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Supporting Information

Penta-fluorophenol: A Smiles rearrangement-inspired cysteine-selective fluorescent probe for imaging of human glioblastoma

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This PDF file includes:

1. Materials and Methods

General information

The chemical reagents were purchased from Aldrich (US), TCI (Japan), Alfa Aesar (US), and Acros Organics (US). Commercially available reagents and anhydrous solvents were used without further purification. ER-Tracker™ Red (BODIPY™ TR Glibenclamide, Invitrogen, US) was used for the cellular co-localization imaging experiment. Dulbecco's modified Eagle's media (DMEM) and fetal bovine serum (FBS) for cell culture (U87 MG, HeLa, HEK293, and COV-318) were purchased from Hyclone (Utah, US). Penicillin-streptomycin for U87 MG, HeLa, HEK293 and COV-318 cell line was purchased from Gibco Industries Inc. (Auckland, NZ). Opti-MEM medium, FBS and antibiotic-antimycotic for SNU4098 cell line were purchased from Gibco Industries Inc. (Auckland, NZ). Tribromoethanol and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). Species used to perform the screening of metal ions, and amino acid; $PbCl_2$, $FeCl_3$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $Hg(NO_3)_2$, $CdCl_2$, $NiCl_2$, KCl, AgCl, $CoCl_2$, NaCl, $AuCl_3$, L-glutamine, L-cysteine, DL-homocysteine, L-glutathione, human serum albumin (HSA) and L-lysine. The pH range was 4–10 including biological pH (7.4) for the pH screening. The pH buffers were purchased from Daejung Chemicals & Metals co., LTD (Rep. of Korea). The cell culture dish (SPL Life Science, #20060, Rep. of Korea) was purchased for the applications. Chemical reactions were performed under an argon atmosphere. Thin-layer chromatography (TLC) was performed using pre-coated silica gel 60F-254 glass plates (Merck KGaA, Germany). ^{1}H NMR and ^{13}C NMR spectra were measured with a Bruker AVANCE III 400 MHz (US). In case of NPO-C, ^{1}H NMR spectra was measured with a JNM-ECZ500R (500 MHz, US). In the NMR spectra, the chemical shifts (δ) are reported in ppm, multiplicities are indicated by s (singlet), d (doublet), t (triplet), dd (double of doublets), and m (multiplet). ^{19}F NMR spectra was obtained on a Bruker NMR spectrometer 500 MHz (US). ^{19}F NMR spectra are referenced to residual hexafluoro-benzene (-164.90 ppm). ^{1}H NMR and ^{13}C NMR Spectra are referenced to residual chloroform (7.24 ppm) and DMSO (2.50 ppm) in ^{1}H NMR. High-resolution mass spectra of NPO-B were recorded on a JEOL JMS-700 spectrometer at Korea Basic Science Center, Kyung-pook National University, and the values are reported in units of mass-to-charge (*m/z*). High-resolution mass spectra of NPO-A and NPO-C were recorded on a JEOL JMS-700 spectrometer at Center for Research Facilities, Kyung-Hee University, and the values are reported in units of mass-to-charge (*m/z*). Liquid chromatography-mass spectrometry (LC-MS) spectra were measured on an Agilent HP 1260 system (Agilent Technologies, Santa Clara, US). Fluorescence images were visualized by a confocal laser scanning microscope (CLSM, LSM-800, Carl Zeiss, Germany). Tissue images were visualized using two-photon microscopy (TPM, TCS SP5, Leica microsystem, Germany).

Synthesis of NPO series

NPO series were prepared with the reaction of 4-chloro-7-dinitrobenzofurazan and phenol derivatives in the presence of base. See details in Supplementary Information, Scheme S1.

UV/Vis and fluorescence spectroscopic methods

UV/Vis absorption spectra were obtained using spectrophotometer (Agilent Technologies Cary 8454, US). Fluorescence emission spectra were recorded on a spectro-fluorophotometer (SHIMADZU CORP. RF-6000, Japan) with a 1 cm standard quartz cell (internal volume of 0.1 mL, Hellma Analytics, Jena, Germany). The stock solution of NPO series was prepared in dimethyl sulfoxide (DMSO, 10 mM). The cuvette for absorbance and fluorescence measurements was made from quartz and has an internal volume of 100 μL.

Quantum chemical calculation

All calculations were performed using the density functional theory (DFT) method with the APFD functional and 6-31g(d) basis set as implemented in the Gaussian 16 package. The optimized structures and electronic energies of NPO-Cys and NPO-Hcy in the intramolecular substitution reaction (S-bound compounds are converted into N-bound compounds) were obtained in Figure S10. Since the intramolecular substitution reactions of NPO-Cys and NPO-Hcy are accompanied by the proton transfer between NPO-Cys (or NPO-Hcy) and water molecules, we calculated the intrinsic reaction coordinate (IRC) for the intramolecular substitution reaction of NPO-Cys with 3 water molecules and NPO-Hcy with 4 water molecules. In our calculation, the transition state (TS) is properly found such that the TS has an imaginary frequency vibration that is associated with breaking the C-S bond and forming the C-N bond in the intramolecular substitution reaction of NPO-Cys and NPO-Hcy. After the TS was found, the IRC calculation was carried out from the TS to both the reactant (S-bound compound) and to the product (N-bound compound). The N-bound compounds in the IRC calculation were further optimized to obtain the fully relaxed N-bound compounds.

Cell culture

Human primary glioblastoma cell line (U87 MG), immortalized human cervical cancer cell line (HeLa), human ovarian cancer cell line (COV-318), and human embryonic kidney cell line (HEK293) were obtained from Korean Cell Line Bank. Cells were cultured in Dulbecco's modified Eagle's media (Hyclone, US) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Gibco). Glioblastoma cell line (SNU4098) was isolated from human GBM tissues (patient-derived clinical sample) and it was obtained from Korean Cell Line Bank. The SNU4098 cells were cultured in Opti-MEM, supplemented with 5% fetal bovine serum (Gibco™, US) and 1% antibiotic-antimycotic (Gibco™, US). Cell lines were kept in humidified air, containing 5% CO_2 at 37 °C.

Cytotoxicity

The cytotoxicity of NPO-B against SNU4098, U87 MG, HeLa, COV-318 and HEK293 cell line was evaluated using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Tech. Inc, Japan) according to the manufacturer's instructions. The cells (5×10^3 cells per well) were seeded in 96-well plates and incubated for 24 h at 37 °C in a humidified 5% CO_2 incubator. The cells were then treated with 10, 20, and 50 μM concentrations of the NPO-B and the cell toxicity was measured after a 30 min incubation. Later, NPO-B was then removed by washing in PBS (3 times), followed by changing serum free media. CCK-8 solution (10 μL, 10× working concentration) in serum free media was added to each well of a 96-well plate, followed by incubation for 2 h at 37 °C. After 2 h, the absorbance was measured at a wavelength of 450 nm

using a microplate reader (Multiskan FC, Thermo Fisher, MA, USA). The percentage of cell cytotoxicity was calculated using the formula; Cell viability (%) = (Mean OD of sample × 100) / (Mean OD of the control group) (OD: optical density).

Confocal laser scanning microscopy (CLSM) cell imaging

Approximately 2×10^5 cells were seeded on 35-mm glass confocal dishes (SPL Life Science, Rep. of Korea) and incubated for 24 h. At 80% confluency, media was changed to serum-free media. After 30 min, cells were treated with NPO-B (30 μM) for 30 min at 37 °C in an incubator at 5% CO_2. Cell media was washed three times with PBS. After washing, cells were treated with 4% formaldehyde for 10 min, and then the solution was removed by washing PBS (3 times). The prepared samples were imaged using CLSM.

Co-localization assay

Approximately 2×10^5 cells (SNU4098, U87MG) and 1×10^5 cells (HeLa) were seeded on 35-mm glass confocal dishes and incubated for 24 h. At the 80% confluency, media was changed to a serum-free media. After 30 min, cells were treated with NPO-B (30 μM) for 30 min at 37 °C in 5% CO_2 incubator. Cells were washed with PBS and treated with organelle tracker in serum free media for 10 min at 37 °C in 5% CO_2 incubator [Notice: tracker treatment concentration and volume, 2 μL (1000× working concentration) of ER-Tracker™ Red, 1 μL of MitoTracker™ Deep Red FM (2000× working concentration), 1 μL of LysoTracker™ Deep Red (2000× working concentration)]. Cell media were washed three times with PBS. After washing, the cells were treated with 4% formaldehyde for 10 min, and then the solution was removed by washing in PBS. DAPI was treated with cell in PBS for 10 min. After DAPI treatment, cells were washed three times with PBS. The prepared samples were turned into imaging using CLSM.

Fluorescence of reaction between NPO-B and cysteine in dish, cell and biopsy

(1) Reaction between vial and cell. Approximately 1×10^6 cells (U87MG and HEK293) were seeded on 6 well plate (SPL Life Science, Rep. of Korea). After incubation at 37 ºC incubator containing 5% CO_2 for 24 h, media was washed with PBS, and refill the fresh media (Serum-free). For 30 min, the cells were starved in serum free media. Afterward, cells were treated with cysteine dependent on concentration (0 - 1 mM) for 1 hours. The media with cysteine residue was washed with PBS (2 times), and cell were treated with NPO-B in fresh media (30 μM, Serum-free) for 30 min. Afterward, the media was washed with PBS to remove NPO-B residue. And then, the cells were harvested by trypsin-EDTA and were wash with PBS to removed trypsin-EDTA using centrifugation (3 min, 1300 rpm, 25 ºC). The cell membrane can be permeable by treating tritonX-100 solution for 3 min. The prepared samples were detected on spectrometer under the excitation 478 nm and the samples were compared to reaction (NPO-B with cys) within vial.

(2) Reaction between dish and biopsy. To confirm fluorescence spectra in biopsy after reaction with NPO-B, we performed lambda scan on confocal microscopy (LSM-510, Carl Zeiss, Germany). Briefly, human GBM biopsy (Patient ID: 18-104) was washed with PBS (1 times wash to reduce damage of tissue) on 6 well dish. Afterward, NPO-B (100 μM, solution: PBS)

was treated on the biopsy for 30 min in 37 ºC shacking incubator (100 rpm). After incubation, to remove floating of NPO-B, biopsy was washed with PBS (3 times). In dish, NPO-B (100 μM, solution: PBS) was reacted with cysteine (500 μM) for 30 min in 37 ºC shacking incubator (100 rpm). The prepared samples were scanned using confocal microscopy to obtain whole wavelength.

Animals

6-week-old female BALB/c nu/nu mice (Taconic, provided by Daehan Biolink Co., Ltd., Eumseong, Rep. of Korea) were housed at an ambient temperature of 23 ± 1 °C and relative humidity of 60 ± 10% under a 12 h light/dark cycle, and were allowed free access to water and food. All of the experiments performed with mice were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996 and protocols approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(SE)-19-002).

Intracranial xenograft (tumor implantation) animal model

U87MG and SNU4098 cell lines were used for the xenograft. Mice were anesthetized with tribromoethanol (312.5 mg/kg, *i.p.*) and mounted in a stereotaxic apparatus (myNeuroLab, St. Louis, MO, USA). Each mouse received a unilateral injection of 1.0×10^6 U87MG cells or SNU4098 cells per 5 μL in medium without FBS, according to the following coordinates: anteroposterior: -3.0 mm from bregma; mediolateral: 1.8 mm from bregma; and dorsoventral: −3.0 mm from the skull. The flow for the injection was regulated using an electronic pump at 1 μL/min for 5 min and followed by 2 min with a needle at the injection site to avoid reflux. Sham operations followed the same procedure for the infusion of the medium without FBS. After surgery, mice were allowed to recover from anesthesia in a temperature-controlled chamber and then placed in individual cages.

Mouse tissue sample preparation

On the 28th day after cell implant, mice were anesthetized and transcardially perfused with 0.05 M PBS. The mouse organs were dissected to perform further *ex vivo* studies.

***In vivo* distribution analysis of NPO-B**

To investigate the distribution of NPO-B by the fluorescence tissue imaging system (FTIS, VISQUE® InVivo Elite, Vieworks Co. Ltd, Rep. of Korea), mice were arranged by 3 groups (n=3 per group) as follows; (1) Sham group (Intracranially medium without FBS-injected group), (2) U87MG group (Intracranially U87MG cells in medium without FBS-injection) and (3) SNU4098 group (Intracranially SNU4098 cells in medium without FBS-injection). On the 28th (Sham, U87MG) or 56th (SNU4098) day after the surgery, mice were anesthetized to inject the NPO-B intravenously (5 mg/kg, diffused in PBS containing 1% DMSO). At 45 min circulation point after the injection, mice were dissected to isolate the seven organs: brain, lung, heart, liver, spleen, colon and kidney, in order to perform further *ex vivo* studies.

***Ex vivo* tissue fluorescence imaging**

VISQUE® InVivo Elite (Vieworks Co., Ltd., Rep. of Korea) was used as an *ex vivo* fluorescence tissue imaging system (FTIS). The imaging experiment was carried out in a dark room (wrapped in aluminum foil). (A) Animal samples: After intravenously injection of NPO-B, mouse organs (brain, lung, heart, liver, spleen, colon and kidney) were isolated and washed with PBS buffer (3 times). Then, the *ex vivo* tissue imaging experiment for tissues was conducted. Brain: lens zoom: 6×, focus: 0.0 cm, iris: F3.6, mode: GFP, LED: Blue, type: single-frame, exposure time: 5 sec, binning: 1×1, intensity min=170.0, max=480.0). Other organs: lens zoom: 3×, focus: 1.0 cm, iris: F3.6, mode: GFP, LED: Blue, type: single-frame, exposure time: 5 sec, binning: 1×1, intensity min=170.0, max=480.0. (B) Human samples: Human tissue samples were washed with PBS buffer (3 times) and treated with NPO-B (100 μM) for 30 min. After incubation, the tissue samples were washed with PBS buffer (3 times) to remove remaining NPO-B. Next, the tissue samples were washed again with PBS buffer (3 times) and treated with 4% PFA to fix for 30 min. The imaging experiment was conducted, washing the tissues with PBS buffer (3 times) again before imaging. Condition: lens zoom: 2.5×, focus: 1.5 cm, iris: F2.5, mode: GFP, LED: Blue, type: single-frame, exposure time: 1 sec, binning: 1×1, intensity min=1031.0, max=3218.6.

TPM tissue imaging

The imaging experiment was carried out in a dark room; tissues samples were wrapped in aluminum. The scanning two-photon microscopy (TPM, Leica, Nussloch, Germany) equipped with a Titanium Sapphire laser (Chameleon vision, Coherent) and 25× water immersion objective lens was used for *ex vivo* imaging of these tissue samples. Two-photon excitation at 900 nm with laser poser of approximately 50 mW at the focal plane provided the best image quality. Fluorescence intensity of TPM images were analyzed using Leica software and Image-J. (A) Animal samples: Mice were anesthetized and perfused transcardially with PBS buffer (0.05 M). The mice were dissected to isolate organs: brain, lung, liver, kidney, spleen, colon, heart and stomach and to perform further ex vivo studies. Each organ was washed with PBS buffer (3 times). The mice tissues were placed in dry ice for 5 min. After this, frozen organs were cut into several pieces by surgical blade (NO.11, Reather safety razor Co., LTD, Japan). The sliced tissue samples showed an average thickness of about 100 μm, and were transferred into a 24 well plate (SPL Life Science, Rep. of Korea), and washed with PBS buffer (3 times). Next, NPO-B (100 μM) was treated and incubated for 1 h at 37 °C in a shaking incubator. After incubation, the tissue samples were washed with PBS buffer (3 times), and treated with 4% PFA for the tissue fixation. Two-photon microscopy (TPM) imaging experiments were conducted with additional washing of tissues with PBS buffer (3 times). (B) Human samples: Patient-derived human clinical samples (GBM) assay with TPM used the same method described above.

Haematoxylin and eosin (H&E) staining of GBM clinical samples

Tissue samples were obtained from the Department of Neurosurgery, Seoul National University Hospital. The histopathology of the tumor was reviewed by two neuropathologists and classified according to the 2016 World Health Organization classification of tumors of the CNS. Samples were first immersed in PBS and fixed in a 4% paraformaldehyde solution. Then the tissues were routinely processed and embedded in paraffin. Sections of formalin-fixed paraffin-

embedded (FFPE) tissue (4 μm) were prepared. After rehydration, sections were stained with hematoxylin (Merck, USA) and eosin (BBC biochemical, USA) for histologic assessment. The tissue slices were stained with hematoxylin for 5 min and immersed briefly in a 1% hydrochloric acid (HCl) in ethanol (EtOH) solution. They were then treated with eosin for 15 sec. Stained tissue sections were mounted with Leica's CV ultra-mounting medium (Leica, Germany). TissueFaxs (TissueGnostics, Austria) was used to observe the staining.

Human GBM samples

The human GBM tissue samples were snap-frozen in liquid nitrogen immediately during the surgery and were stored at -80 °C. This study was approved by the Institutional Review Boards (IRB) of Seoul National University Hospital (#H-1404-056-572).

Immuno-toxicity analysis

C57BL/6J mice were kept in a specific pathogen-free condition in the animal facility at Seoul National University College of Medicine (Rep. of Korea). Six-to eight-week old male mice weighing 20–25 g was used for the experiments. The animal protocol for the experiments was reviewed and approved by Ethics Committee of Seoul National University.

NPO-B treatment. NPO-B (5 mg/kg) and LPS (extracted from *Escherichia coli*, Sigma, St. Louis, MO, with dose of 10 μg/animal) were injected intravenously through the lateral tail vein. The same volume of 1% DMSO was injected as the control. For the measurement of cytokine, blood was collected 2 h after injection. After 6 h treatment with NPO-B, liver injury was quantified by measuring the plasma enzyme activities of ALT and AST using a kit (Biotron Diagnostics, Ins., Hemet, CA, USA) according to the manufacturer's instructions.

Measurement of cytokines. Blood was collected from the intra-orbital plexus with a heparinized capillary tube, and the plasma was obtained by centrifugation. The amounts of cytokines in the plasma were measured by ELISA according to the manufacturer instructions. The TNF-α, and IL-6 ELISA kits were purchased from BioLegend (San Diego, CA, USA). The IL-1β ELISA kit was purchased from R&D system (Minneapolis, MN, USA).

Isolation of splenocytes and inguinal lymph node. Mice were euthanized, and the spleen and inguinal lymph node were aseptically removed and placed into a washing medium containing cold RPMI1640 (WELGENE, Daegu, Rep. of Korea) with 100 U/mL of penicillin and 100 μg/mL of streptomycin. The homogenized spleen and inguinal lymph node were passed through a 70-μm cell strainer and centrifuged at 600 G for 10 min. The resulting pellet was harvested and re-suspended in RBC lysis buffer. The cells were counted after washing them in the medium.

Flow cytometry. Freshly isolated splenocytes were re-suspended in cold fluorescence activated cell sorting (FACS) buffer containing 0.5% BSA and blocked at RT for 10 min with a TruStain FcX™ antibody (BioLegend, San Diego, CA, USA). Then, the cells were stained with anti-CD3, CD4, CD8, CD25, CD69, NK1.1, CD19 and CD11b antibodies (BD Biosciences, USA) and placed on ice for 30 min. After washing with the cold FACS buffer, cells were analyzed by FACSCalibur (BD Biosciences, USA). FlowJo software (Tree Star, Inc., Ashland, OR, USA) was used for the data analysis.

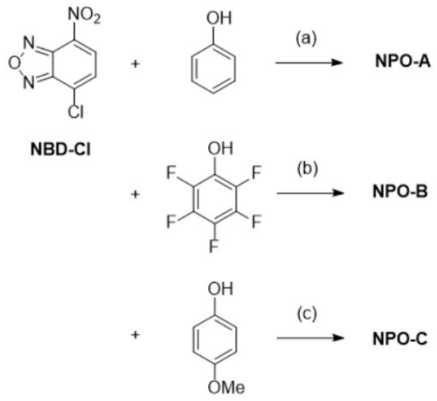
Local lymph node assay (LLNA): BrdU-ELISA method. The mice were intraperitoneally exposed to 0.5 mL of BrdU (250 mg/kg) (Sigma, USA) to be incorporated into proliferating

lymph node cells. After 16 h, the mice were euthanized, and the inguinal lymph nodes were collected. Lymphocytes (1×10^5/well) were seeded on 96-well v-shape bottom plate. The cell proliferation was evaluated by ELISA using Cell Proliferation ELISA, BrdU (colorimetric) kit (Cell signaling, Danvers, MA, USA) in accordance with the manufacturer's instruction. Absorbance was measured using a spectro-photometer at 450 nm to obtain BrdU labeling index. Stimulation index was calculated as the ratio of the BrdU labeling index for each treatment group to that for the control group.

Examination of liver and kidney injury. After the mice were anesthetized with Zoletil (25 mg/kg) and Rompun (10 mg/kg), the portal vein was cannulated, and the liver was perfused with sterile PBS. Liver and kidney tissues removed were fixed in 4% paraformaldehyde for immunohistochemistry, then embedded in paraffin. The sections were stained with hematoxylin and eosin (H&E) for histological examination.

Statistical analysis. Data were presented as mean ± SDs. An unpaired two-tail *t*-test was used to compare the two groups [CTL *vs.* NPO-B or NPO-B *vs.* LPS]. P-values of <0.05 were considered statistically significant. Statistical tests were carried out using GraphPad InStat version 5.01 (GraphPad Software, Le Jolla, CA, USA).

2. Synthesis of NPO Series



Scheme S1. Syntheses of compounds NPO series: (a) EtOH, TEA, 25 °C, 12 h; (b) DMF, DIPEA, K_2CO_3, 60 °C, 10 min; (c) DMF, DIPEA, K_2CO_3, 25 °C, 20 min.

Synthesis of NPO-A

Triethylamine (TEA, 413 μL, 0.003 mol) was added to a solution of 4-chloro-7-nitrobenzofurazan (200 mg, 0.001 mol) and phenol (280 mg, 0.003 mol) in ethanol (EtOH, 10.0 mL) at 25 °C was added. The resulting mixture was then stirred at 25 °C for 12 h. The reaction mixture was partitioned between organic solvent (ethyl acetate; EtOAc) and deionized water (DI H_2O), and then the organic layer was separated. The aqueous layer was extracted with a solvent twice, and the combined organic extracts were washed with brine, and dried over anhydrous sodium sulfate (Na_2SO_4) at 25 °C for 30 min. The solvent evaporated under reduced pressure, and the crude mixture was purified by flash silica gel column chromatography (eluent: EtOAc/n-hex = 2/8, v/v) to give NPO-A as an orange color solid (12.1 mg, 4.71%). ^{1}H NMR (DMSO, 400 MHz, 292.2 K): δ 8.65 (d, 1H, J=8.30 Hz), 7.60 (d, 2H, J=6.91 Hz), 7.43 (d, 3H, J=7.21 Hz), 6.68 (d, 1H, J=8.71 Hz). ^{13}C NMR ($CDCl_3$, 400 MHz, 294.2 K): δ 133.57, 131.00, 127.49, 121.13, 107.73. HRMS: m/z calcd for $C_{12}H_7N_3O_4$, 257.0437; found, 257.0439.

Synthesis of NPO-B

N,N-diisopropylethylamine (DIPEA, 300 μL) and K_2CO_3 (207 mg, 1.50 mmol) was added to a solution of 4-chloro-7-nitrobenzofurazan (200 mg, 0.001 mol) and pentafluorophenol (552 mg, 0.003 mol) in N,N-dimethylformamide (DMF, 30.0 mL) at 25 °C. The resulting mixture was then stirred at 60 °C for 10 min. The reaction mixture was partitioned between organic solvent (EtOAc) and deionized water (DI H_2O), and then the organic layer was separated. The aqueous layer was extracted with solvent twice, and the combined organic extracts were washed with brine, and dried over anhydrous sodium sulfate (Na_2SO_4) at 25 °C for 30 min. The solvent evaporated under reduced pressure, and the crude mixture was purified by flash silica gel column chromatography (eluent: EtOAc/n-hex = 1/9, v/v). NPO-B was produced as a dark yellow solid (110.2 mg, 31.75%). ^{1}H NMR ($CDCl_3$, 400 MHz, 298.2 K): δ 8.50 (d, 1H, J=8.06 Hz), 6.83 (d, 1H, J=8.30 Hz). ^{13}C NMR ($CDCl_3$, 400 MHz, 294.2 K): δ 109.41, 127.39, 128.79, 130.96 131.45, 132.31, 132.66, 137.14, 140.14, 141.73, 142.67, 144.09, 150.70. ^{19}F NMR

(hexafluoro-benzene, 500 MHz): δ -155.7493 (d, 2F), -157.9279 (t, 1F), -162.5784 (t, 2F). HRMS: m/z calcd for $C_{12}H_2F_5N_3O_4$, 346.9965; found, 346.9964.

Synthesis of NPO-C

N,*N*-diisopropylethylamine (DIPEA, 100 μL) and K_2CO_3 (31.0 mg, 0.025 mmol) was added to a solution of 4-chloro-7-nitrobenzofurazan (30.0 mg, 0.015 mmol) and 4-methoxyphoenol (55.9 mg, 0.045 mol) in N,N-dimethylformamide (DMF, 3.0 mL) at 25 °C was added. The resulting mixture was then stirred at 25 °C for 20 min. The reaction mixture was partitioned between organic solvent (EtOAc) and deionized water (DI H_2O), and then the organic layer was separated. The aqueous layer was extracted with solvent twice, and the combined organic extracts were washed with brine, and dried over anhydrous sodium sulfate (Na_2SO_4) at 25 °C for 30 min. The solvent evaporated under reduced pressure, and the crude mixture was purified by flash silica gel column chromatography (eluent: EtOAc/n-hex = 2/8, v/v) to give NPO-C as a dark yellow solid (3.9 mg, 9.07 %). ^{1}H NMR ($CDCl_3$, 500 MHz, 298.2 K): δ 8.413 (d, 1H, J=8.00 Hz), 7.17 (d, 2H, J=9.5 Hz), 7.01 (d, 2H, J=9.00 Hz), 6.52 (d, 1H, J=8.5 Hz), 3.85 (s, 3H). ^{13}C NMR ($CDCl_3$, 400 MHz, 294.2 K): δ 158.31, 155.13, 146.09, 145.21, 144.19, 133.87, 130.33, 121.95, 116.04, 115.69, 114.77, 107.44, 55.84. HRMS: m/z calcd for $C_{13}H_9N_3O_5$, 287.0542; found, 287.0547.

3. Supporting Figures

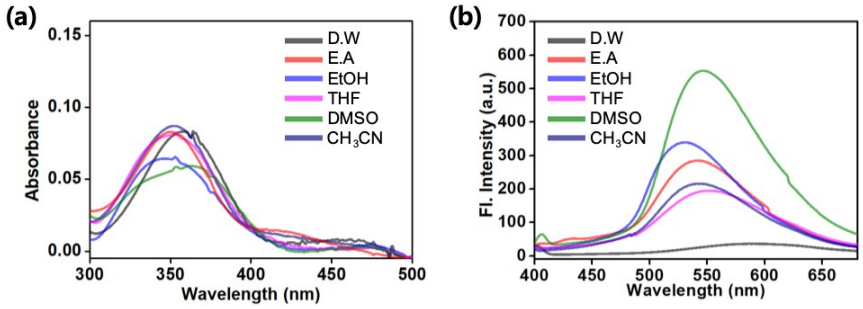


Fig. S1. Absorption and emission spectra of NPO-B (10 μM) in various solvents at 25 ºC. (a) Absorption and (b) Emission spectra. The emission spectra were measured under excitation at the maximum absorbance in each solvent. D.W., deionized water; E.A., ethyl acetate; EtOH, ethanol; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; CH_3CN, acetonitrile.

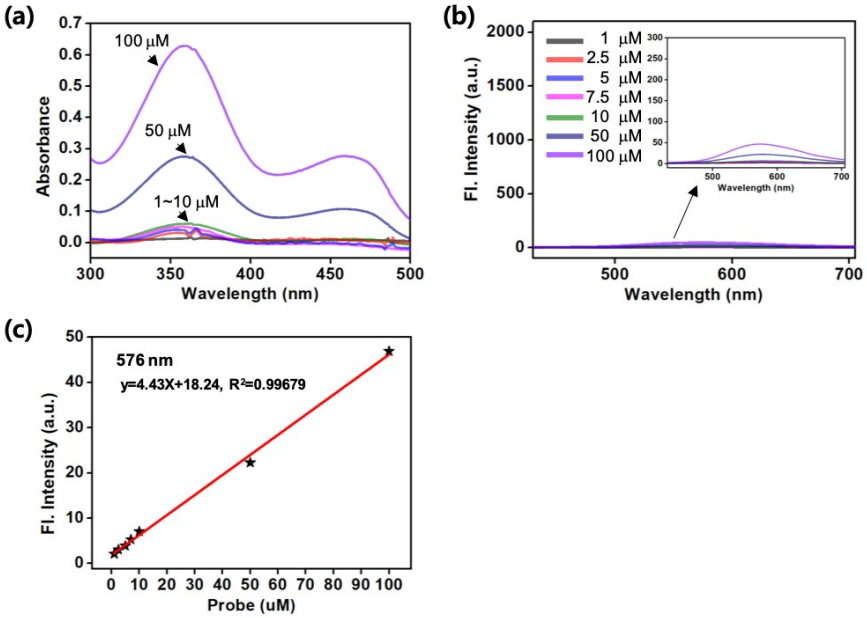


Fig. S2. Absorption and emission spectra of NPO-B. (a) Absorption and (b) Emission spectra of NPO-B (1–100 μM) in the deionized water (DI H_2O). (c) Fluorescence intensity plots of NPO-B (1–100 μM) at 576 nm. All data was collected at room temperature, and the emission spectra were measured under excitation at the maximum absorbance in each concentration of NPO-B.

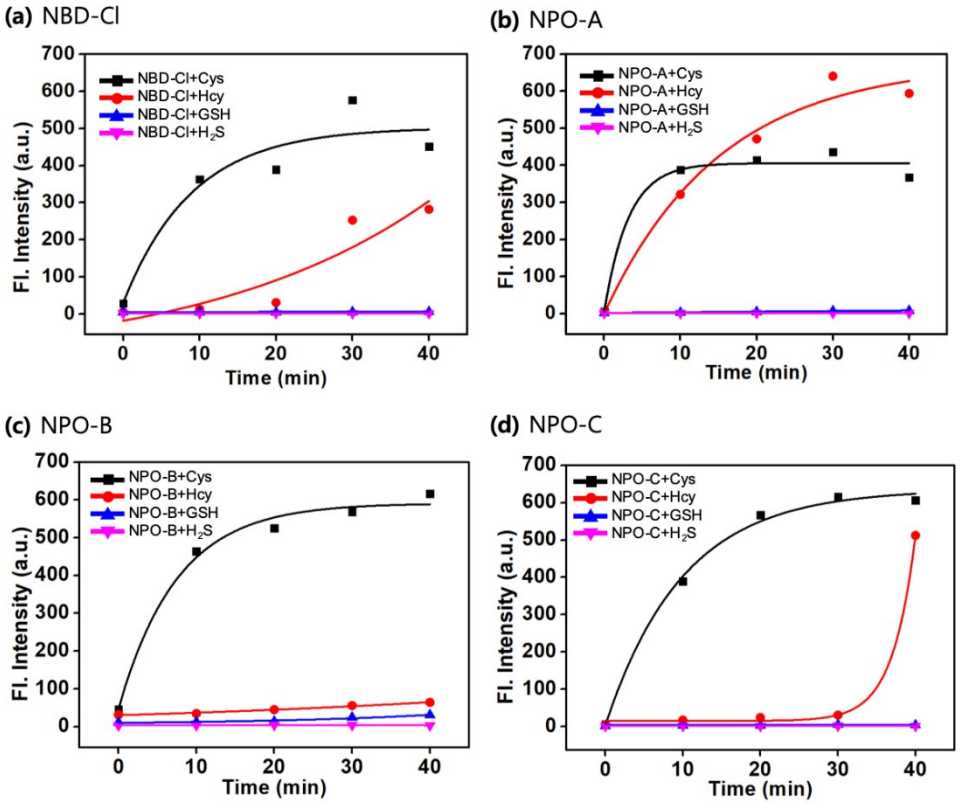


Fig. S3. A plot of time dependent (0–40 min) fluorescence intensity of NPO series (10 μM) in DI. H_2O at 550 nm, maximum emission peak. (a) NBD-Cl, (b) NPO-A, (c) NPO-B, (d) NPO-C. All the reactions of NPO series with thiols (50 μM in DI. H_2O) were conducted at 37 ºC in a shaking incubator.

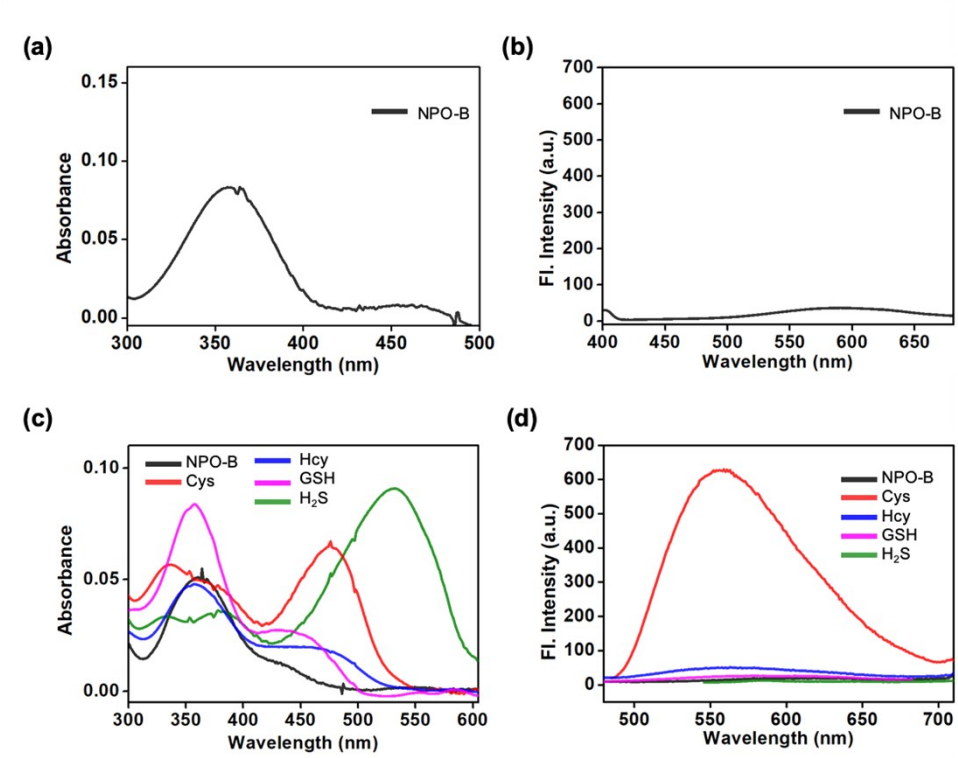


Fig. S4. Absorption and emission spectra of NPO-B with and without thiols. (a, b) Absorption and emission spectra of NPO-B (10 μM in DI. H_2O) at 1 min, 37 ºC in a shaking incubator. (c, d) Absorption and emission spectra of NPO-B (10 μM in DI. H_2O) with various thiols (50 μM) at 30 min, 37 ºC in a shaking incubator. The emission spectra of fluorescence were measured under excitation at the maximum absorbance peak.

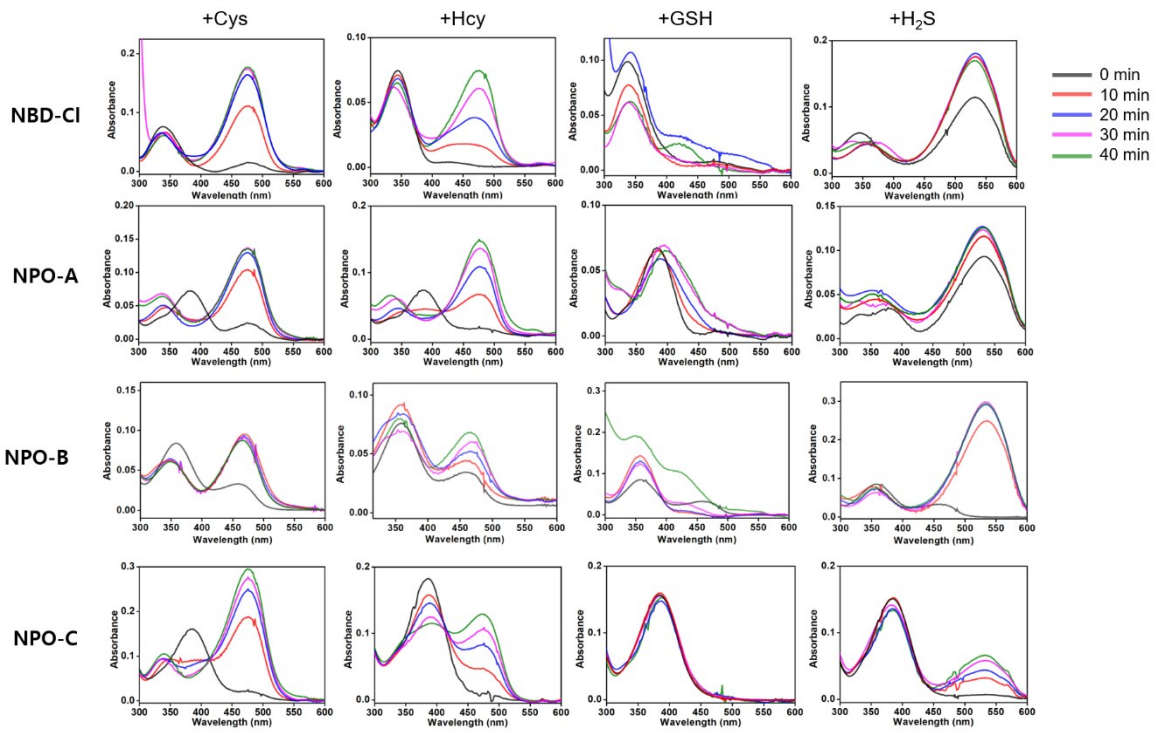


Fig. S5. Time dependent (0–40 min) absorption spectra of NPO series (10 μM in DI. H_2O) with thiols (50 μM). All the reactions of NPO series with thiols were conducted at 37 ºC in a shaking incubator.

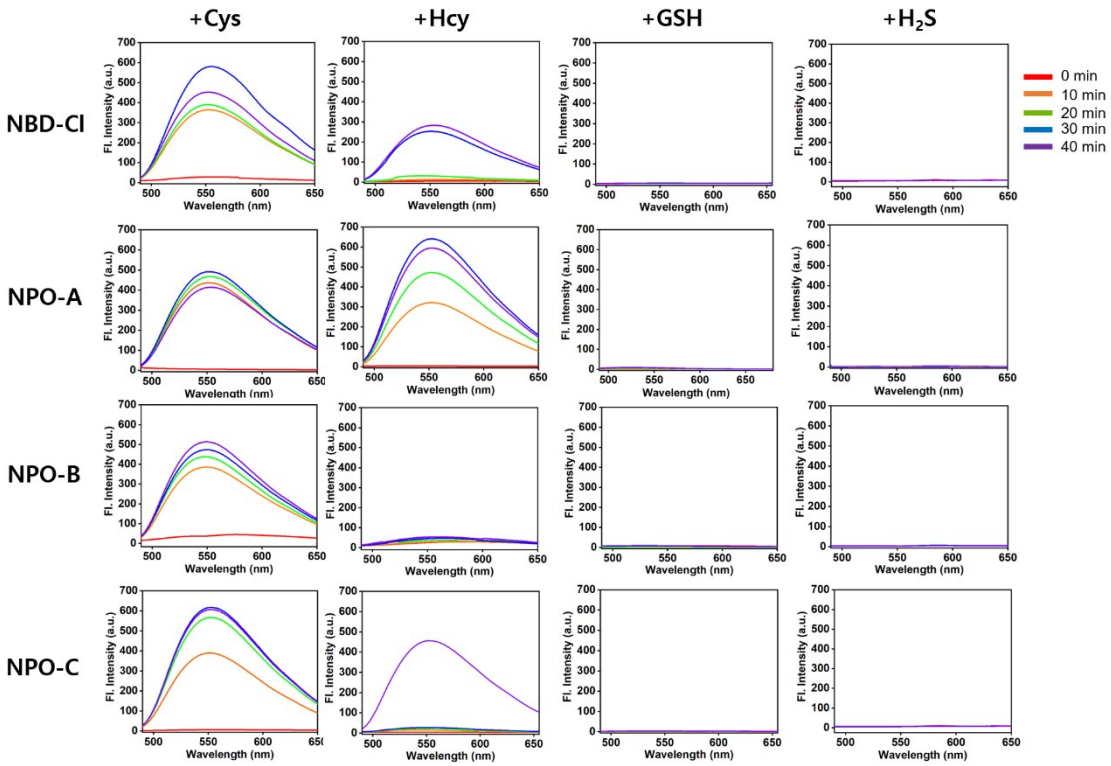


Fig. S6. Time dependent (0–40 min) emission spectra of NPO series (10 μM in DI. H_2O) with thiols (50 μM). All the reactions of NPO series with thiols were conducted at 37 ºC in a shaking incubator. The emission spectra were measured under excitation at 474 nm.

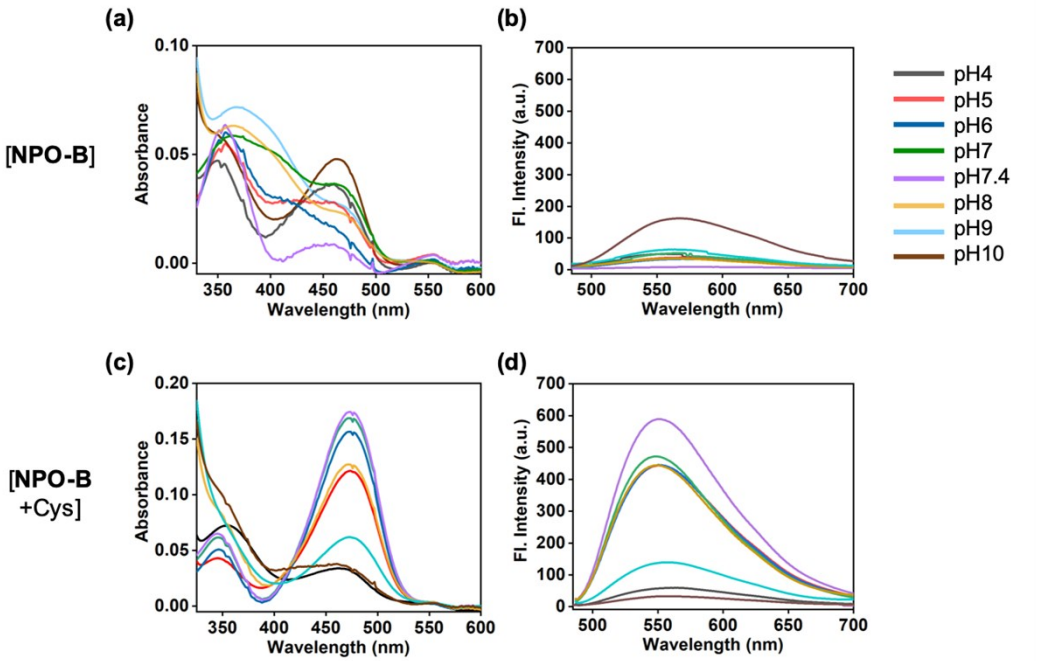


Fig. S7. Absorption and emission spectra of NPO-B with and without cysteine in various pH (pH 4, 5, 6, 7, 7.4, 8, 9, and 10). (a, b) Absorption and emission spectra of NPO-B (10 μM in each pH buffer) for 1 min incubation at 37 ºC. (c, d) Absorption and emission spectra of NPO-B (10 μM in each pH buffer) with cysteine (50 μM) for 30 min incubation at 37 ºC. The emission spectra were measured under excitation at the maximum absorbance peak.

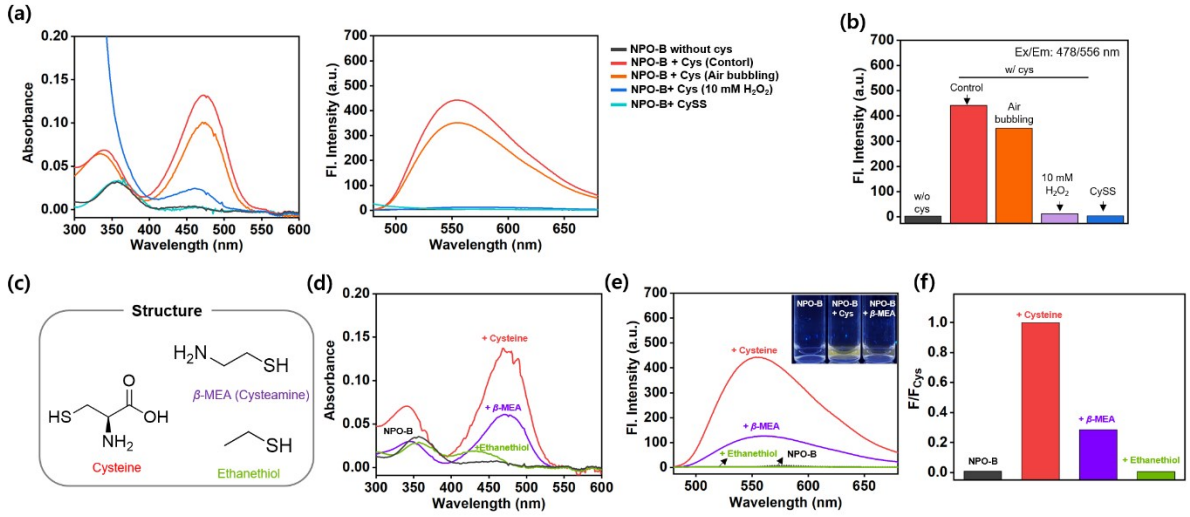


Fig. S8. (a) Absorption and emission spectra of NPO-B (10 μM) with cysteine under the oxidative condition. Oxidative condition set: (i) cysteine (50 μM) containing DI H_2O (200 μL) was bubbled with air (speed = 1.6 mL/min). (ii) cysteine (50 μM) containing DI H_2O (200 μL) with hydrogen peroxide (H_2O_2, 10 mM). (b) Emission intensity (at 556 nm) plot from the panel (a). (c) The chemical structure of cysteine, cysteamine, and ethanethiol. (d, e) Absorption and emission spectra of NPO-B with cysteine or other thiol substances. Inset photo: vials under UV lights (365 nm). (f) Normalized emission intensity (at 556 nm) plot from the panel (e). All emission spectra were measured under the excitation at 478 nm after incubation for 30 min at 37 °C.

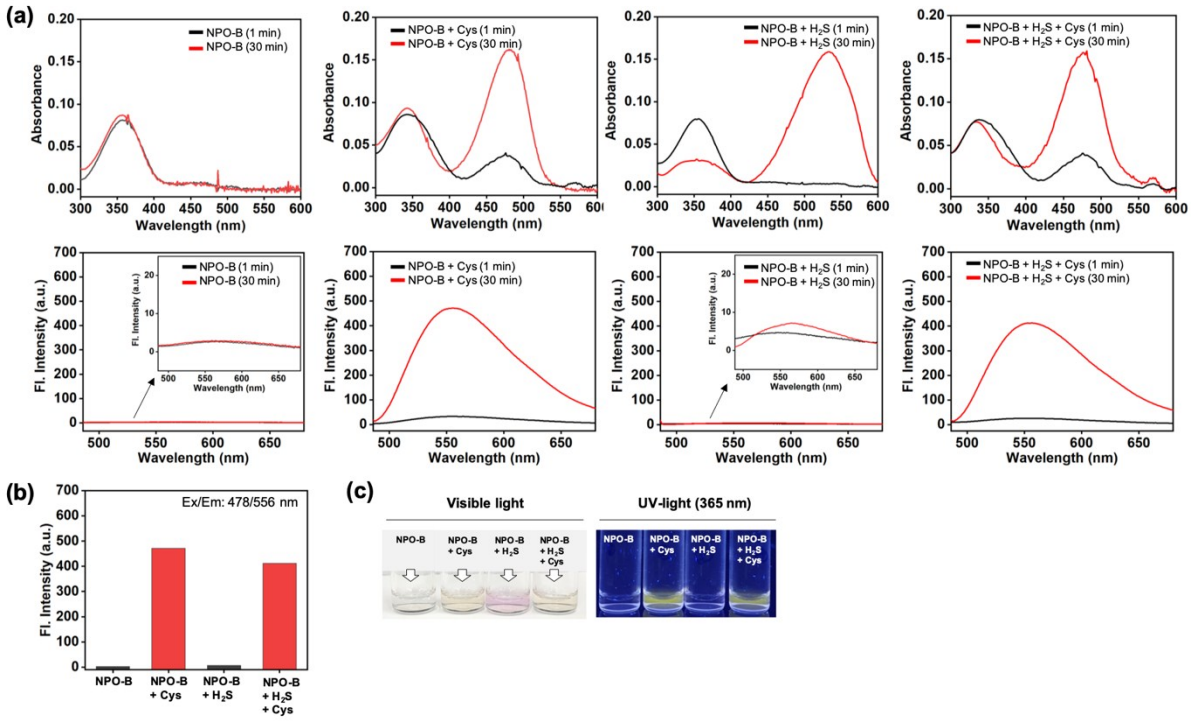
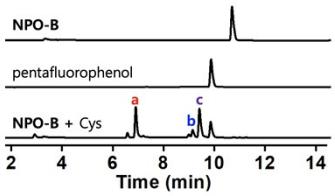
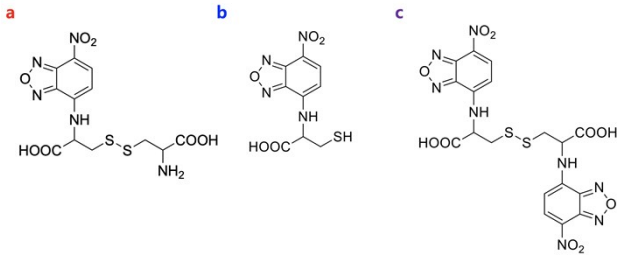


Fig. S9. (a) Absorption (upper) and emission spectra (bottom) before and after incubation of NPO-B (10 μM) with Cys (50 μM), H_2S (50 μM), and their mixture, in DI H_2O. The emission spectra were obtained under the excitation at 478 nm after 37 °C incubation. (b) Emission intensity plot from the panel (a). (c) Photos of vials (panel (a)) under the visible (left) and UV light (right, excitation: 365 nm).

(a)



(b)



(c)

	pos (g/mol)	neg (g/mol)	Exact Mass (g/mol)
a	404.0	402.0	403.33
b	285.0	283.0	284.02
c	Not detected	565.0	566.03

Fig. S10. LC-MS analysis of reaction product. (a) Spectra of LC-MS for NPO-B, pentafluorophenol, and reaction mixture of NPO-B (1 mM) and cysteine (Cys, 5 mM) in DI. H_2O at 37 ºC for 1 h. (b) Chemical structure of reaction product in panel (a). (c) Positive, negative and exact mass data for each compound in panel (a) and (b).

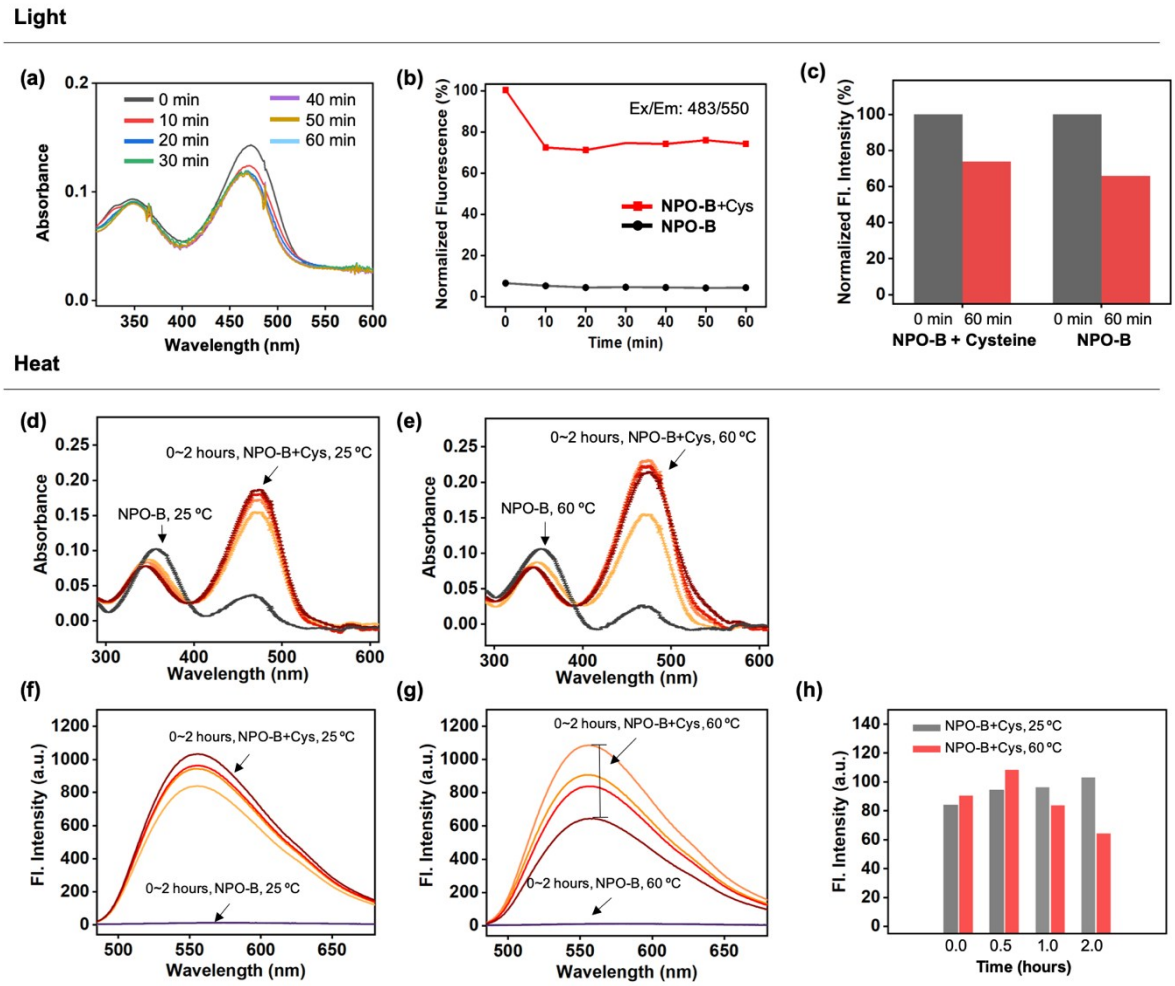
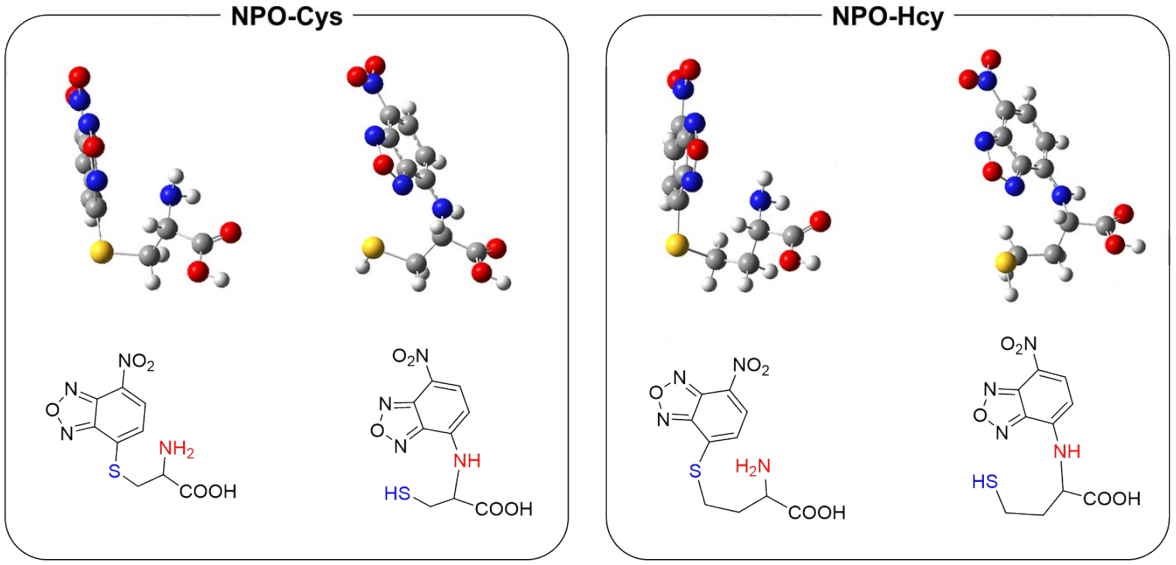


Fig. S11. Stability of NPO-B with and without Cys in DI H_2O. (a) Absorption spectra of the reaction mixture of NPO-B and Cys under UV irradiation (3 W) at 25 ºC for 1 h. The mixture was prepared with the reaction of NPO-B (10 μM in DI H_2O) with cysteine (50 μM) at 37 ºC shaking incubator for 30 min. (b) A fluorescence intensity plot of NPO-B with and without Cys under UV irradiation (3 W) at 25 ºC for 1 h. The "NPO-B+cys" set was prepared with the reaction of NPO-B (10 μM in DI H_2O) with cysteine (50 μM) at 37 ºC in a shaking incubator for 30 min. (c) Normalized intensity (point: 0 min of NPO-B+cys and NPO-B, 60 min of NPO-B+cys and NPO-B) of panel (b). (d) Absorption and (f) emission spectra of the reaction mixture of NPO-B and Cys under DI H_2O at 25 ºC for 2 h. (e) Absorption and (g) emission spectra of the reaction mixture of NPO-B and Cys under DI H_2O at 60 ºC for 2 h. (h) The fluorescence intensity plot for panel (f) and (g). All emission spectra were acquired under excitation at the maximum absorption wavelength.



	NPO-Cys		
	Energy (Hartree)	Relative energy (kJ/mol)	ΔE (kJ/mol)
S-bound	-1379.349547	0	-27.65
N-bound	-1379.35916	-27.65	

	NPO-Hcy		
	Energy (Hartree)	Relative energy (kJ/mol)	ΔE (kJ/mol)
S-bound	-1340.063005	0	-25.24
N-bound	-1340.073537	-25.24	

Fig. S12. The optimized structures of NPO-Cys and NPO-Hcy before and after the intramolecular substitution reaction (S-bound is converted into N-bound) obtained by the DFT method (APFD/6-31g(d)).

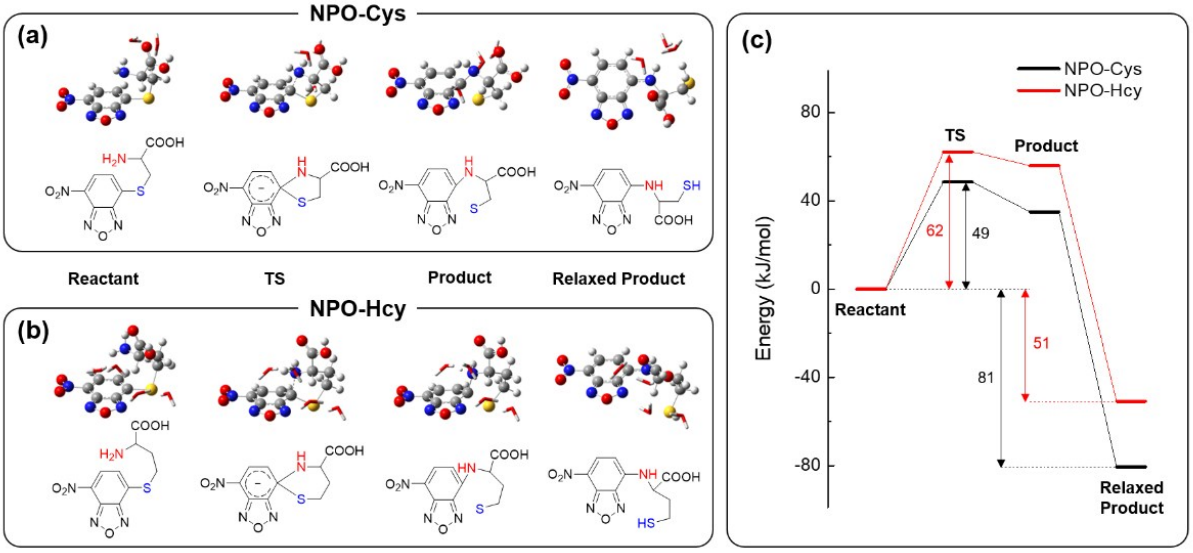


Fig. S13. Results of quantum chemical calculation. (a) and (b) The structures of NPO-Cys and NPO-Hcy with water molecules in the intramolecular substitution reaction (i.e., S-bound compounds are converted into N-bound compounds). Note that NPO-Cys and NPO-Hcy are represented by a ball and stick model while water molecules are represented with a tube model. Reactant: S-bound compound, TS: transition state, Product: N-bound compound, Relaxed Product: Final relaxed N-bound compound. (c) The energy diagram of the IRC for the intramolecular substitution reaction of NPO-Cys and NPO-Hcy.

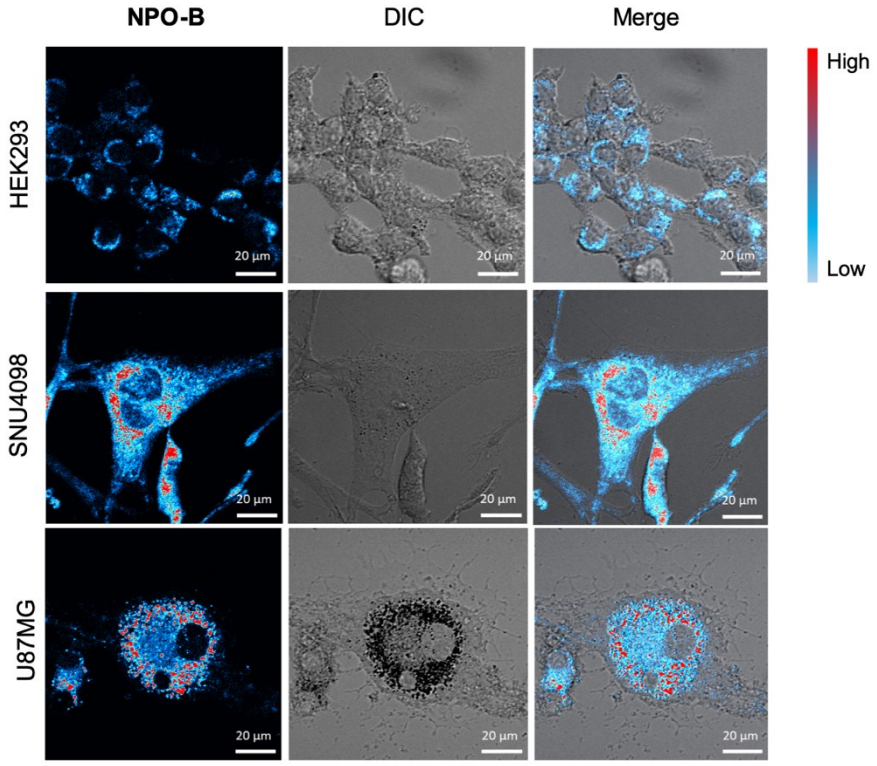


Fig. S14. Representative CLSM images of HEK293, COV-318 and HeLa with treatment of NPO-B. Cyan-to-white pseudo colored was applied to improve the contrast. All experiments were conducted after treating of NPO-B (30 μM) to each cell at 37 ºC, 5% incubator for 30 min. Excitation wavelength λ_{ex} = 488 nm; detection band filter = 500–700 nm; Scale bar = 20 μm.

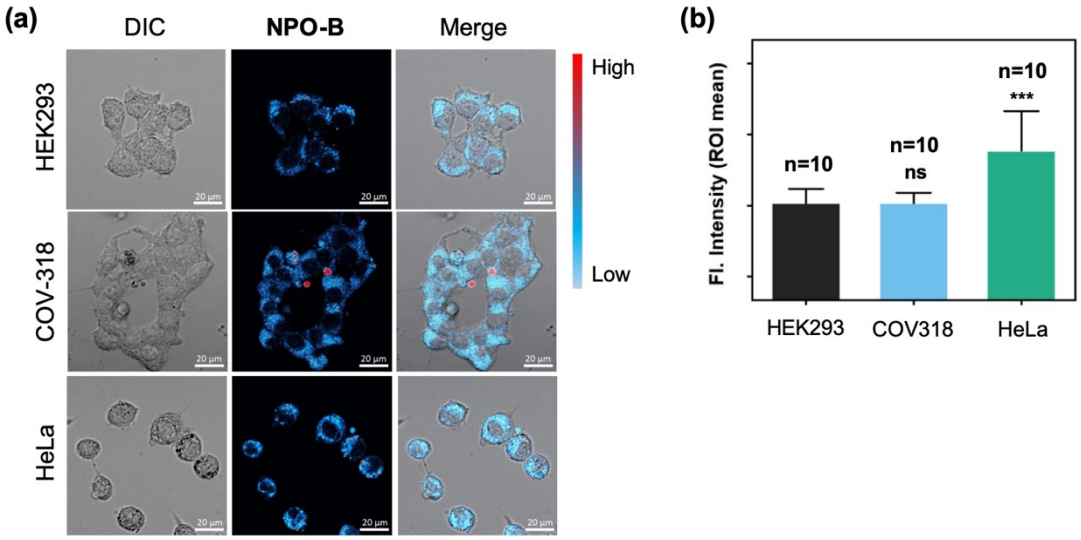


Fig. S15. Representative CLSM images for HEK293, COV-318, and HeLa with treatment of NPO-B. (a) CLSM image of each cell lines after treatment of NPO-B. Cyan-to-white pseudo colored was applied to improve the contrast. (b) Relative fluorescence intensities in each cells. Each error bar represents mean ± SD, n=number of cells, ns=non-significant, ***P < 0.001. All experiments were conducted after treating of NPO-B (30 μM) to each cell at 37 ºC, 5% incubator for 30 min. Excitation wavelength λ_{ex} = 488 nm; detection band filter = 500–700 nm; Scale bar = 20 μm. ROI mean was obtained on Image J program by drawing whole cell based on DIC image by hand.

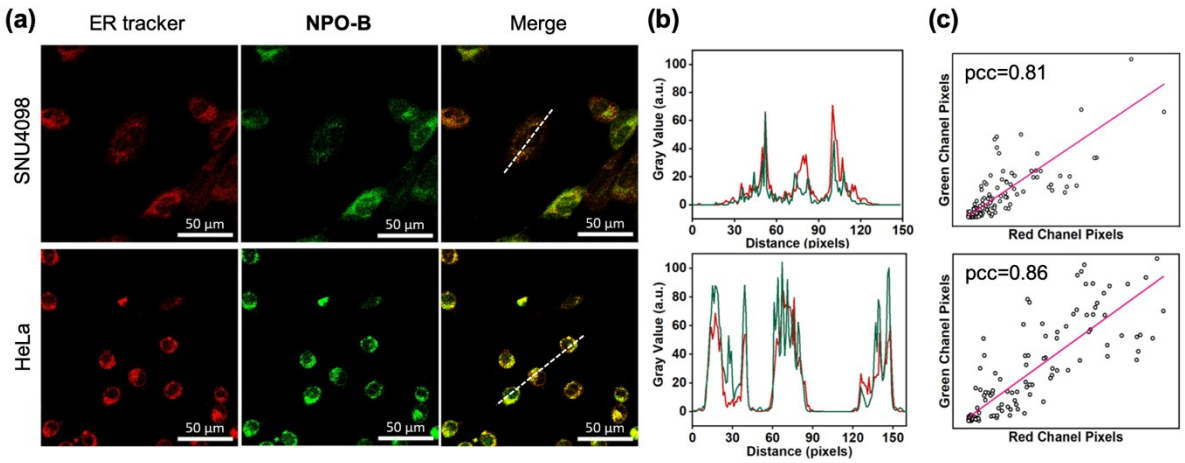


Fig. S16. Co-localization analysis of NPO-B in SNU4098 and HeLa cell line. (a) CLSM images of NPO-B (green, λ_{ex} = 488 nm) with ER-tracker (red, λ_{ex} = 590 nm). White lines: ROI region to calculate the (b) pixel intensity distribution and (c) PCC value. (b) Pixel intensities corresponding to the region marked in white lines in each CLSM image. (c) Profile plot of pixel intensity from panel (b) between green and red channels. Scale bar: 50 μm. This experiment was conducted after treating of NPO-B (30 μM) and ER-tracker (2 μg/mL) to each cell at 37 ºC, 5% incubator for 30 min. Pixel intensities was obtained by Image-J program.

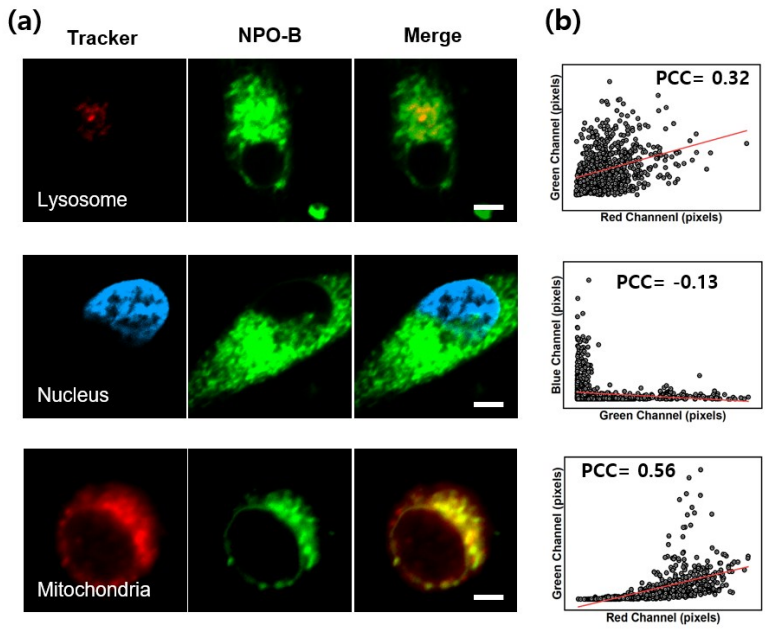


Fig. S17. Co-localization analysis of NPO-B in the U87MG cell lines. (a) CLSM images of U87MG treated NPO-B with each tracker [Notice: Lysosome tracker (red), DAPI (blue), Mitochondria tracker (red); Band gap: blue (λ_{ex} = 405 nm, 405-474 nm), green (λ_{ex} = 488 nm, 500-610 nm), red (λ_{ex} = 561 nm, band gap = 620-700 nm)]. (b) Profile plots of whole cell to obtain PCC. This experiment was conducted after treating of NPO-B (30 μM) and each tracker to each cell at 37 ºC, 5% incubator for 30 min. Working concentration of each tracker was shown at Material & Method part.

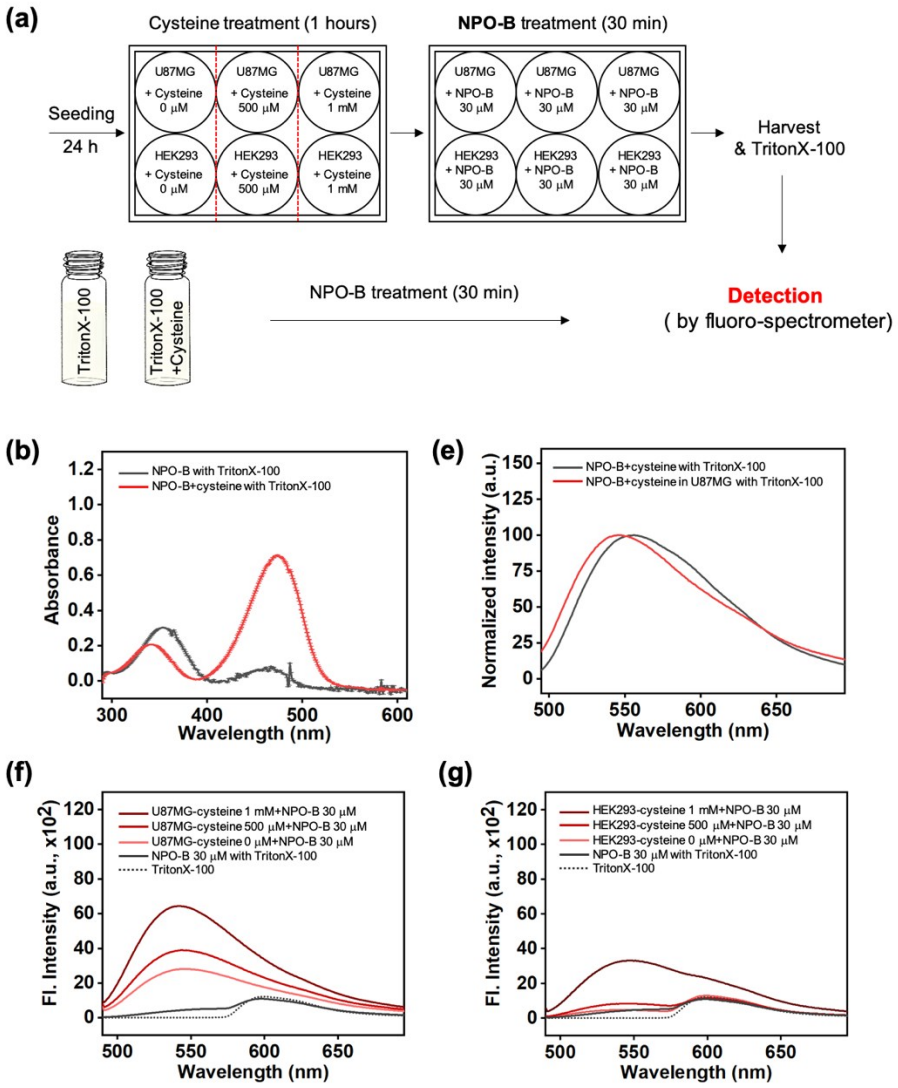


Fig. S18. The experiment to identify emission spectra in vial and in cells. (a) Procedures of whole experiment. (b) Absorption spectra of two condition (NPO-B and NPO-B + cysteine) with tritonX-100 in vial (glass, 4 mL). (c) Absorption spectra of NPO-B, NPO-B + cysteine within U87MG. (d) Absorption spectra of NPO-B, NPO-B + cysteine within HEK293. (e) Normalized intensity spectra in vial and cells. (f) Emission spectra of NPO-B, NPO-B + cysteine within U87MG. (d) Emission spectra of NPO-B, NPO-B + cysteine within HEK293. All emission spectra was exitated under the 478 nm, maximum absorption point. All solvent was using 100 μL of tritonX-100.

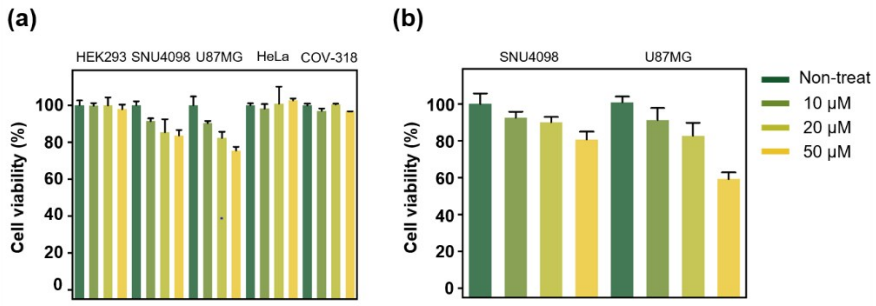


Fig. S19. Cell viability assay with CCK-8 for NPO-B in various cells (HEK293, SNU4098, U87MG, HeLa and COV-318). (a) Cell viability plot of NPO-B. (b) Cell viability plot of pentafluorophenol. All cytotoxicity assay with CCK-8 was performed after treating of NPO-B at 37 ºC, 5% incubator for 30 min. Experiments were performed in triplicate; each bar represents mean ± SEM. The absorption of CCK-8 solution was measured at 450 nm.

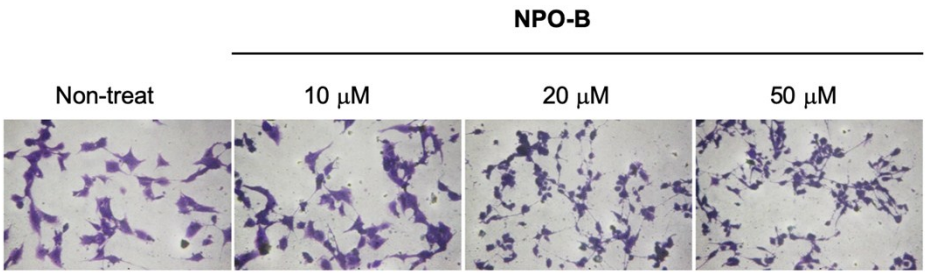


Fig. S20. Morphological changes of U87MG cell line in crystal violet dye, after being treated with NPO-B, at different concentrations, at 37 ºC, 5% incubation for 30 min. All images were produced under an optical microscope at 200× magnification.

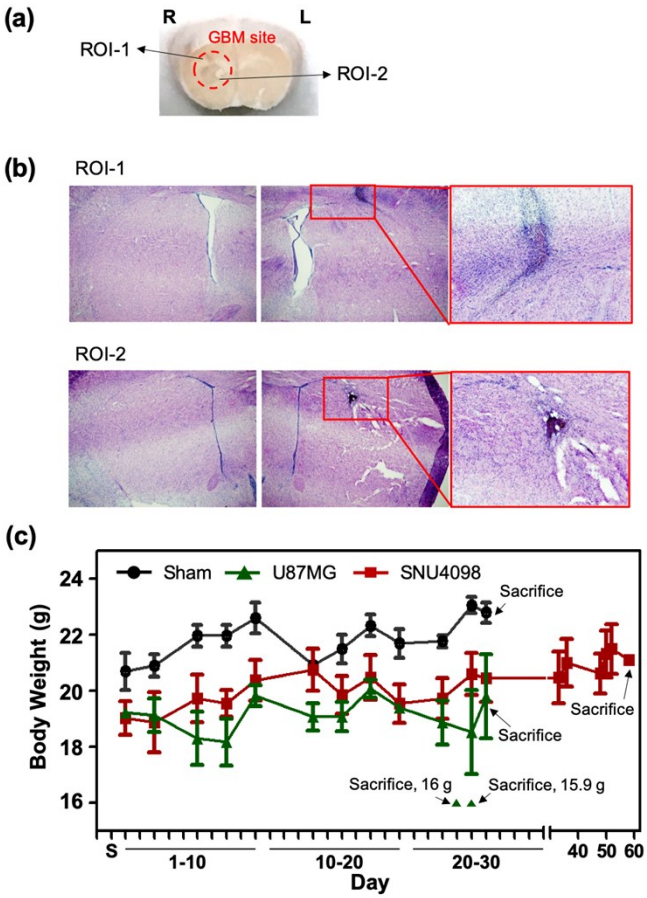


Fig. S21. Coronal sectional view of GBM xenograft mouse brain and H&E-stained tissue sections. (a) Coronal sectional view of SNU-4098 implanted GBM xenograft mouse model brain. (b) H&E-stained tissue sections at ROI-1 and ROI-2. The mice brains were isolated 21 days after the GBM cell implant surgery. Magnification: 40×, inset red-box: 100×. (c) Body weight changes for sham (control), U87MG, and SNU4098. Each error bar represents mean ± SD.

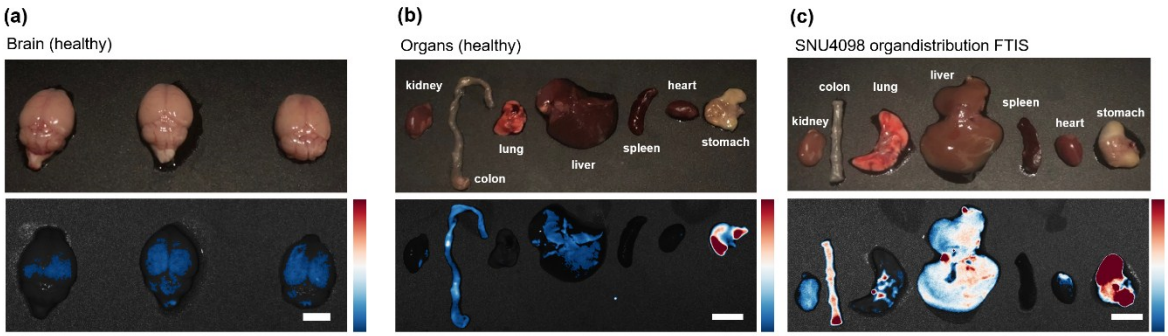


Fig. S22. *Ex vivo* FTIS images of mouse (healthy) organs. (a) Image of brain (sham group, upper) and FTIS image of brain (sham group, bottom). Scale bar = 0.5 cm. (b) Image of organs (sham group, upper) and FTIS image of organs (sham group, bottom). Scale bar = 1.2 cm. (c) Image of organs (SNU4098 xenograft model group, upper) and FTIS image of organs (SNU4098 xenograft model group, bottom). Scale bar = 1.2 cm. All experiments were conducted after the intravenous injection of NPO-B (5 mg/kg, PBS containing 1% DMSO) and 45 min circulation time. All FTIS images were taken at an exposure time of 5 sec.

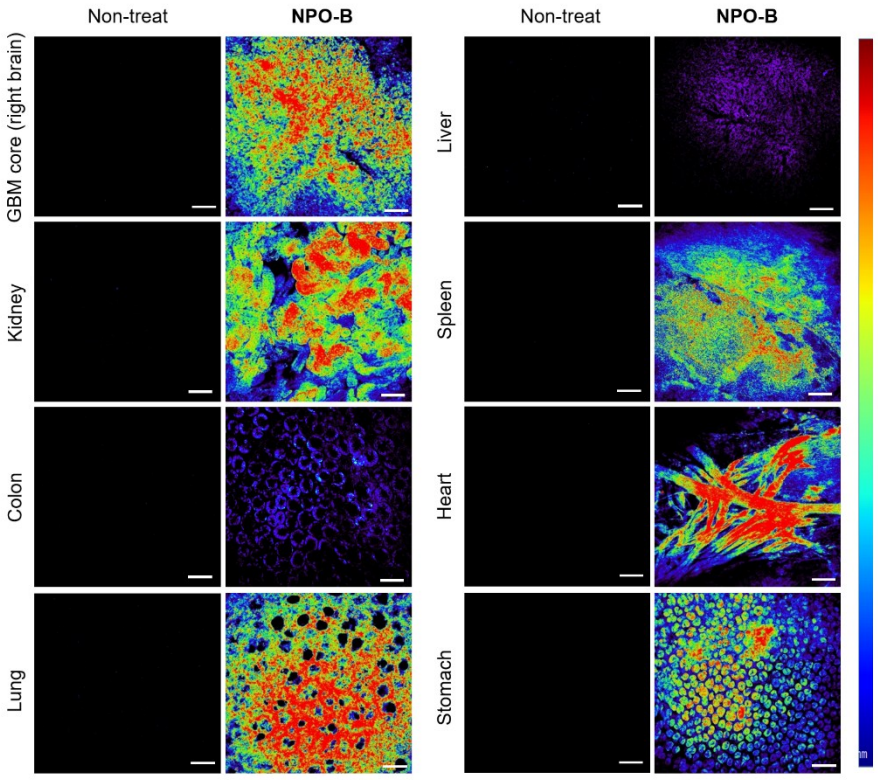


Fig. S23. TPM images of mouse organs after the treatment of NPO-B to GBM xenograft mouse model (U87MG cell line implanted). The organs were incubated with NPO-B (100 μM) for 60 min at 37 °C. The signals were collected in the wavelength range of 458-527 nm, under excitation at 900 nm with laser power of approximately 50 mW at the focal point. All images were overlaid by pseudo-color. Scale bars = 100 μm. The images were acquired at a middle depth (~50 μm) of the tissues.

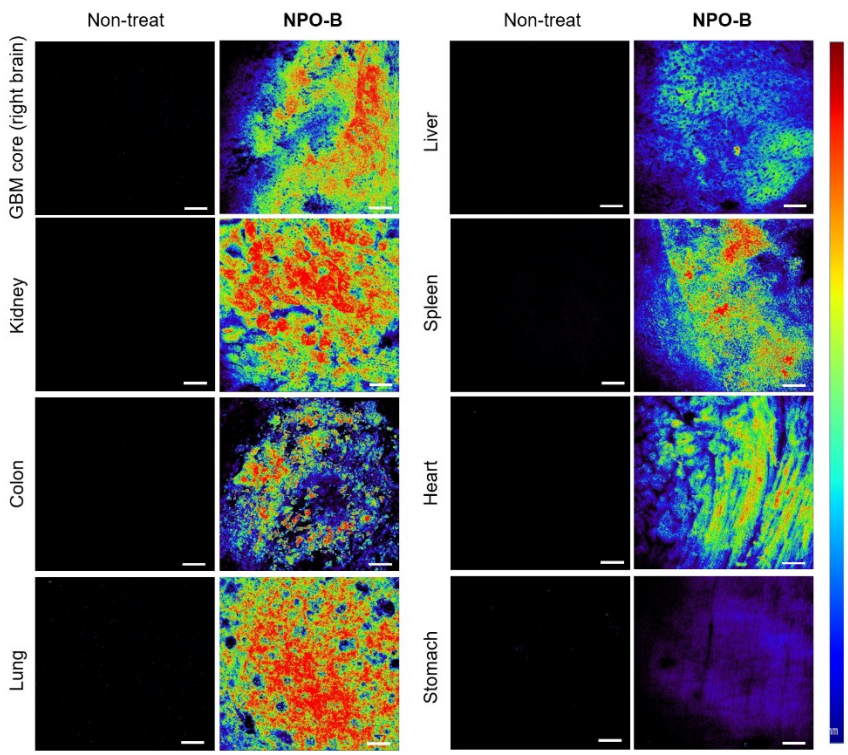


Fig. S24. TPM images of mouse organs after treatment of NPO-B to GBM xenograft mouse model (SNU4098 cell line implanted). The organs were incubated with NPO-B (100 μM) for 60 min at 37 °C. The signals were collected in the wavelength range of 458-527 nm, under excitation at 900 nm with laser poser of approximately 50 mW at the focal point. All images were overlaid by pseudo-color. Scale bars = 100 μm. The images were acquired at a middle depth (~50 μm) of the tissues.

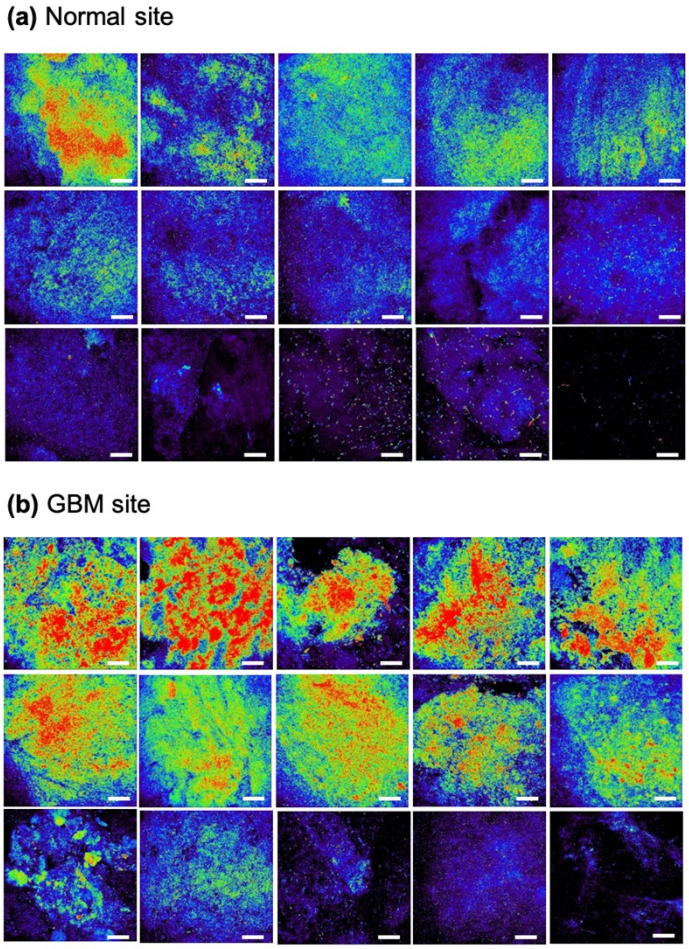


Fig. S25. TPM images of human clinical brain tissue samples treated with NPO-B (100 μM) for (a) normal tissues (n=15) and (b) glioblastoma core tissues (n=15). The signals were collected in the wavelength range of 458-527 nm, under excitation at 900 nm with laser poser of approximately 50 mW at the focal point. All images were overlaid by pseudo-color. Scale bar = 100 μm. The images were acquired at a middle depth (~50 μm) of the tissues.

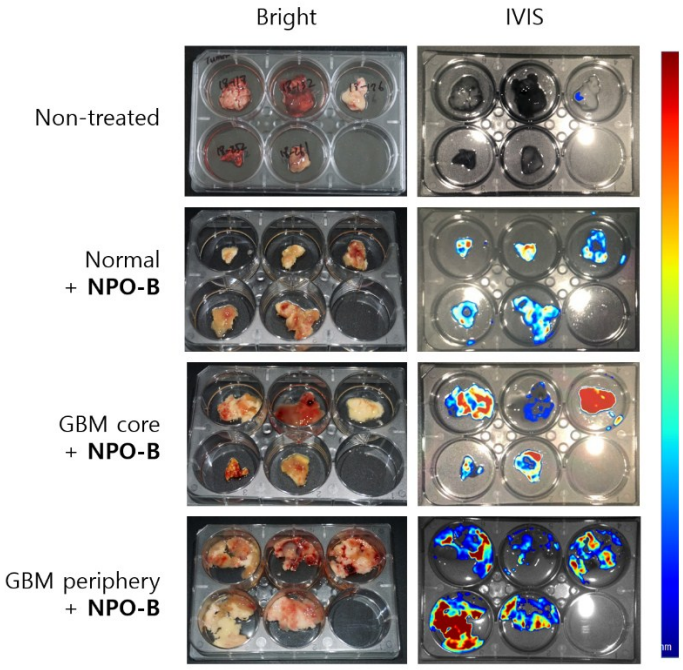


Fig. S26. FTIS images of human clinical brain tissue samples with NPO-B (100 μM) in a 6-well plate. All experiments were performed at room temperature after the treatment of NPO-B at 37 ℃ in a shaking incubator for 30 min. GFP exposure time = 1 sec.

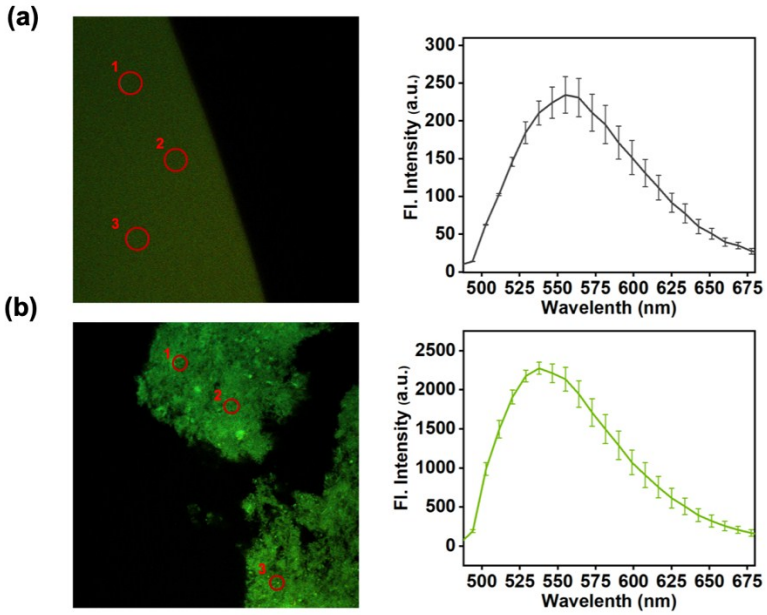


Fig. S27. Lambda scan analysis with a confocal microscope. (a) Mixure solution of 100 μM NPO-B with 500 μM cysteine on dish (Left, red circles indicate ROI regions to analyze fluorescence spectra). Flurorescence intensity histogram of ROI regions in left image (Right). (b) Human GBM biopsy (Patient ID: 18-104, patient) after reaction with 100 μM NPO-B for 30 min. (Left, red circles indicate ROI regions to analyze fluorescence spectra). Flurorescence intensity histogram of ROI regions in left image (Right). All measurement was obtained under the excitation 488 nm and magnification was 200x.

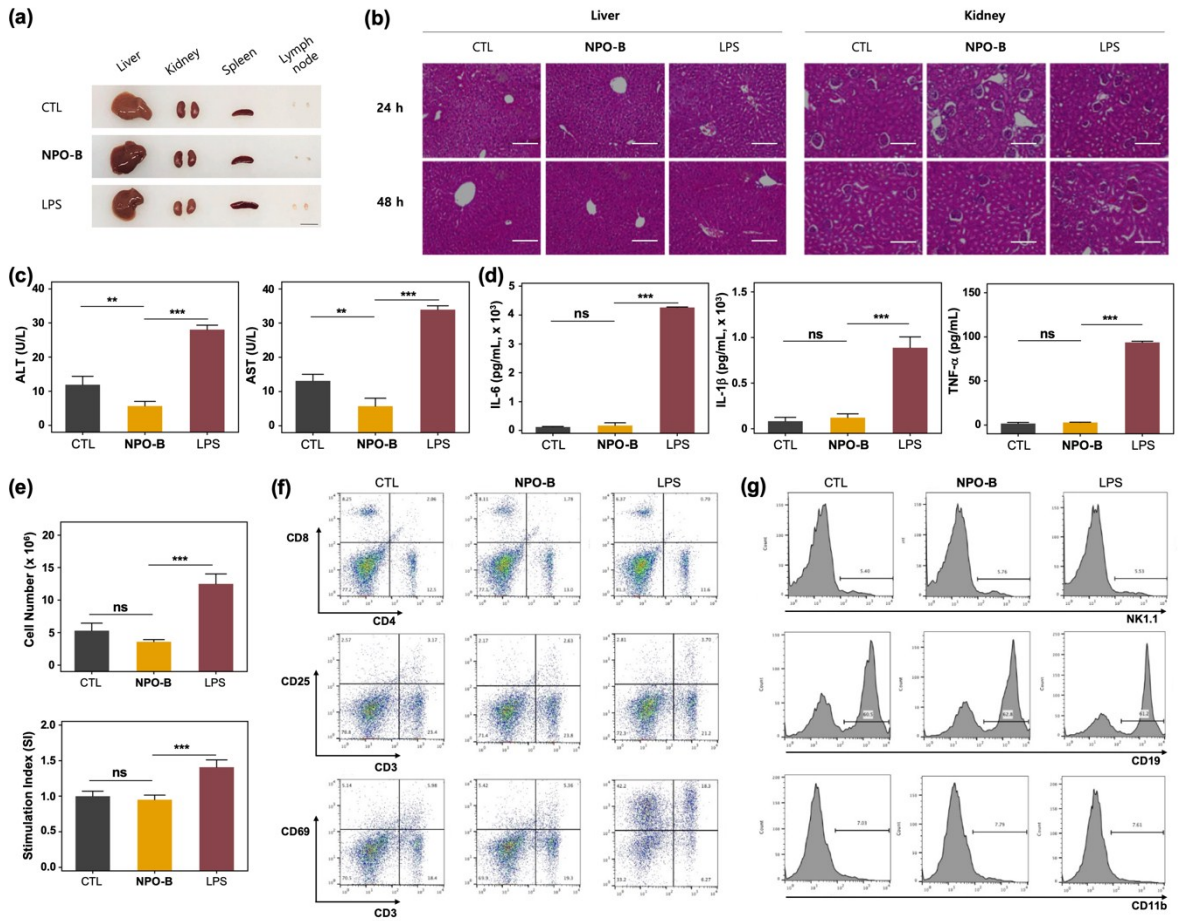


Fig. S28. Immuno-toxicity analysis of NPO-B. (a) Actual sizes of the representative liver, kidney, spleen and inguinal lymph node from the mouse (C57BL/6J). NPO-B (5 mg/kg) and LPS (10 μg/hd) was treated i.v., respectively. Scale bars = 1 cm. (b) H&E stained images of the mouse liver and kidney tissue after treatment (i.v.) of NPO-B and LPS. Scale bar = 125 μm. (c) Six hours or (d) Two hours after NPO-B and LPS administration, mice blood was analyzed for ALT/AST assay, and IL-6, IL-1β and TNF-α ELISA. (e) The number of cells and the stimulation index (SI) following BrdU ELISA in inguinal lymph nodes. (f, g) Splenocytes were stained with anti-CD3, CD4, CD8, CD25, CD69, NK1.1, CD19 and CD11b, and analyzed by flow cytometry. The cell population data of splenocytes based on the results of (f–g) is presented at Fig. S29. Abbreviation notice: control (CTL), lipopolysaccharides (LPS), ns (non-significant).

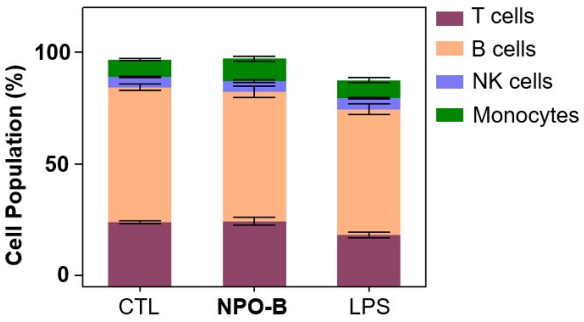
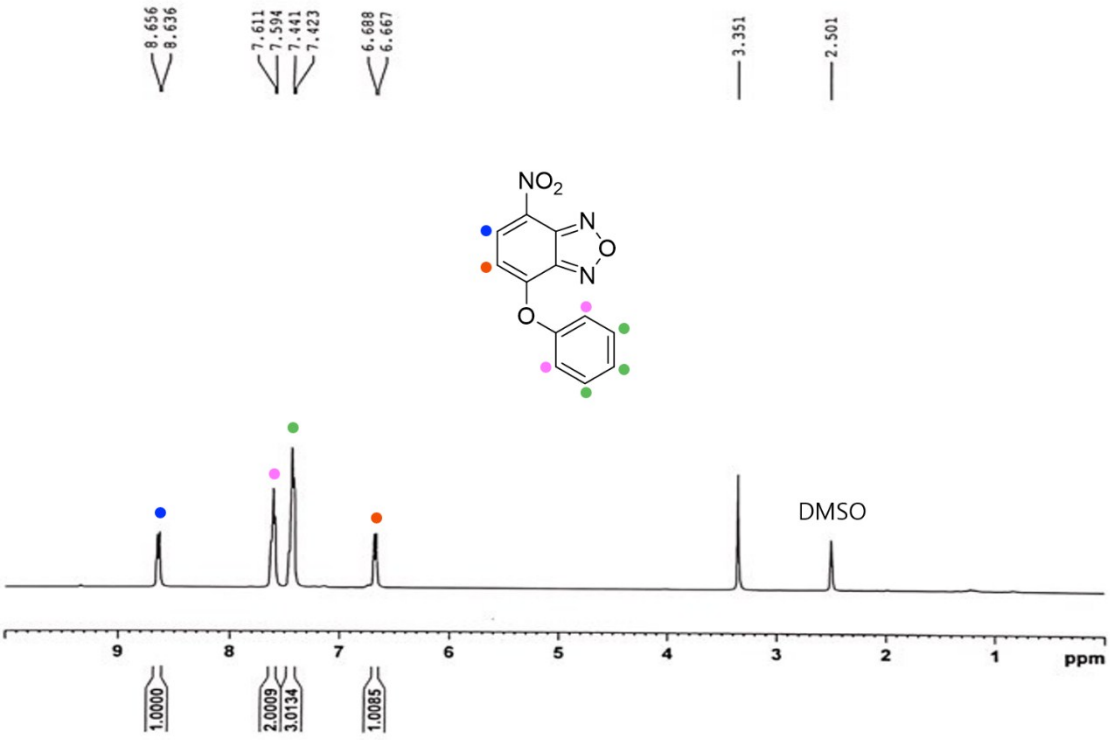
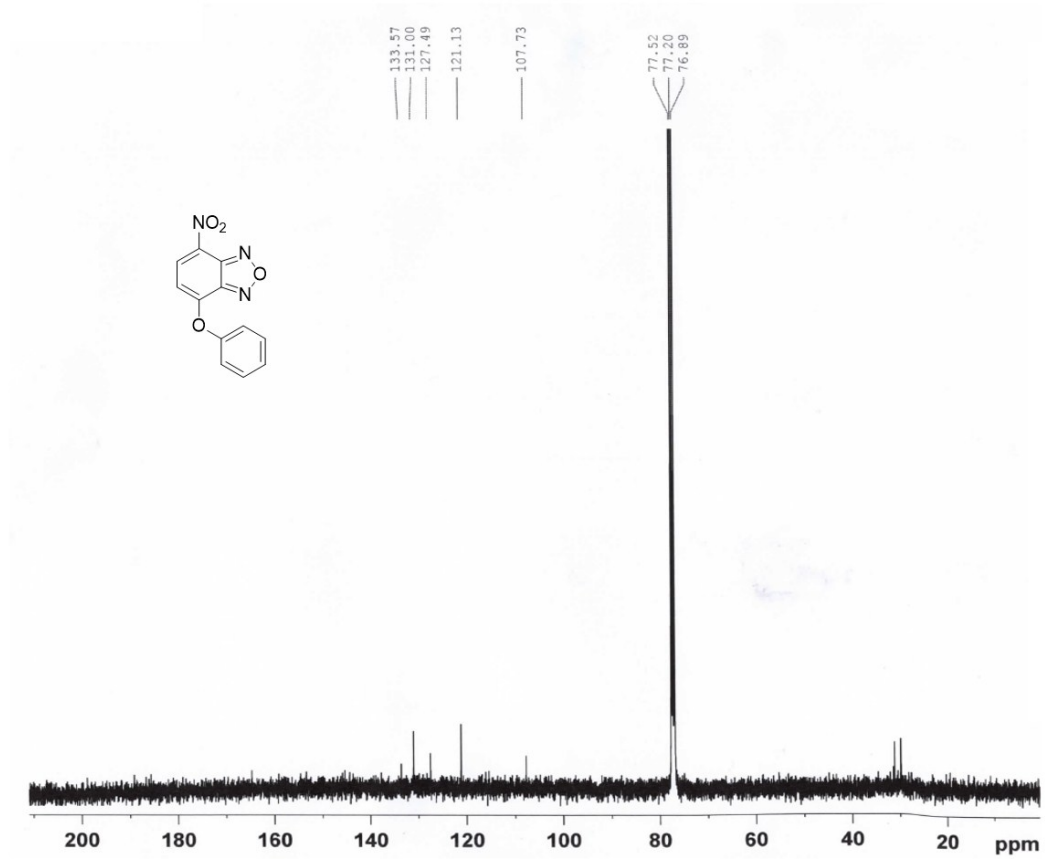


Fig. S29. The cell population of splenocytes based on the results of Fig. S28f and Fig. S28g.

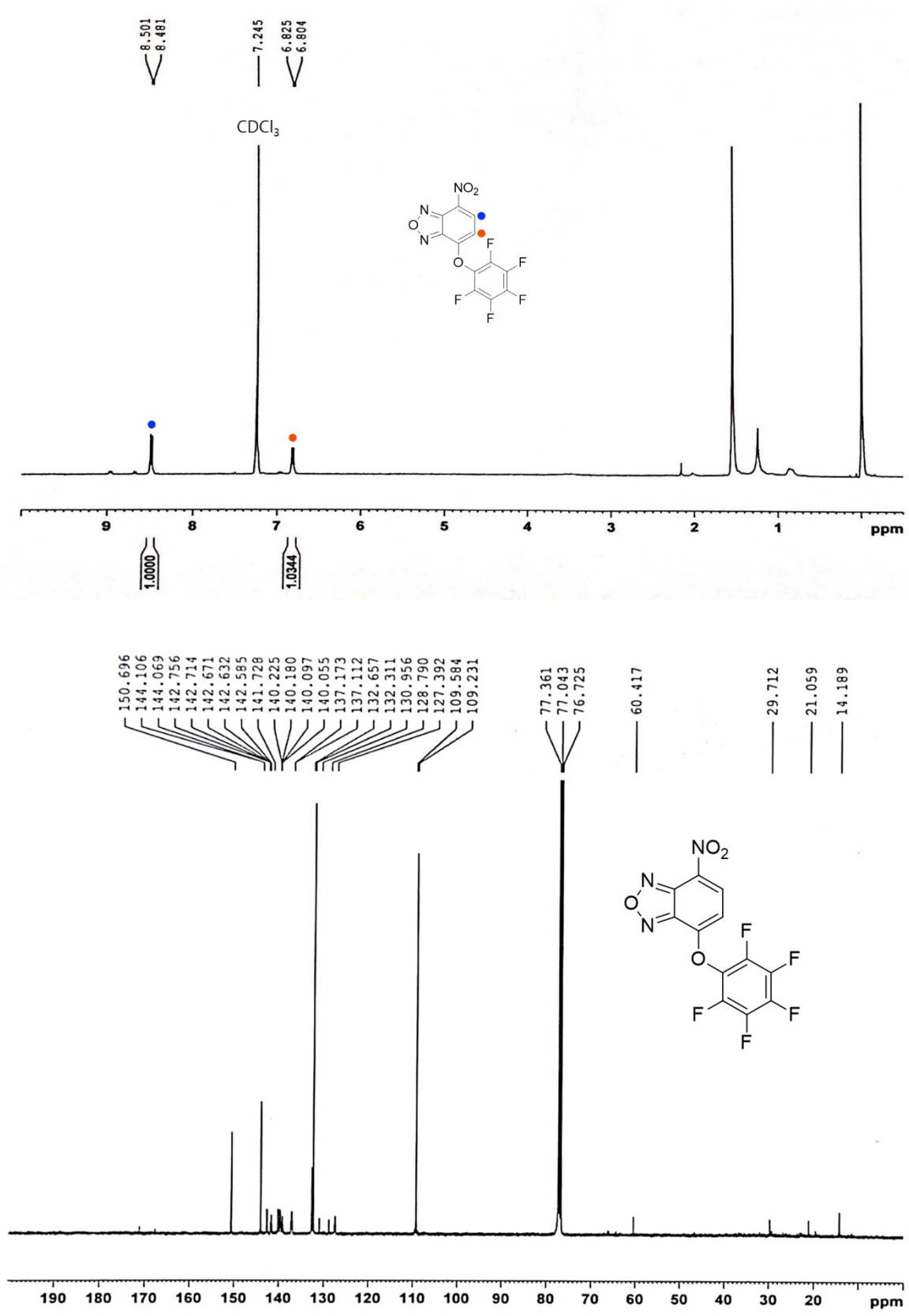
4. NMR (^{1}H, ^{13}C, ^{19}F) Spectra and HRMS of NPO Series

^{1}H and ^{13}C NMR for NPO-A

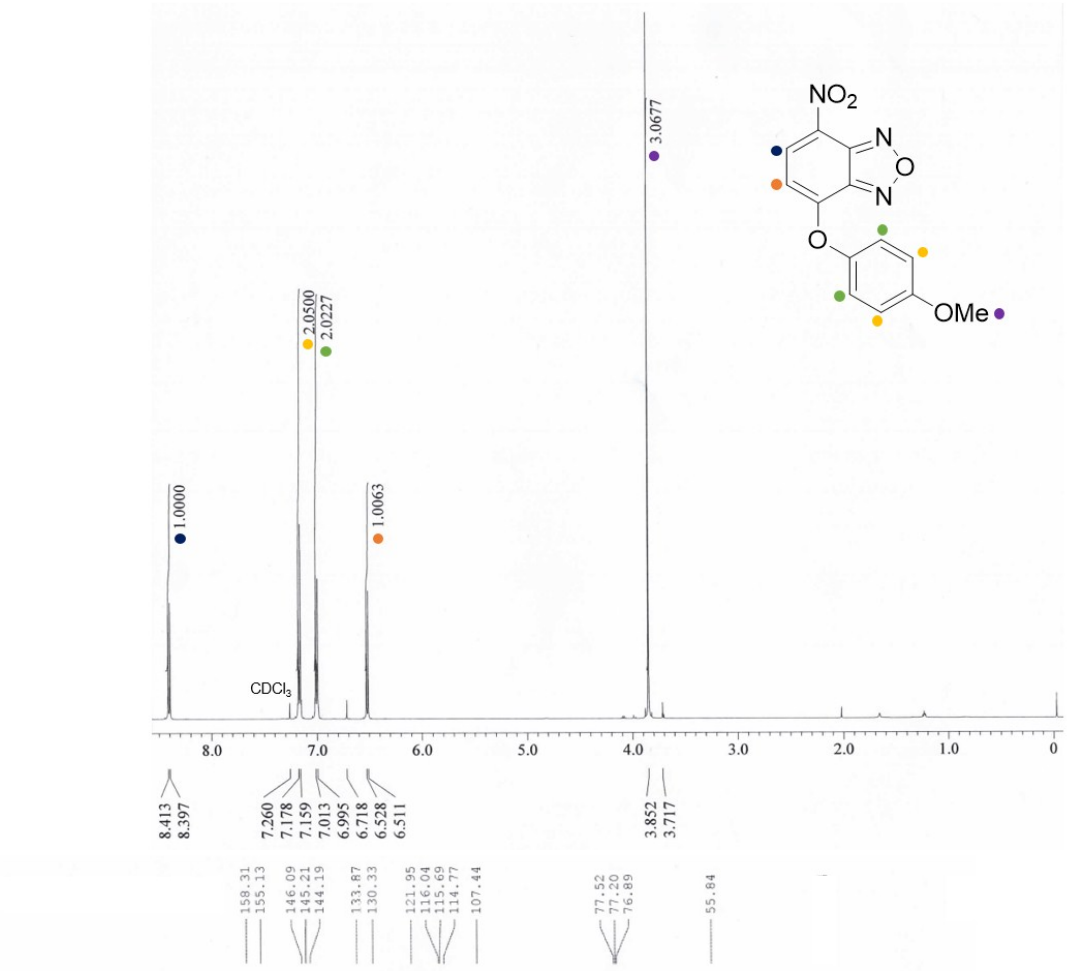


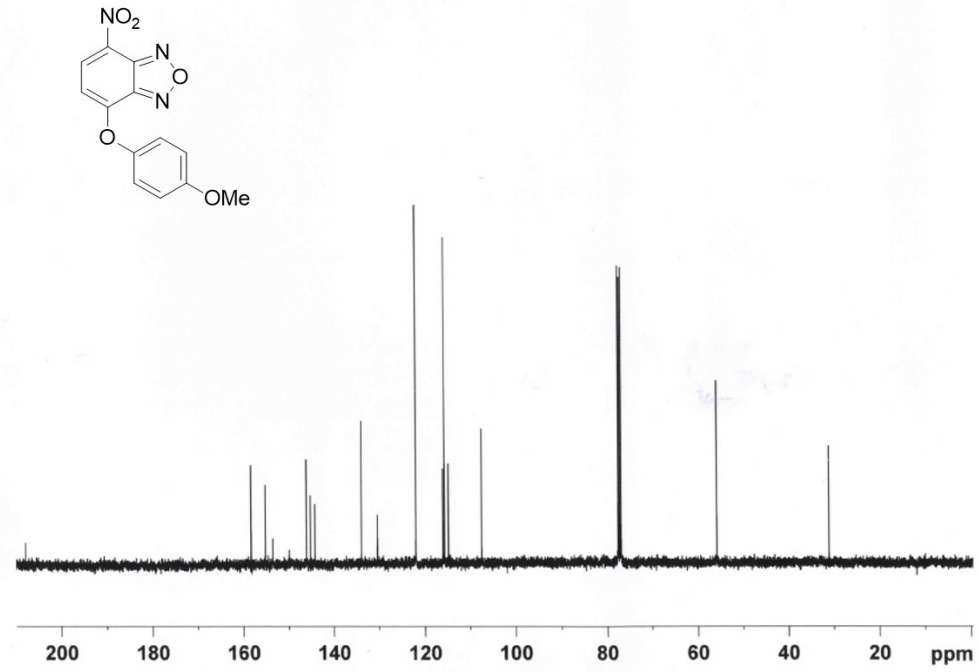


^{1}H and ^{13}C NMR for NPO-B

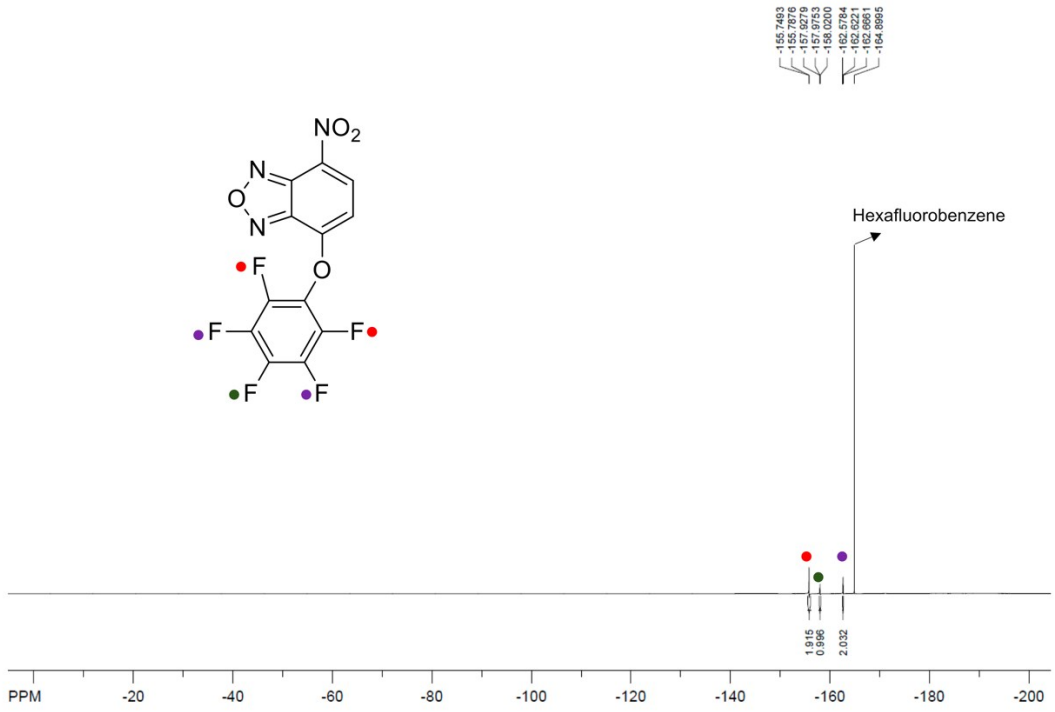


^{1}H and ^{13}C NMR for NPO-C

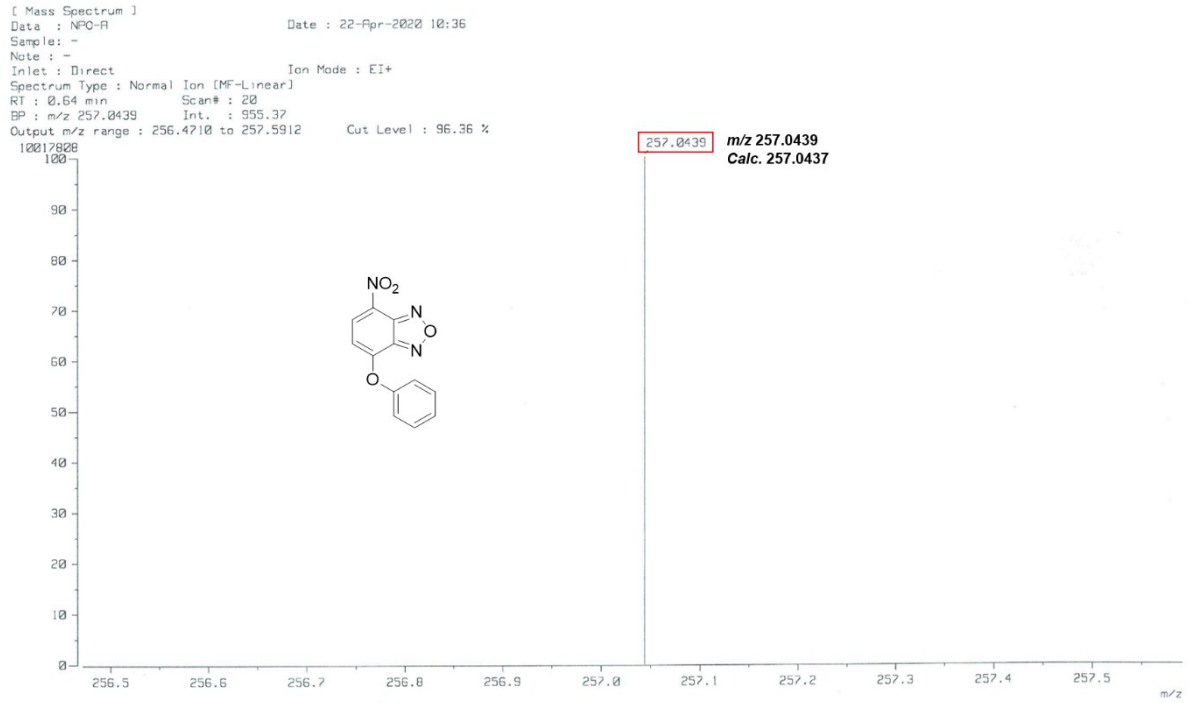


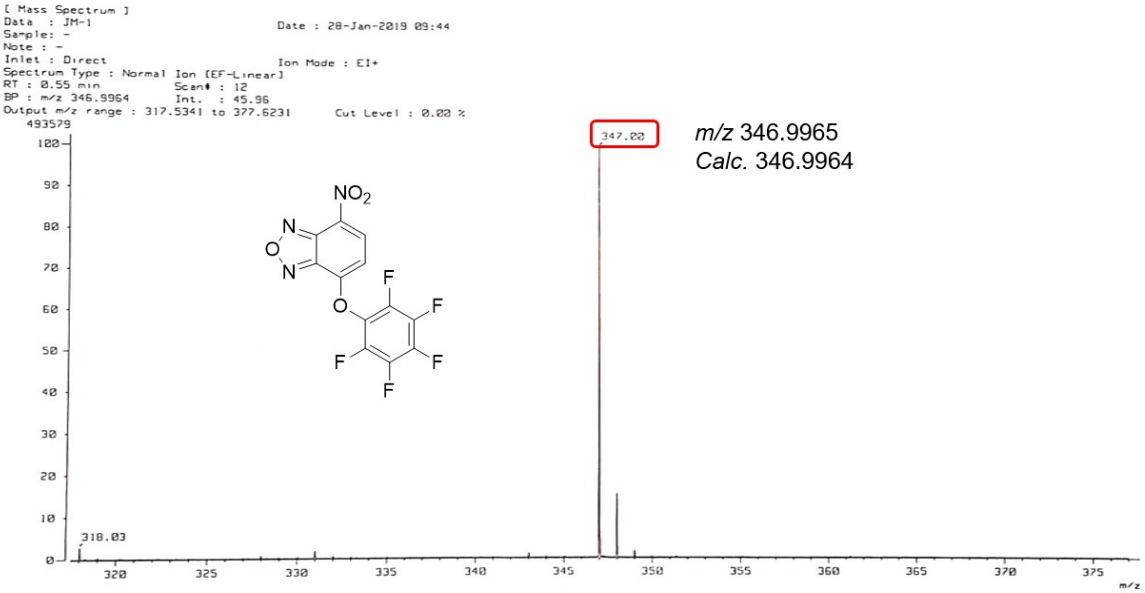


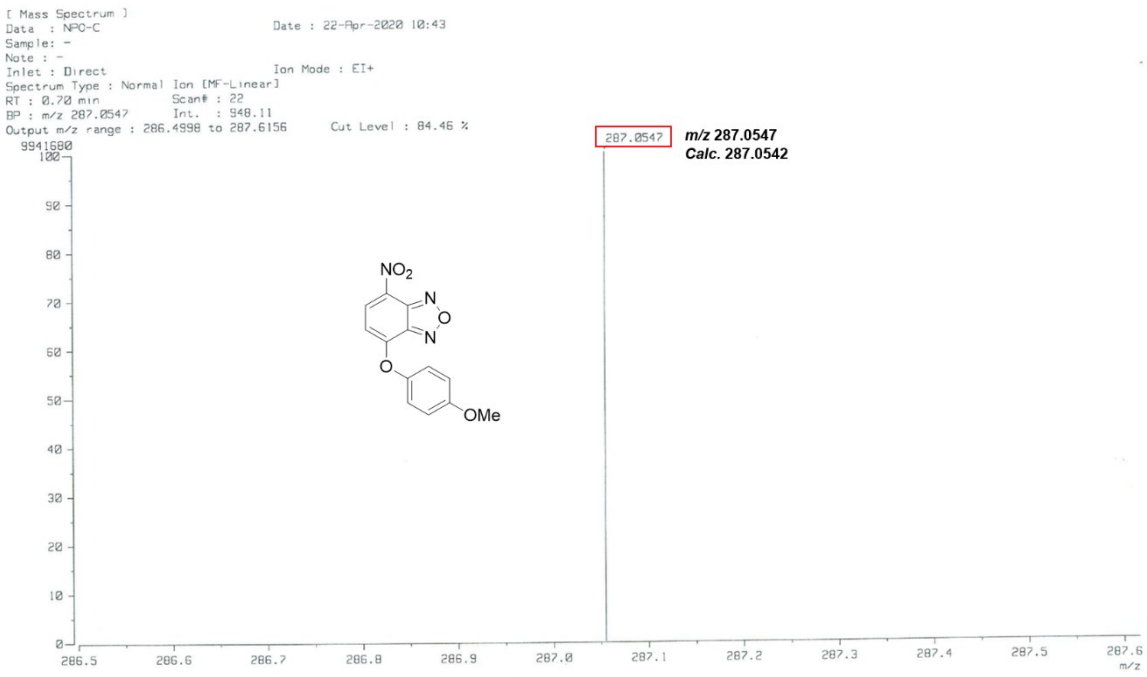
^{19}F NMR for NPO-B



HR-mass for NPO-A, NPO-B and NPO-C






[Mass Spectrum]
Data : NPC-C
Date : 22-Apr-2020 10:43
Sample: -
Note : -
Inlet : Direct
Ion Mode : EI+
Spectrum Type : Normal Ion [MF-Linear]
RT : 0.70 min
Scan# : 22
BP : m/z 287.0547
Int. : 948.11
Output m/z range : 286.4998 to 287.6156
Cut Level : 84.46 %
9941680
287.0547
m/z 287.0547
Calc. 287.0542
NO2
N
O
N
O
OMe
100
90
80
70
60
50
40
30
20
10
0
286.5
286.6
286.7
286.8
286.9
287.0
287.1
287.2
287.3
287.4
287.5
287.6
m/z

5. Supporting Tables

Table S1. Electronic energies of reactant, TS, product, and relaxed product in the IRC for the intramolecular substitution reaction of NPO-Cys and NPO-Hcy.

	NPO-Cys	
	Energy (Hartree)	Relative energy (kJ/mol)
Reactant	-1569.171773	0
TS	-1569.153281	48.6
Product	-1569.158437	35.0
Relaxed product	-1569.202458	-80.6
	NPO-Hcy	
	Energy (Hartree)	Relative energy (kJ/mol)
Reactant	-1684.838588	0
TS	-1684.814884	62.2
Product	-1684.817277	56.0
Relaxed product	-1684.857938	-50.8

Table S2. The list of patient information (human clinical samples; Korean)

	Date	Patient ID	Sex	Age
Normal brain tissue	2018-05-19	18-146	M	46
	2018-08-31	18-237	F	26
	2018-01-03	18-002	F	34
	2018-06-07	18-157	F	38
	2017-09-13	17-213	F	42
	2017-02-02	17-014	M	55
	2016-09-02	16-171	M	59
	2016-03-18	16-050	F	49
	2016-04-08	16-082	M	45
	2016-10-06	16-200	M	40
	2015-11-13	15-240	F	51
	2015-05-15	15-100	M	54
	2015-07-30	15-159	M	52
	2015-09-04	15-182	F	28
	2015-12-16	15-261	M	53
Tumor brain tissue (GBM)	2018-04-17	18-104	M	80
	2018-06-22	18-176	F	74
	2018-07-13	18-192	M	75
	2018-09-21	18-261	F	59
	2018-05-09	18-132	F	64
	2018-06-07	18-156	M	35
	2018-01-26	18-030	M	75
	2018-03-15	18-077	M	59
	2018-04-19	18-109	F	70
	2018-04-27	18-118	F	83
	2018-05-16	18-145	F	31
	2018-07-06	18-186	M	61
	2018-09-05	18-242	F	63
	2018-09-12	18-251	F	81
	2018-09-12	18-252	F	66
Tumor-normal interface tissue (GBM)	2018-10-30	18-276	M	37
	2018-06-14	18-163	M	48
	2018-01-26	18-030	M	75
	2018-03-15	18-077	M	59
	2018-04-19	18-109	F	70