Supporting Information for

A Mitochondrial-Targeted Prodrug for NIR Imaging Guided and Synergetic NIR Photodynamic-Chemo Cancer Therapy

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EXPERIMENTAL SECTION

Reagents and apparatus. All chemicals were purchased from commercial suppliers and used without further purification. Water was purified and doubly distilled by a Milli-Q system (Millipore, USA).The fluorescence measurements were conductedat room temperature on a Fluoromax-4 spectrofluorometer (HORIBA JobinYvon, Edison, NJ). Mass spectra were performed using an LCQ Advantage ion trap mass spectrometer (Thermo Finnigan).NMR spectra were recorded on a Bruker DRX-400 spectrometer using TMS as an internal standard. Thin layer chromatography (TLC) was conducted using silica gel 60 F254, and column chromatography was carried out over silica gel (200-300 mesh), both of them were obtained from Qingdao Ocean Chemicals (Qingdao, China).The pH was measured with a Mettler-Toledo Delta 320 pH meter. Fluorescence images of cells were obtained using an Olympus FV1000-MPE multiphoton laser Scanning confocal microscope (Japan).

Singlet oxygen detection. Singlet oxygen probe (MNAH) and 9, 10-diphenylanthracene (DPHA) were employed to evaluate the singlet oxygen generation. **PNPS** was mixed with MNAH (at a final concentration of 5 μM) or DPHA (at a final concentration of 50 μM) in PBS buffer (20 mM, pH 7.4). The solutions were irradiated on a 50 W white light for different time. MNAH fluorescence was excited with an excitation wavelength of 440 nm and an emission wavelength from 460-600 nm. DPHA absorbance was measured. The SOG of samples was quantified by comparing the MNAH fluorescence enhancement or DPHA absorbance decrease with the background or control samples. For intracellular ROS generation, HeLa cells were cultured in DMEM at 37 °C. After 80% confluence, the cells incubated with **PNPS** (5 μM) for 2 h in dark, DCF-DA (2 μM) was loaded into the cells. After 20 min incubation, cells were then exposed to white light irradiation for 30 min. For **PNPS**, the excitation was 635 nm, and the band filter was 560 nm; for DCF-DA detection, the excitation was 488 nm, and the emission filter was 500-560 nm.

Cell culture and imaging. HeLa, HepG2, HCT116 and HL-7702 cells were maintained in

DMEM medium with 10% fetal bovine serum (FBS, GIBCO) and 0.5 mg/mL penicillin-streptomycin at 37 °C under a 5% CO_2 atmosphere. When the cell density reached 90% of confluence, a subculture was done and the medium was changed approximately every day. Cells were first seeded in a 20 mm glass-bottom dish plated and grown to around 80% confluency for 24 h before the experiment. Then cells were washed with 1 mL of PBS twice. **PNPS** was added to the culture media for 0.5 or 2 h, and cells were again washed twice with 1 mL of PBS. Fluorescence imaging of cells was carried out using an Olympus FV1000 MPE multiphoton laser scanning microscope (Japan). The fluorescence for **PNPS** was excited at 635 nm, and emission was collected at 680-760 nm. For co-localization study, cells were first incubated with **PNPS** (5 μM) for 30 min, and then 500 nM of Mito-Tracker green was added and incubated at 37 °C for another 30 min. After washing with DPBS, cells were imaged.

Flow cytometric analysis. HeLa cells, HepG2 cells and HL-7702 cells in 6-well plate were precultured for 24 h respectively and incubated with **PNPS** for the designated time. After incubation, the cells were treated with trypsin, washed with medium twice and subjected to flow cytometry analysis using Cyan-LX (DakoCytomation). The cells without any treatment were used as control. The mean fluorescence was determined by counting 10,000 eventson the BD FACSVerse™ flow cytometer.

Cell cytotoxicity assay. The cytotoxicity of the compounds was evaluated using the MTS assay. The HeLa, HepG2 cells and HL-7702 cells were seeded in 96-well plates at a density of 8000 cells/well. After incubation for 24 h, the medium was replaced with freshly prepared solution of **NPS-H_2O_2** and **PNPS** at different concentrations and further incubated at 37 °C for 3 h in the dark. Fresh medium was added and further incubated for 24 h. For the phototoxicity assay using **NPS-H_2O_2** and **PNPS**, after incubation with **NPS-H_2O_2** and **PNPS** at different concentrations in the dark for 3 h, fresh medium was added and the cells were irradiated with white light for 3 h. After 24 h incubation, the cell medium was replaced with 100 μL of fresh culture medium and 20 μL of MTS solution. After 1 h incubation, therapeutic

efficacy on cells was assayed by measuring the absorbance at 490 nm, using a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT).

Cell apoptosis detection. HeLa or HL-7702 cells were first seeded in a 20 mm glass-bottom dish plated and grown to around 80% confluency for 24 h before the experiment. The culture medium was removed, and the cells were rinsed with DPBS. Following by incubation of the cells with 5′-DFUR (10 μM), **NPS-H_2O_2** (10 μM) and **PNPS** (10 or 20 μM) at 37 °C for 3 h in the dark, the medium was removed and the cells were washed twice with DPBS. For pretreatment with H_2O_2, the HL-7702 cells were first incubated with H_2O_2 (100 μM) for 30 min. For the phototoxicity study, the cells were next irradiated with white light for 3 h and further incubated for 8 h in the dark. For the dark control, the cells were further incubated for 12 h in the dark. The cellular apoptosis imaging measurement was carried out based on standard detection kit (PI stain) according to manufacturer's protocol. The cells were then imaged immediately by Olympus FV1000 MPE multiphoton laser scanning microscope (Japan).

Animal model. Male Balb/c mice (~3 weeks old) were obtained from Hunan SJA Laboratory Animal Co., Ltd. and used under protocols approved by Hunan University Laboratory Animal Center. To generate the HCT116 murine tumor model, ~2×10^7 HCT116 cells in~100 μL DPBS were subcutaneously injected in the left upper extremity of each mice. The mice were treated when the tumor volumes approached ~70 mm^3. All living cells and live animal operations were in accord with institutional animal use and care regulations, according to protocol No. SYXK (Xiang) 2008-0001, approved by Laboratory Animal Center of Hunan.

***In vivo* imaging.** Before *in vivo* imaging, the mice were anesthetized by inhalation of 5% isoflurane in 100% oxygen. The nude mice were imaged using a Caliper VIS Lumina XR small animal optical *in vivo* imaging system. Imaging mode for all experiments was set as excitation scan, and Input/Em was chosen as 675 nm for excitation with Cy5.5 filter for emission channel.

Synthesis

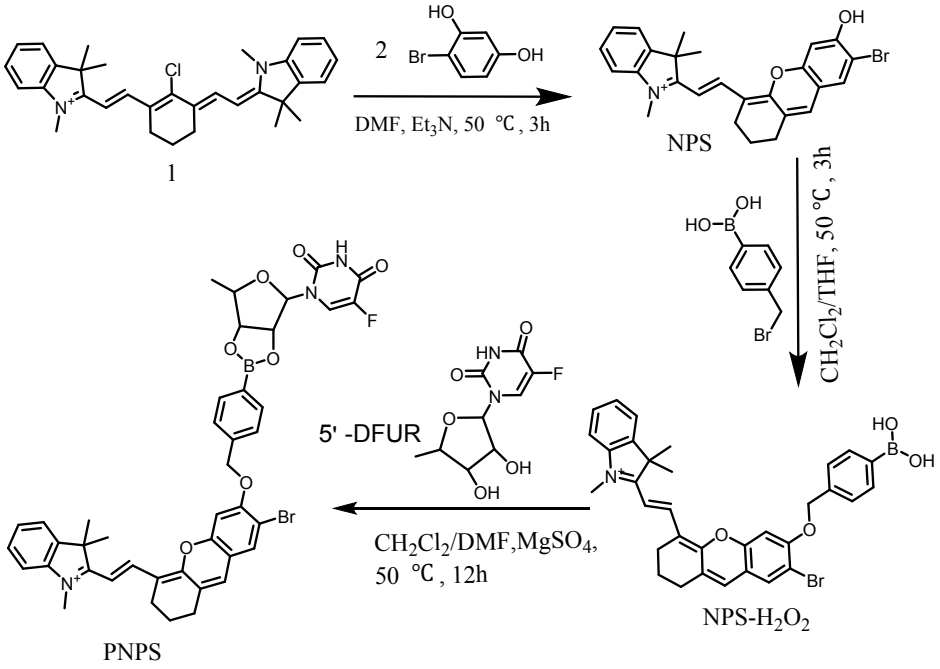

Fig. S1 Synthetic route for theranostic prodrug **PNPS**.

Synthesis of compound 1. Compound **1** was synthesized according to a literature procedure.[11] HNMR($CDCl_3$, 400 MHz) δ (ppm): 8.36 (d, J = 8.35 Hz, 2H), 7.41-7.37 (m, J = 7.39 Hz, 4H), 7.28-7.25 (m, J = 7.27 Hz, 2H), 7.21 (d, J = 7.20 Hz, 2H), 6.23 (d, J = 6.22 Hz, 2H), 3.76 (s, 6H), 2.76 (t, J = 2.75 Hz, 4H), 1.99 (t, J = 1.97 Hz, 2H), 1.73 (s, 12 H). MS (ESI): m/z 483.4, (M), calcd for $C_{32}H_{36}ClN_2^+$ 483.3.

Synthesis of compound NPS. 4-Bromoresorcinol (1.88 g, 10 mmol)and Et_3N (2 mL) were placed in a flask containing DMF (10 mL), and the mixture was stirred at 50℃under nitrogen atmosphere for 10 min. Compound **1** (3.0 g, 5 mmol) in DMF (10.0 mL) was introduced to the mixture via a syringe, and the reaction mixture was heated at 55 °C for 6 h. Then, the reaction solution was poured into ice water and extracted with CH_2Cl_2. The combined organic solution was concentrated under reduced pressure. After purified by the silica gel chromatography (CH_2Cl_2/EtOH, 10:1, v/v), compound **NPS** was obtained as a blue-green solid (2.24g, 38%). ^{1}HNMR($CDCl_3$, 400 MHz) δ(ppm): 8.13(d, J = 8.11Hz, 1H), 7.65(s, 1H), 7.32-7.29 (m, J = 7.30Hz, 3H), 7.10-7.06 (t, J = 7.08Hz, 1H),6.87 (d, J = 6.86Hz,1H), 6.72 (s,

1H), 5.62 (d, *J* = 5.61Hz, 1H), 3.37(s, 3H), 2.70-2.67 (t, *J* = 2.69Hz, 2H), 2.64-2.61(t, *J* = 2.62Hz, 2H), 1.91-1.89 (t, *J* = 1.90Hz, 2H), 1.67 (s, 6 H). MS (ESI): m/z 462.3, [M], calcd. for $C_{26}H_{25}BrNO_2^+$ 462.1.

Synthesis of compound NPS-H_2O_2. Compound **NPS** (0.59 g, 1mmol) and $CsCO_3$ (1.63 g, 5mmol) was dissolved in dry CH_2Cl_2 (20 mL) and stirred at 40℃ under N_2 atmosphere. And 4-boronobenzyl bromide (0.426 g, 2 mmol) in dry THF (10 mL) were added though syringe. After that, the reaction solution was stirred at 50℃ for 3 h. The organic phase was concentrated under reduced pressure. After purified by the silica gel chromatography (CH_2Cl_2/EtOH, 6:1,v/v), compound **NPS-H_2O_2** was obtained as a blue solid (0.18 g, 25%). ^{1}HNMR (CD_3OD, 400 MHz) δ (ppm): 8.73-8.67 (m, *J* = 8.69Hz, 1H), 7.84 (d, *J* = 7.83Hz, 1H), 7.74-7.69 (m, *J* = 7.71Hz, 3H), 7.58-7.50 (m, *J* = 7.54Hz, 5H), 7.58-7.24-7.13 (m, *J* = 7.19Hz, 2H), 6.57-6.53 (d, *J* = 6.55Hz, 1H), 5.39 (s, 2H), 3.89 (s, 3H), 3.34 (s, 2H), 2.77 (t, *J* = 2.75Hz, 2H), 2.72 (t, *J* = 2.71Hz, 2H), 1.93 (t, *J* = 1.91Hz, 2H), 1.82(s, 6 H). MS (ESI): m/z 612.3, [M+H_2O], calcd. for $C_{33}H_{32}BBrNO_4^+$ 596.1.

Synthesis of compound PNPS. Compound **NPS-H_2O_2** (0.145 g, 0.2 mmol)and $MgSO_4$ (3 g) was dissolved in dry CH_2Cl_2 (20 mL) and stirred at 40℃ under N_2atmosphere. And 5'-deoxy-5-fluorouridine (0.246 g, 1 mmol) in dry DMF (5 mL) were added though syringe. After that, the reaction solution was stirred at 40℃ for 10 h. Then, ice water (50 mL) was added, and the reaction solution was extracted with CH_2Cl_2/EtOH. The combined organic phase was dried with Na_2SO_4, and concentrated under reduced pressure. After purified by the silica gel chromatography (CH_2Cl_2/EtOH, 3:1,v/v), compound **NPS-H_2O_2** was obtained as a blue solid (0.068 g, 38%). ^{1}HNMR(CD_3OD, 400 MHz) δ (ppm): 8.61(d, *J* = 8.59 Hz, 1H), 7.75 (d, *J* = 7.74Hz, 2H), 7.65-7.59 (m, *J* = 7.61 Hz, 3H), 7.50-7.40 (m, *J* = 7.44 Hz, 5H), 7.14 (s, 1H), 7.03 (s, 1H), 6.47 (d, *J* = 6.45 Hz, 1H), 5.29 (s, 2H), 5.25 (m, *J* = 5.24 Hz, 1H),3.79 (s, 3H), 2.67 (t, *J* = 2.66 Hz, 2H), 2.62 (t, *J* = 2.61 Hz, 2H), 2.11(t, *J* = 2.09 Hz, 1H), 1.94(d, *J* = 1.93 Hz, 1H), 1.84 (t, *J* = 1.83 Hz, 2H), 1.71 (s, 6 H), 1.52 (m, *J* = 1.50 Hz, 2H),

0.8 (s, 3H). MS (ESI): m/z 804.1758, [M], calcd. for $C_{42}H_{37}BBrFN_3O_7^+$ 804.1886.

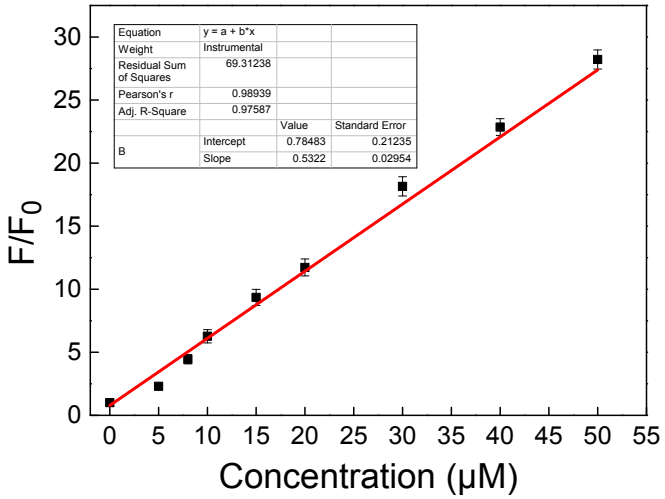

Fig. S2 A plot of fluorescence intensity at 710 nm of **PNPS** (5 μM) vs the H_2O_2 concentration. The linear responses of **PNPS** to H_2O_2 at 5-50 μM. λ_{ex}=680 nm.

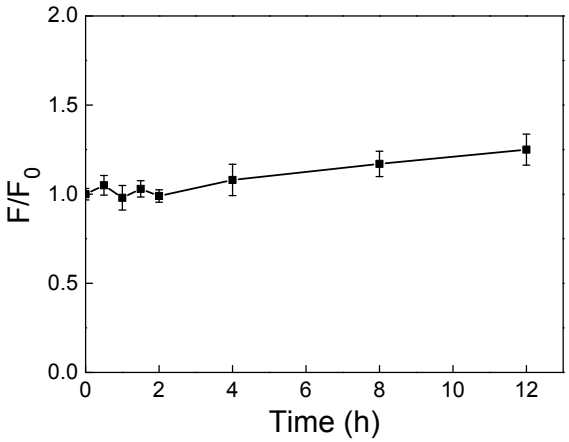

Fig. S3 A plot of fluorescence intensity at 710 nm of **PNPS** (5 μM) vs time in the absence of H_2O_2. The results showed very limited spontaneous signal generation over 12 h.

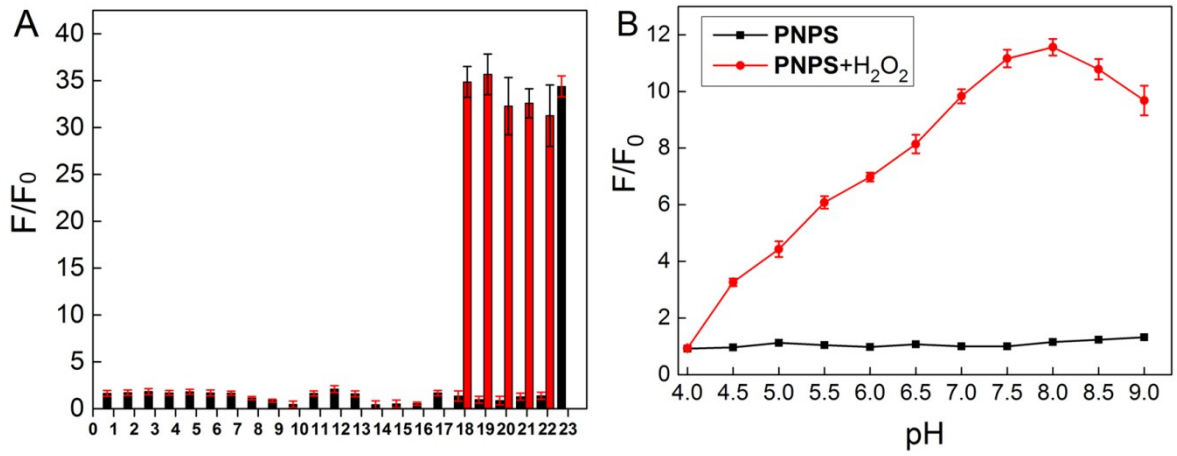

Fig. S4 (A) Fluorescence response of **PNPS** (5 μM) toward various biological compounds.The fluorescence intensity at λ_{em}= 710 nm was plotted versus substances: (1-6, 0.1 mM) NaClO, $O_2^{\bullet-}$, $ONOO^-$, $^1O_2$, t-BuOOH, $NaNO_2$, (7-17, 1mM) $MgCl_2$, $FeSO_4$, $Zn(NO_3)_2$, $CaCl_2$, glucose, L-isoleucine, L-glutamate, GSH, Cys, NaHS, NaN_3, (18) 5% BSA, (19) 5% HSA, (20) 50 U/L Alkaline Phosphatase, (21) 50 U/L γ-glutamyltransferase, (22) 50 U/L β-galactosidase, (18-22 The black bar represents the fluorescence intensity of only a single analyte with probe. The red bar represents the fluorescence intensity of only a single analyte and 100 μM H_2O_2 with probe), (23) 100 μM H_2O_2. (B) Fluorescence intensity at 710 nm of **PNPS** with and without H_2O_2, as a function of pH. Each spectrum was acquired 0.5 h after exposure to analyte at room temperature in PBS buffer (pH 7.4) with λ_{ex} = 680 nm.

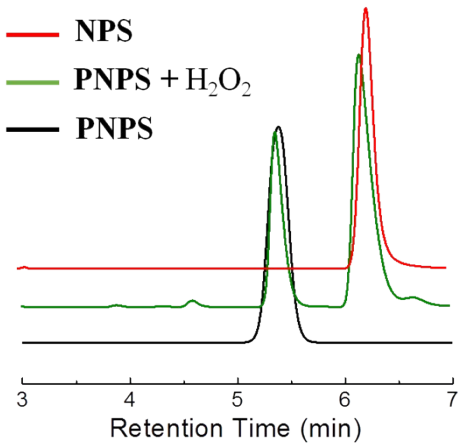

Fig.S5 Reverse-phase HPLC chromatogram of **PNPS**, **PNPS** reaction with 50 μM H_2O_2 for 30 min, and compound**NPS**. Detection wavelength is 600 nm.

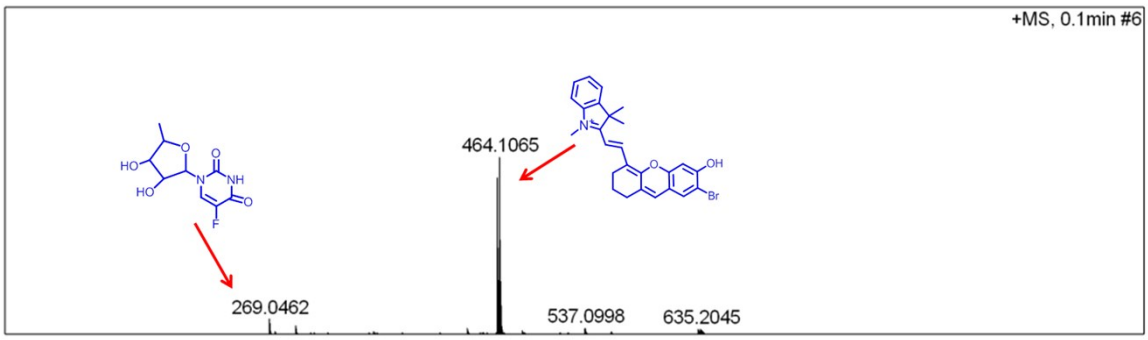

Fig.S6 HRMS spectrum showing the activated product, 5′-DFUR and **NPS**.

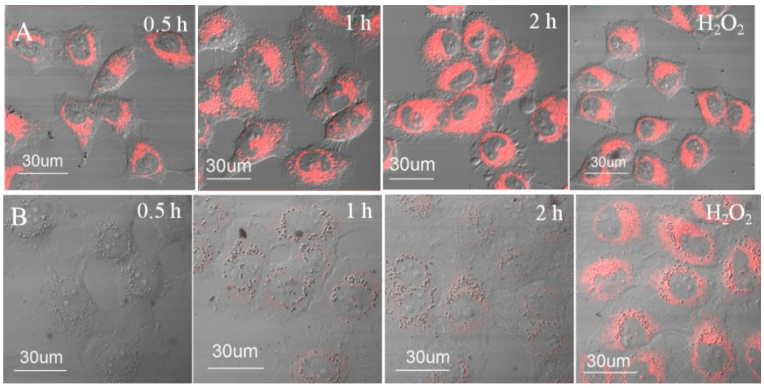

Fig. S7 Cellular fluorescence images of prodrug **PNPS** -treated (A) HeLa and (B) HL-7702 cells. The cells were incubated with **PNPS** (5 μM) for 0.5 h, 1 h and 2 h, respectively. Or the cells were pretreated with H_2O_2 (200 μM) for 0.5 h and then incubated with **PNPS** for another 0.5 h. λ_{ex} = 635 nm, λ_{em} = 680-750 nm.

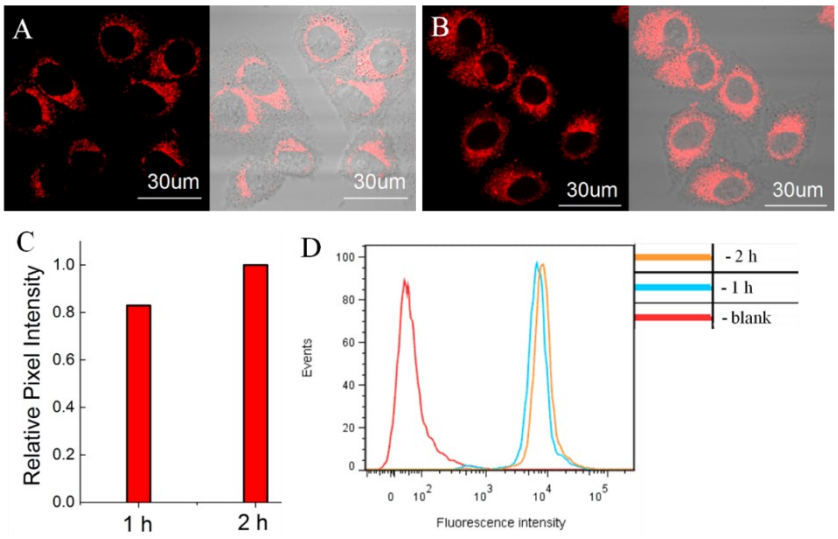

Fig. S8 Cellular fluorescence images of prodrug **PNPS** -treated HepG2 cells. The cells were incubated with **PNPS** (5 μM) for (A) 1 h and (B) 2 h, respectively. (C) Relative pixel intensity (n= 3) from images (A), (B). The pixel intensity from image (B) is defined as 1.0. (D) Flow cytometric analysis of **PNPS** (5 μM) fluorescence after incubated with HepG2 cells. λ_{ex} = 635 nm, λ_{em} = 680-750 nm

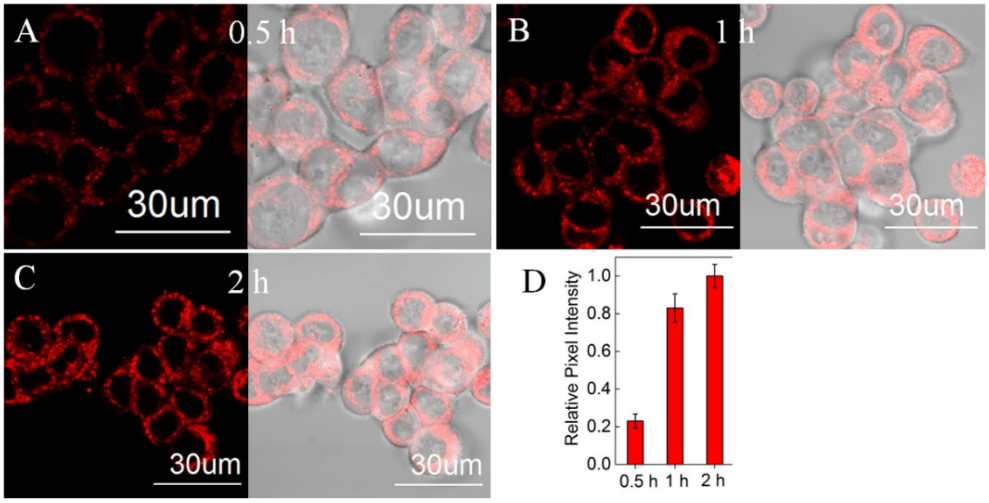

Fig. S9 Cellular fluorescence images of prodrug **PNPS** -treated HCT116 cells. The cells were incubated with **PNPS** (5 μM) for (A) 0.5 h, (B) 1 h and (C) 2 h, respectively. (D) Relative pixel intensity (n= 3) from images (A)-(C). The pixel intensity from image (C) is defined as 1.0. λ_{ex} = 635 nm, λ_{em} = 680-750 nm.

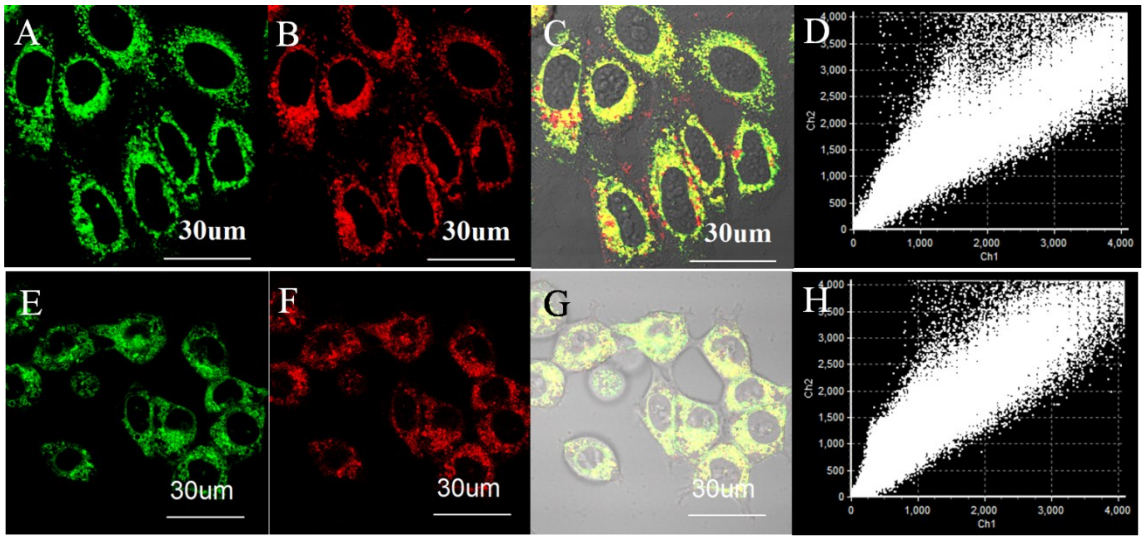

Fig. S10 Fluorescence images of co-localized experiment in HepG2 (A-D) and HCT116 (E-H) cells. The cells were incubated with **PNPS** for 0.5 h and then incubated with MitoTracker Green for another 0.5 h. (A, E) MitoTracker Green (0.5 μM, λ_{ex} = 488 nm, λ_{em} = 500-550 nm); (B, F) Prodrug **PNPS** (5 μM, λ_{ex} = 635 nm, λ_{em} = 680-750 nm); (C) Overlay of (A) and (B); (G) Overlay of (E) and (F). (D, H) Intensity correlation plot of stain, the Pearson's correlation factor for HepG2 and HCT116 cells is 0.943 and 0.958 respectively.

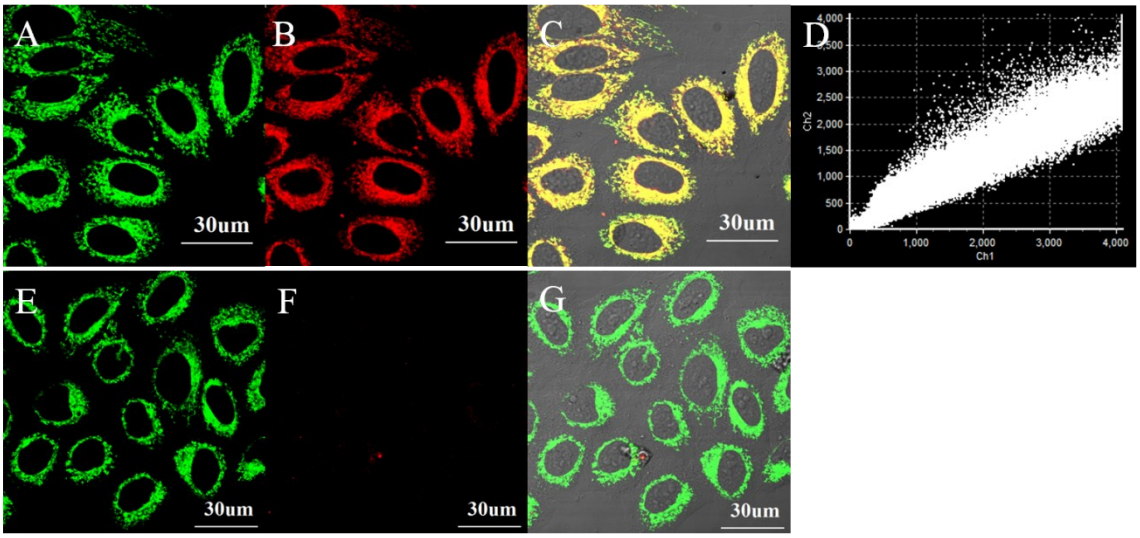

Fig. S11 Fluorescence images of co-localized experiment in HeLa cells. The cells were incubated with **NPS** (A-D) or **PNPS** (E-F) and MitoTracker Green for total 10 min. (A, E) MitoTracker Green (0.5 μM, λ_{ex} = 488 nm, λ_{em} = 500-550 nm); (B) **NPS** (5 μM, λ_{ex} = 635 nm, λ_{em} = 680-750 nm); (C) Overlay of (A) and (B); (D) Intensity correlation plot of stain, the Pearson's correlation factor is 0.960; (F) **PNPS** (5 μM, λ_{ex} = 635 nm, λ_{em} = 680-750 nm). (G) Overlay of (E) and (F).

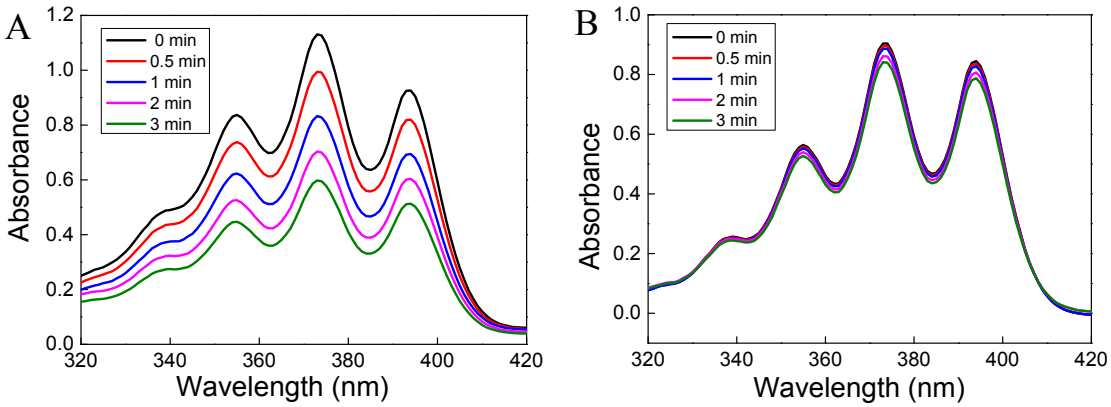

Fig.S12 The UV-vis absorption spectra of DPHA (50 μM) in the presence of **PNPS** (10 μM) after different durations of white light irradiation. (A) In the presence of H_2O_2 (200 μM); (B) In the absence of H_2O_2. The experiments were carried out in PBS/DMF buffer solution (PBS/DMF=2:1, v/v, pH=7.4).

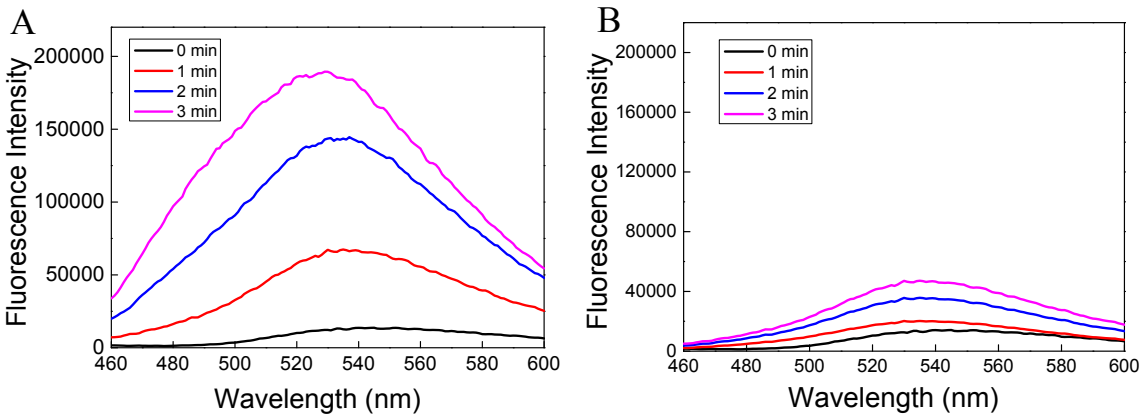

Fig.S13 The fluorescence spectra of MNAH (5 μM) in the presence of **PNPS** (10 μM) after different durations of white light irradiation. (A) In the presence of H_2O_2 (200 μM); (B) In the absence of H_2O_2. The experiments were carried out in PBS/DMSO buffer solution (PBS/DMSO=19:1, v/v, pH=7.4).

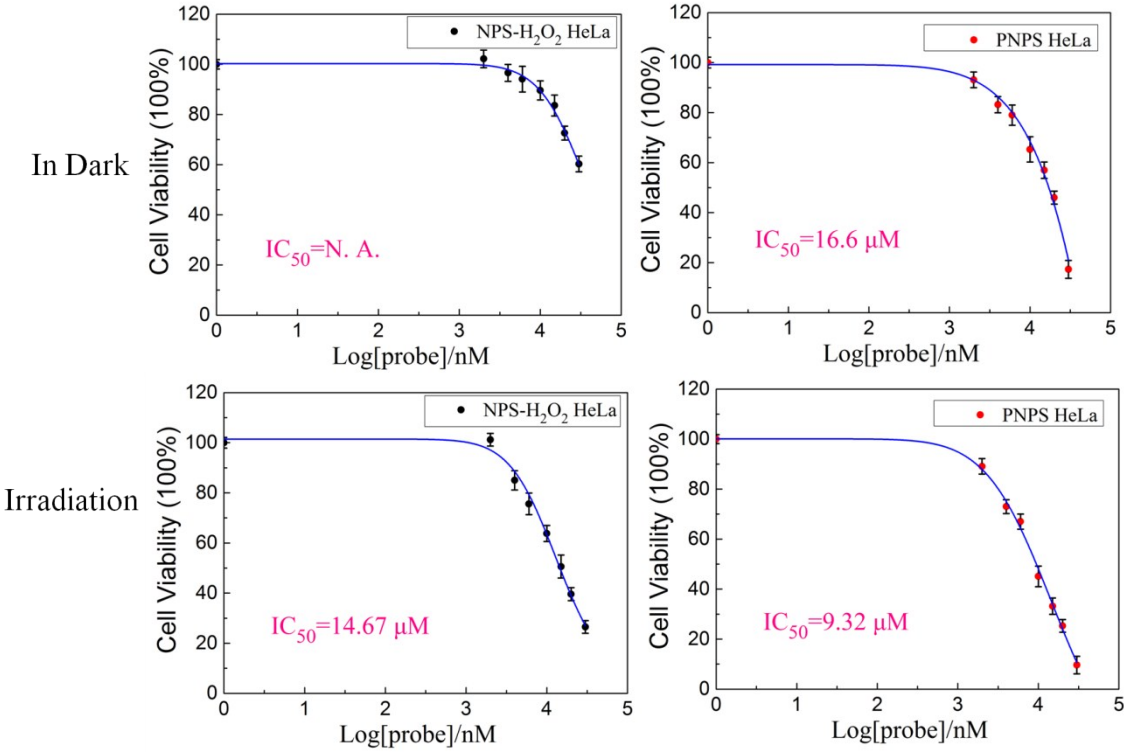

Fig.S14 IC50 of HeLa cells upon treatment with **NPS-H_2O_2** or **PNPS** in dark (●) or under light irradiation (●), N.A. = not available.

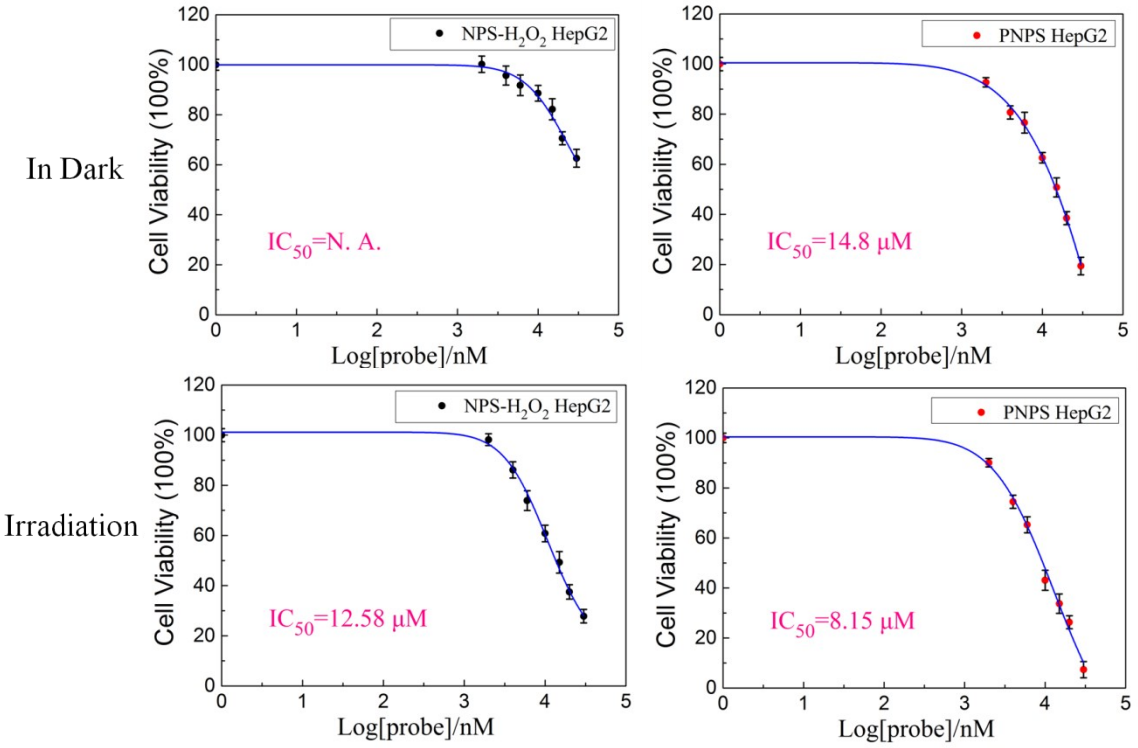

Fig.S15 IC50 of HepG2 cells upon treatment with **NPS-H_2O_2** or **PNPS** in dark (●) or under light irradiation (●), N.A. = not available.

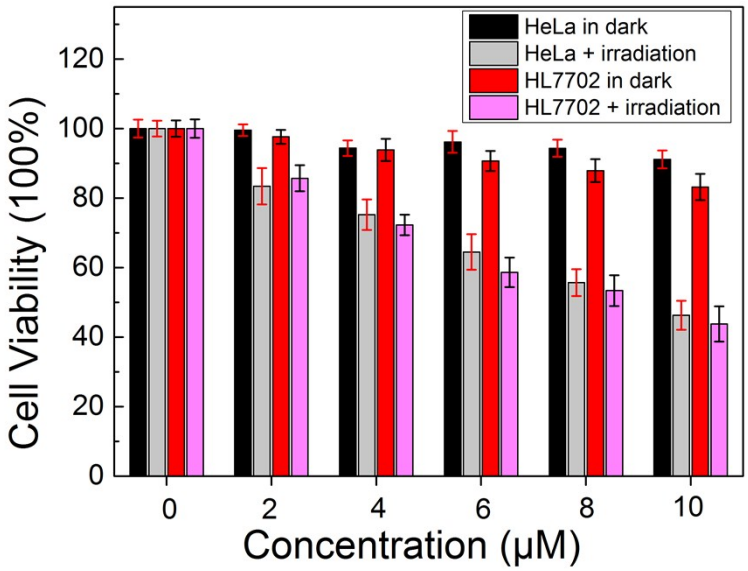

Fig.S16 Viability of HeLa and HL7702 cells upon treatment with different concentrations of **NPS** showed under white light irradiation or in dark.

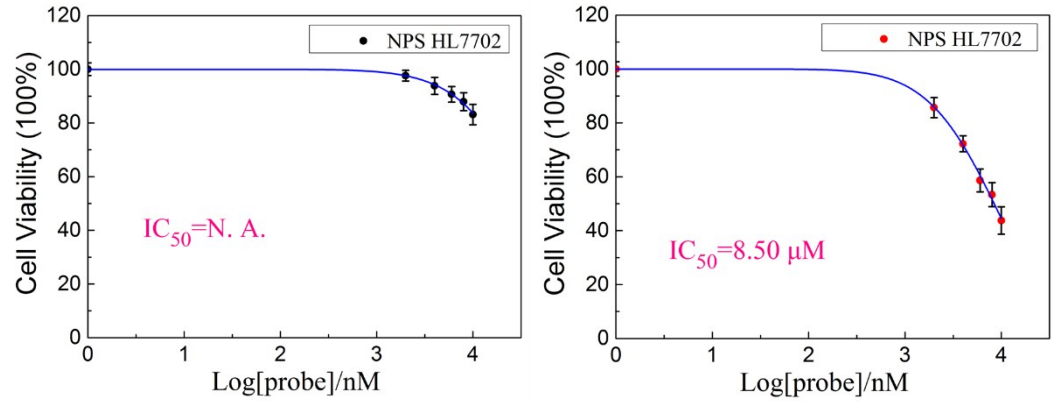

Fig.S17 IC50 of HL7702 cells upon treatment with **NPS** in dark (●) or under light irradiation (●), N.A. = not available.

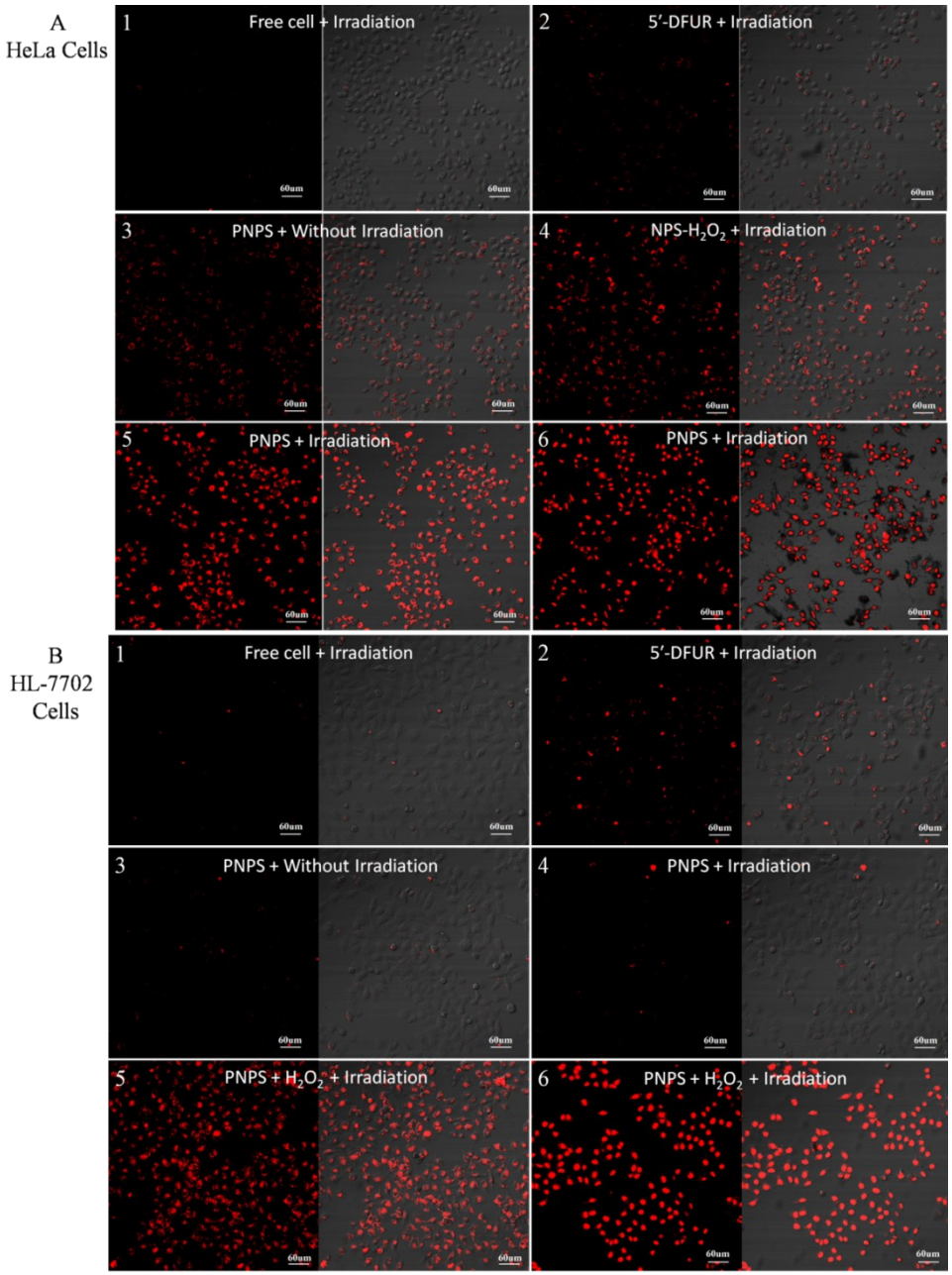

Fig.S18 PI staining experiments of (A) HeLa and (B) HL-7702 cells, the pretreated cells were incubated in dark for 8 h before PI staining. Confocal fluorescence and overlay fluorescence and bright field images of PI stained cells after different incubation. (A1, B1) Free HeLa or HL-7702 cells with white light irradiation for 3 h; (A2, B2) HeLa or HL-7702 cells were incubated with 10 μM 5′-DFUR for 3 h and then with white light irradiation for 3 h; (A3, B3) HeLa or HL-7702 cells were incubated with 10 μM **PNPS** for 3 h; (A4) HeLa cells were incubated with 10 μM **NPS-H_2O_2** for 3 h and then with white light irradiation for 3 h; HeLa cells were incubated with (A5) 10 μM or (A6) 20 μM **PNPS** for 3 h and then with white light irradiation for 3 h; (B4) HL-7702 cells were incubated with 10 μM **PNPS** for 3 h and then with white light irradiation for 3 h; HL-7702 cells were incubated with H_2O_2 for 0.5 h and (B5) 10 μM or (B6) 20 μM **PNPS** for another 3 h and then with white light irradiation for 3 h.

References

1. Y. Li, Y. Sun, J. Li, Q. Su, W. Yuan, Y. Dai, C. Han, Q. Wang, W. Feng and F. Li, *J. Am. Chem. Soc.*, 2015, **137**, 6407-6416.

MS and ^{1}HNMR

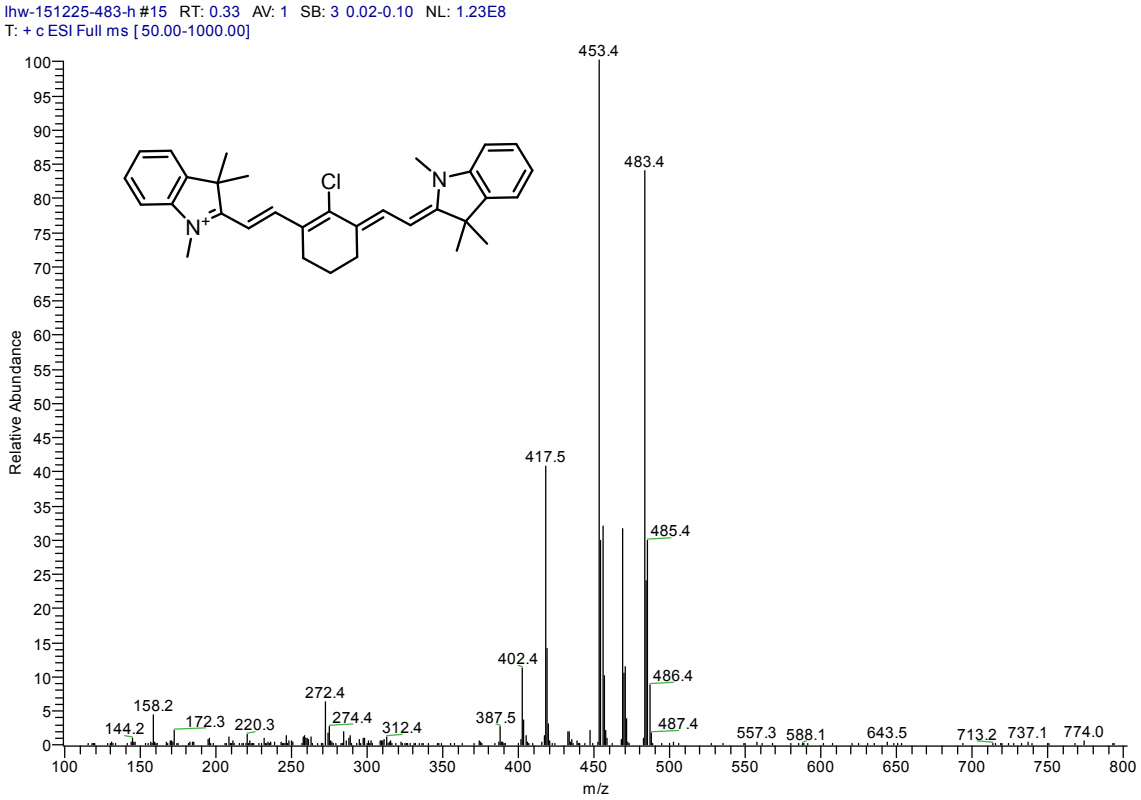

Fig. S19 ESI-MS Spectrum of compound **1**.

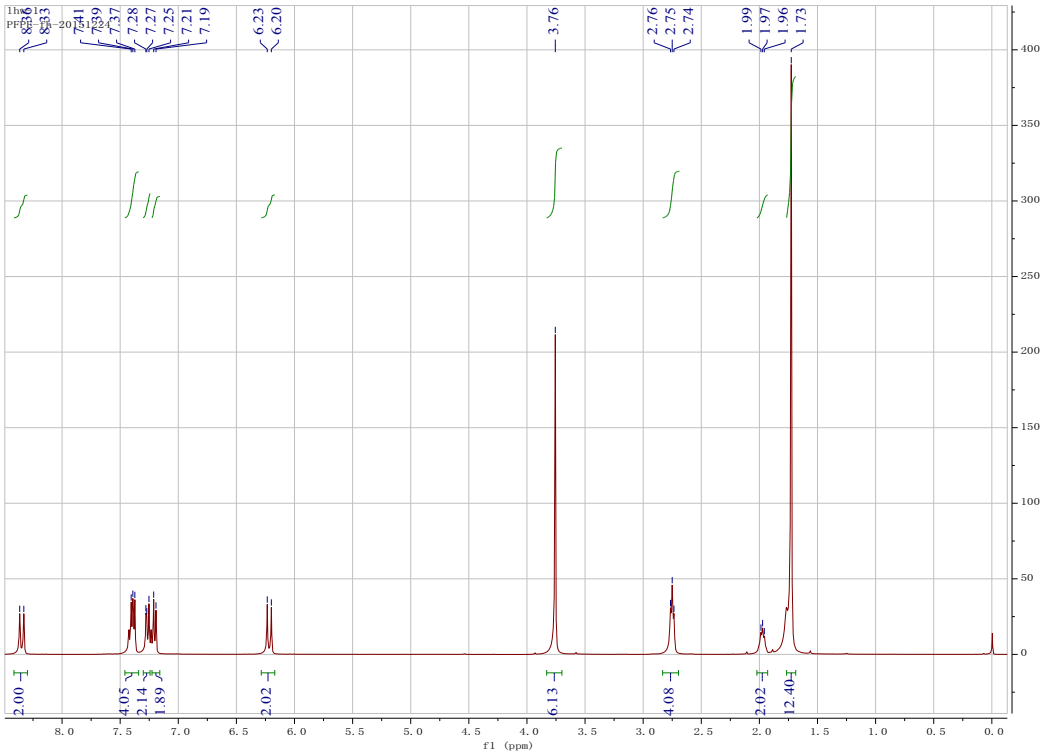

Fig. S20 ^{1}HNMR spectrum of the compound **1**.

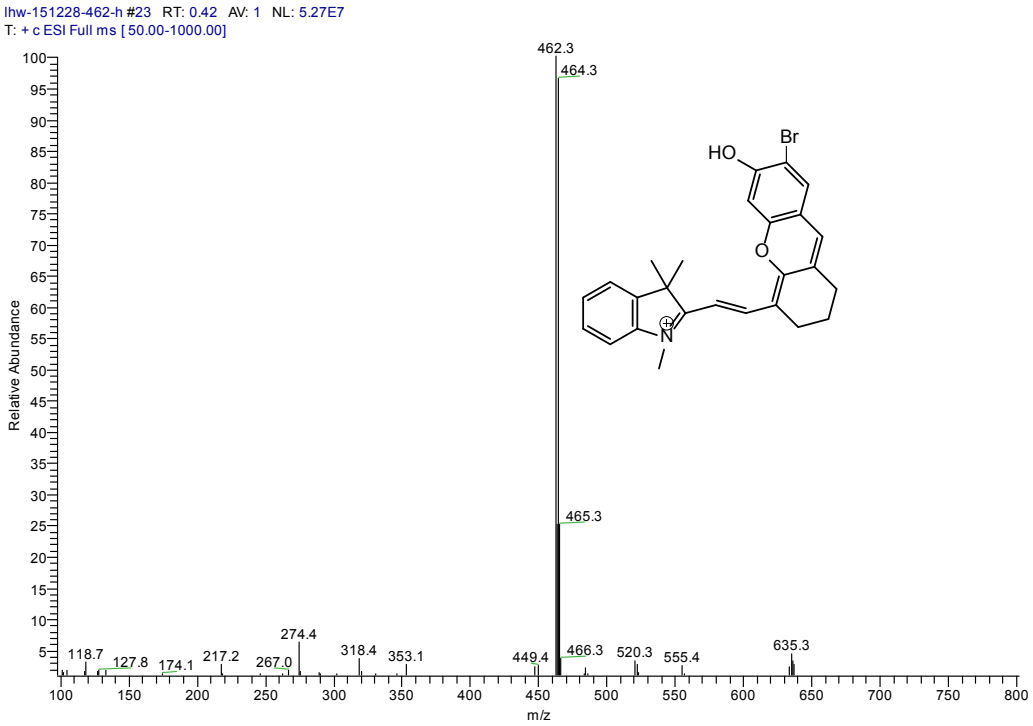

Fig. S21 ESI-MS Spectrum of **NPS**.

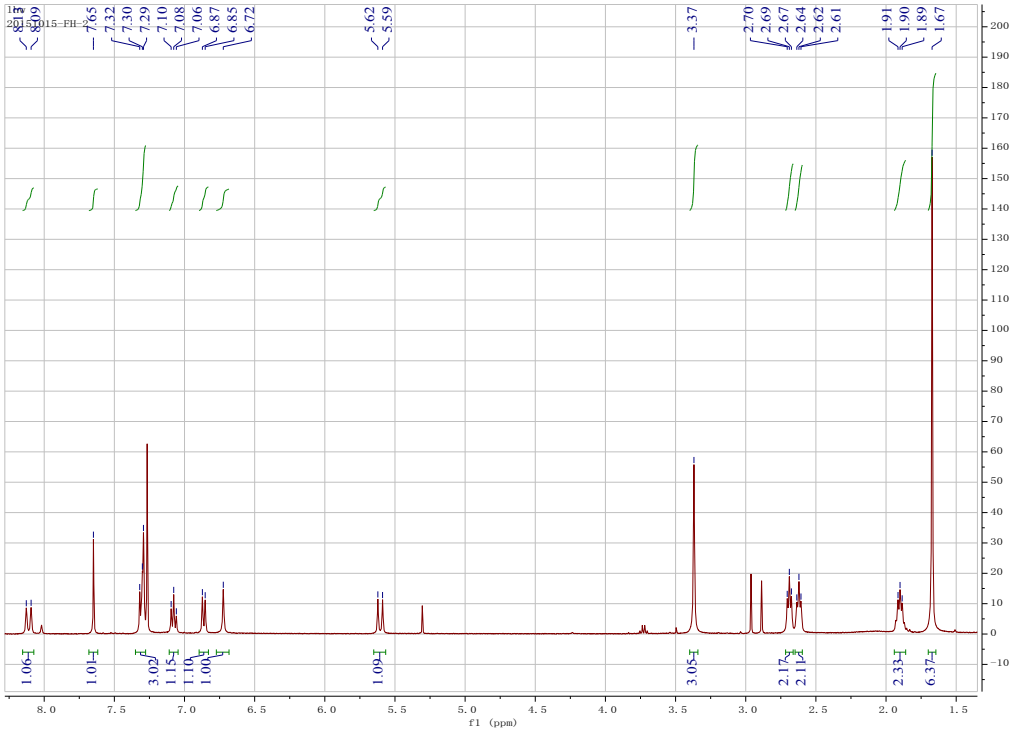

Fig. S22 [1]HNMR Spectrum of **NPS**.

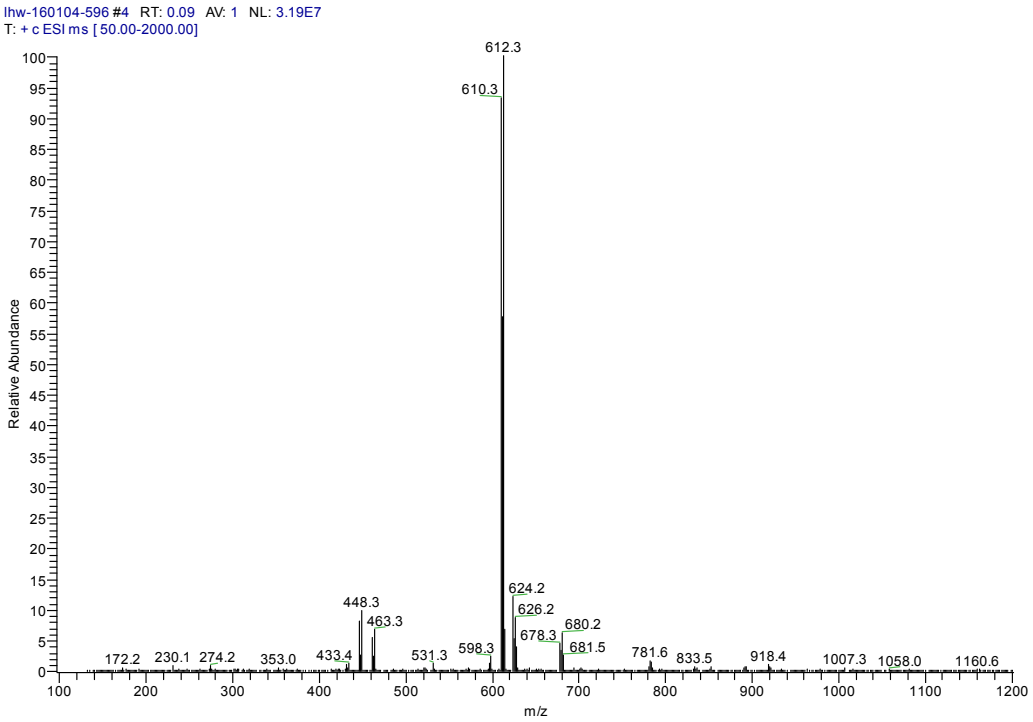

Fig. S23 ESI-MS Spectrum of **NPS-H_2O_2**.

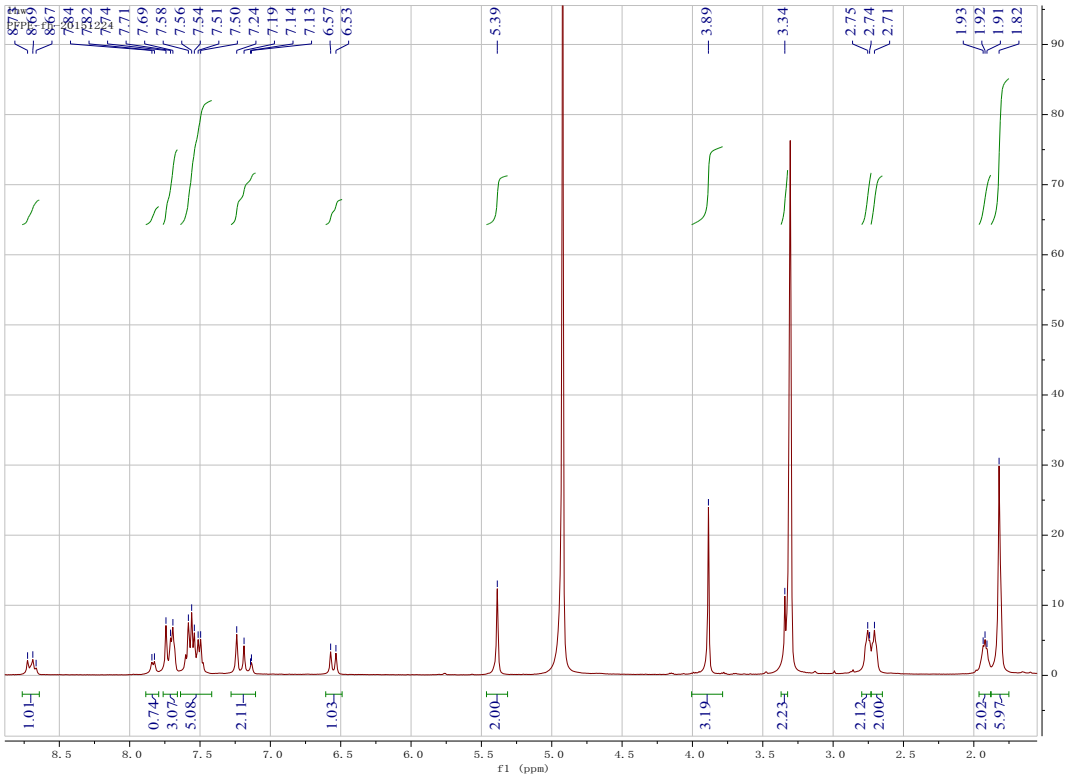

Fig. S24 [1]HNMR Spectrum of **NPS-H_2O_2**.

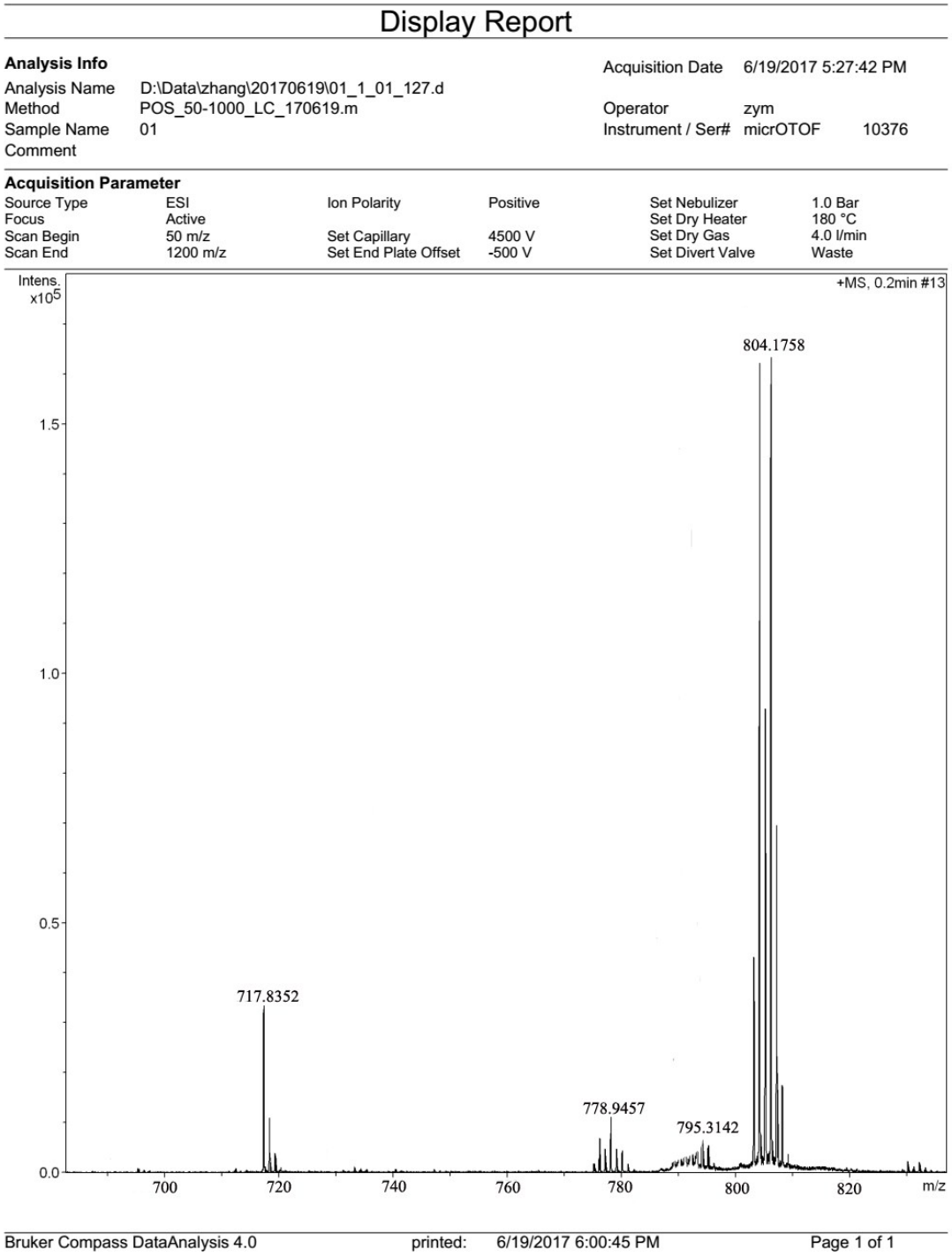

Fig. S25 ESI-HRMS Spectrum of **PNPS**.

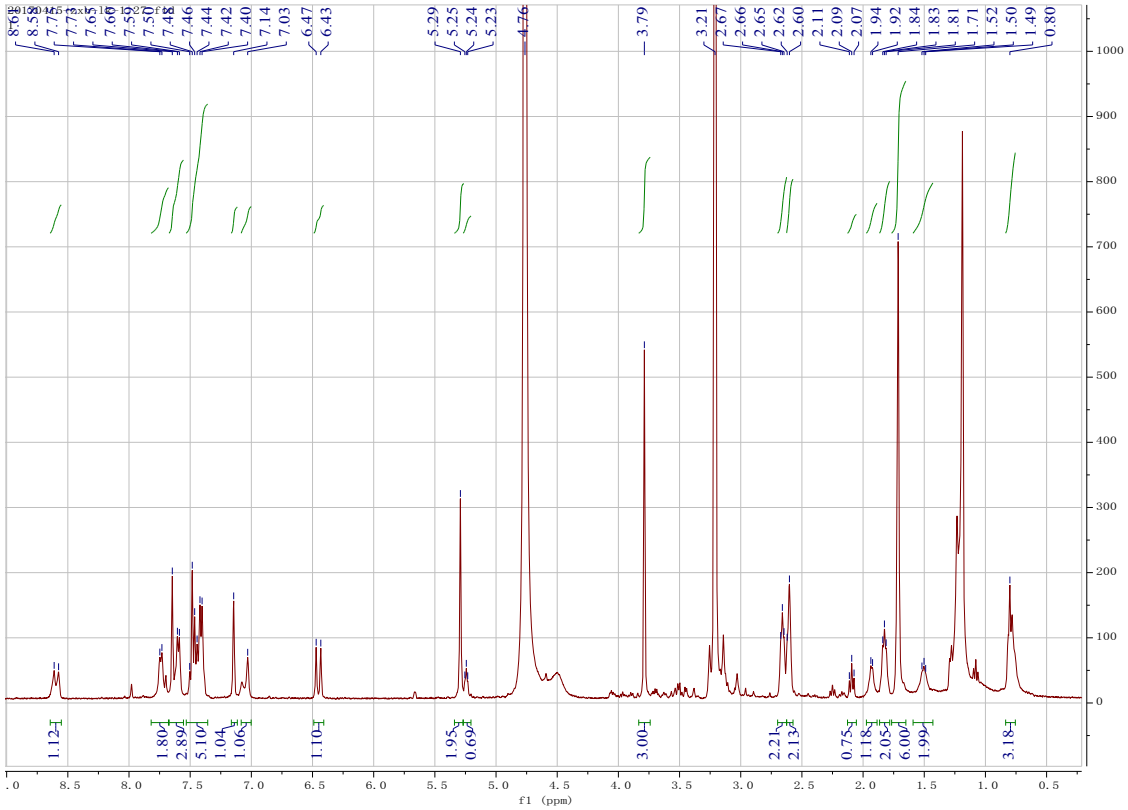

Fig. S26 [1]HNMR Spectrum of **PNPS**.

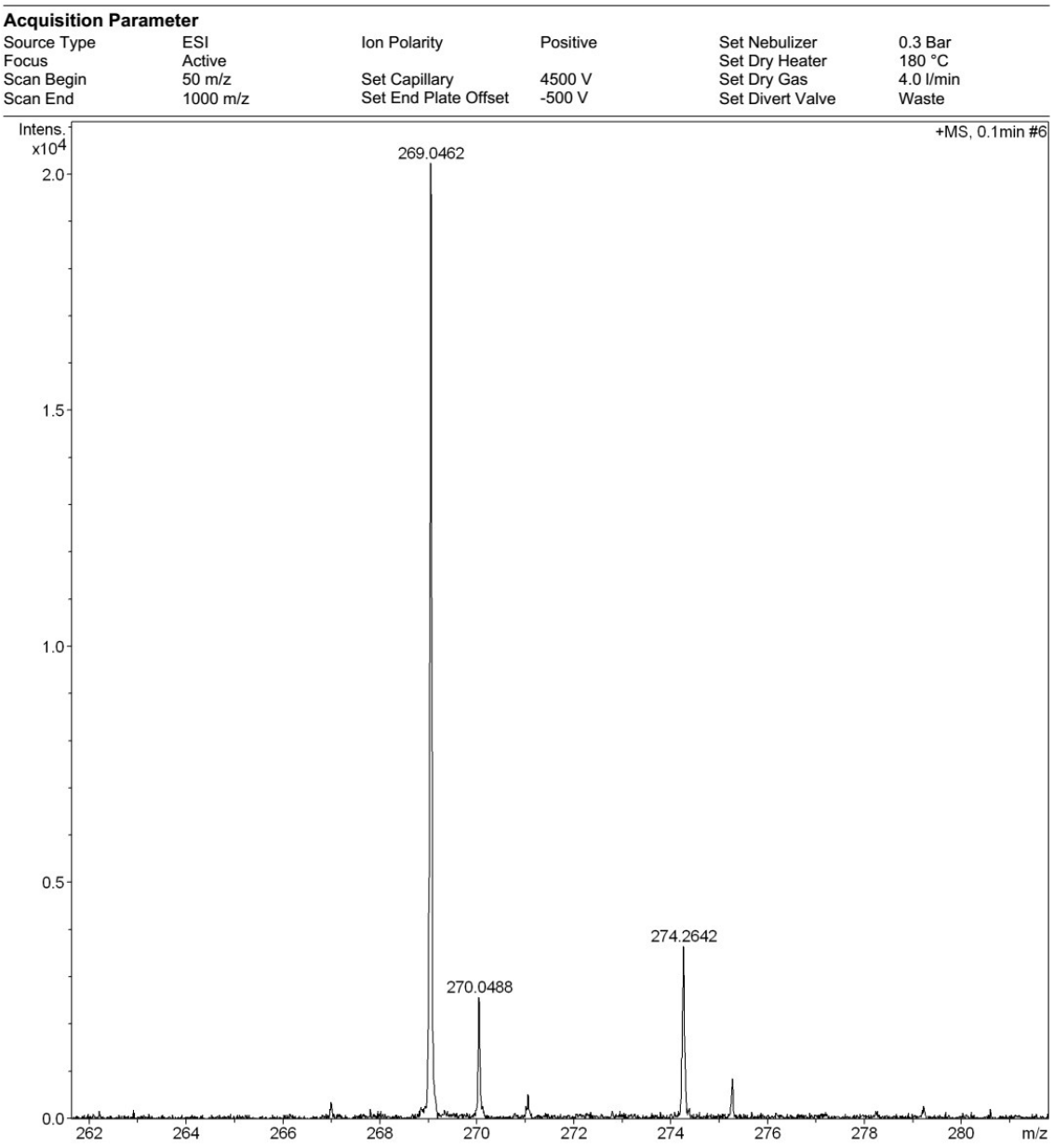

Fig. S27 HRMS Spectrum of reaction solution of **PNPS** and H_2O_2.

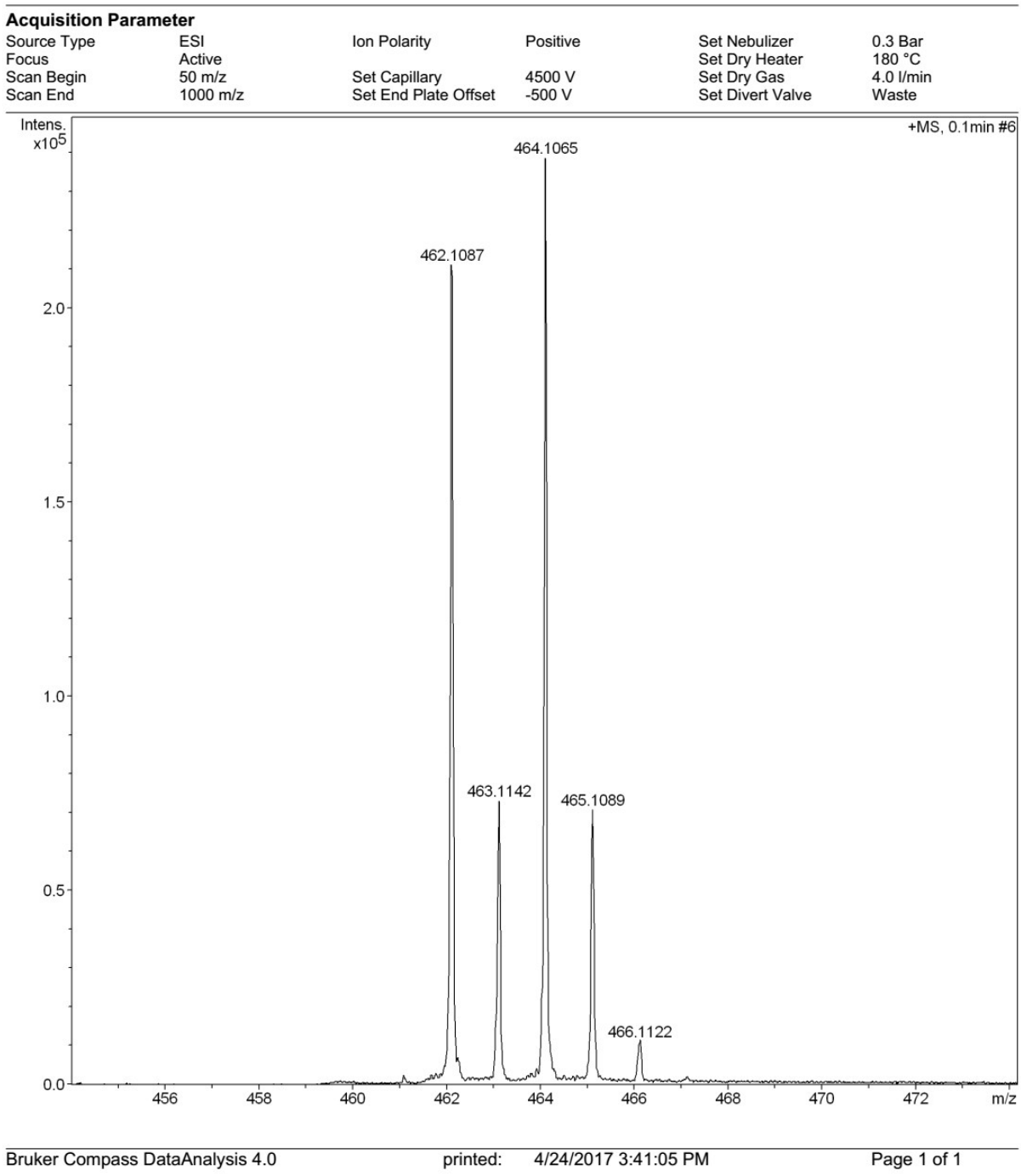

Fig. S28 HRMS Spectrum of reaction solution of **PNPS** and H_2O_2.