Theranostic Endoperoxide Agent with Targeted Singlet Oxygen Release and Concomitant Fluorescence Signal

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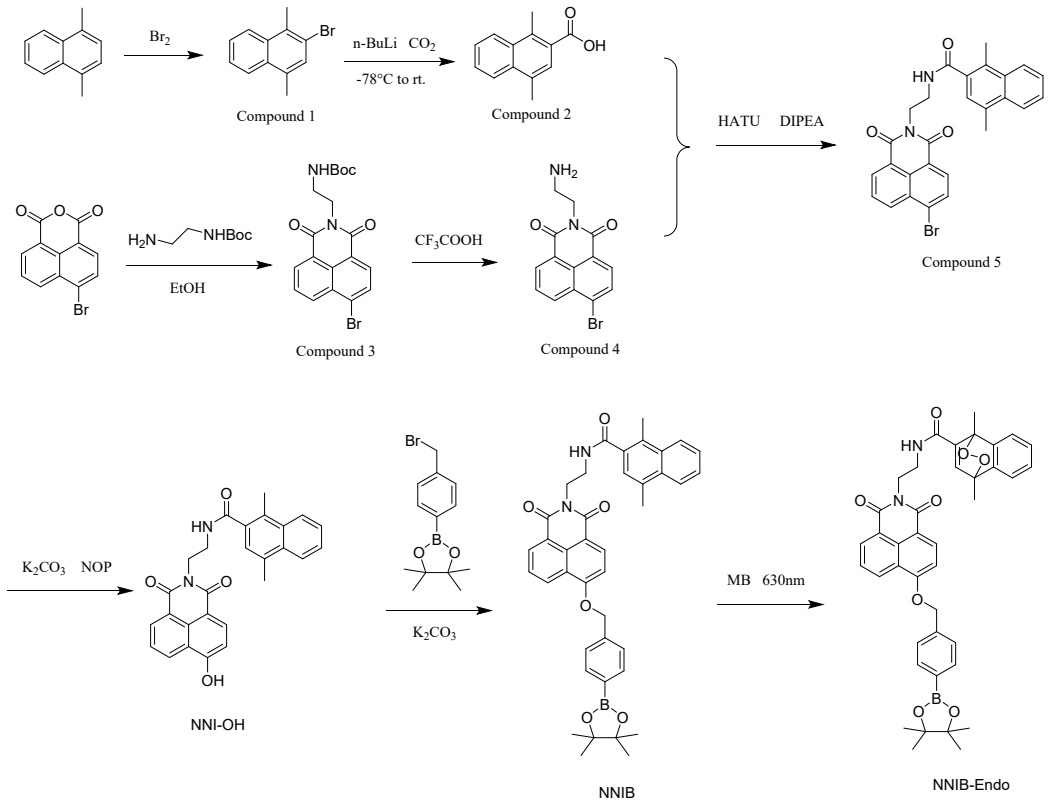
General experimental information

Materials: Unless otherwise specified, all reagents and solvents were purchased from commercial suppliers and used without further purification. Reactions were monitored by thin layer chromatography using Huang-hai TLC Silica gel 60 F-254. Column chromatography was performed by using Mei-gao Silica Gel 60 (particle size: 200-300 mesh). 1,3-Diphenylisobenzofuran (DPBF), were purchased from Shanghai aladdin Co., Ltd (Shanghai, China). Reactive Oxygen Species Assay Kit and Hoechst staining solution were bought from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China).

Instruments: The ^{1}H and ^{13}C NMR spectra were recorded using Bruker Vaian DLG400. Chemical shifts were reported in parts per million (ppm) and coupling constants (*J* values) are given in Hz. Splitting patterns are indicated as follow s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were recorded with Q Exactive Plus (Thermo Fisher, USA). The UV-Vis absorption spectra were performed by using Agilent Cary-3500 UV-Vis spectrophotometer. Fluorescence spectra were determined on Agilent CARY Eclipse spectrophotometer. The MTT assay was performed on a SpectraMax i3x microplate reader (Molecular Devices, USA). Fluorescence imaging was determined on Fluorescence microscope DMi8.

Experimental Procedures

1. Molecular synthesis



Scheme S1. The synthetic route of endoperoxide.

Synthesis of **Compound 1:** 1,4-Dimethylnaphthalene (1.56 g, 10.0 mmol) was dissolved in 15 mL chloroform under Argon atmosphere and exclusion of light. Bromine solution (0.54 mL, 10.5 mmol) was added to the reaction mixture over 10 minutes period of time at 0 °C, in an ice bath. Reaction was allowed to warm at room temperature for 4 hours while it was stirring. Reaction was monitored by TLC. Then, the reaction mixture was diluted with 20 mL chloroform and washed with 25 mL saturated $Na_2S_2O_3$ solution, 25 mL water, and 25 mL saturated NaCl solution, accordingly. Organic layer was combined and dried over anhydrous Na_2SO_4. After removal of the solvent by rotary evaporator, the crude product was purified by silica gel column chromatography with Hexane as the eluent to obtain colorless oil form with 93% yield. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.99 – 7.94 (m, 1H), 7.88 (d, J = 4.2 Hz, 1H), 7.49 – 7.43 (m, 2H), 7.40 (s, 1H), 2.70 (s, 3H), 2.56 (s, 3H).

Synthesis of **Compound 2:** 2-Bromo-1,4-dimethylnaphthalene (1.18 g, 5 mmol) was dissolved in dry tetrahydrofuran (20 mL) under nitrogen atmosphere and cooled to –78 °C, n-BuLi (2.5 M, 4.4 mL, 11 mmol) was added over a period of 20 min. The reaction was warmed to room temperature and then stirred for 15 min. Afterwards it was cooled to –78 °C. Gaseous CO_2 from dry ice which was dried by passing over sicapent was added for 2 h, then the reaction was warmed to room temperature. Ethyl acetate (15 mL) was added after the completion of the reaction, tetrahydrofuran was removed under reduced pressure. The residue was diluted with ethyl acetate (55 mL) and extracted with 0.25 M NaOH solution (5 x 15 mL). The aqueous phase was washed with hexane (30 mL) and then acidified with conc. HCl under stirring. The precipitate was filtered off and washed thoroughly with water. The product was dried in vacuo. The product was

isolated as a white solid. Yield: 726 mg, 73%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.28 – 8.23 (m, 1H), 8.12 – 8.06 (m, 1H), 7.73 – 7.65 (m, 2H), 7.63 (d, J = 1.1 Hz, 1H), 2.86 (s, 3H), 2.68 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.72, 133.47, 133.25, 132.91, 132.18, 129.92, 127.51, 126.81, 126.48, 126.14, 125.01, 19.34, 15.87.

Synthesis of **Compound 3:** 4-bromo-1,8-naphthalic anhydride (0.554 g, 2 mmol) and N-Boc-Ethylenediamine (0.385 g,2.4 mmol) was dissolved into 15 mL ethanol. The solution was refluxed for 3h at 90 °C. After cooling to room temperature, yellow solid was obtained after vacuum filtration followed by washing with 30 mL cold ethanol. Yield: 671 mg, 81%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.60 – 8.56 (m, 1H), 8.48 (d, J = 8.5 Hz, 1H), 8.33 (d, J = 7.9 Hz, 1H), 7.96 (d, J = 7.9 Hz, 1H), 7.76 (dd, J = 8.5, 7.3 Hz, 1H), 4.28 (t, J = 5.8 Hz, 2H), 3.46 (q, J = 5.8 Hz, 2H), 1.20 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.93, 162.91, 155.03, 132.35, 131.19, 130.36, 130.07, 129.56, 129.36, 128.03, 127.05, 121.89, 121.03, 78.14, 39.01, 38.50, 27.18.

Synthesis of **Compound 4:** To a solution of compound 3 (0.671 g, 1.6 mmol) in 10 ml dry DCM, trifluoroacetic acid (1.0 ml) was added dropwise at 0 °C with constant stirring. After that, the reaction mixture was allowed to come to room temperature and stirred under inert atmosphere for 3h. Then, the solvent was evaporated to obtain the desired product as white solid which was used in the next step without further purification. Yield: 0.511 g (quantitative yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.56 (dd, J = 7.9, 4.8 Hz, 2H), 8.32 (d, J = 7.8 Hz, 1H), 8.22 (d, J = 7.9 Hz, 1H), 8.04 – 7.98 (m, 1H), 7.95 (s, 2H), 4.31 (t, J = 5.9 Hz, 2H), 3.17 (q, J = 5.8 Hz, 2H). ^{13}C NMR (126 MHz, DMSO) δ 163.47, 163.42, 132.67, 131.50, 131.32, 130.87, 129.74, 129.14, 128.78, 128.40, 122.91, 122.12, 37.55, 37.45.

Synthesis of **Compound 5**: Compound 4 (159.4 mg, 0.5 mmol), DIPEA (174 μL, 1 mmol) and compound 2 (100 mg, 0.5 mmol) were stirred in acetonitrile (10 mL) respectively for 5 minutes and then HATU (228 mg, 0.6 mmol) was added. The resulting mixture was stirred at rt for 5 h. The precipitate was filtered off and washed thoroughly with cold acetonitrile. The product was dried in vacuo and isolated as a white solid. Yield: 150 mg, 60%. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.61 (d, J = 7.2 Hz, 1H), 8.53 (d, J = 8.4 Hz, 1H), 8.37 (d, J = 7.8 Hz, 1H), 7.97 (t, J = 8.8 Hz, 2H), 7.89 (d, J = 6.7 Hz, 1H), 7.79 (t, J = 7.9 Hz, 1H), 7.52 – 7.42 (m, 2H), 7.09 (s, 1H), 6.28 (s, 1H), 4.46 (d, J = 5.7 Hz, 2H), 3.86 (d, J = 6.9 Hz, 2H), 2.53 (s, 3H), 2.51 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 169.69, 163.18, 163.13, 134.32, 132.36, 131.97, 131.85, 131.33, 131.28, 131.23, 130.72, 129.72, 128.78, 128.72, 128.44, 126.04, 126.01, 124.95, 124.89, 124.28, 123.08, 122.30, 36.60, 18.72, 14.98.

Synthesis of **NNI-OH**: A solution of Compound 5 (150 mg, 0.3 mmol), K_2CO_3 (62 mg, 0.45 mmol) and 2-hydroxyisoindoline-1,3-dione (58.5 mg, 0.36 mmol) dissolved in 3 mL DMSO was vigorously stirred at 120 °C for 5 h. Then the mixture was cooled to room temperature, poured into 25 mL ice water, and acidified with HCl solution (1 M) until a solid product precipitated out. The resulting precipitated solid was purified by column chromatography to give **NNI-OH** as a yellow solid. Yield: 110 mg, 84%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.84 (s, 1H), 8.53 (ddd, J = 8.5, 7.7, 1.2 Hz, 2H),

8.43 – 8.36 (m, 2H), 8.06 (dd, *J* = 6.5, 3.3 Hz, 1H), 7.99 (dd, *J* = 6.4, 3.3 Hz, 1H), 7.79 (dd, *J* = 8.3, 7.3 Hz, 1H), 7.58 (dt, *J* = 6.5, 3.3 Hz, 2H), 7.18 (d, *J* = 8.2 Hz, 1H), 7.16 (s, 1H), 4.31 (dd, *J* = 6.4, 4.8 Hz, 2H), 3.64 (q, *J* = 5.8 Hz, 2H), 2.56 (s, 3H), 2.51 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.29, 164.59, 163.91, 160.62, 135.06, 133.91, 132.60, 132.47, 131.86, 131.50, 129.92, 129.37, 129.24, 126.64, 126.61, 126.09, 125.63, 125.57, 124.90, 122.90, 122.71, 113.50, 110.36, 37.45, 37.43, 19.34, 15.62.

Synthesis of **NNIB**: To a solution of **NNI-OH** (50 mg, 0.114 mmol) in CH_3CN (5 mL) were added K_2CO_3 (32 mg, 0.224 mmol) and 4-(bromomethyl)benzene boronic acid pinacol ester (34 mg, 0.114 mmol). The reaction mixture was heated to reflux overnight. After removal of solvent, the residues were purified by silica gel column chromatography to afford pure product. Yield: 48 mg, 65%.^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.58 – 8.50 (m, 2H), 8.43 (d, *J* = 8.3 Hz, 1H), 7.97 – 7.91 (m, 1H), 7.90 – 7.85 (m, 1H), 7.81 (d, *J* = 7.6 Hz, 2H), 7.62 (t, *J* = 7.8 Hz, 1H), 7.44 (t, *J* = 6.7 Hz, 4H), 7.10 (s, 1H), 7.00 (d, *J* = 8.3 Hz, 1H), 6.46 (t, *J* = 4.7 Hz, 1H), 5.30 (s, 2H), 4.44 (t, *J* = 5.4 Hz, 2H), 3.83 (q, *J* = 5.2 Hz, 2H), 2.50 (s, 6H), 1.28 (s, 12H). ^{13}C NMR (101 MHz, CDCl3) δ 170.19, 164.13, 163.49, 159.11, 137.36, 134.27, 132.81, 132.61, 131.89, 131.75, 131.51, 131.00, 128.98, 128.72, 128.59, 128.23, 125.69, 125.16, 125.13, 124.95, 124.30, 123.70, 123.50, 122.74, 121.09, 113.91, 105.62, 82.97, 69.89, 38.90, 38.23, 28.68, 23.85, 18.25, 14.57.

Synthesis of **NNIB-Endo**: Precursor (Compound **NNIB**, 50 mg) was dissolved in 3 mL DCM and the reaction mixture was cooled down to 0 °C. Catalytic amount of methylene blue (0.5 mg, 1% equiv.) was added and the reaction mixture was stirred under oxygen atmosphere with the irradiation using an 18 W red light (630 nm) for 4 h. When the reaction completed monitored by ^{1}H NMR, methylene blue was removed by activated carbon and the solvent was removed in vacuo to give the endoperoxide. ^{1}H NMR (400 MHz, Chloroform-d) δ 8.55 (dd, J = 14.9, 7.9 Hz, 2H), 8.45 (d, J = 8.2 Hz, 1H), 8.03 (s, 1H), 7.82 (d, J = 7.7 Hz, 2H), 7.64 (t, J = 7.9 Hz, 1H), 7.44 (d, J = 7.7 Hz, 2H), 7.23 (q, J = 4.8, 4.1 Hz, 1H), 7.17 (d, J = 3.5 Hz, 2H), 7.03 (d, J = 8.4 Hz, 1H), 6.76 (s, 1H), 6.53 (s, 1H), 5.33 (s, 2H), 4.35 (q, J = 5.0 Hz, 2H), 3.66 (t, J = 6.8 Hz, 2H), 1.86 (s, 3H), 1.80 (s, 3H), 1.29 (s, 12H)

2. Kinetic experiments of the endoperoxides

With the intention of getting more qualitative results, the NMR investigations of two systems were performed as a function of time, the solvent being $CDCl_3$. It can be observed that these endoperoxides have different half-lives respectively according to appearance/disappearance of their normalized integral values of the selected peaks. The rate constant and half-life calculations were done in accordance to the first-order reaction rate equations. The equations are given below:

$$\ln[A]_t = -kt + \ln[A]_{sum} \quad , \quad t_{1/2} = 0.693/k \qquad \text{Equation (1)}$$

In the following NMR spectra, it is possible to observe the evolution of peaks (2.0-1.5 ppm) which belong to precursor due to endoperoxide cycloreversion. While the peaks of parent compound increase, the peaks of endoperoxide (6.7-6.5, 2.7-2.5 ppm)

decrease.

3. Photophysical characterization of NNIB-Endo and NNI-OH

The stock solution of **NNIB-Endo** or **NNI-OH** (1 mM) was obtained by dissolving **NNIB-Endo** or **NNI-OH** in DMSO. The stock solution of H_2O_2 was diluted to 4 mM stepwise. **NNIB-Endo** and H_2O_2 were mixed in a solution composed of different organic solvents and PBS (10 mM, pH = 7.4) in a volume ratio of 1 : 1. The final concentration of **NNIB-Endo** was 20 μM, and H_2O_2 was 8.0×10^{-4} - 2.0×10^{-6} M. The UV-Vis absorption spectra were performed by using Agilent Cary-3500 UV-Vis spectrophotometer. The fluorescence spectra were obtained on a Agilent CARY Eclipse spectrophotometer with the excitation slit and emission slit set at 5.0 nm.

4. Selectivity of NNIB-Endo toward H_2O_2

The selectivity of **NNIB-Endo** for H_2O_2 was evaluated against biologically relevant analytes, including cations (e.g., Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}), anions (e.g., Cl^-, Br^-, CO_3^{2-},HCO_3^-,CH_3COO^-, SO_4^{2-}), reactive oxygen species (ROS; e.g., ·OH, NO_2^-, ClO^-), and amino acids (e.g., cysteine, glutathione), by monitoring fluorescence intensity changes under standardized conditions (pH 7.4, 37°C). Fluorescence responses were measured before and after the addition of each analyte. Cations, anions, and amino acids exhibited negligible changes in fluorescence intensity.

5. Cell Viability Assay

MTT assays were used to investigate the cytotoxicity of **NNIB-Endo**、**NNIB** and **NNI-OH**. HepG2 cells、MCF-7 cells 、A549 cells and 4T1 cells were cultured in DMEM/F12 medium or RPMI-1640 medium (10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin) in a cell incubator at 37 °C under 5% CO_2. Approximately 6×10^3 cells (each well) were added to a sterile 96- well plate and cultured for 24 h. Then, the samples were added (The stock solution was prepared in DMSO, and the final concentration of DMSO was maintained at 0.1%), and the mixture was incubated for another 24 h. After that, 10 μL MTT solutions (5.5 mg/mL) were added and cultured for 4 h. The supernatant was removed and replaced with 100 μL DMSO to dissolve the formazan crystals. After shaking at 37 °C for 5 min, the absorbance at 570 nm was recorded. The samples of each group were carried out with six replicates. All the experimental values were obtained by subtracting the background signals of the media without cells. The cell viability was calculated according to the following equation:

$$Cell\ Viability\ （\%）= \frac{100\% \times （OD_{Treated} - OD_{Background})}{OD_{Control} - OD_{Background}}$$

where $OD_{Treated}$ was obtained in the presence of inhibitors.

6. Fluorescence imaging of sample in cells

HepG2 cells were seeded in a 48-well plate with a density of 10^4 cells per well and

incubated for 24 h. The cells were then treated with 10 μM of different compound respectively in DMEM for 4 or 8 h. Cell imaging was conducted after washing the cells with PBS 3 times.

7. Fluorescence Imaging of Intracellular H_2O_2

Super-resolution fluorescence imaging technology was also employed to assess the ability of endoperoxide to response to endogenous H_2O_2 in cancer cells. HepG2 cells (cancer cells) and HUVEC cells (normal cells) were transferred to a 48-well plate and cultured for 24 hours. Subsequently, the cells were divided into four groups. The first group (HepG2 cells) and second group (HUVEC cells) were not pretreated, while the third group (HUVEC cells) received a pretreatment with H_2O_2 (25 μM) for 2 hours, the fourth group (HUVEC cells) received a pretreatment with PMA (5 μg/mL) for 2 hours. After washing three times with PBS buffer, cells in all four groups were incubated with **NNIB-Endo** (10 μM) for 4 hours. Fluorescence imaging was performed using a fluorescence microscope (DMi8).

8. Detection of intracellular singlet oxygen generation

Next, we used 2,7-dichlorofluorescein diacetate (DCFH-DA) to track the production of ROS in cancer cells. DCFH-DA is nonfluorescent which emits bright fluorescence in the presence of ROS in cancer cells. HepG2 cells (1.5×10^5 cells/well) were cultured in glass-bottom dishes for 24 h to ensure adhesion. Cells were subsequently incubated with 25 μM endoperoxide probes for 25 min at 37°C. After medium removal and three PBS washes, intracellular ROS levels were assessed by treatment with 10 μM DCFH-DA in DMEM (25 min, 37°C), followed by PBS washing. Nuclei were counterstained with Hoechst 33342 (10 μg/mL, 10 min) prior to final PBS washes. Fluorescence imaging was performed using a fluorescence microscope (DMi8).

9. In vitro cytotoxicity

Cell apoptosis analysis was evaluated via a fluorescence microscope (DMi8). HepG2 cells were seeded in a 48-well plate with a density of 10^4 cells per well and incubated for 24 h. The cells were then treated with 10 μM of different compound respectively in DMEM for 8 h. After incubation for a further 8 h, the cells were carefully washed with PBS and stained with Calcein AM and propidium iodide (PI) for 25 min. Finally, fluorescence imaging of the cells was performed by DMi8.

The apoptosis and necrosis of the cells were measured using the Annexin V-FITC Apoptosis Detection Kit (BestBio). HepG2 cells were seeded in a 6-well plate with a density of 10^6 cells per well and incubated for 24 h. The cells were then treated with 10 μM of different compound respectively in DMEM for 8 h. After 8 h further incubation, the cells were collected and washed with PBS three times. Further incubation was performed with Annexin V and PI at room temperature for 30 min. At last, cells were assessed by flow cytometry analysis.

5×10^3 per dish HepG2 cells were seeded with DMEM medium containing 10%

FBS and 1% penicillin on Nunclon Sphera 96U Bottom Plate. The multicellular tumor spheroids were incubated at 37 °C. The culture medium was replaced every two days. Annexin-FITC/PI Apoptosis Detection Kit was used for detection of **NNIB-Endo** induced multicellular tumor spheroids apoptosis. The multicellular tumor spheroids were incubated onto the Nunclon Sphera 96U Bottom Plate for 4 days, then they were treated with 10 μM of different compound respectively at 37 °C for 8 hours. After washing twice of PBS, the multicellular tumor spheroids were stained with Annexin-FITC/PI Apoptosis Detection Kit according to the manufacture instructions. The fluorescence was observed through the fluorescence microscope (DMi8).

10. Western blotting analysis

4T1 cells were cultivated in 6-well plates until they attained an estimated confluence of 80%. The cells were further incubated with PBS, **NNI-OH**, **NNIB**, **NNIB-Endo** for an additional 8 h at a temperature of 37 ℃ respectively. Following the incubation period, the cells were ruptured, and the levels of proteins were measured using a BCA kit. The proteins obtained were isolated using SDS-PAGE and subsequently deposited onto PVDF membranes. Seal the membrane using a solution of skim milk powder with a concentration of 5% for a duration of 1 h. The samples were thereafter kept at a temperature of 4 ℃ overnight and exposed to primary antibodies targeting Caspase-3, and β-actin. On the following day, horseradish peroxidase was combined with a secondary antibody (specifically, goat against rabbit) at a dilution of 1:5000. The mixture was then incubated at room temperature for a duration of 1 h. Following the washing step using TBST, protein bands were detected using a chemiluminescence detection reagent.

11. Scratch Wound Healing Assay

HepG2 cells were seeded in 6-well plates at a density of 8×10^5cells per well, and the following day the confluent monolayer was scratched with a 20 μL tip. Washing twice with PBS and refreshing medium with 10% FBS was followed. Each well was treated again with the PBS, **NNI-OH**, **NNIB**, **NNIB-Endo** respectively and incubated at 37 °C under 5% CO_2. After 0 h, 24 h and 48 h snapshots of the scratch were taken with the microscope (10×) (Olympus IX81, Tokyo, Japan) with cell F© software. Migration was calculated as $(1-\frac{A_X}{A_0})\%$, where A_0 and A_x represented the empty scratch area at 0 h and 24 h, 48 h, respectively.

12. Migration assay

The transwell migration assay was used to evaluate the migration inhibition of **NNI-OH**, **NNIB**, **NNIB-Endo**. Briefly, the upper chambers with polycarbonate membranes containing 8 μm pores were washed with PBS and incubated in 1% bovine serum albumin (BSA) for 1 h. HepG2 cells were seeded into the upper chamber of the transwell, 10% fetal bovine serum was placed in the lower chamber as attractant. Then, the cells were allowed to grow at 37 °C in serum-free medium. An equal volume of **NNI-OH**, **NNIB**, **NNIB-Endo** or PBS was added as a compared group and a blank control group. After 24 h, the upper chambers were washed with PBS and the cells on the upper surface were scrubbed off with humid cotton buds. Then, the cells on the

bottom surface were fixed with paraformaldehyde for 20 min and stained with 0.1% crystal violet solution for 30 min. Number of cells that migrated through the polycarbonate membrane was counted in six fields with an optical microscope. The inhibition rate was calculated compared with blank control group.

13. *In vivo* anti-tumor study

For *in vivo* antitumor evaluation, 4T1 tumor-bearing mice were randomly allocated into four experimental groups (n=6 per group) when tumor volumes reached ~200 mm^3. Treatments administered via injection included: physiological saline (vehicle control), **NNI-OH**, **NNIB**, and **NNIB-Endo** (The stock solution was prepared in DMSO, and the final concentration of DMSO was maintained at 0.1%). Tumor dimensions (width [W] and length [L]) and body weights were systematically monitored at regular intervals, with tumor volumes calculated using the formula $V = (W^2 \times L)/2$. After 12 days of treatment, all animals were euthanized for collection of tumor tissues and major organs (heart, liver, spleen, lungs, kidneys). Histopathological evaluations were performed through hematoxylin and eosin (H&E) staining, Ki67 proliferation analysis, and TUNEL apoptosis detection, with tissue sections examined by light microscopy.

Results and Discussion

a) **b)**

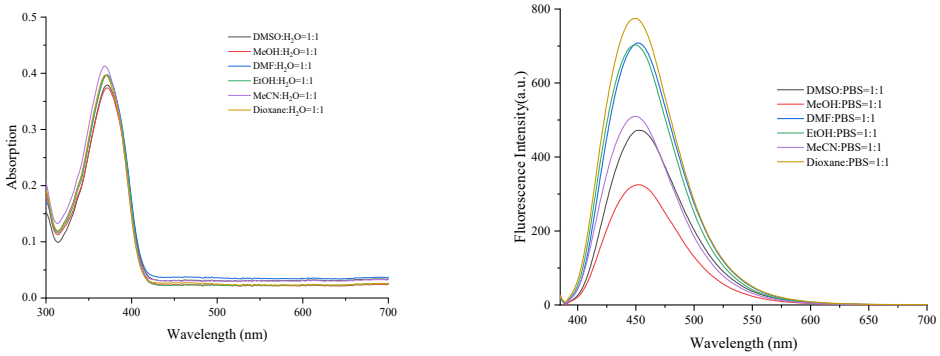


Figure S1. Photophysical characterization of **NNIB-Endo** (20 µM): a) Absorbance b) Fluorescence spectrum in different solvent systems (For fluorescence measurements: λ ex= 375 nm slit:5 nm at 25℃.)

Table S1. Photophysical characterization data of **NNIB-Endo**

Solvent system	λmax / nm	Imax / a.u.
DMSO/PBS	452	472
MeOH/PBS	451	324
DMF/PBS	452	708
EtOH/ PBS	448	702
MeCN/ PBS	448	509
Dioxane/ PBS	449	774

a) **b)**

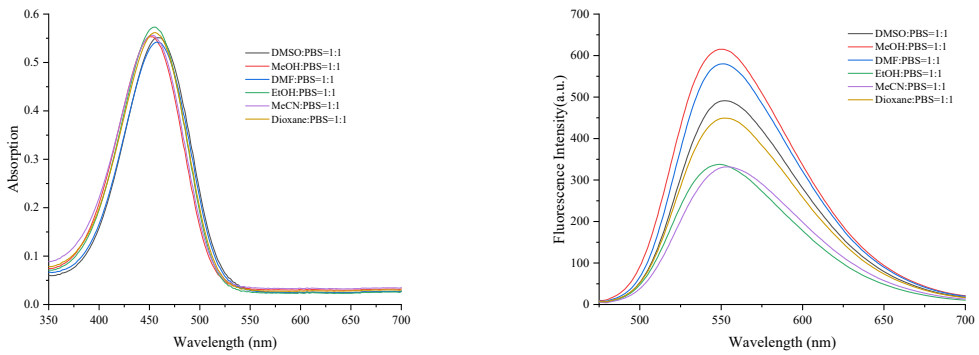


Figure S2. Photophysical characterization of **NNI-OH** (20 µM): a) Absorbance b) Fluorescence spectrum in different solvent systems (For fluorescence

measurements: λ ex= 450 nm slit:5 nm at 25℃.)

Table S2. Photophysical characterization data of **NNI-OH**

Solvent system	λmax / nm	Imax / a.u.
DMSO/PBS	550	490
MeOH/PBS	550	615
DMF/PBS	551	580
EtOH/ PBS	545	336
MeCN/ PBS	555	331
Dioxane/ PBS	552	449

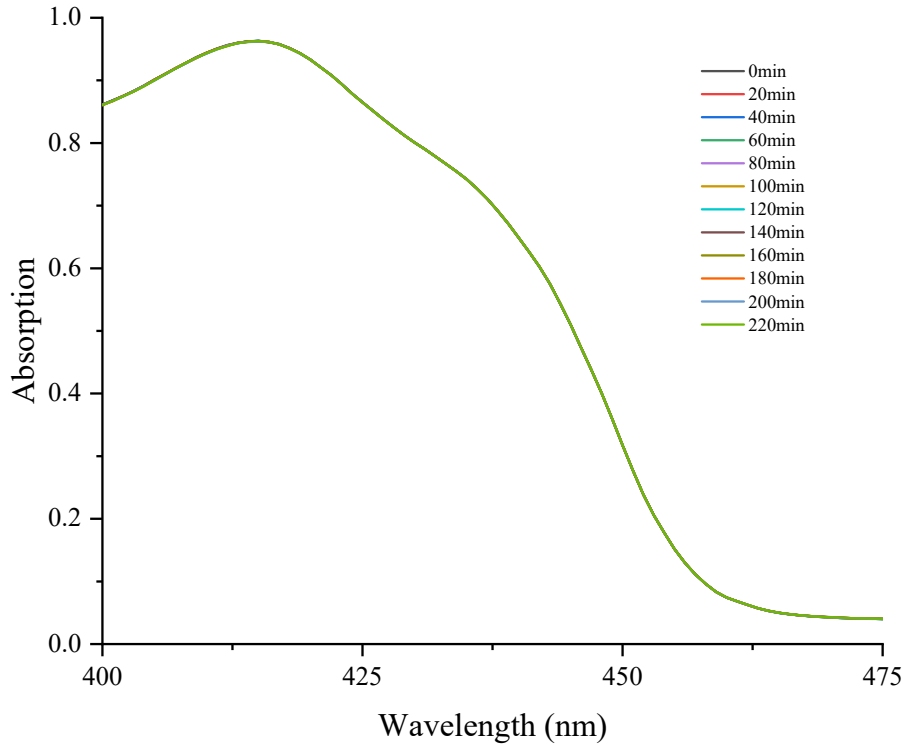


Figure S3. Time dependent UV-Vis spectra of DPBF (50 μM) in the presence of 500 μM **NNIB** in DMF at 37 ℃.

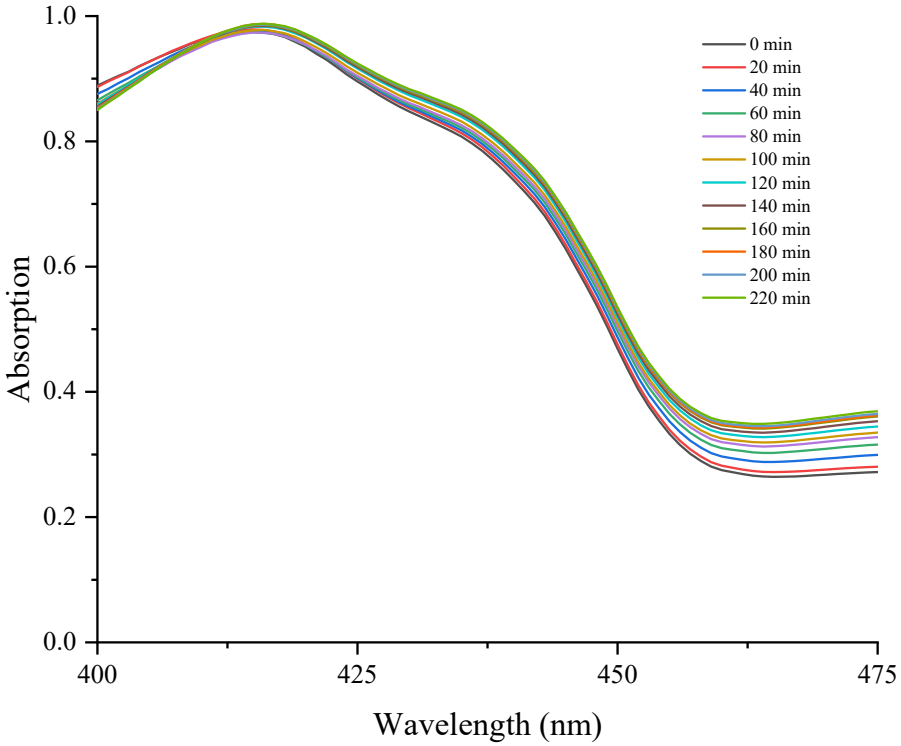


Figure S4. Time dependent UV-Vis spectra of DPBF (50 μM) in the presence of 500 μM **NNI-OH** in DMF at 37 ℃.

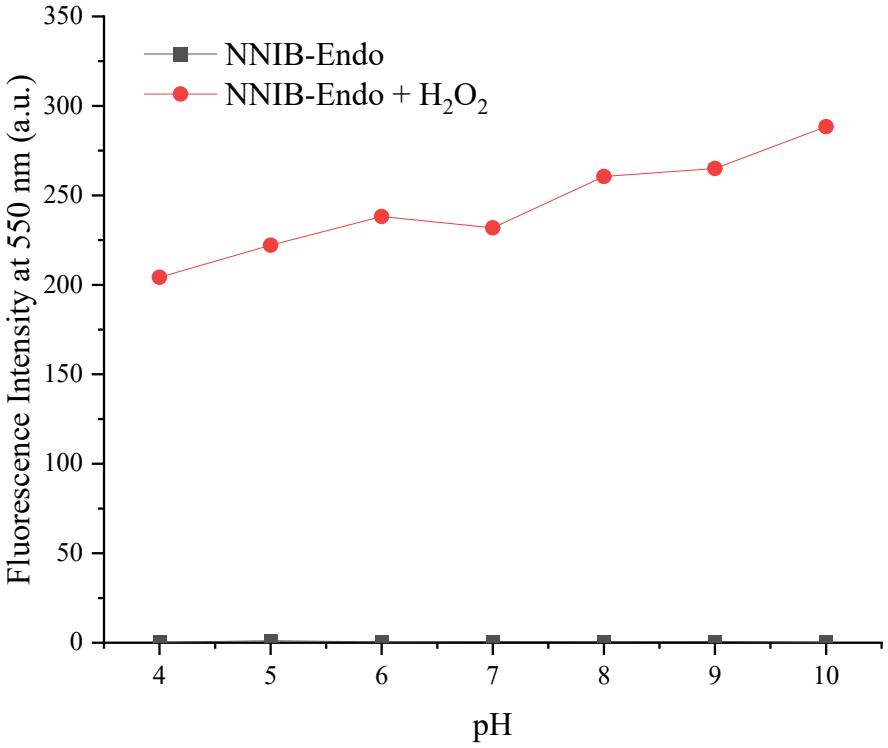


Figure S5. Fluorescence intensity changes at 550 nm before (black line) and after (red line) the response of probe **NNIB-Endo** and H_2O_2 under different pH buffers.

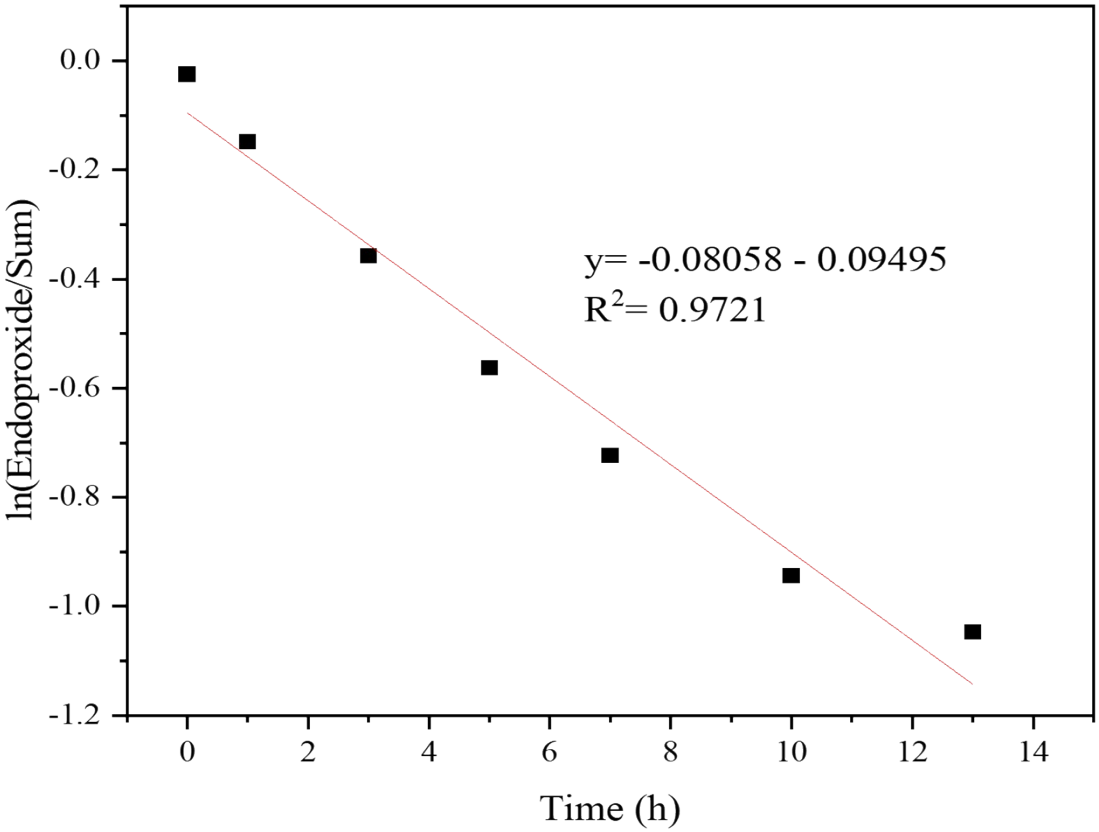


Figure S6. Cycloreversion of endoperoxide **NNIB-Endo** over time at 37 °C. The values were obtained from ^{1}H NMR in $CDCl_3$. The half-life was calculated as 8.6 h according to the equation (1).

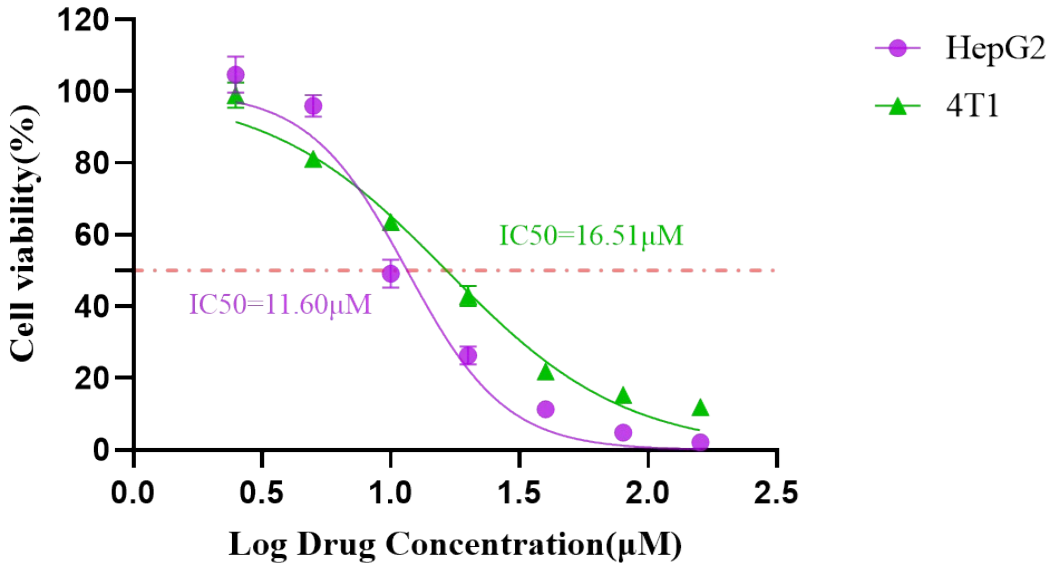


Figure S7. IC50 values of HepG2 cells and 4T1 cells after incubation with **NNIB-Endo**

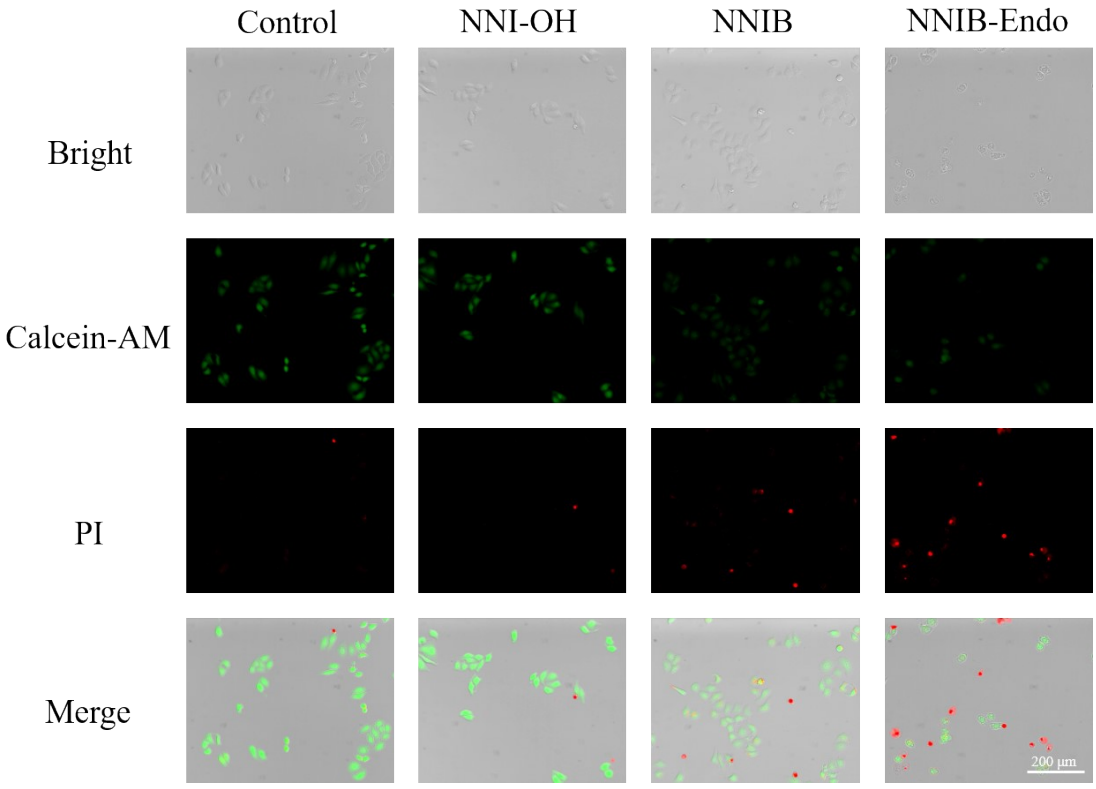


Figure S8. Fluorescence imaging of Calcein-AM and PI stained HepG2 cells after different treatments. Calcein-AM: λex = 488 nm, λem = 500–550 nm. PI: λex = 561 nm, λem = 590–640 nm. Scale bar = 200 μm.

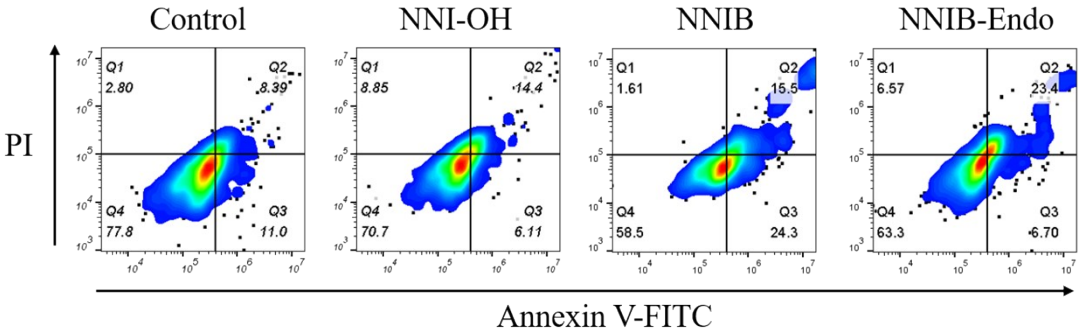


Figure S9. Flow cytometry evaluation of HepG2 cells with the different treatments.

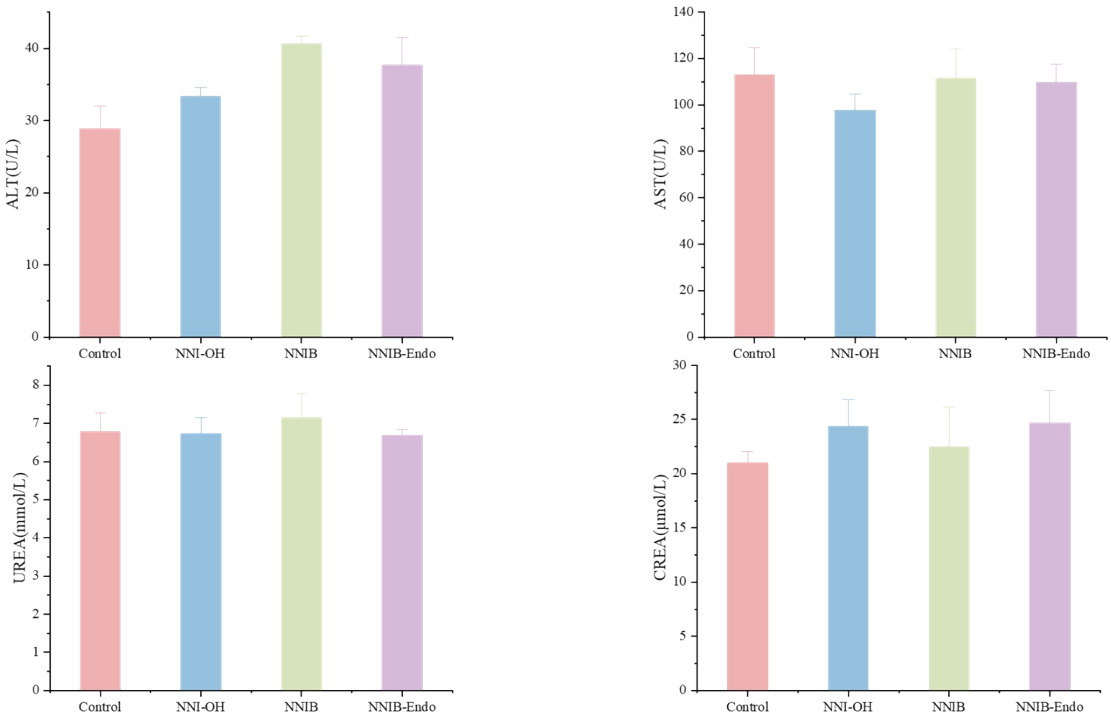


Figure S10 Serum biochemistry study: ALT, AST, BUN, CREA. Data are presented as mean ± SD (n = 3).

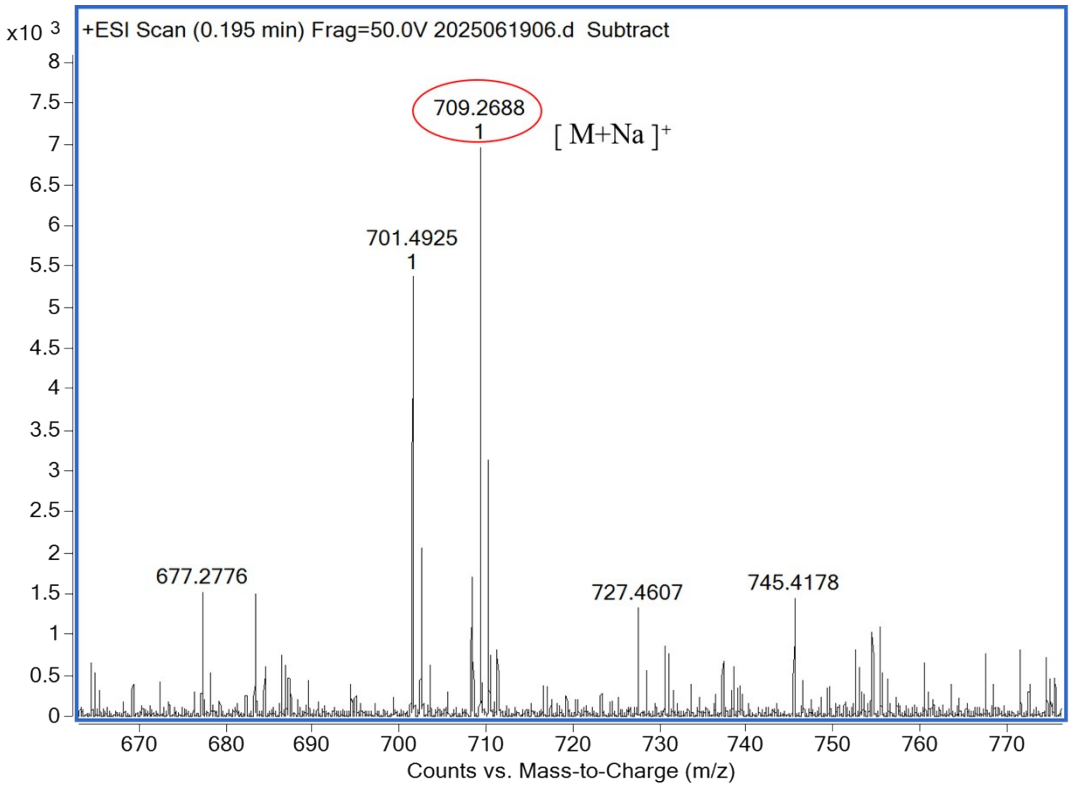


Figure S11 Mass spectrum of the endoperoxide **NNIB-Endo**.

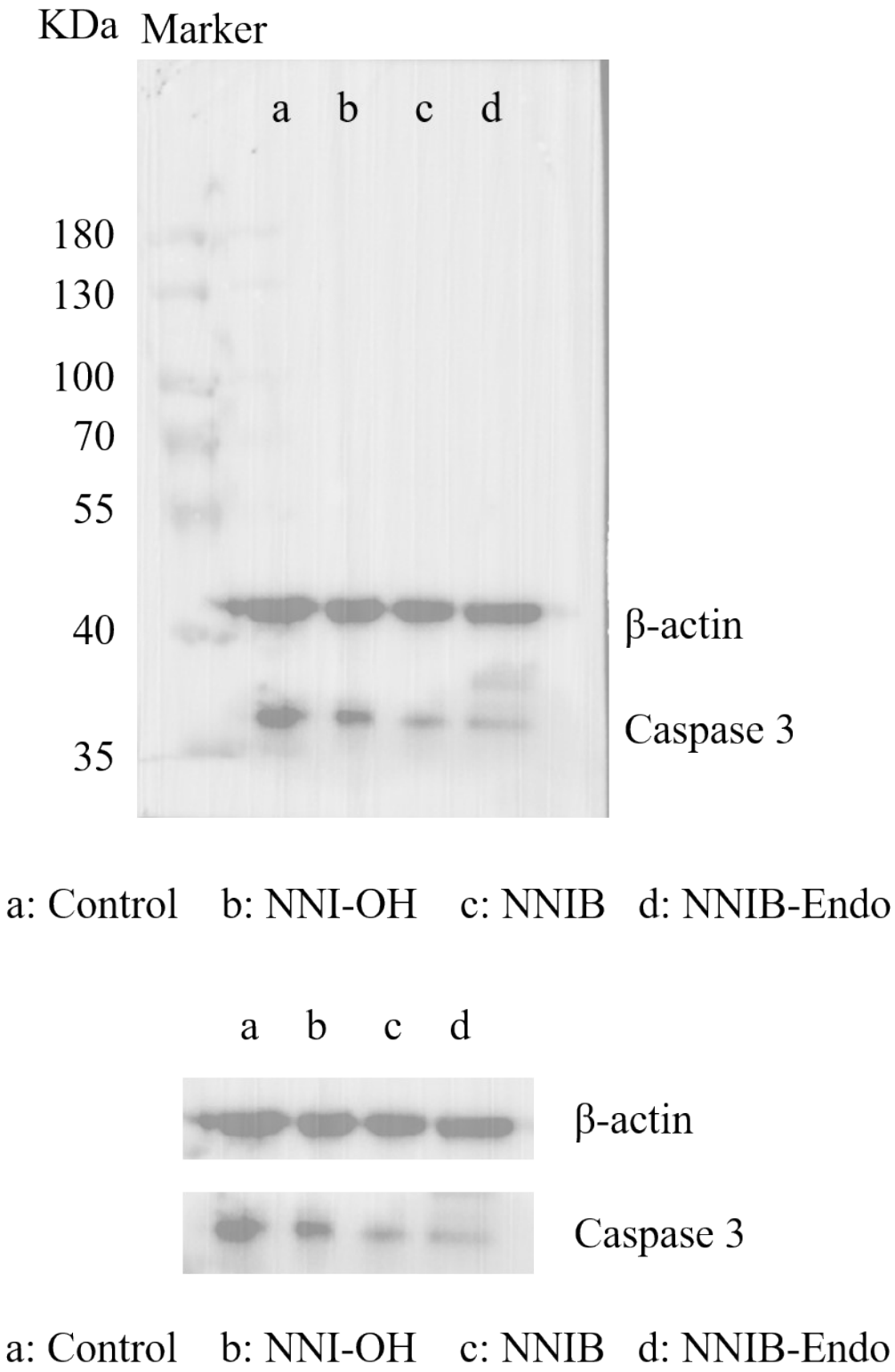


Figure S12 Uncropped Western blot picture with HepG2 cells under different treatments.

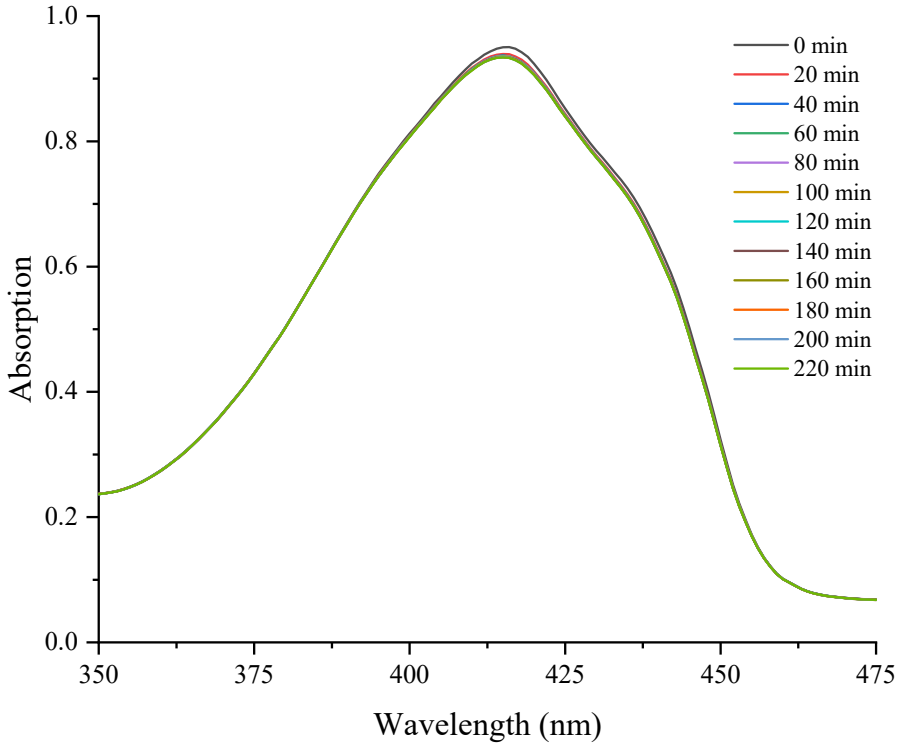


Figure S13. Time dependent UV-Vis spectra of DPBF (50 μM) in DMF at 37 ℃.

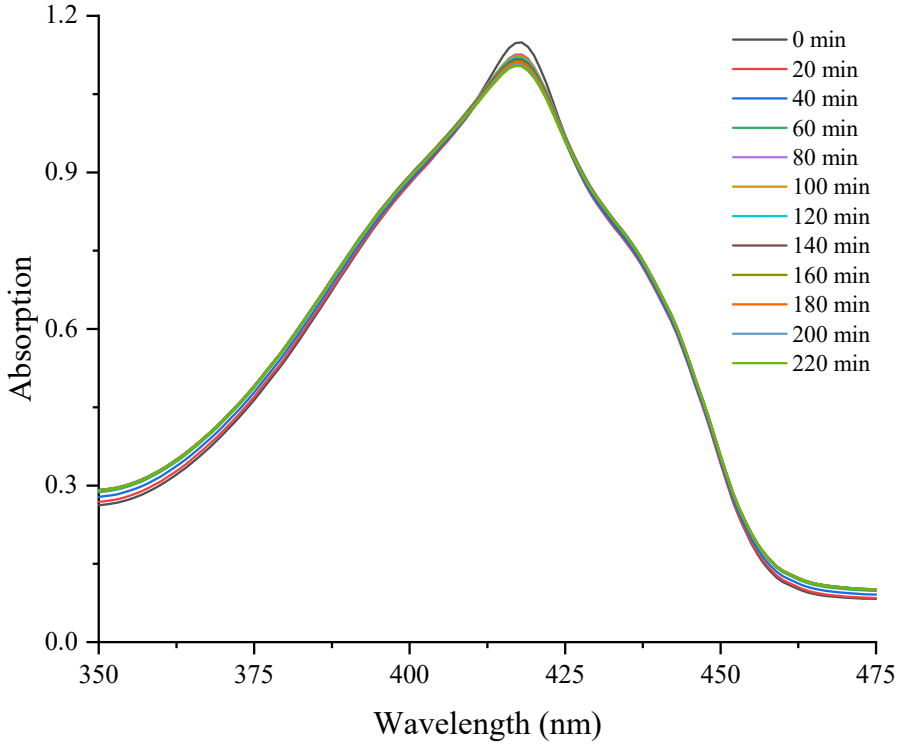


Figure S14. Time dependent UV-Vis spectra of DPBF (50 μM) in the presence of 500 μM H_2O_2 in DMF at 37 ℃.

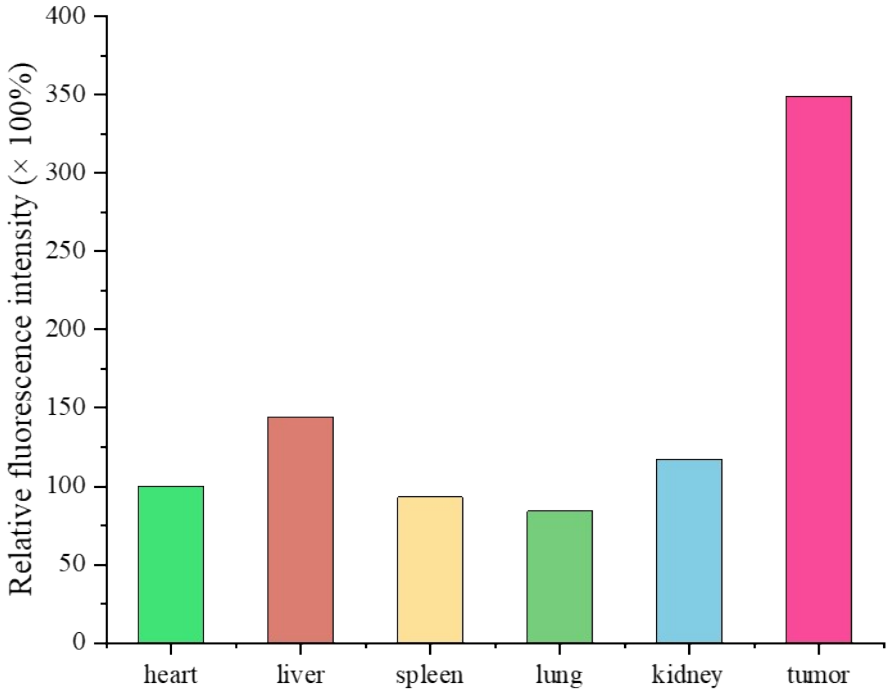


Figure S15 Relative fluorescence intensity in heart, liver, spleen, lung, kidney and tumor tissues.

NMR Spectrum

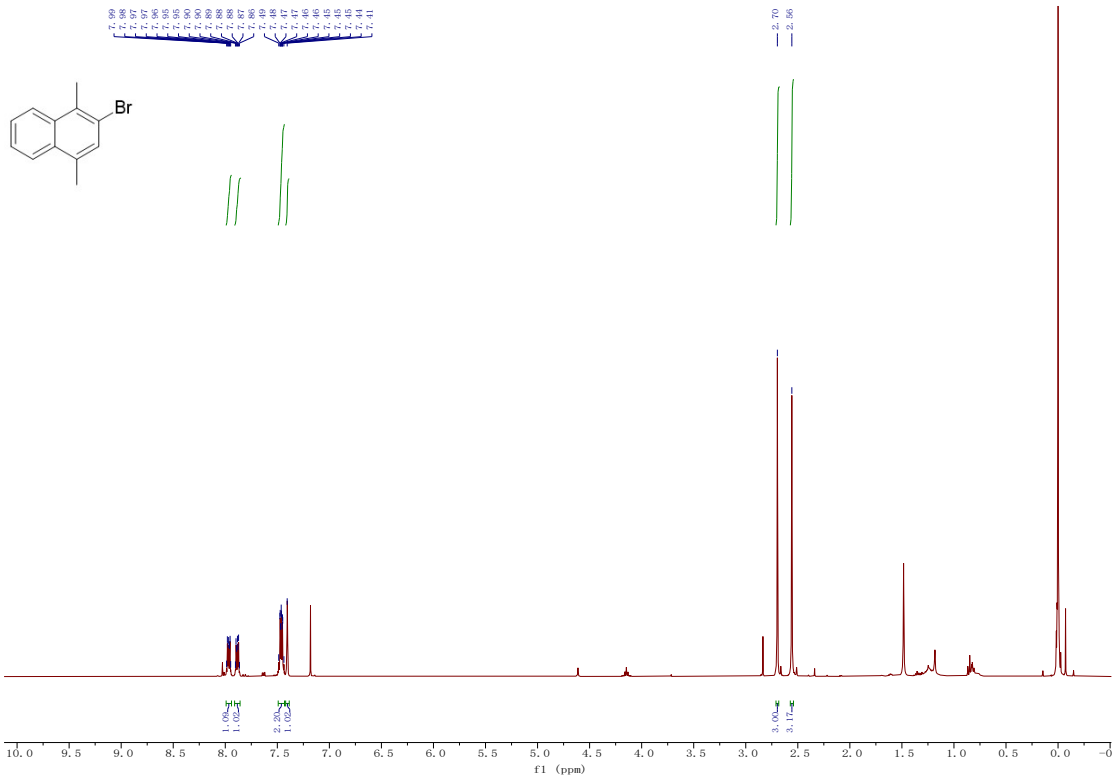


Figure S12 ^{1}H-NMR of compound 1

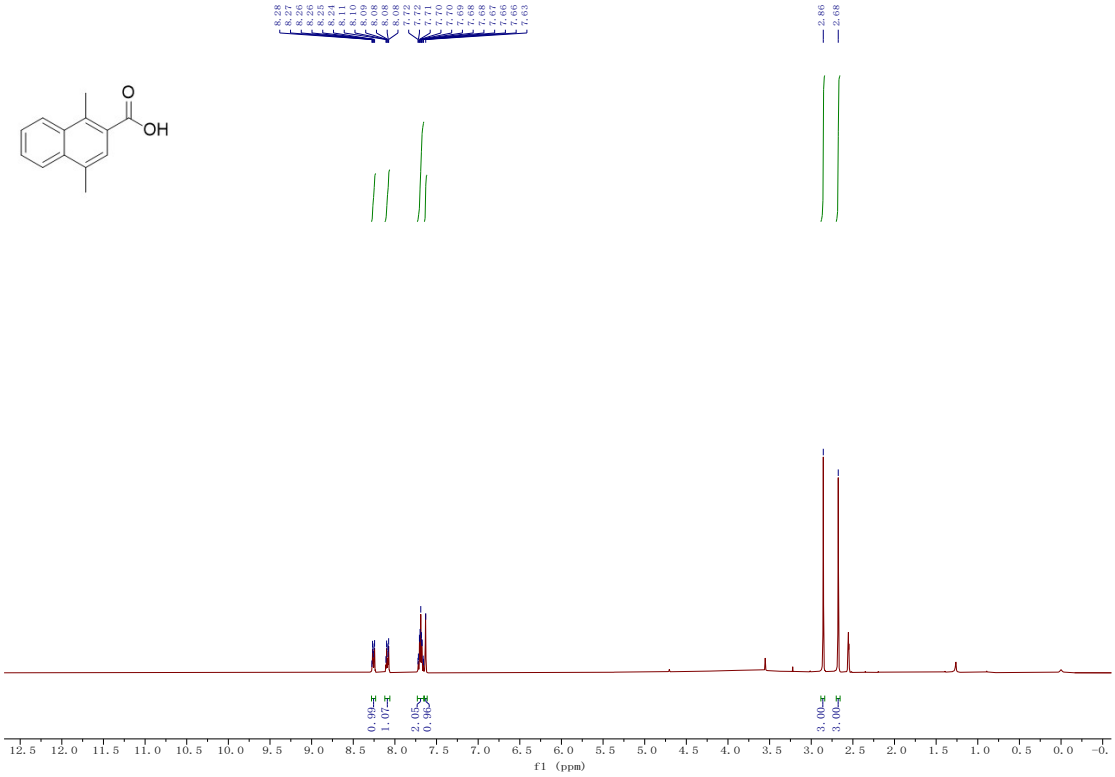


Figure S13 ^{1}H-NMR of compound 2

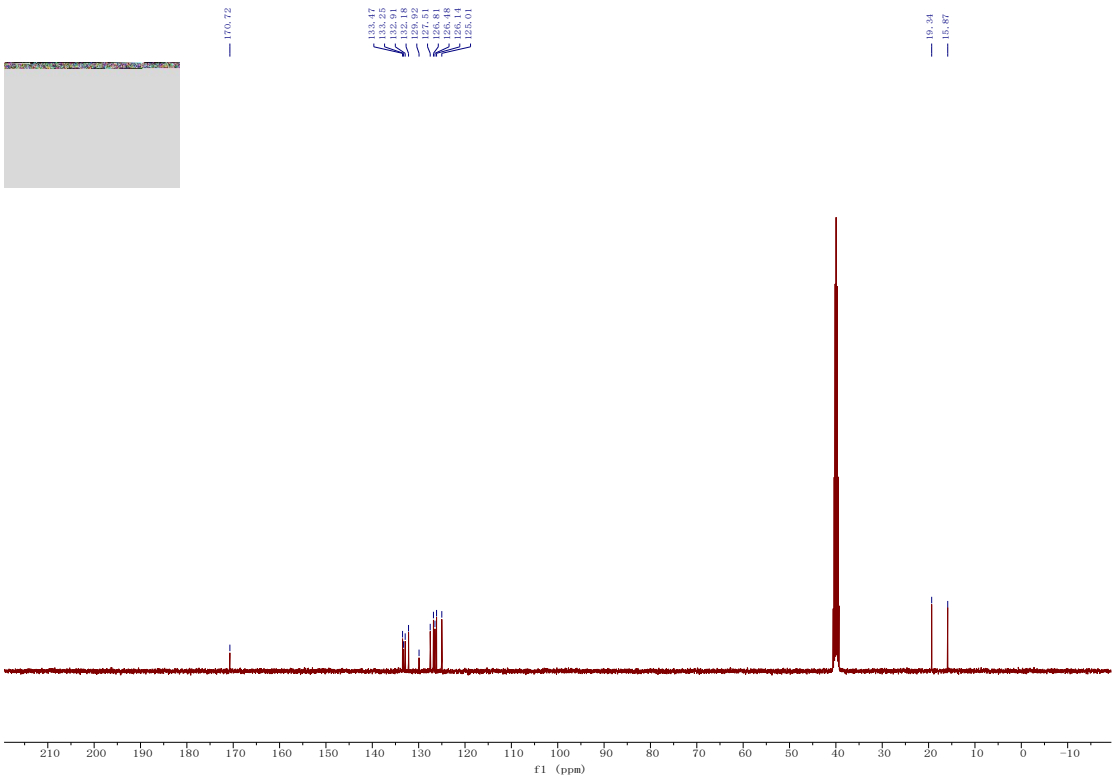


Figure S14 ^{13}C-NMR of compound 2

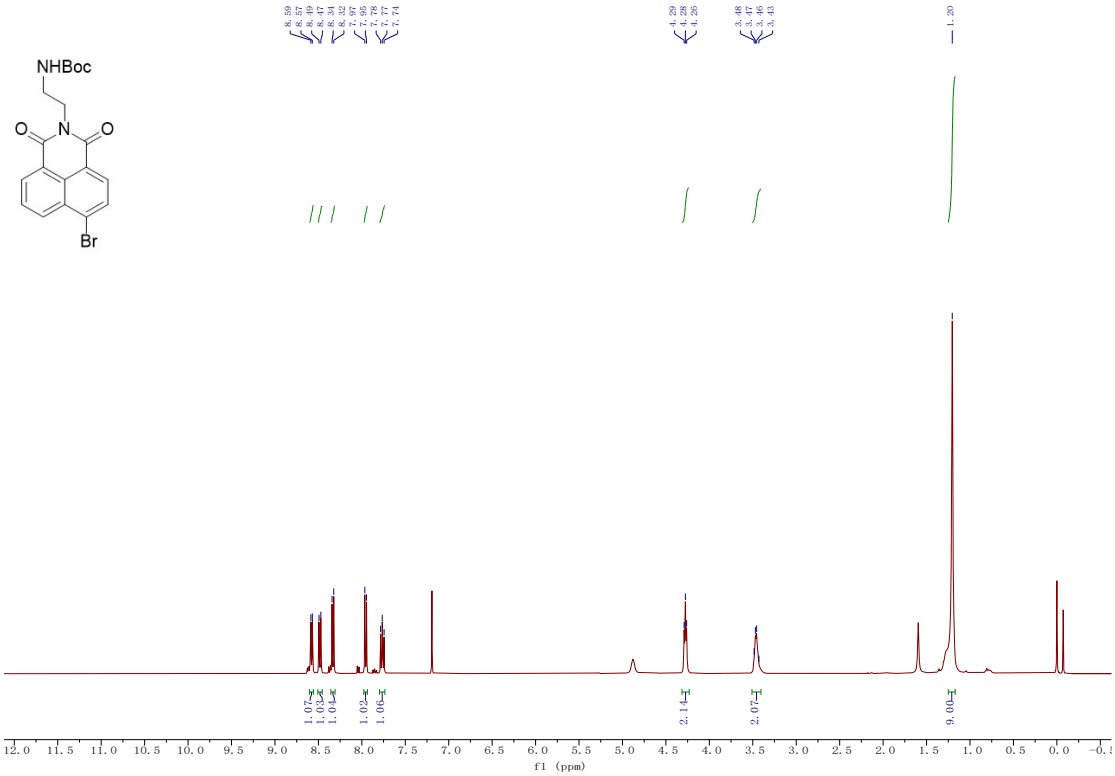


Figure S15 ^{1}H-NMR of compound 3

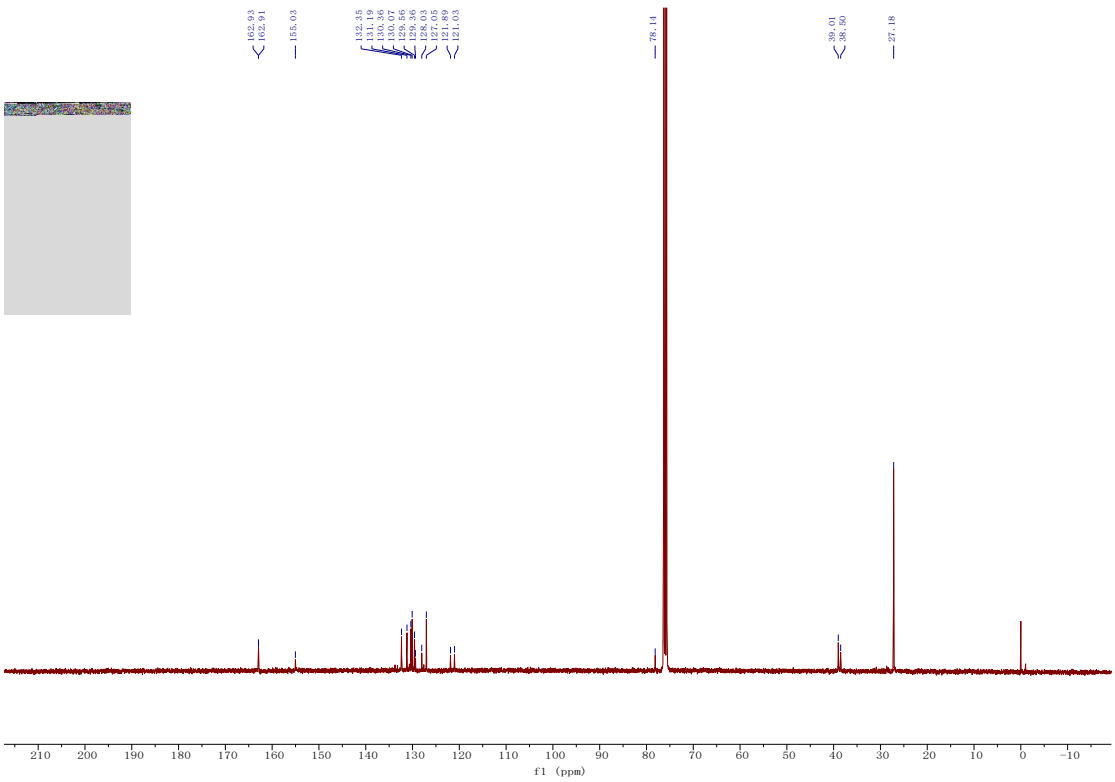


Figure S16 ^{13}C-NMR of compound 3

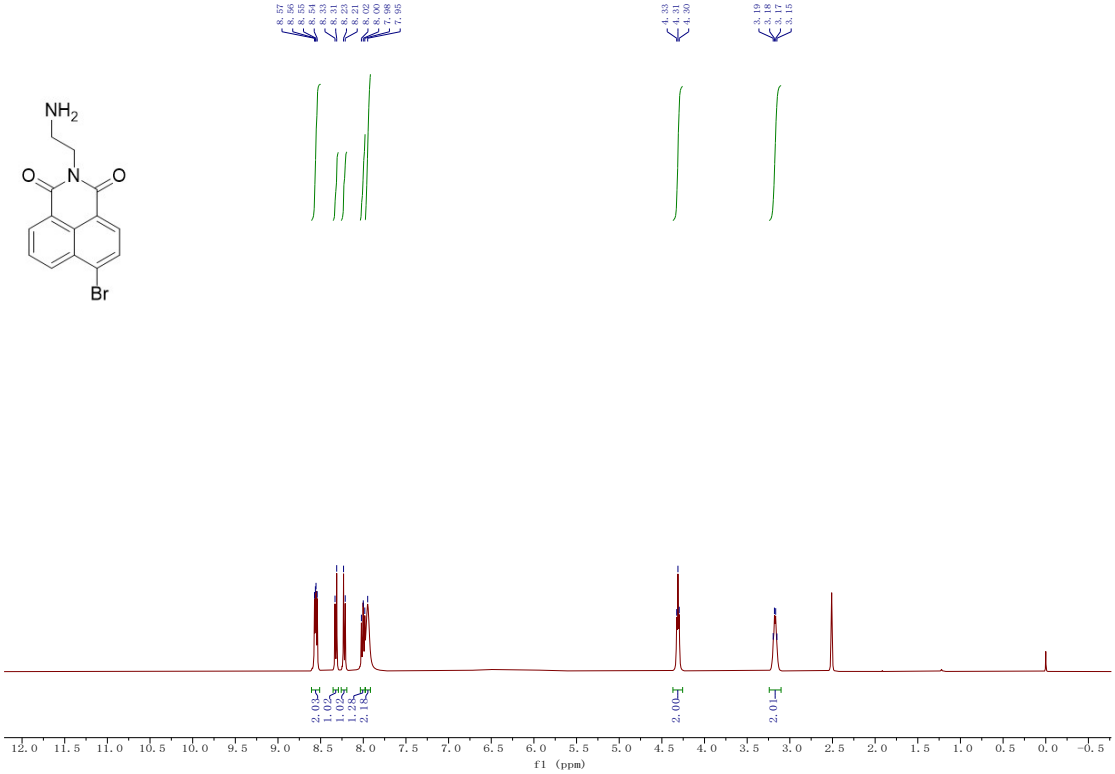


Figure S17 ^{1}H-NMR of compound 4

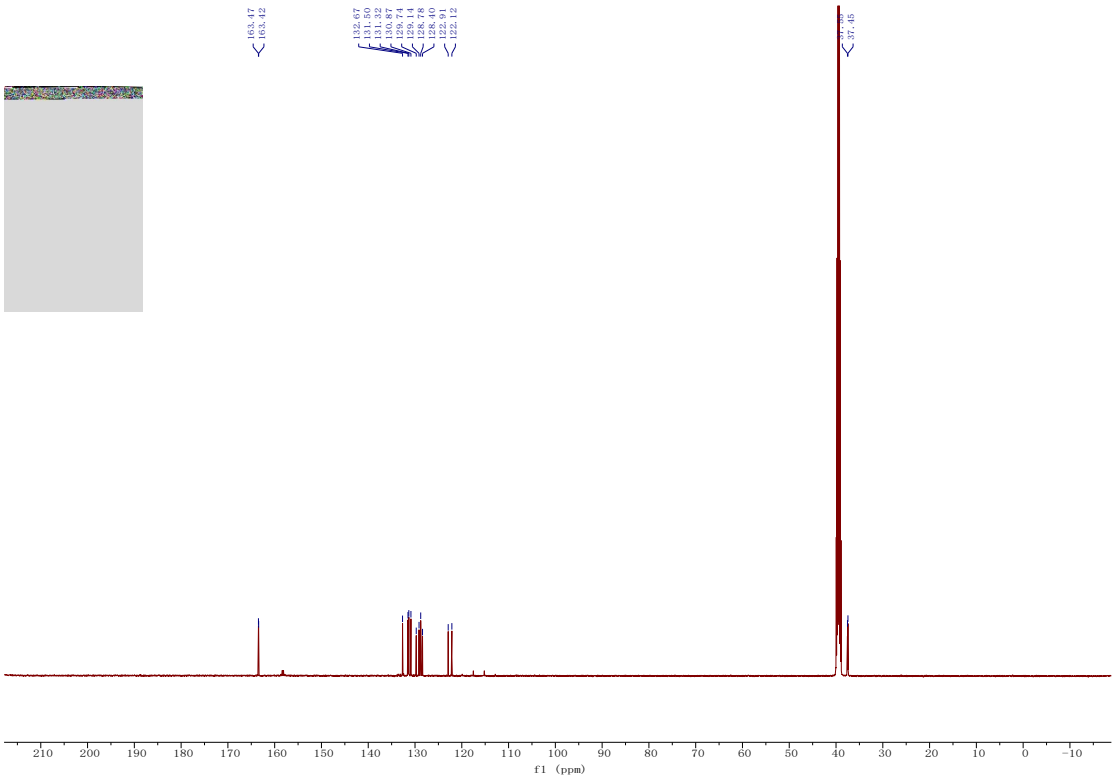


Figure S18 ^{13}C-NMR of compound 4

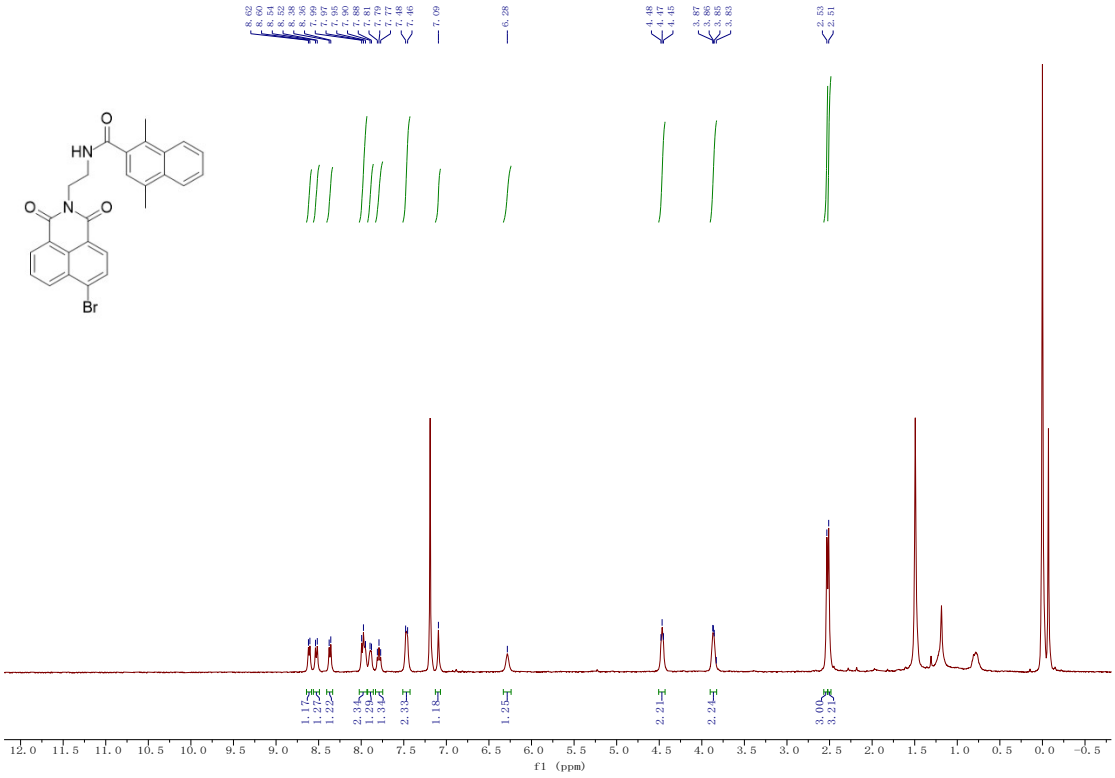


Figure S19 ^{1}H-NMR of compound 5

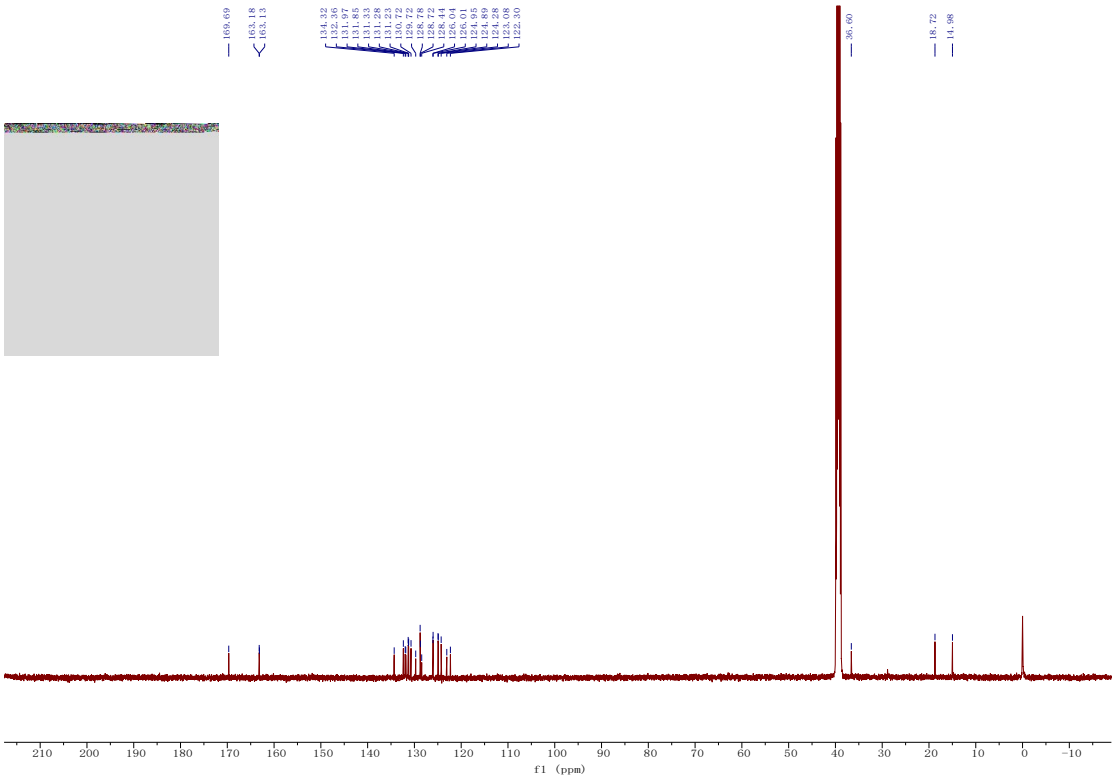


Figure S20 ^{13}C-NMR of compound 5

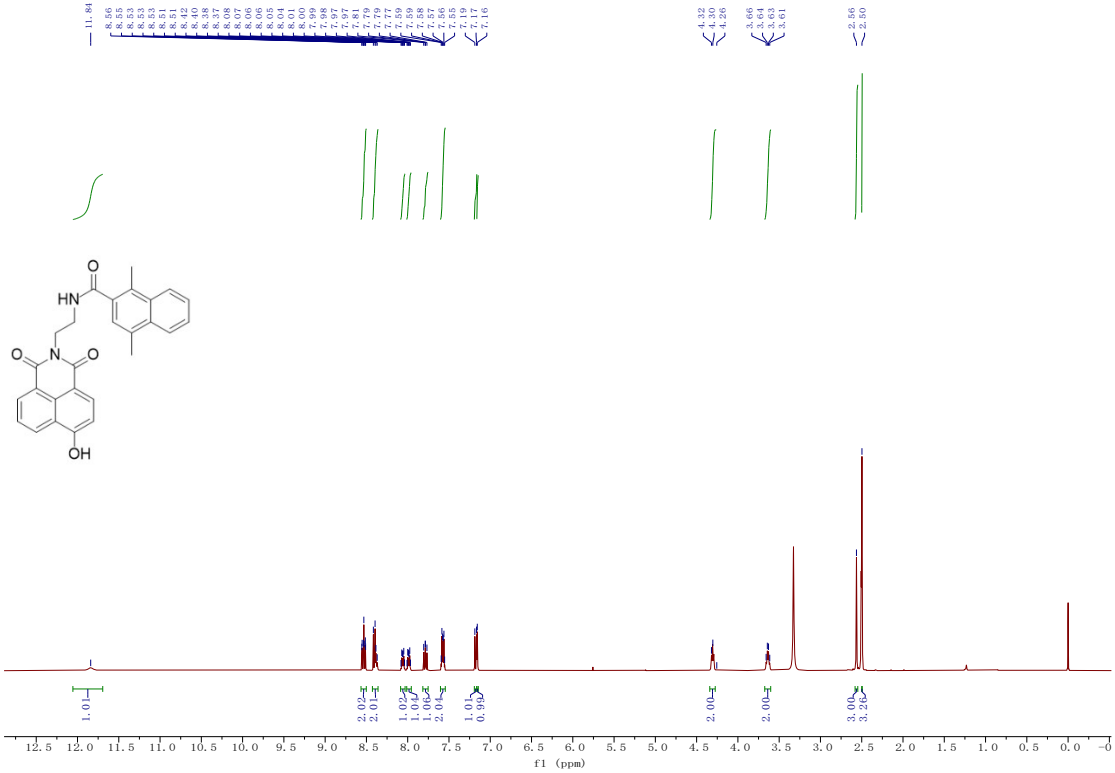


Figure S21 ^{1}H-NMR of compound **NNI-OH**

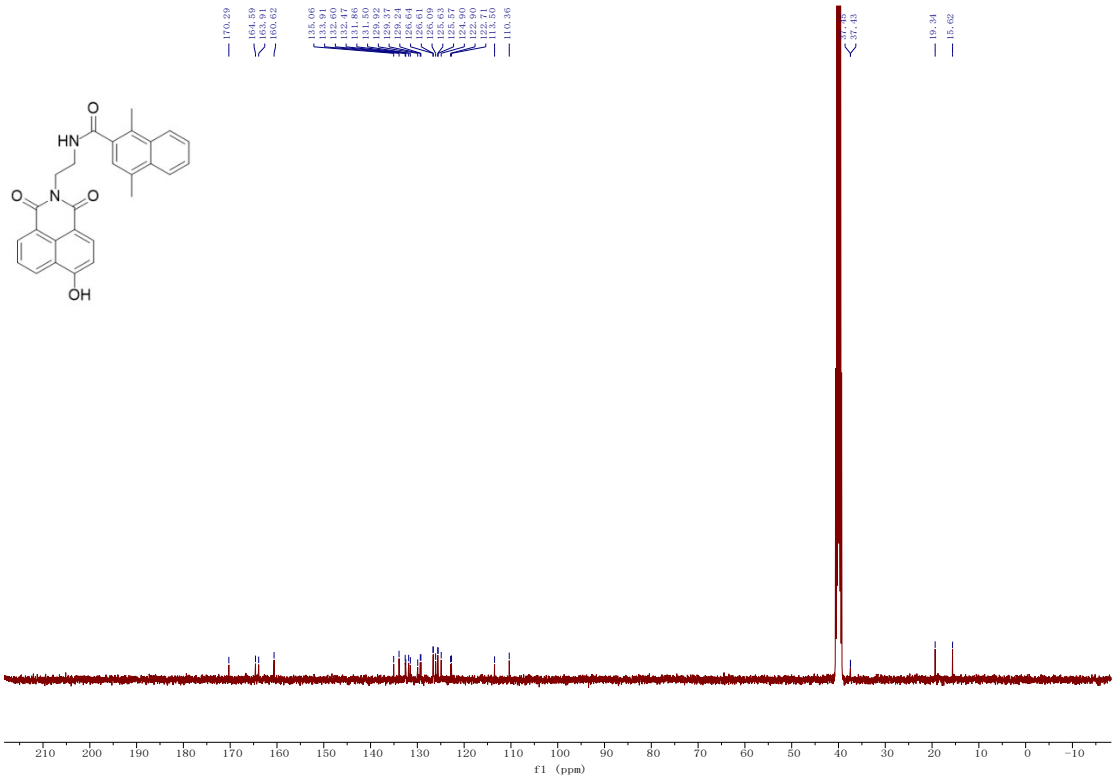


Figure S22 ^{13}C-NMR of compound **NNI-OH**

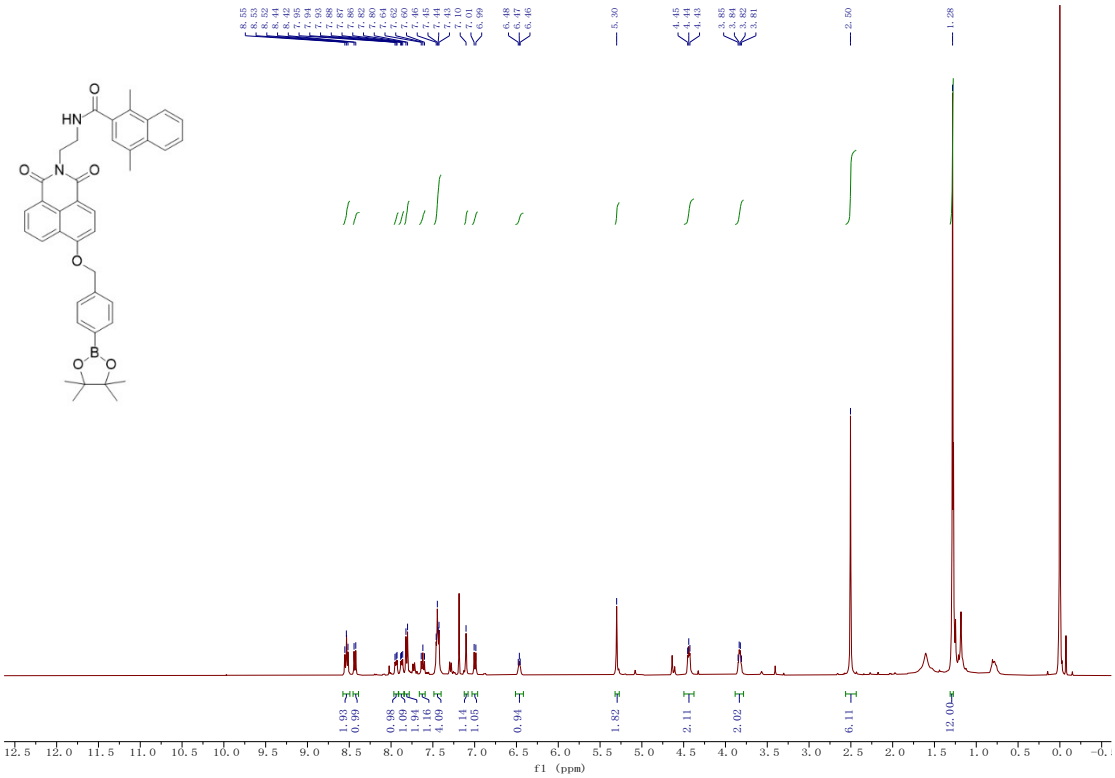


Figure S23 ^{1}H-NMR of compound **NNIB**

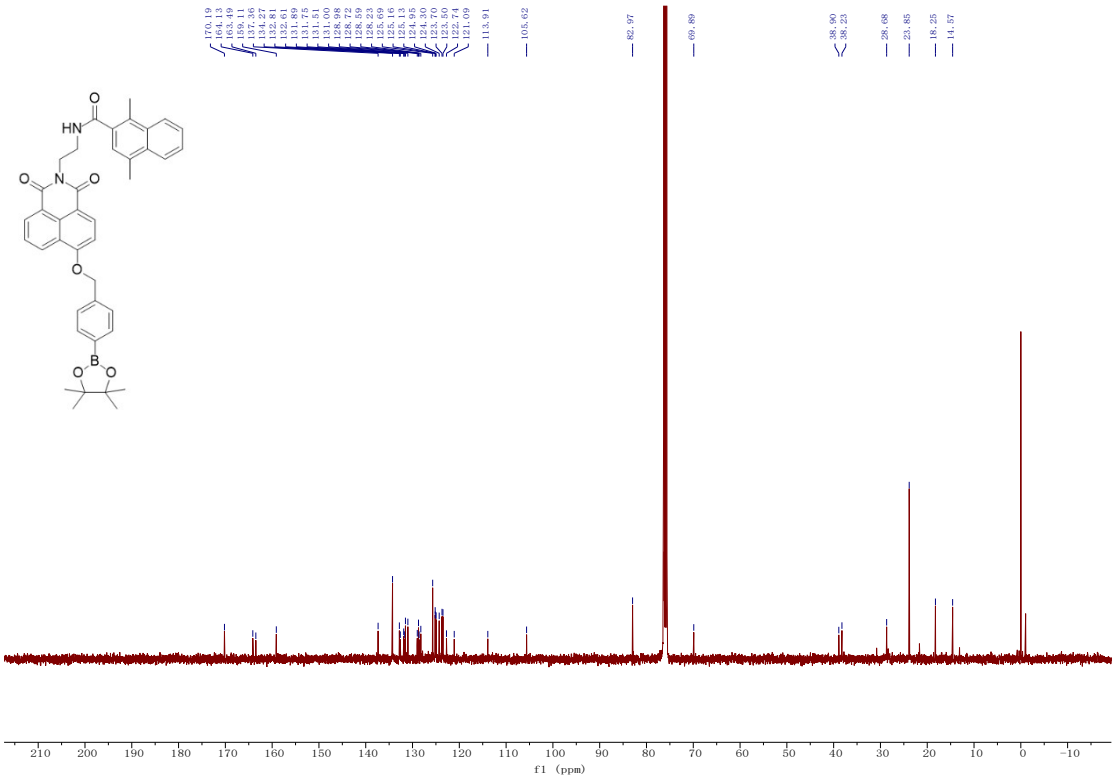


Figure S24 ^{13}C-NMR of compound **NNIB**

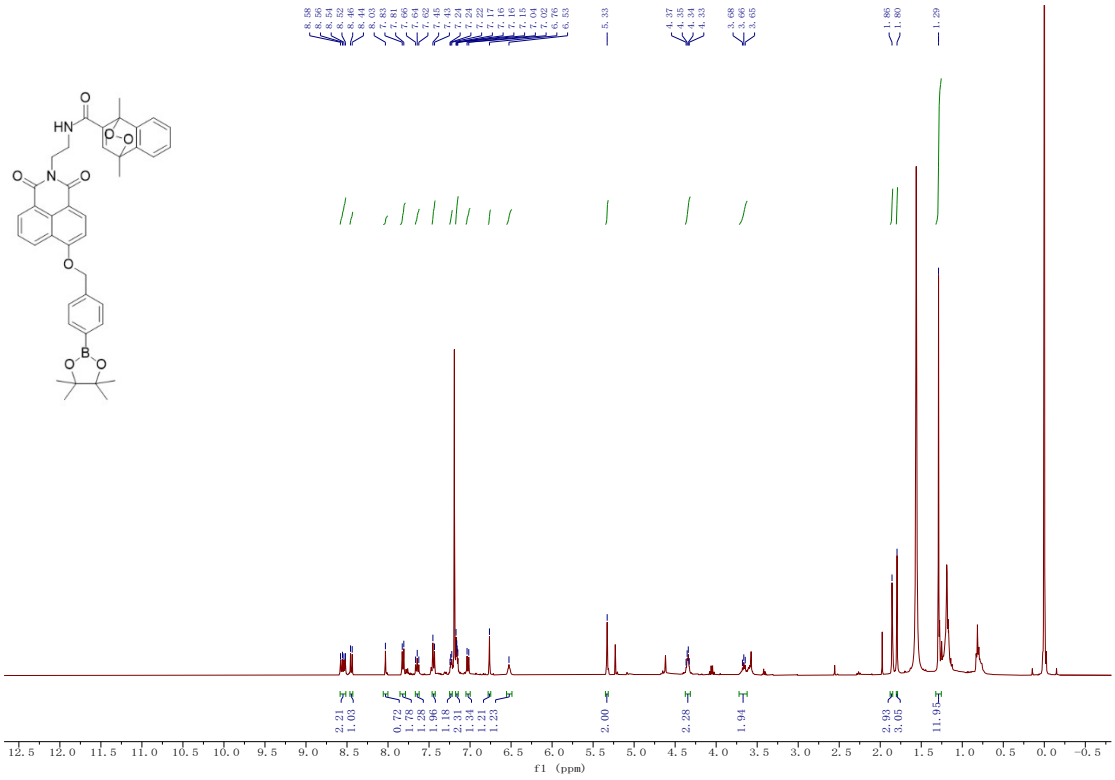


Figure S25 ^{1}H-NMR of compound **NNIB-Endo**