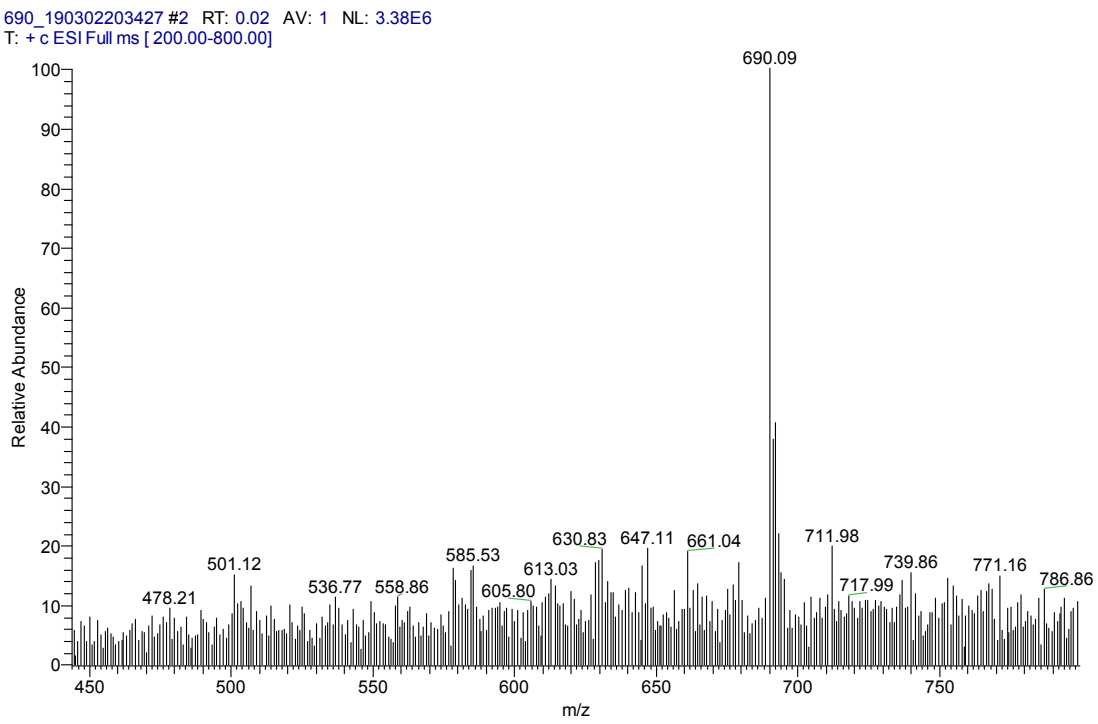


Fig. S6 ESI-MS spectrum of NIR-PAP.

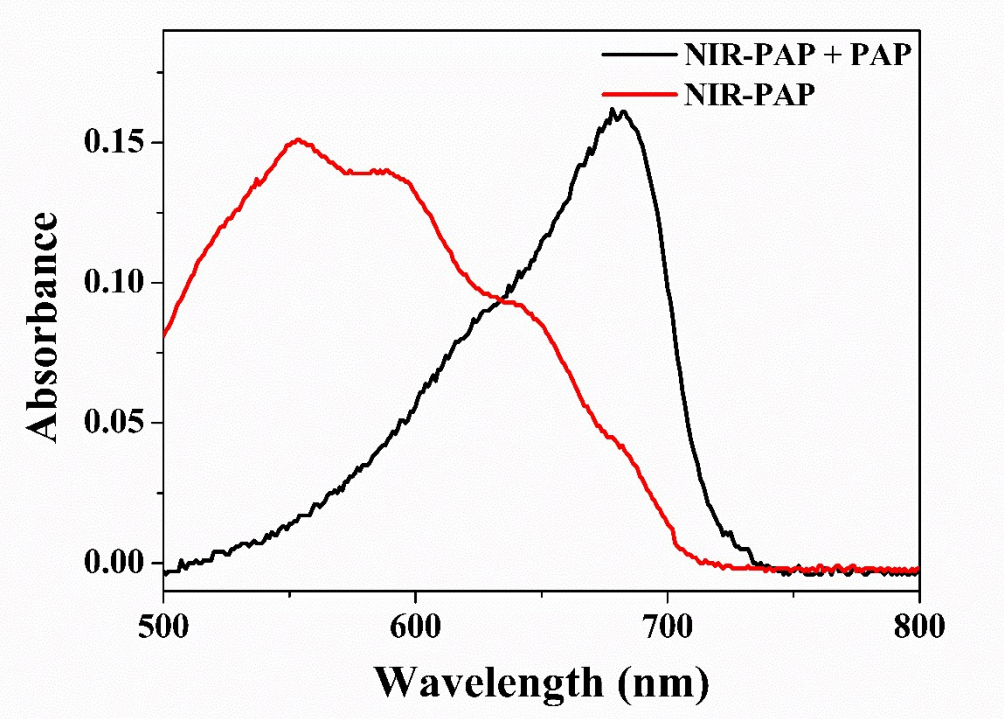


Fig. S7 UV-vis absorption spectra of NIR-PAP (10 μM) before (red line) and after (black line) being reacted with PAP (4.0 U/mL) at 37 °C for 2 h.

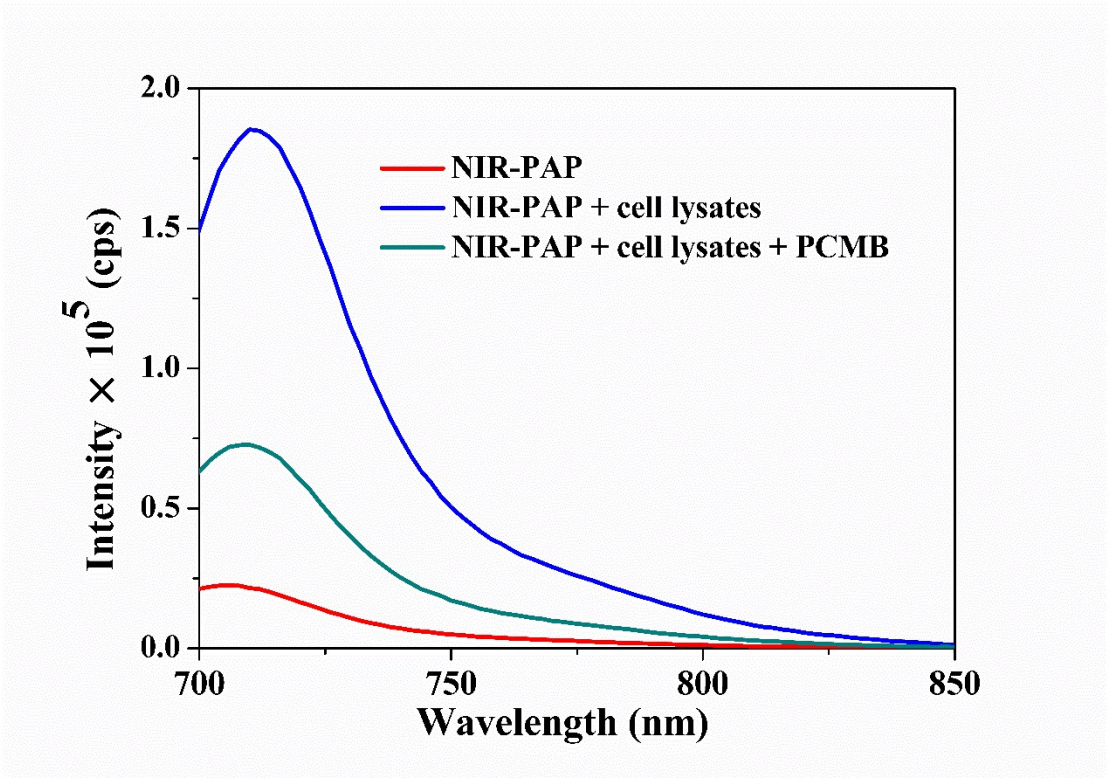


Fig. S8 Fluorescence responses of probe to HepG2 cell lysates. (red line) NIR-PAP (5 μM) alone; (blue line) NIR-PAP (10 μM) + cell extract (10 μL); (green line) NIR-PAP (10 μM) + cell extract (10 μL) + PCMB (200 μM).

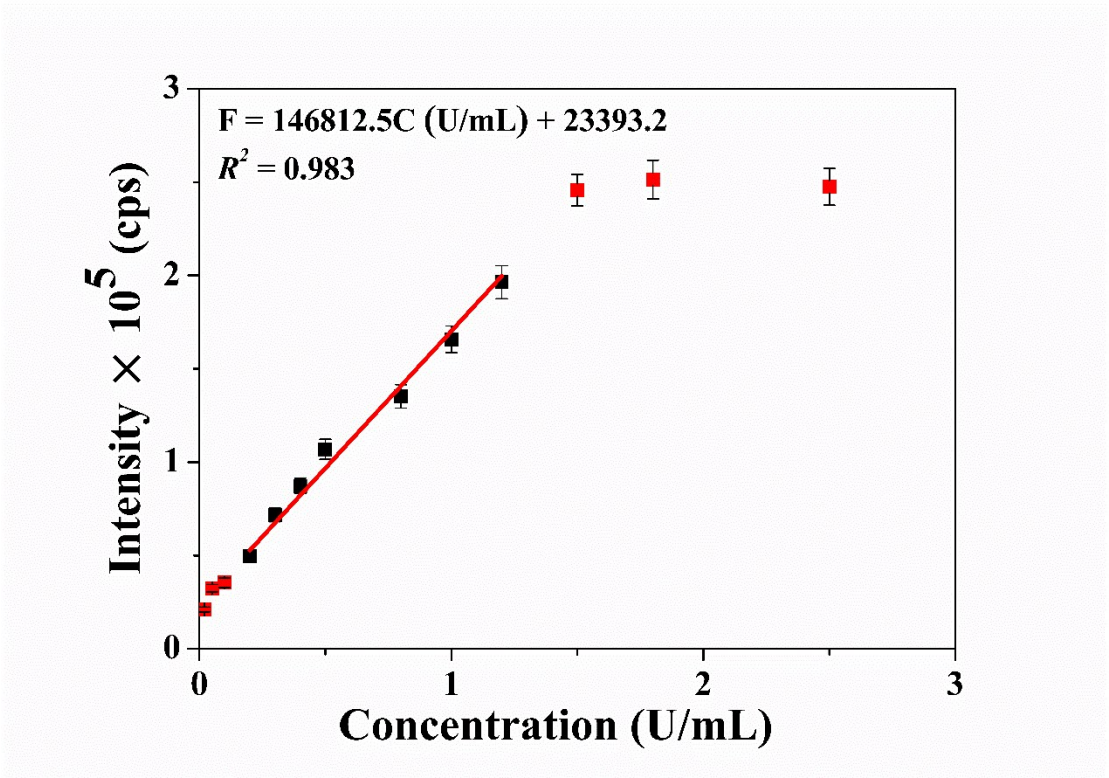


Fig. S9 Linear fitting of fluorescence intensity (F) toward the concentration (C) of PAP from 0.2 to 1.2 U/mL. $\lambda_{ex/em}$ = 680/715 nm.

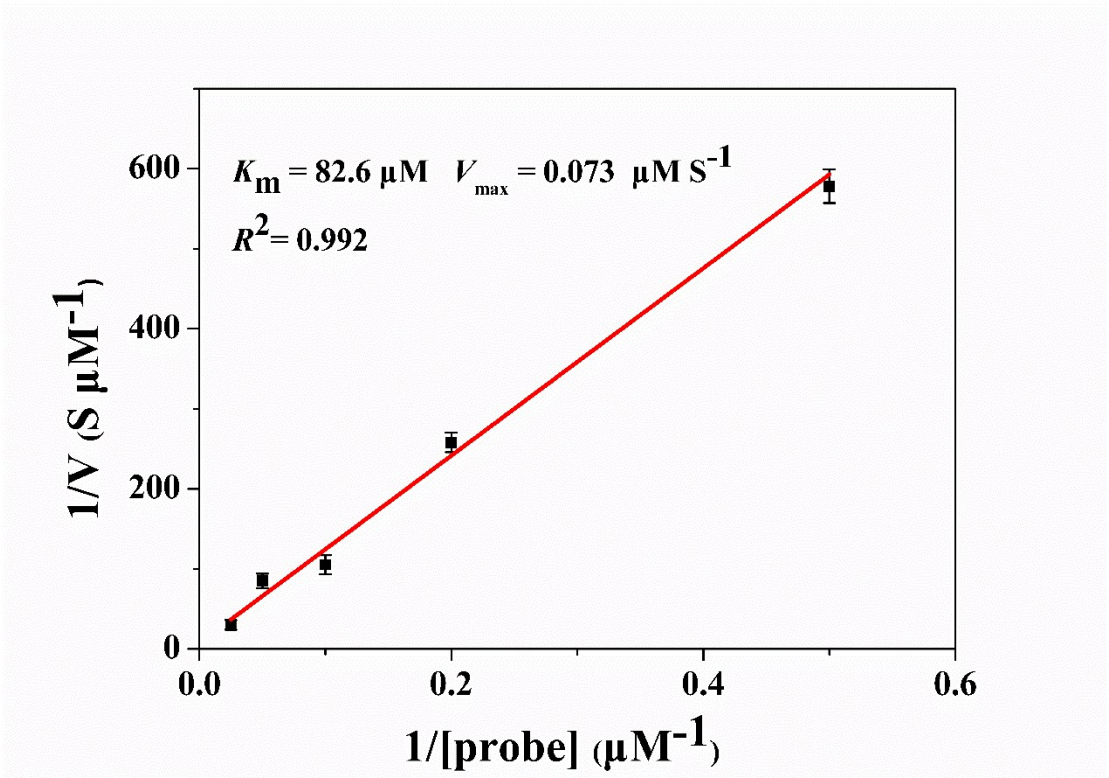


Fig. S10 Lineweaver-Burk plot for the enzyme-catalyzed reaction. The Michaelis-Menten equation was described as: $V = V\text{max}\ [\text{probe}] / (K\text{m}+[\text{probe}])$, where V is the reaction rate, [probe] is the concentration of NIR-PAP (substrate), and Km is the Michaelis constant. Conditions: 1.5 U/mL PAP, 2 - 40 μM of NIR-PAP, λex/em = 680/715 nm.

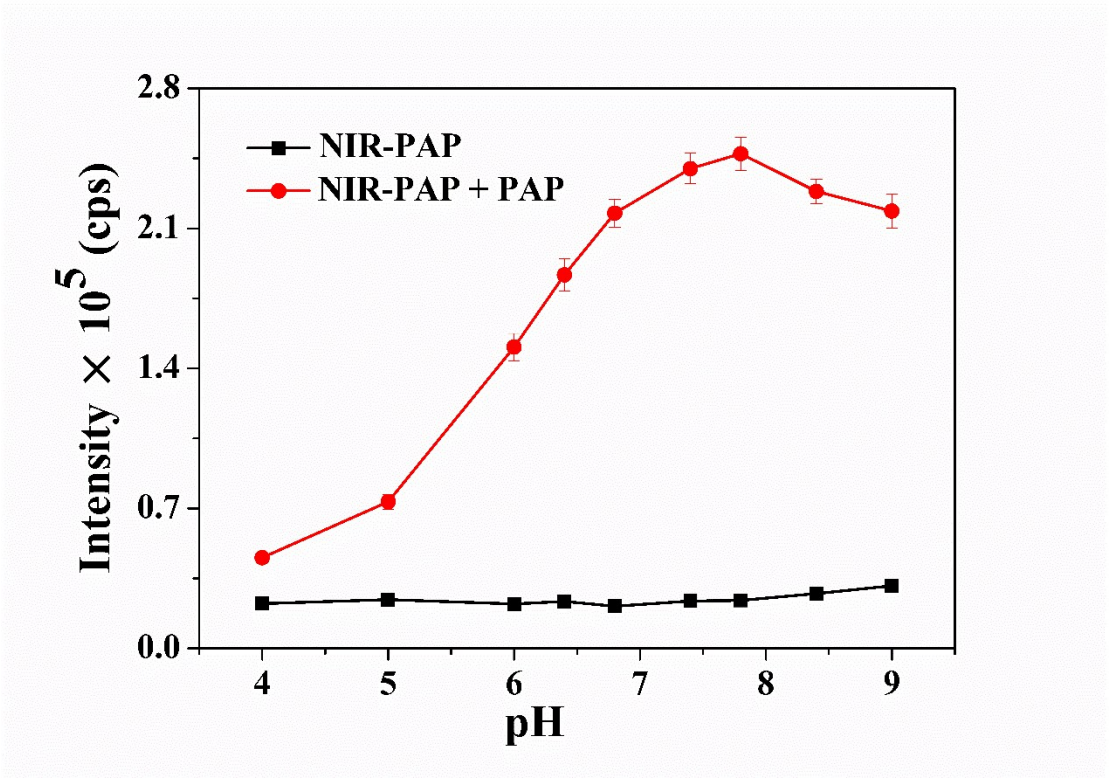


Fig. S11 Effect of pH value on the fluorescence intensity of NIR-PAP (5 μM) and its response toward PAP (1.5 U/mL) at 37 °C for 2 h. λex/em = 680/715 nm.

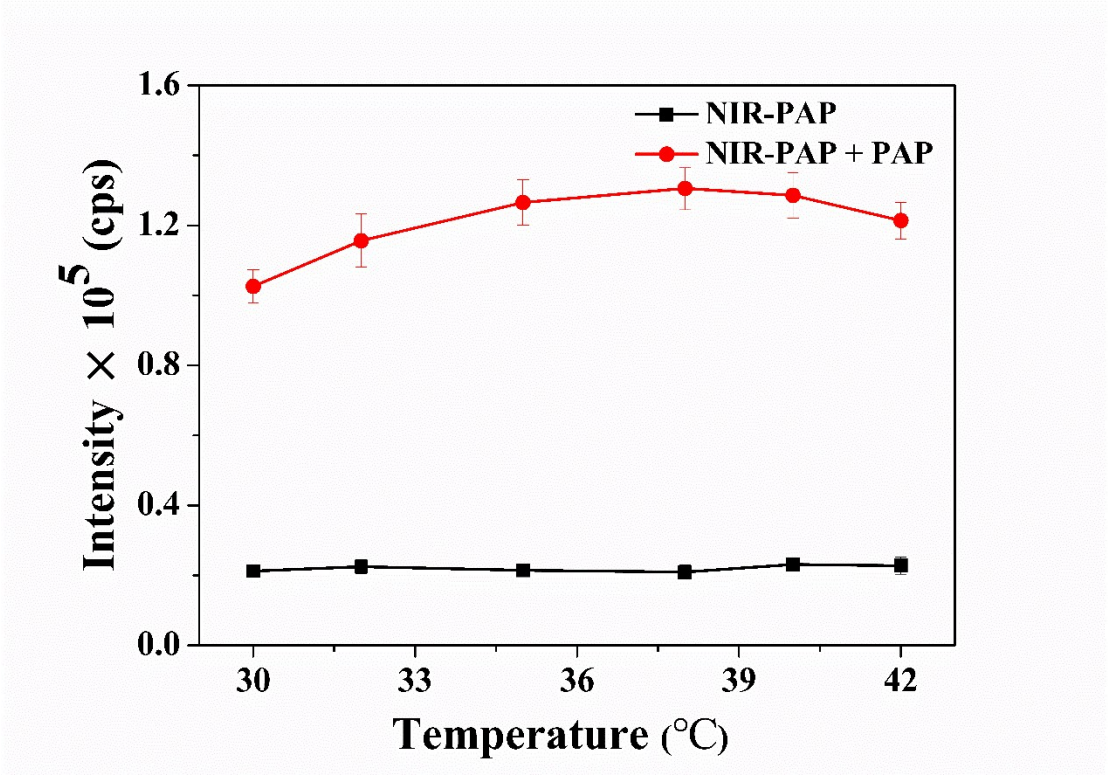


Fig. S12 Effect of temperature on the fluorescence intensity of NIR-PAP (5 μM) and its response with PAP (0.8 U/mL) at 37 °C for 2 h. λex/em = 680/715 nm.

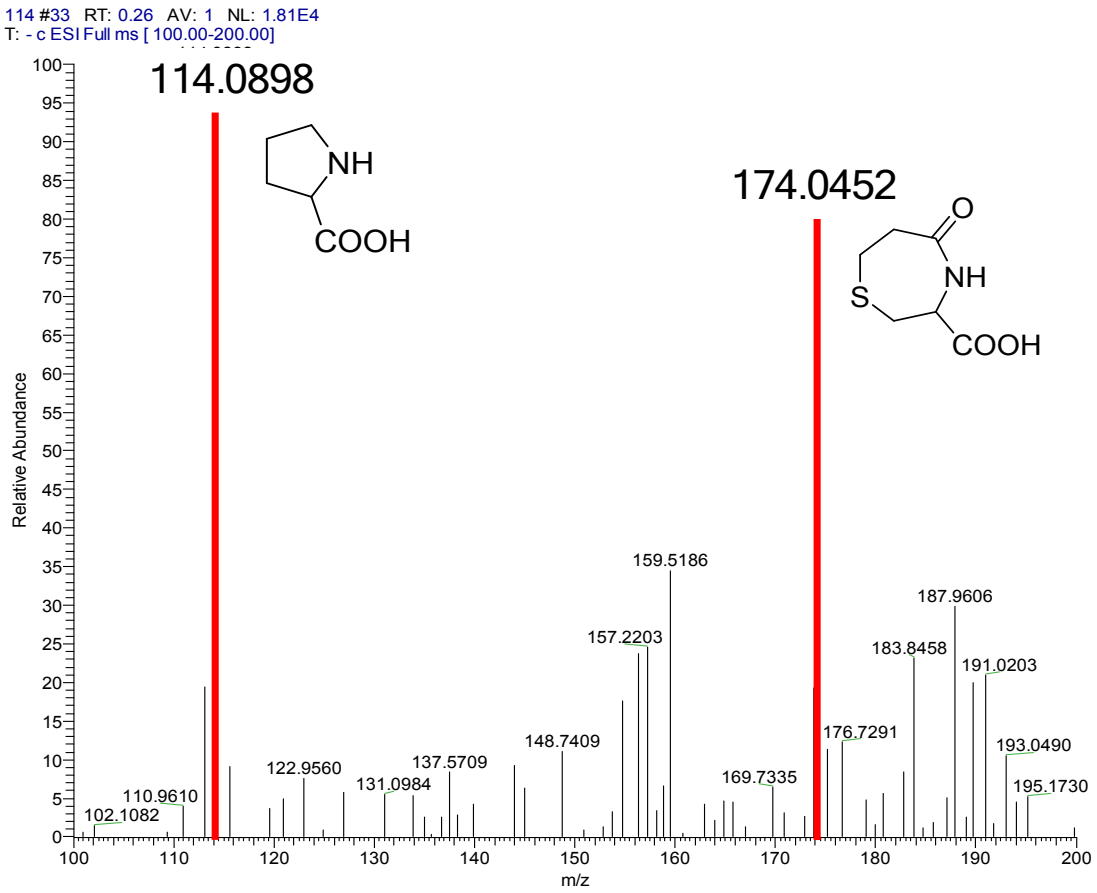


Fig. S13 ESI-MS spectrum of the reaction by-products of NIR-PAP and PAP.

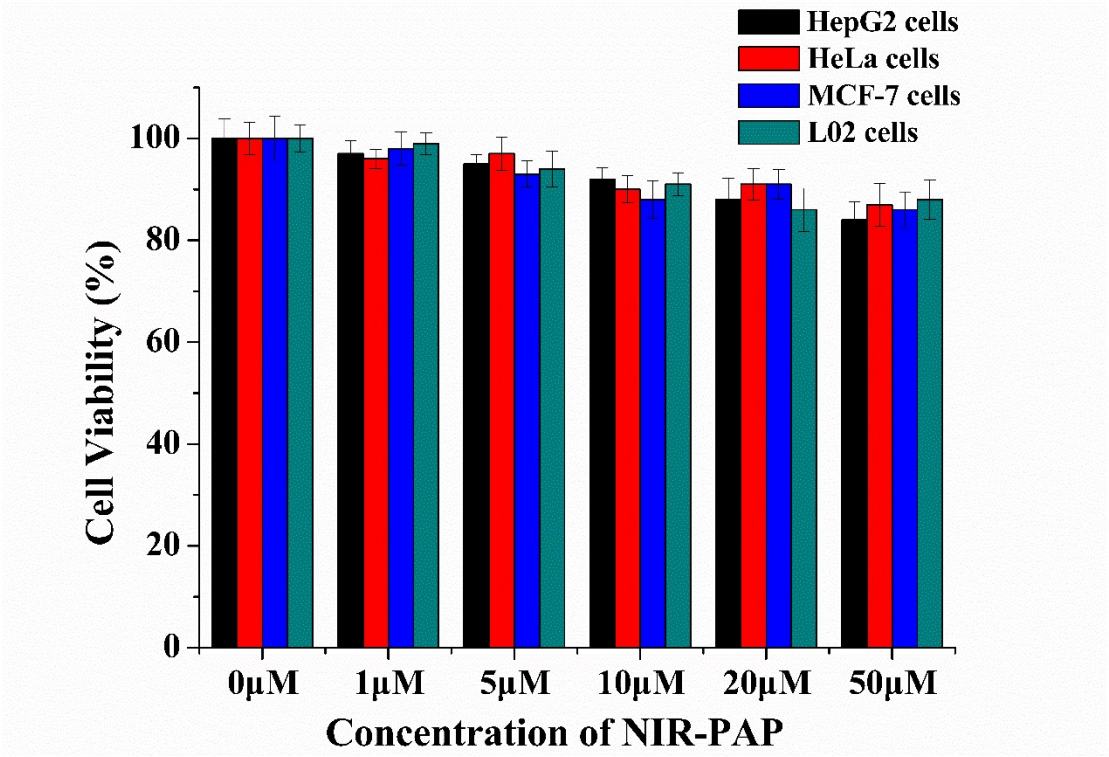


Fig. S14 Effects of NIR-PAP (0, 1, 5, 10, 20 and 50 μM) on the viability of HepG2 cells, HeLa cells, MCF-7 cells and L02 cells. The viability of cells without NIR-PAP is defined as 100%. The results are the means ± SD of three experiments.

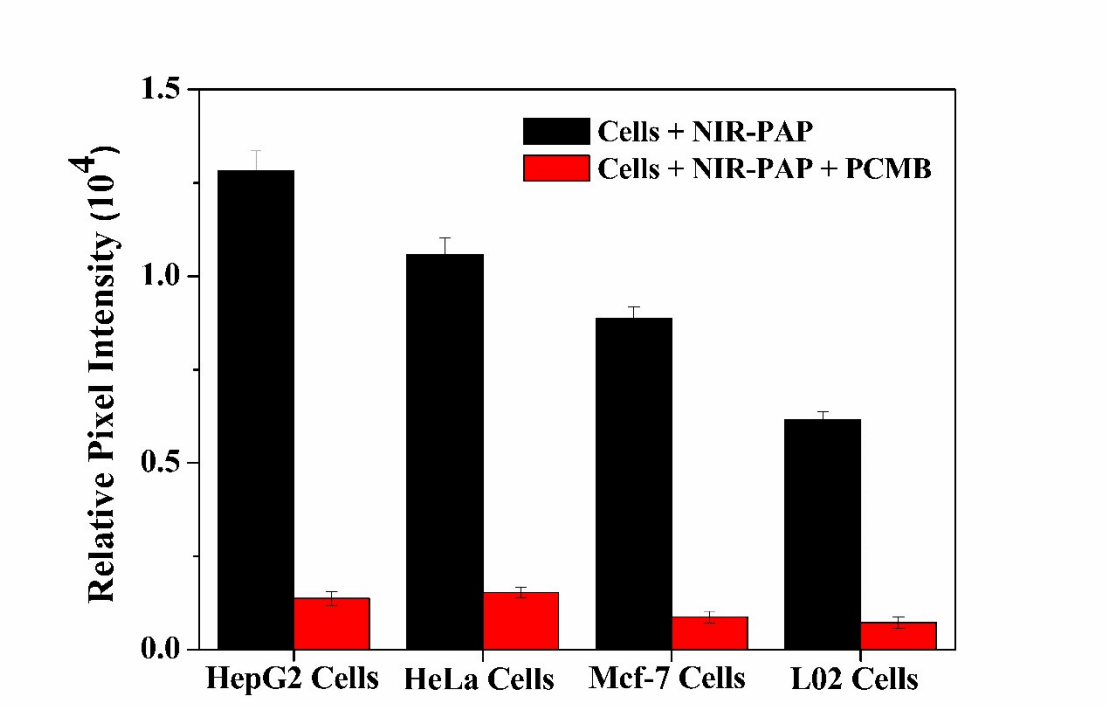


Fig. S15 Relative pixel intensity of the corresponding fluorescence images in different cells in Fig. 3 in the manuscript.

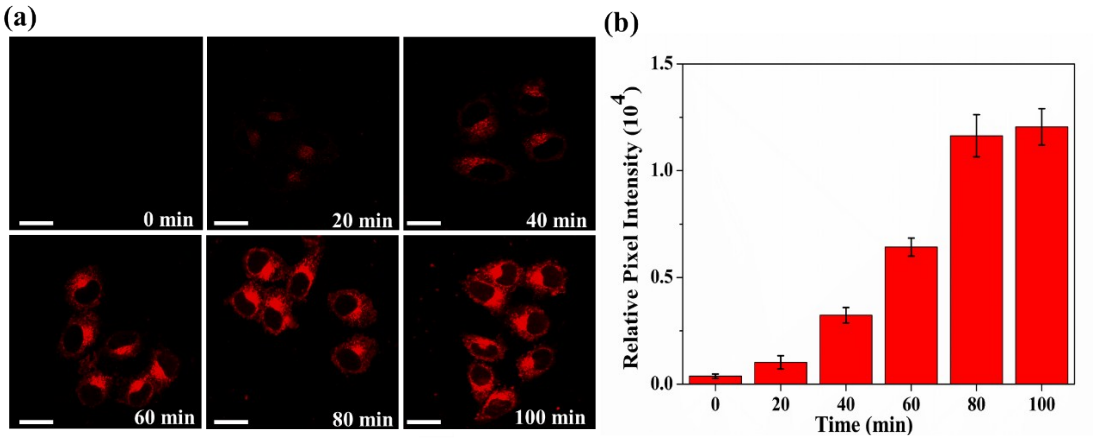


Fig. S16 (a) Confocal fluorescence images of HepG2 cells incubated with NIR-PAP (10 μM) obtained at different time intervals (0, 20, 40, 80 and 100 min). (b) Relative pixel intensity of the corresponding fluorescence images in (A). Scale bar=20 μm.

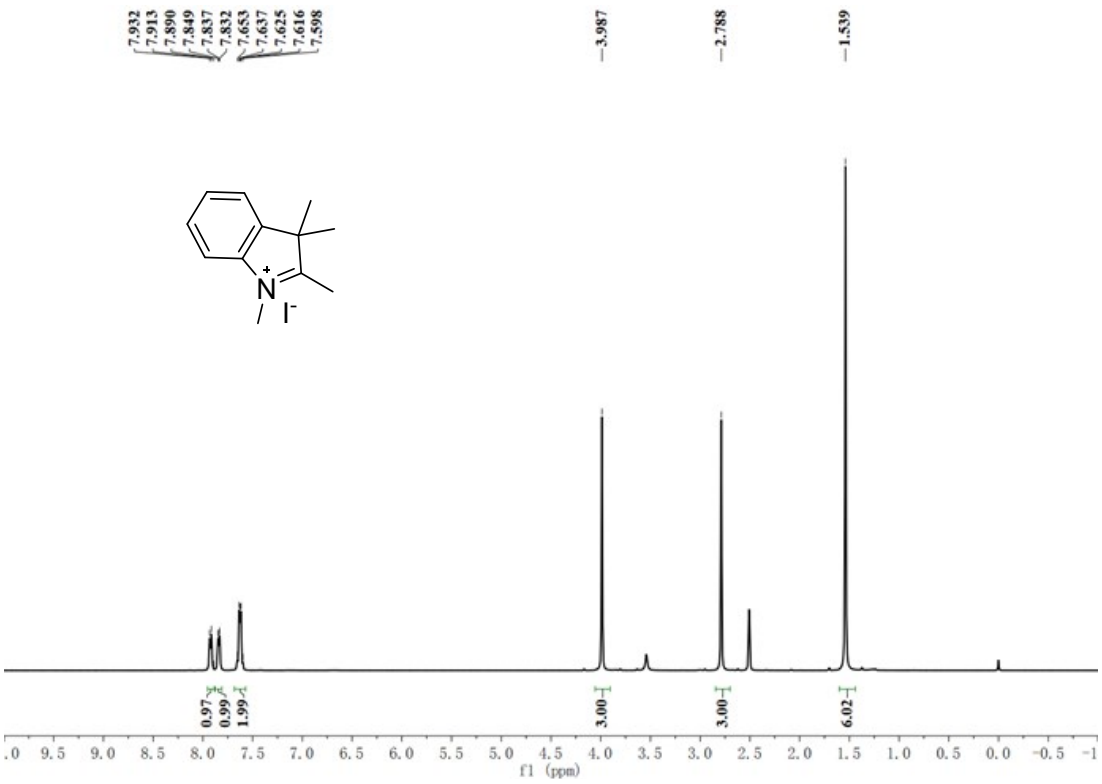


Fig. S17 ^{1}H NMR spectrum of Compound 1 in DMSO-d_6.

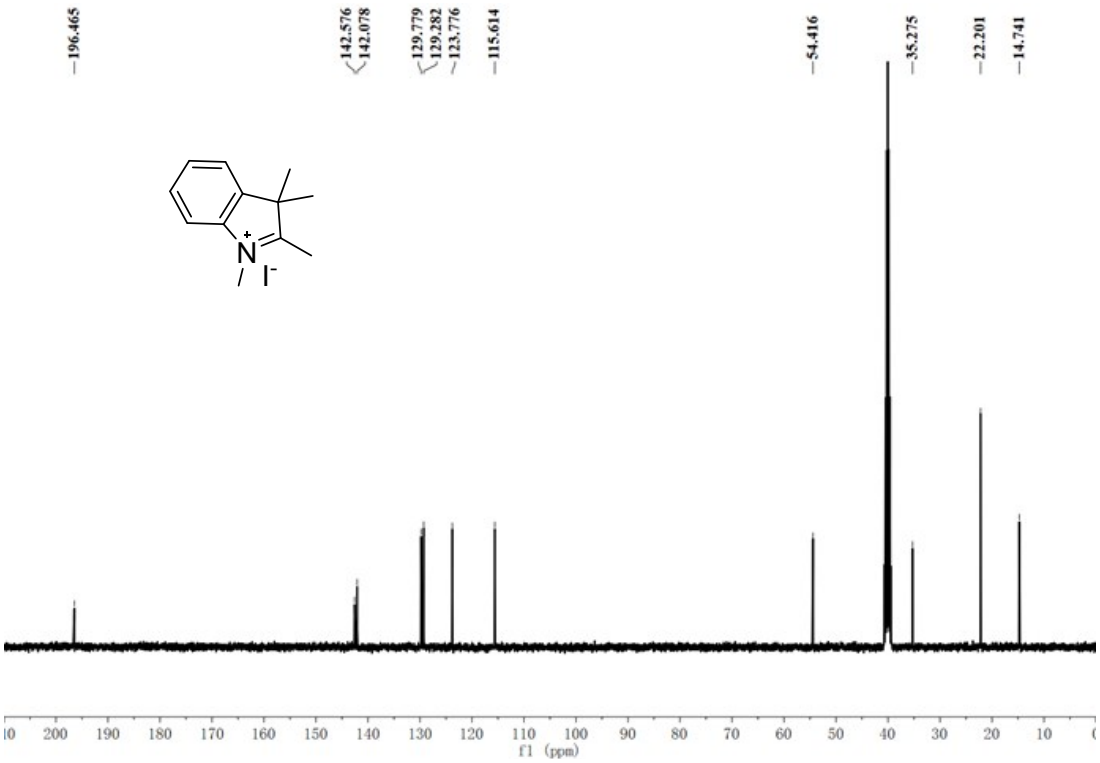


Fig. S18 ^{13}C NMR spectrum of Compound 1 in DMSO-d_6.

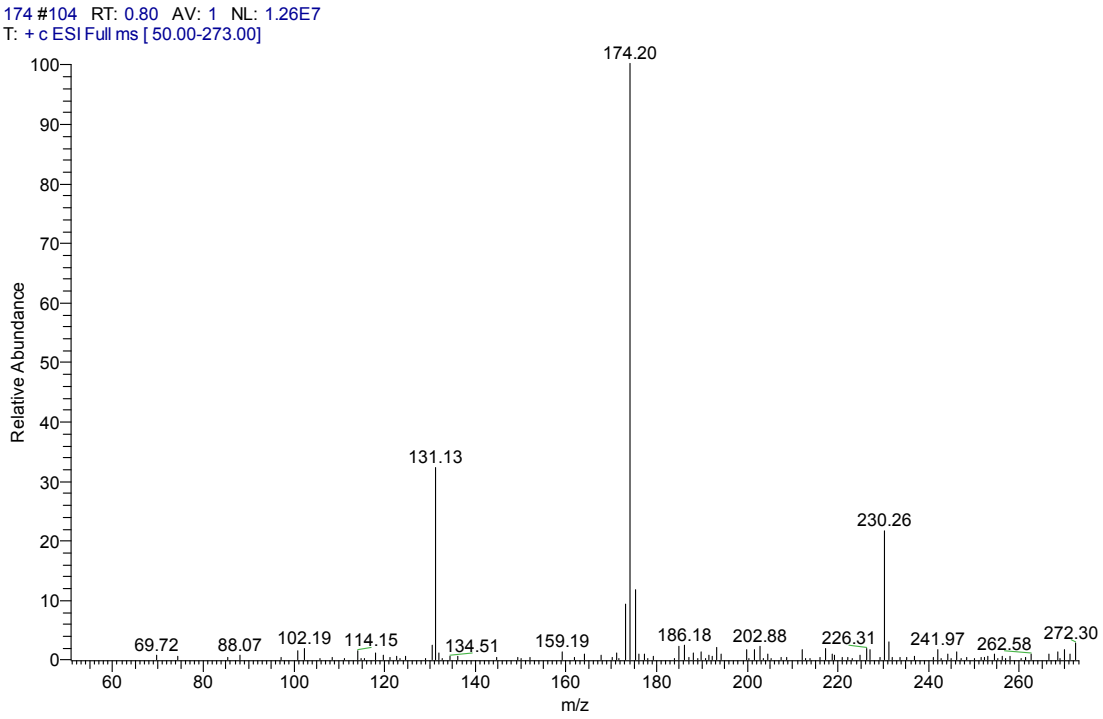


Fig. S19 ESI-MS spectrum of Compound 1.

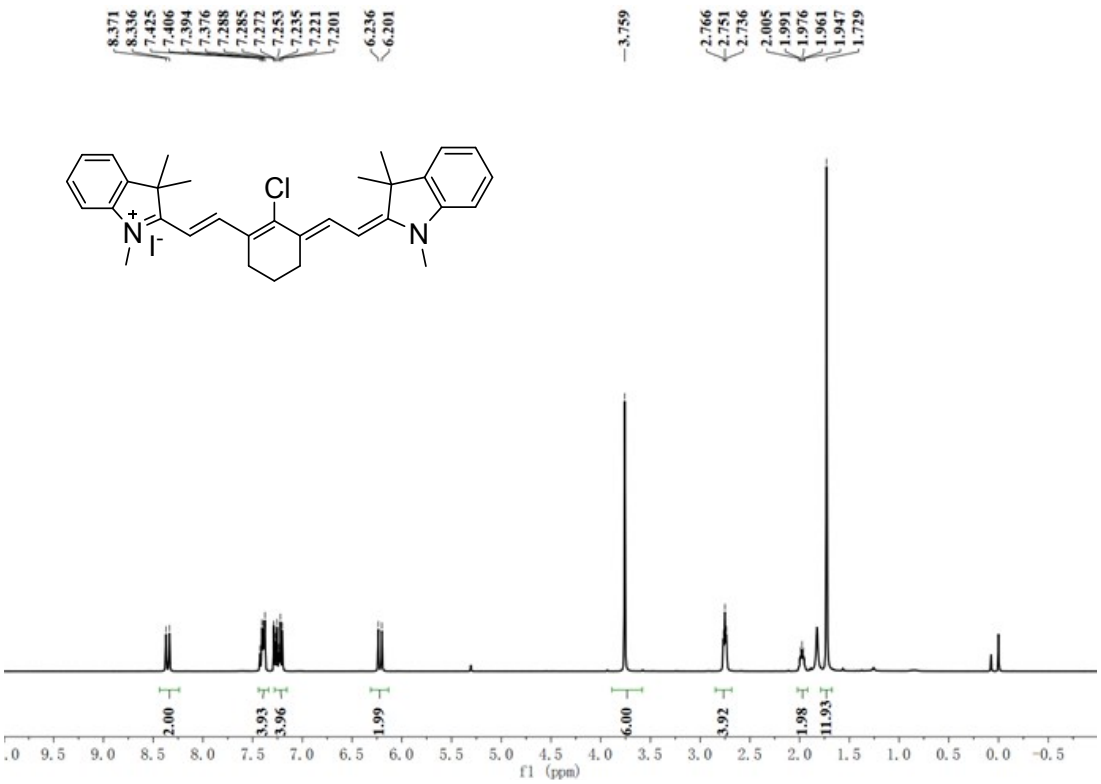


Fig. S20 ^{1}H NMR spectrum of Compound 3 in $CDCl_3$.

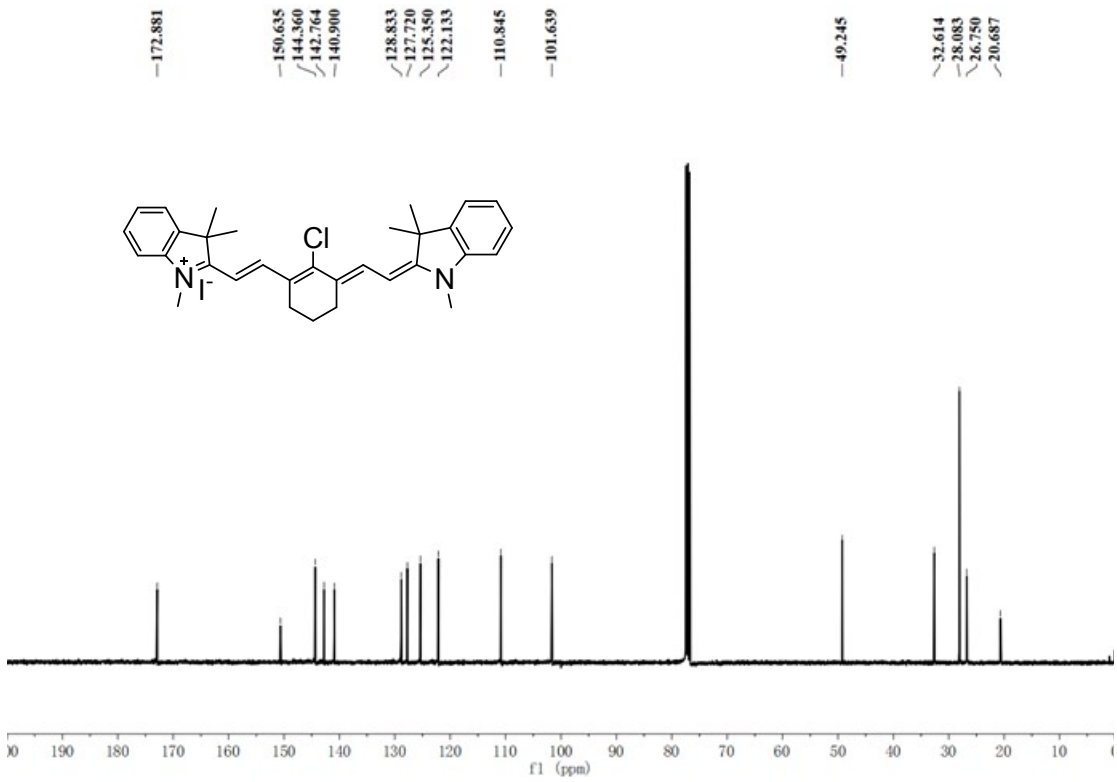


Fig. S21 ^{13}C NMR spectrum of Compound 3 in $CDCl_3$.

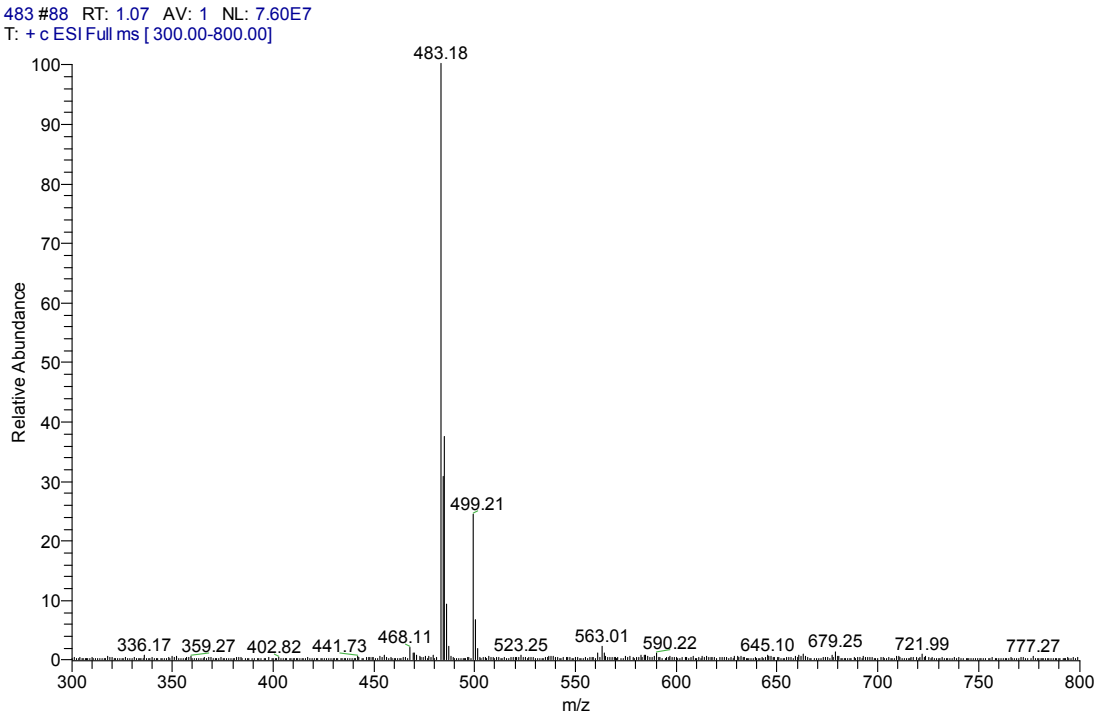


Fig. S22 ESI-MS spectrum of Compound 3

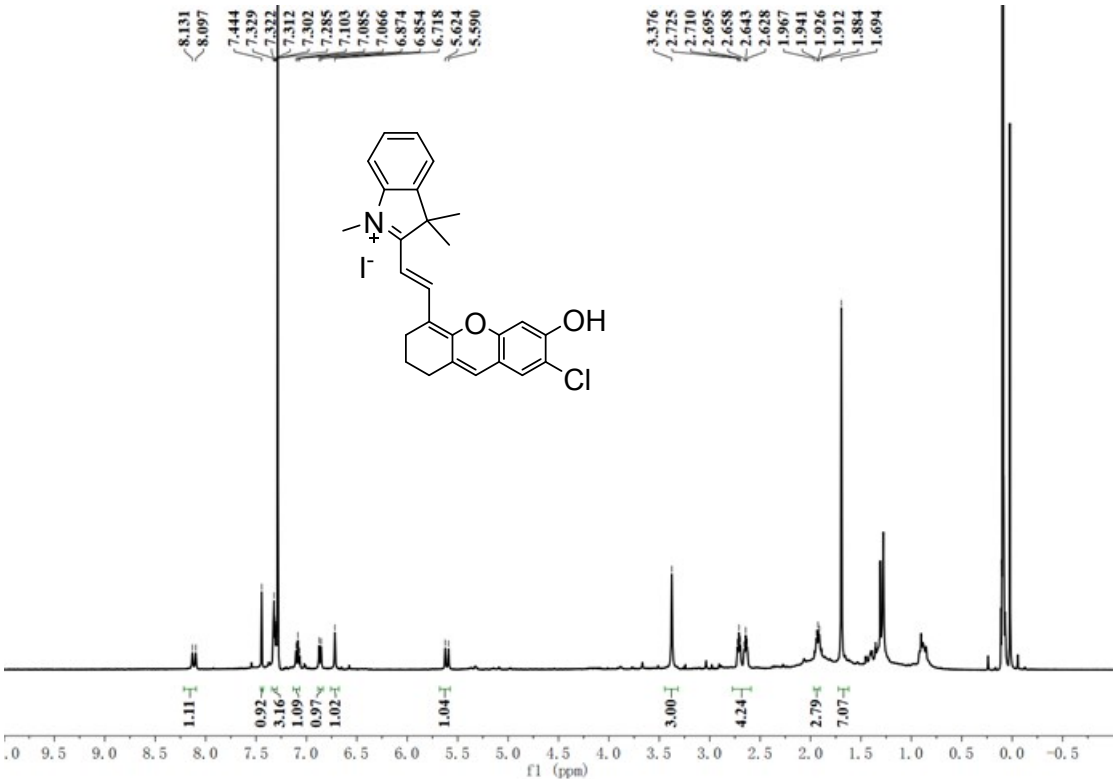


Fig. S23 ^{1}H NMR spectrum of Hcy-OH in $CDCl_3$.

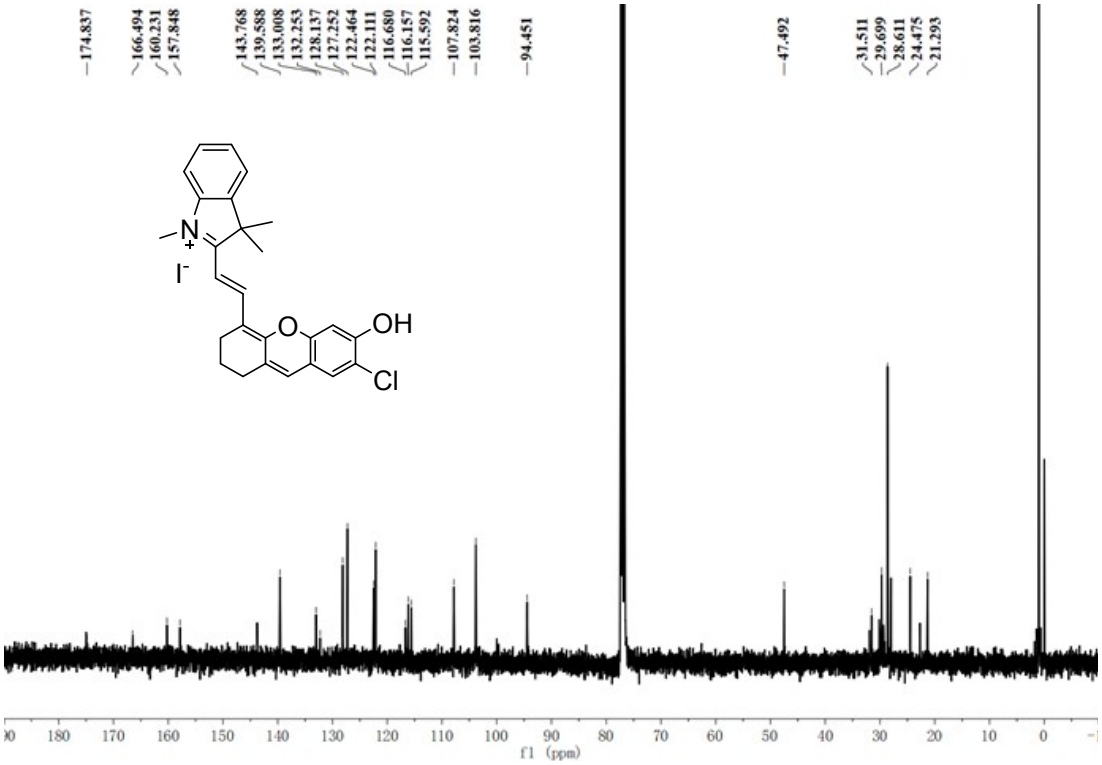


Fig. S24 ^{13}C NMR spectrum of Hcy-OH in $CDCl_3$.

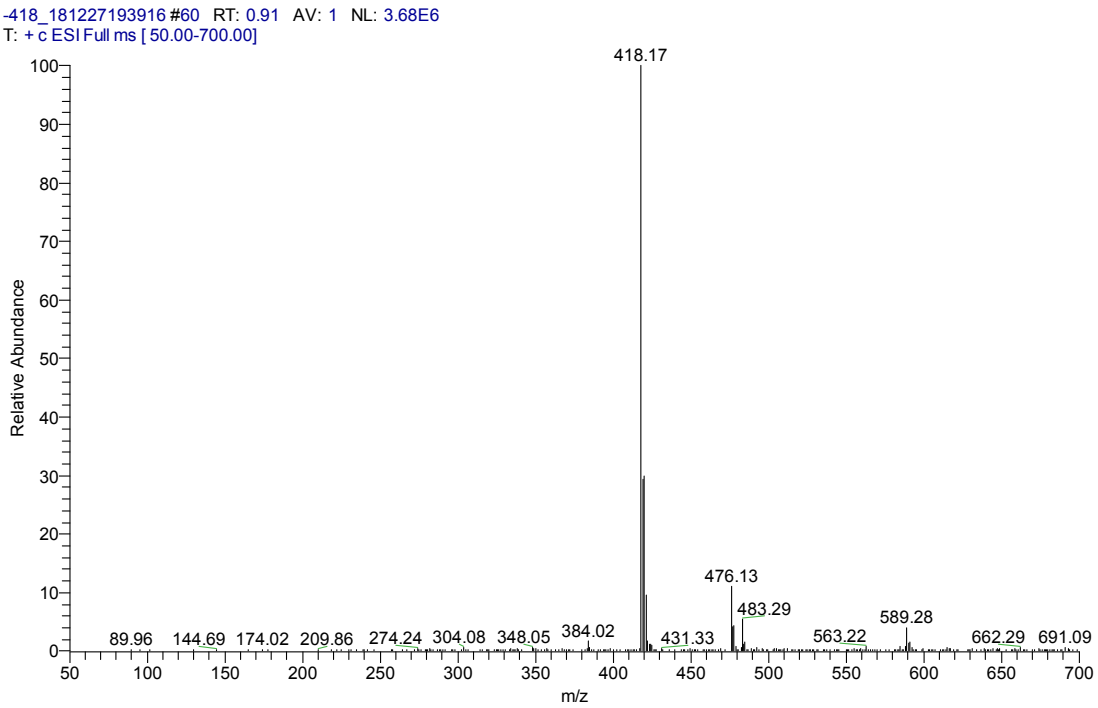


Fig. S25 ESI-MS spectrum of Hcy-OH.