Supporting Information

Self-Calibrating Bipartite Fluorescent Sensor for Nitroreductase Activity and Its Application to Cancer and Hypoxic Cells

Shin A Yoon,[a] Jieun Chun,[b] Chulhun Kang*[b] and Min Hee Lee*[a]

[a]*Department of Chemistry, Sookmyung Women's University, Seoul 04310, Korea*
[b]*The School of East-West Medical Science, Kyung Hee University, Yongin 17104, Korea*

*Corresponding authors: minheelee@sookmyung.ac.kr (M. H. Lee), kangch@khu.ac.kr (C. Kang)

Contents

1. Materials and instrumentation

All reagents, such as NADH and nitroreductase (NTR) from *Escherichia coli*, metals (chloride salts of K^+, Na^+, Ag^+, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Ba^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Ni^{2+}, Co^{2+}, and Cu^+ ions), anions (tetrabutylammonium (TBA) salts of $H_2PO_4^-$, HSO_4^-, ClO_4^-, AcO^-, CN^-, OH^-, F^-, Cl^-, and I^- ions), reactive oxygen species (ClO^-, H_2O_2, $^{\bullet}O_2^-$, $^{\bullet}OH$, t-Bu$O^{\bullet}$, t-BuOOH, NO, and $ONOO^-$), thiols (cysteine (Cys), homocysteine (Hcy), glutathione (GSH), and NaHS), bovine serum albumin (BSA), glucose, ascorbic acid, glycine, and other chemicals for synthesis, buffer solution were purchased from Aldrich (Aldrich, St. Louis, MO, USA), Alfa (Alfa, Heysham, LA3 2XY, United Kingdom) and TCI (TCI, Tokyo, Japan), and used as received. Stock solutions of metal chloride salts, thiols and reactive oxygen species (ROS) were prepared in deionized water. ROS stock solutions were prepared by using literature procedures.[1] HPLC analyses were carried out on Shimadzu HPLC (Shimadzu LC 6AD) with a Thermo Scientific Acclain™ 120 C18 (3 μm, 120 Å, 2.1 × 150 mm) column. The flow rate was 0.5 mL/min. Buffer A (water with 0.1% v/v trifluoroacetic acid) and Buffer B (acetonitrile with 0.1% v/v trifluoroacetic acid) were used as the mobile phase. NMR spectra were recorded on Bruker (500 MHz) instrument. HR-ESI-MS data were obtained using liquid chromatography mass spectrometer (LC/MS) at the Korea Basic Science Institute (Seoul).

2. UV/Vis absorption and fluorescence spectroscopic methods

All UV/Vis absorption and fluorescence spectra were recorded on UV-2600 (Shimadzu Corporation, Kyoto, Kyoto Prefecture, Japan) and RF-6000 (Shimadzu Corporation, Kyoto, Kyoto Prefecture, Japan) spectrophotometer, respectively. Stock solutions of compounds were prepared

in DMSO (HPLC grade). All data were recorded in PBS solution (10 mM, pH 7.4) containing 10% (v/v) of DMSO. Excitation wavelength was 430 nm.

3. Enzyme kinetics

Fluorescence responses at 550 nm of probe **1** (1-17 μM) to NTR (0.1 μg/mL) with NADH (500 μM) was recorded for 5 min (time interval of 0.1 s). All data were obtained in PBS solution (10 mM, pH 7.4) containing 10% (v/v) of DMSO at 37 ℃ with an excitation of 430 nm. The fluorescence units were converted to concentration by relating the fluorescence signals derived from the known concentrations of compound **2**. Plot of velocity (μmol min^{-1} $mgNTR^{-1}$) *vs.* [probe **1**] (μM) was used to obtain K_m and V_{max} values from nonlinear least-squares analysis for Michaelis-Menten kinetics (OriginPro 8.0). From this, k_{cat} and k_{cat}/K_m values were obtained.

4. Cell culture and confocal microscopic methods

A human cervical cancer cells (HeLa) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Gibco® fetal bovine serum (FBS), and 100 U/mL penicillin-streptomycin. Adenocarcinoma human alveolar basal epithelial cells (A549) and human hepatocellular liver carcinoma cells (HepG2) were cultured Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% FBS, and 100 U/mL penicillin-streptomycin. Mouse embryonic fibroblast cells (NIH-3T3) were cultured in DMEM supplemented with 10% Gibco® bovine calf serum (BCS), and 100 U/mL penicillin-streptomycin. At 2 days before the microscopic experiments, the cells were transferred on cover glass-bottom dish. The cells were seeded at 10^5 per dish and maintained at 37 °C in a humidified atmosphere consisting of 5% (v/v) CO_2 containing air. All cells were purchased from Korean Cell Line Bank (Seoul, South Korea).

For cell culture media, DMEM, RPMI, FBS, trypsin 0.25%-EDTA and penicillin-streptomycin were purchased from BIOWEST (Nuaillé-France). The clear and adhesion-typed confocal dishes (diameter = 35 mm) were purchased from SPL (Phocheon-si, Korea).

5. MTT assay

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells at 1.5×10^4/mL were treated with different concentrations of probe **1** in 96-well plates for 24 h at 37 °C. Then, a solution of MTT in serum free media (5 mg/mL) was added to each well, which was then further incubated for 3 h. The water-insoluble formazan was formed during the incubation, and then DMSO was added to each well. The amount of formazan was then measured by checking the absorbance at 540 nm using a Spectra Max i3x microplate reader (Molecular devices, San Jose, CA). MTT was purchased from Sigma-Aldrich (St. Louis, MO) and used as received without further purification.

6. Co-staining of probe and organelle trackers and confocal microscopic methods

Organelle tracking dyes for co-localization experiments were purchased from Invitrogen. HeLa cells were pretreated with ER-Red (0.7 μM), Lyso-Red (0.05 μM), or Mito-Red (0.1 μM), respectively, in DMEM media for 15 min at 37 °C. The cells were subsequently incubated with probe **1** (5 μM) in DMEM media for 20 min at 37 °C, and then washed with PBS. The excitation wavelength and filter set were 405 nm with a 505-600 nm band path (BP) and 555 nm with a 605-800 nm BP for probe 1 and organelle trackers, respectively. Fluorescence images were obtained using Zeiss LSM-700 with 40x objective (1x and 1.5x zoom) (Carl Zeiss, Oberkochen, Germany). Image analysis was performed using ZEN software and autoQuant X3.

7. $CoCl_2$-induced hypoxic HeLa cells and inhibitory assay methods

Cobalt chloride hexahydrate ($CoCl_2$) and dicoumarol (Dic) were purchased from Sigma-Aldrich (St. Louis, MO) and used as received without further purification. Stock solution of $CoCl_2$ and dicoumarol were prepared in deionized water. HeLa cells were pretreated with $CoCl_2$ (50 μM) in DMEM media for 24 h at 37 °C. The cells were subsequently incubated with or without dicoumarol (500 μM) for 4 h at 37 °C. Then, probe **1** (0.5 μM) was added to each cell for 30 min at 37 °C. The excitation laser was 405 nm and emission filters were 420-475 nm BP and 505-600 nm BP for blue and green channels. Image analysis was performed using ZEN software and Image J software.

8. Supplementary data

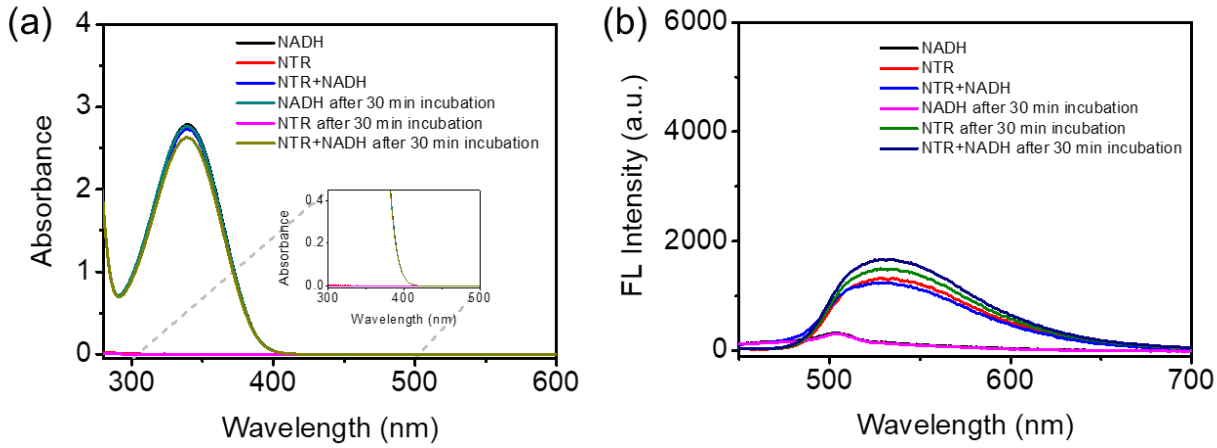

Figure S1. (a) UV/Vis absorption and (b) fluorescence spectra of NTR (5 μg/mL), NADH (500 μM) and NTR/NADH, respectively. All data were obtained in PBS (10 mM, pH 7.4) solution containing 10% (v/v) of DMSO using an excitation at 430 nm before and after 30 min incubation at 37 ℃.

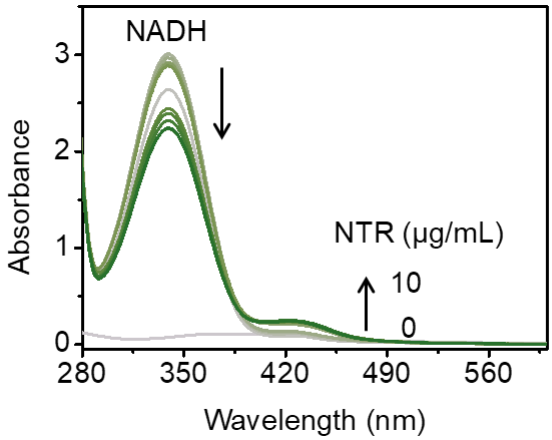

Figure S2. UV/Vis absorption of **1** (10 μM) to different concentration of NTR (0-10 μg/mL) in presence of NADH (500 μM). All data were obtained in PBS (10 mM, pH 7.4) solution containing 10% (v/v) of DMSO after 30 min incubation at 37 °C using an excitation at 430 nm.

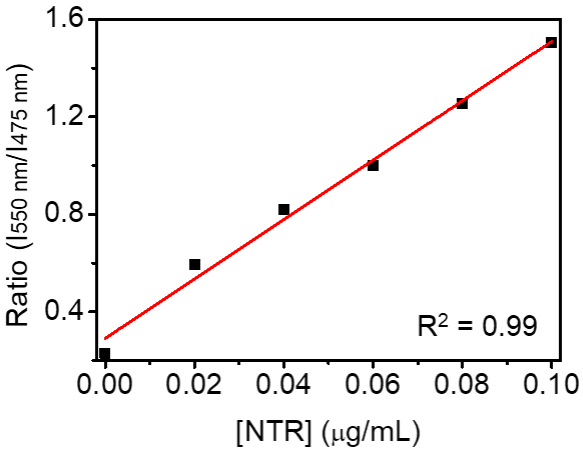

Figure S3. Plot of fluorescence intensity ratio ($I_{550\ nm}/I_{475\ nm}$) *vs.* [NTR] in the presence of NADH (500 μM). All data were obtained in PBS (10 mM, pH 7.4) solution containing 10% (v/v) of DMSO after 30 min incubation at 37 °C using an excitation at 430 nm.

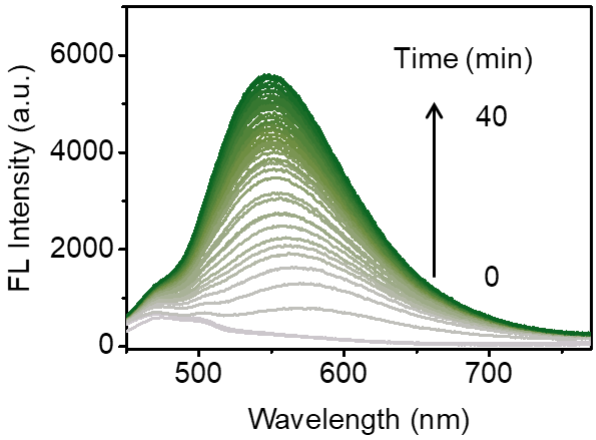

Figure S4. Time-dependent fluorescence increase of probe **1** (10 μM) upon the addition of NTR (5 μg/mL) and NADH (500 μM). All data were obtained in PBS (10 mM, pH 7.4) solution containing 10% (v/v) of DMSO at 37 ℃ using an excitation of 430 nm.

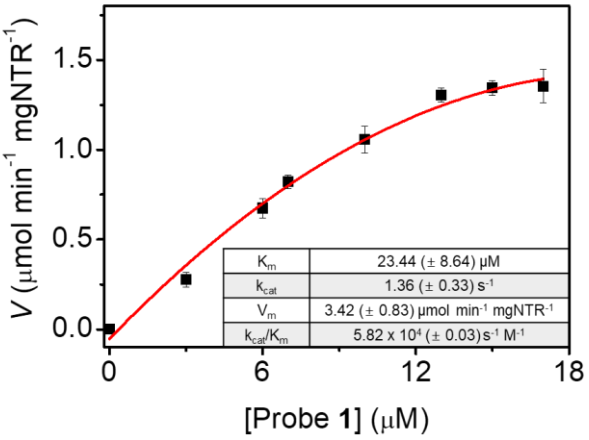

Figure S5. Enzyme kinetics of NTR (0.1 μg/mL) in the presence of NADH (500 μM) with different concentration of probe **1** (mean ± s.d., n = 3). All data were obtained in PBS (10 mM, pH 7.4) solution containing 10% (v/v) of DMSO after 30 min incubation at 37 °C using an excitation at 430 nm.

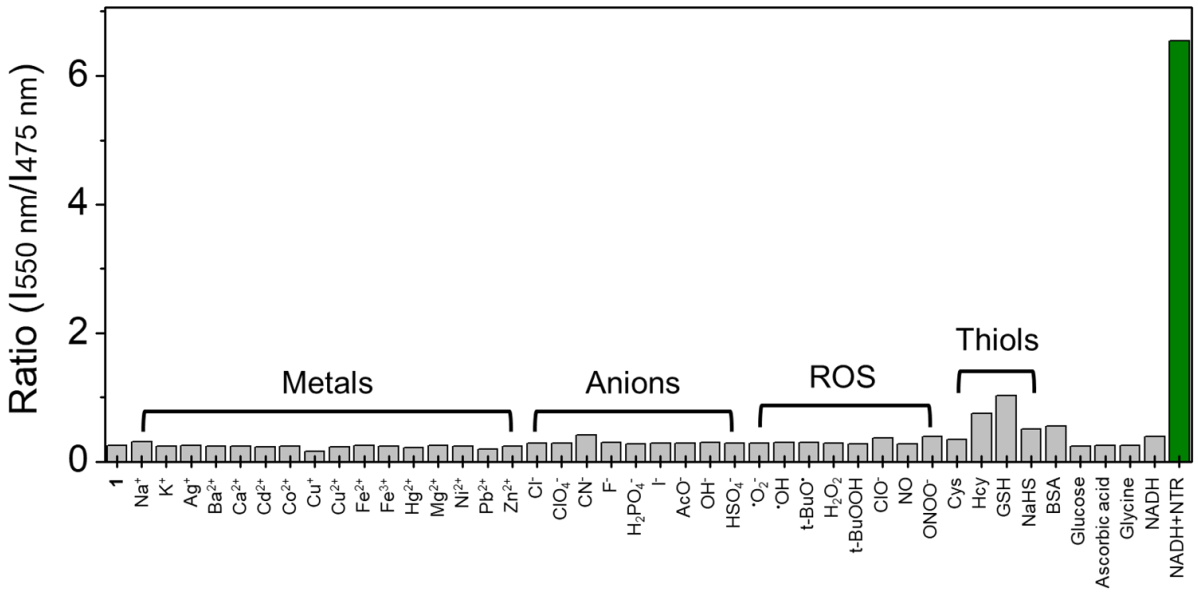

Figure S6. Fluorescence intensity ratio ($I_{550\ nm}/I_{475\ nm}$) of probe **1** (10 μM) in the presence of various analytes, such as NTR (5 μg/mL)/NADH (500 μM), metal ions (200 μM, respectively), anions (200 μM, respectively), ROS (200 μM, respectively), thiols (200 μM, respectively), BSA (1 μg/mL), glucose (1 mM), ascorbic acid (1 mM), and glycine (200 μM). All data were obtained in PBS (10 mM, pH 7.4) solution containing 10% (v/v) of DMSO after 30 min incubation at 37 °C using an excitation at 430 nm.

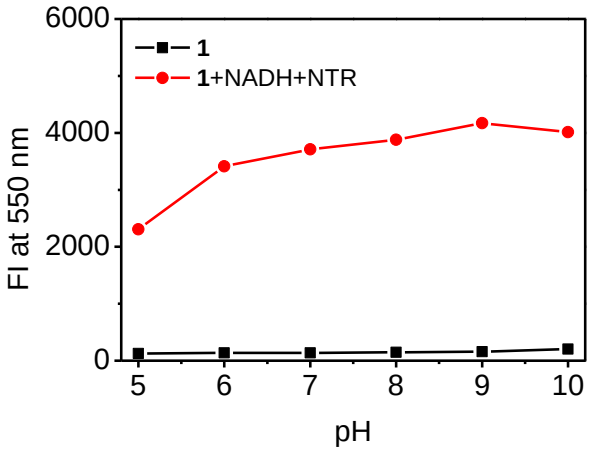

Figure S7. Fluorescence response of probe **1** (10 μM) in the absence and presence of NTR (5 μg/mL) and NADH (500 μM) at different pH values. All data were recorded in different pH buffer solutions containing 10% (v/v) of DMSO using an excitation at 430 nm after 30 min incubation at 37 ℃.

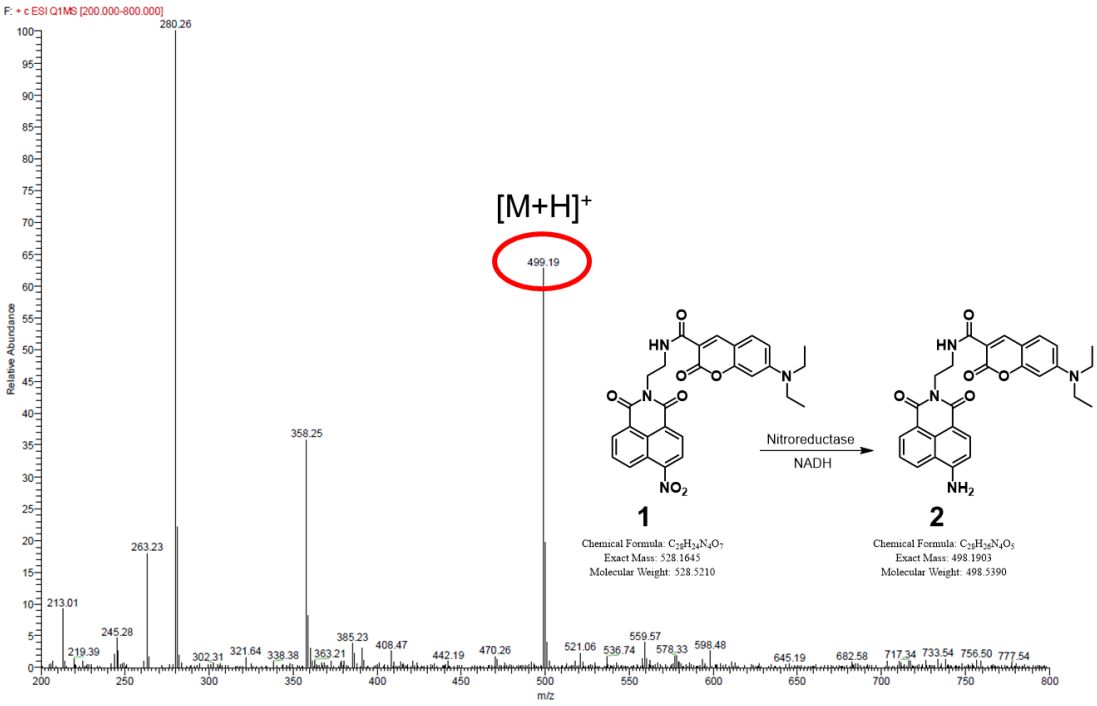

Figure S8. LC-ESI-MS spectrum of probe **1** after incubation with NTR and NADH in buffer solution (Retention time at 11.09 min).

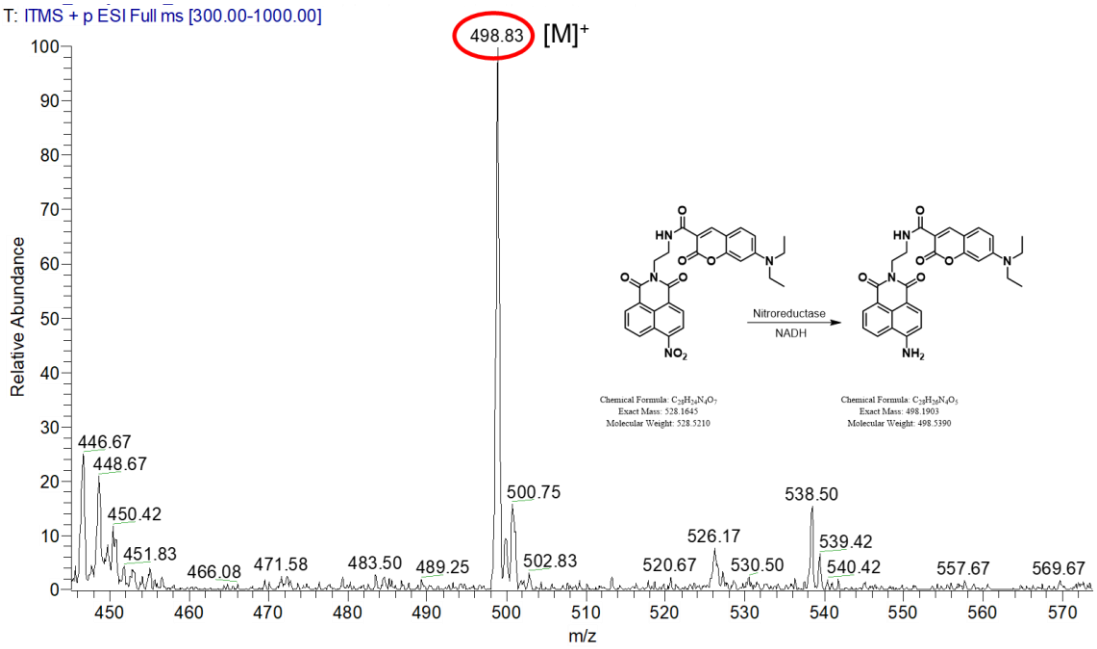

Figure S9. LC-ESI-MS spectrum of lysate of the probe **1**-treated cells (Retention time at 11.09 min).

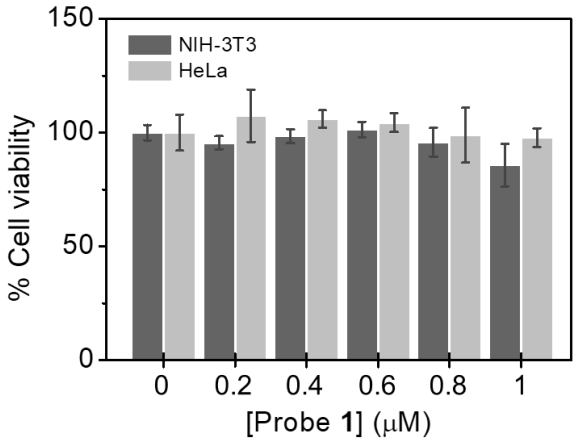

Figure S10. Cell viabilities of probe **1** for 24 h in NIH-3T3 and HeLa cells, respectively. Error bars indicate standard deviation (SD, n = 4).

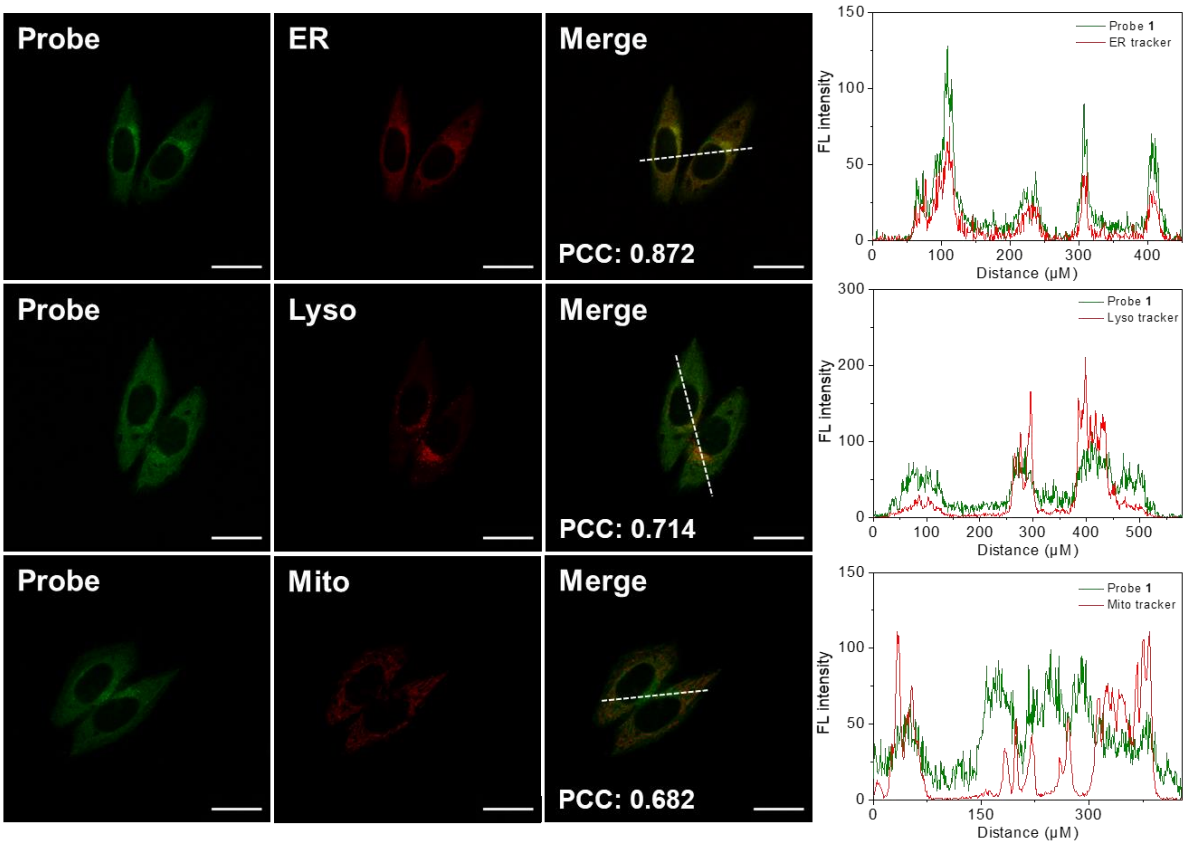

Figure S11. Co-staining of probe **1** and organelle trackers in HeLa cells. Cells were stained with probe **1** (0.5 μM, 30 min) and Mito-Tracker (0.1 μM, 15 min), Lyso-Tracker (0.05 μM, 15 min), and ER-Tracker (0.7 μM, 15 min). The PCC values indicating the colocalization between probe **1** and organelle trackers were obtained using autoQuant X3. Line profiles of fluorescence intensity of probe **1** and each organelle tracker were obtained by following the white line in the merged images. The excitation laser and filter sets were 405 nm with a 505-600 nm band path (BP) and 555 nm with a 605-800 nm BP for probe **1** (green channel) and organelle trackers (red channel), respectively. Scale bar: 20 μm.

9. ^{1}H, ^{13}C NMR and HR-ESI-MS spectra

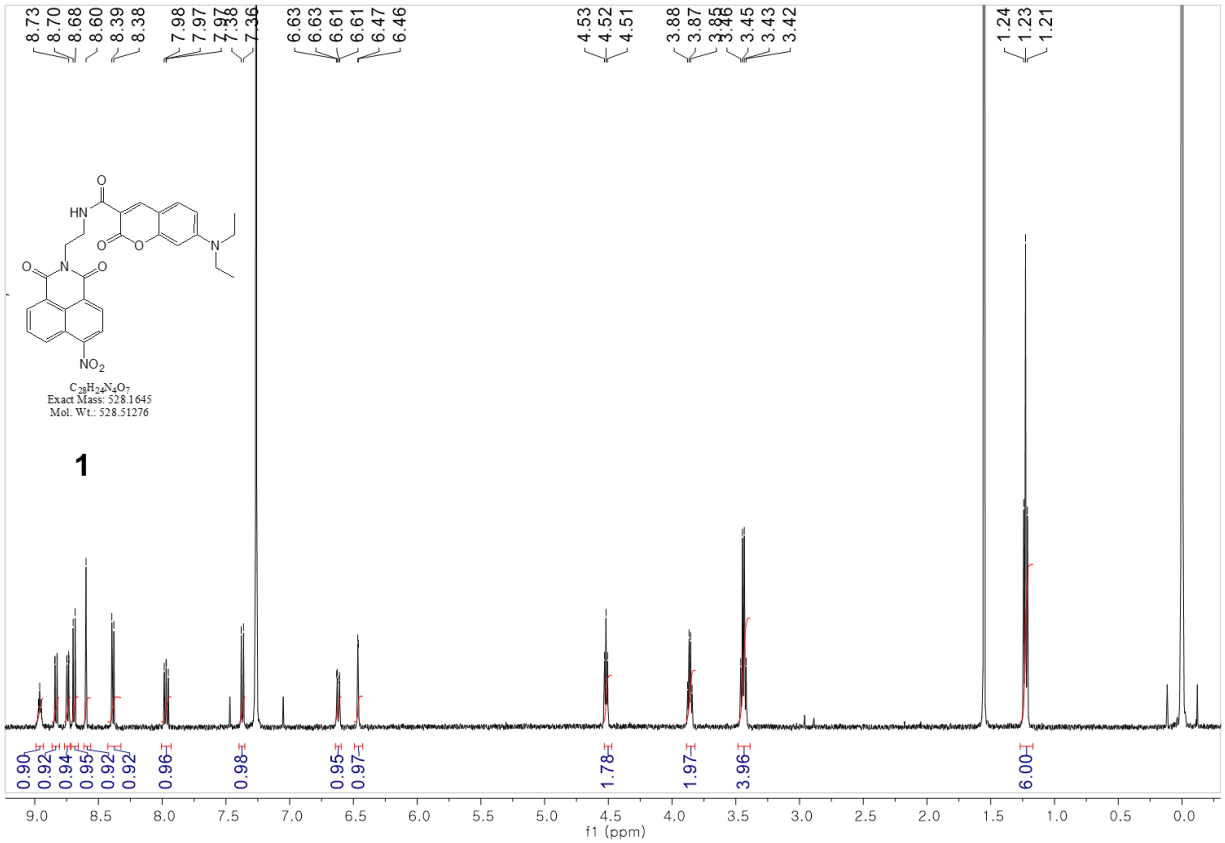

Figure S12. ^{1}H NMR spectrum of **1** recorded in $CDCl_3$.

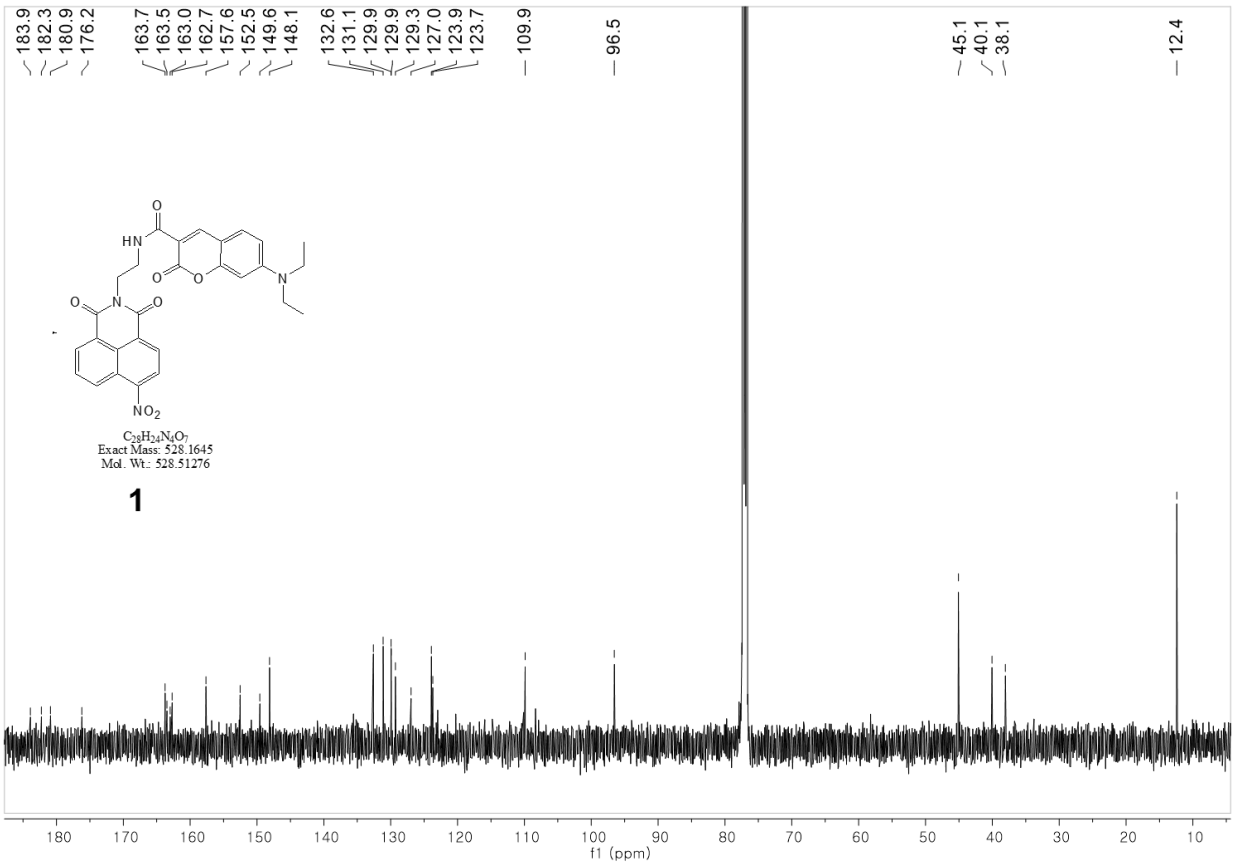

Figure S13. ^{13}C NMR spectrum of **1** recorded in $CDCl_3$.

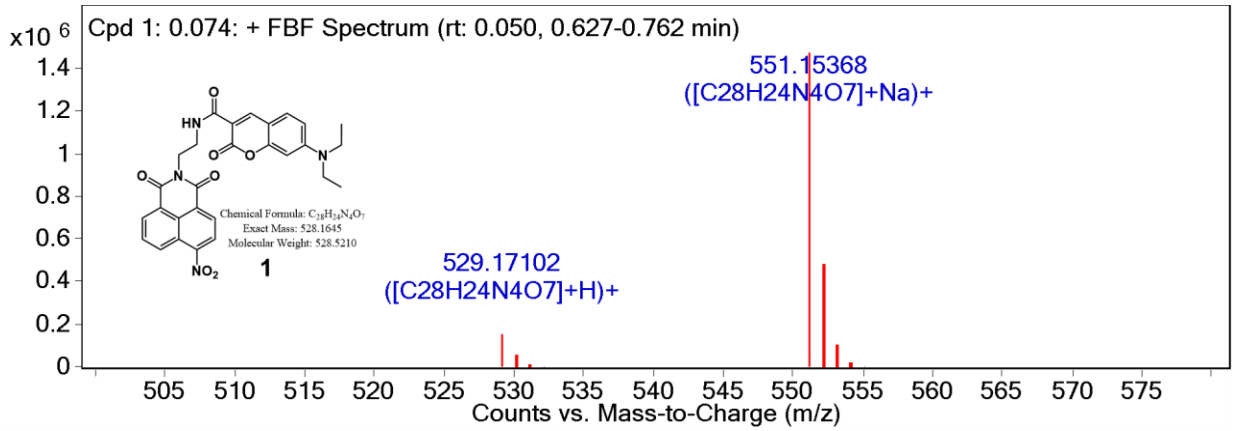

Figure S14. HR-ESI-MS spectrum of **1**.

10. Reference

1. Oushiki, D.; Kojima, H.; Terai, T.; Arita, M.; Hanaoka, K.; Urano, Y.; Nagano, T. Development and application of a near-infrared fluorescence probe for oxidative stress based on differential reactivity of linked cyanine dyes. *J. Am. Chem. Soc.* **2010**, *132*, 2795-2801.