Supporting Information for

A Fluorescent Probe for the Selective Detection of Cellular Peroxynitrite Based on Oxidative Cleavage of an Arylboronate

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1. Synthesis of Compounds

Methods and Materials. All reagents were of the highest commercial quality and used as received without further purification. All solvents were spectral grade unless otherwise noted. Anhydrous DMF, anhydrous ethanol, and anhydrous methanol were obtained as a sure seal bottle from Aldrich Co. Inc. (Milwaukee, WI). Aqueous solutions were freshly prepared with deionized water from a water purification system. (Human Corp. Korea). 2,3,6,7-Tetrahydro-8-hydroxy-1*H*,5*H*-benzo[*ij*] quinolizine-9-carboxaldehyde, 4-bromomethylphenylboronic acid pinacol ester, and benzothiazole-2-acetonitrile were obtained from Aldrich Co. Inc. (Milwaukee, WI).

Synthetic manipulations that required an inert atmosphere (where noted) were carried out under argon using standard Schlenk techniques. NMR (^{1}H and ^{13}C) spectra were recorded on JEOL 400 MHz spectrometers. The ^{1}H and ^{13}C chemical shifts were reported as δ in units of parts per million (ppm), referenced to the residual solvent. Splitting patterns are denoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). High-resolution mass spectra were obtained with JMS-700 (JEOL, Japan) using an electron impact source with an ionizing voltage of 70 V. Absorption spectra were obtained on a Optizen 2120UV. Fluorescence measurements were recorded on a Hitachi F-7000 fluorescence spectrophotometer using 10 mm quartz cuvettes with a path length of 1 cm. Fluorescence quantum yields were determined by standard method, using fluorescein (Φ_F = 0.95 in 0.1 N NaOH) as standards. The reaction with various ROS was measured by monitoring changes in fluorescence intensity upon addition of each analyte using a Synergy Mx Microplate Reader (Bio Tek, USA).

Synthesis of Probe 1 and compound 3

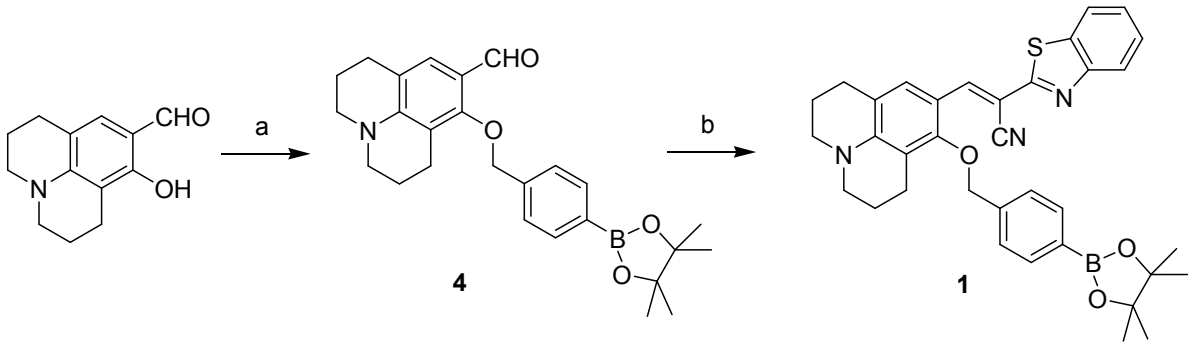
Compound 4: Commercially available 2,3,6,7-tetrahydro-8-hydroxy-1*H*,5*H*-benzo[*ij*]quinolizine-9-carboxaldehyde (70 mg, 0.322 mmol), 4-bromomethylphenylboronic acid pinacol ester (94.7 mg, 0.319 mmol), and K_2CO_3 (88.2 mg, 0.638 mmol) were dissolved in anhydrous DMF (1 mL) at room temperature under argon atmosphere. The reaction mixture was stirred for 5 hours at 60 ^{o}C. After cooling to room temperature, the mixture was extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The crude product was purified by preparative thin-layer chromatography (PTLC) using 5:1 hexanes : ethyl acetate as the mobile phase to afford **4** as a white solid (84 mg, 61% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 9.99 (s, 1 H), 7.83 (d, J = 8 Hz, 2 H), 7.45 (d, J = 8 Hz, 2 H), 7.35 (s, 1 H), 4.92 (s, 2 H), 3.27 (q, J = 6 Hz, 4 H), 2.72 (t, J = 6 Hz, 4H), 1.95 (m, 4 H), 1.35 (s, 12 H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 187.7, 159.4, 149.1, 139.9, 135.1, 127.2, 127.1, 117.4, 117.0, 112.6, 83.9, 50.1, 49.8, 27.5, 25.0, 21.5, 21.3, 20.8; HR-MS (EI): calcd. for $C_{26}H_{32}BNO_4$ $[M]^+$ 433.2424, found 433.2425.

Probe 1: To a stirred solution of compound **4** (40 mg, 0.092 mmol) and benzothiazole-2-acetonitrile (16.2 mg, 0.093 mmol) in ethanol (4 mL) at room temperature under argon was added piperidine (11 μL, 0.110 mmol). The reaction mixture was allowed to stir at room temperature for 4 hours. Following the removal of the solvent under reduced pressure, the crude product was purified by recrystalization using dichloromethane and hexane to afford **1** as an orange solid (32.9 mg, 61% yield). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.33 (s, 1 H), 8.10 (s, 1 H), 8.01 (d, J = 8 Hz, 1 H), 7.84 (dd, J = 8 Hz, 3 H), 7.54 (d, J = 8 Hz, 2 H), 7.44 (t, J = 8 Hz, 1 H), 7.32 (t, J = 8 Hz,1 H), 4.85 (s, 2 H), 3.31 (q, J = 6 Hz, 4 H), 2.79 (t, J = 6 Hz, 4 H), 1.98 (m, 4 H), 1.35 (s, 12 H); ^{13}C-NMR (100 MHz, $CDCl_3$): δ = 166.1, 157.3, 154.2, 148.2, 141.9, 139.8, 135.4, 134.6, 127.8, 126.9, 126.5, 125.0, 123.2, 121.5, 118.3, 113.4, 113.1, 96.9, 84.1, 50.4, 50.0, 27.8, 25.1, 21.8, 21.6, 21.0; HR-MS (EI): calcd. for $C_{35}H_{36}BN_3O_3S$ $[M]^+$ 589.2570, found 589.2570.

Compound 3[1]**:** To a stirred mixture of 2,3,6,7-tetrahydro-8-hydroxy-1*H*,5*H*-benzo[*ij*]quinolizine-9-carboxaldehyde (100 mg, 0.46 mmol) and benzothiazole-2-acetonitrile (80 mg, 0.46 mmol) in dry MeOH (10 mL) at room temperature under argon atmosphere was added piperidine (0.45 mL, 4.6

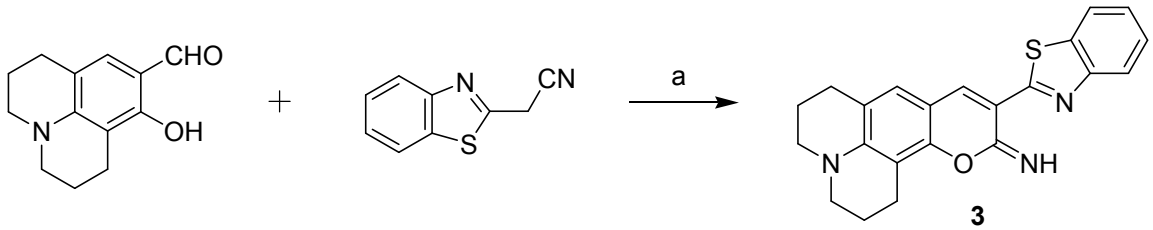
mmol). The resulting solution was stirred at room temperature for 4 hours. The precipitate was collected by filtration, washed with methanol, and dried under high vacuum to afford **3** as an orange solid (139.8 mg, 81%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ = 8.02 (d, J = 8 Hz, 1 H), 7.89 (d, J = 8 Hz, 1 H), 7.47 (t, J = 7 Hz, 1 H), 7.34 (t, J = 7 Hz, 1 H), 6.89 (s, 1 H), 3.31-3.27 (m, 4 H), 2.88 (t, J = 6 Hz, 2 H), 2.75 (t, J = 6 Hz, 2 H), 2.02-1.96 (m, 4 H).

(a) Synthesis of probe 1



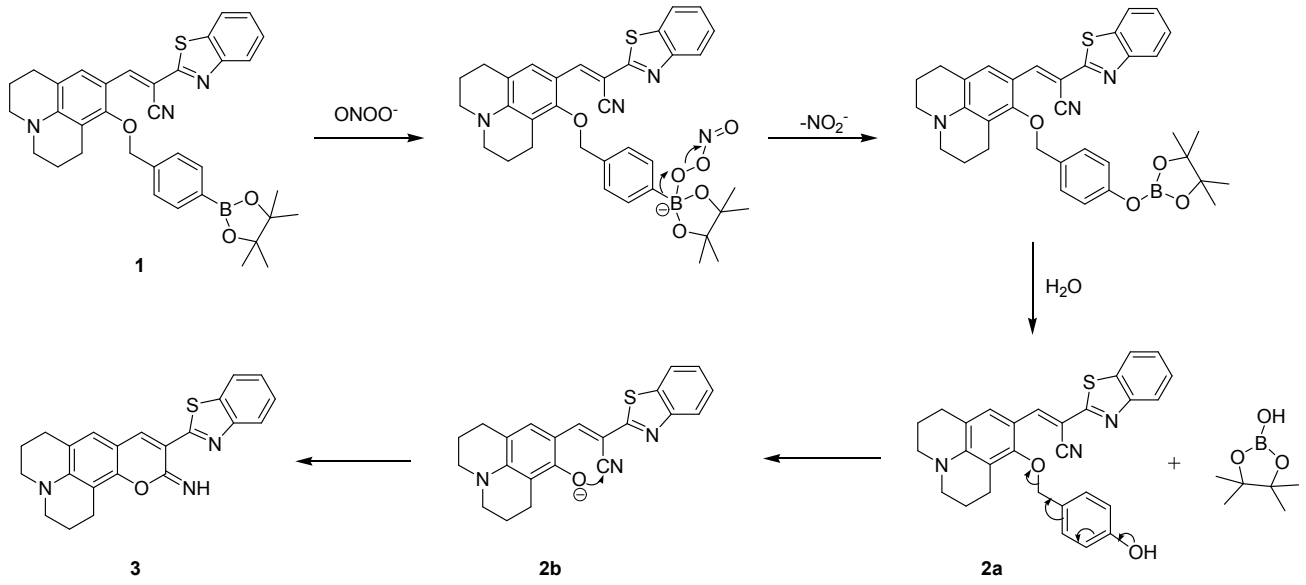
Scheme S1 Synthetic scheme of probe **1**. (a) 4-bromomethylphenylboronic acid pinacol ester, K_2CO_3, DMF, 5 hours, 60 °C, 61%; (b) benzothiazole-2-acetonitrile, piperidine, EtOH, 4 hours, r.t., 61%.

(b) Synthesis of compound 3[1]



Scheme S2 Synthetic scheme of compound **3**. (a) piperidine, dry MeOH, 4 hours, r.t., 81%.

(c) Proposed sensing mechanism



Scheme S3 Proposed boronates-oxidation mechanism by $ONOO^-$.

2. Studies of Photophysical Properties

Table S1 Photophysical properties of probe **1** and **3**

Compound	Solvent	$\lambda_{abs.\ max}$, nm	ε, $M^{-1}cm^{-1}$	$\lambda_{em.\ max}$, nm	Φ_{FL} [b]
1	C_2H_5OH	482	26680	536	0.01
1	buffer[a]	468	20370	646	0.01
3	C_2H_5OH	480	37160	530	0.96
3	buffer[a]	500	29500	540	0.62

[a]Data were obtained in phosphate buffer (10 mM, pH = 7.4)/Ethanol (9:1, v/v). Excited at 430 nm for **1** and **3** in various solvents. [b]Quantum yields vs. Fluorescein in 0.1N NaOH (Φ_F = 0.95)[2]

Absorption and emission spectra of probe 1 and compound 3

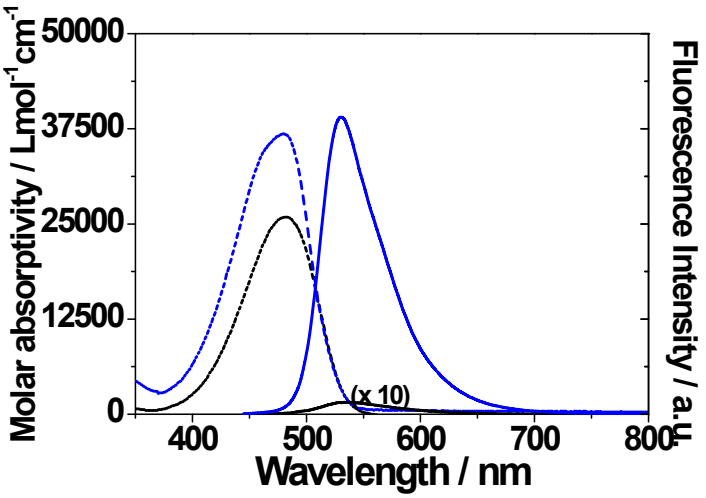


Fig. S1 Absorption (dash-lines) and emission (solid-lines) spectra of probe **1** (black), and **3** (blue) in EtOH. Excited at 430 nm. [**1**] = [**3**] = 10 μM. The emission spectrum of **1** is magnified 10-fold.

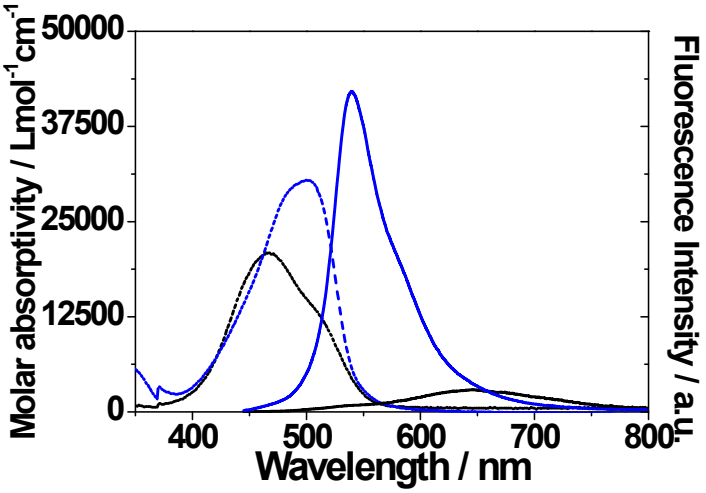


Fig. S2 Absorption (dash-lines) and emission (solid-lines) spectra of probe **1** (black), and **3** (blue) in phosphate buffer (10 mM, pH = 7.4, 25 °C) containing 10% EtOH as a cosolvent. Excited at 430 nm.

[**1**] = [**3**] = 10 μM.

3. Fluorometric Assay Studies

A solution of probe **1** (0.1 mM in ethanol, 20 μL) was diluted with in phosphate buffer (10 mM, pH = 7.4, 160 μL) at 37 °C. Each analyte was prepared as concentration of 1 mM in deionized water. Analytes were added to probe solution in 96-well flat bottom microplates, and the reactions were monitored at 37 °C for 30 minutes. The fluorescence signal for each well was measured at 540 nm (λ_{ex} = 430 nm). Various reactive oxygen species (ROS) were prepared as follows: Superoxide ($\cdot O_2^-$) was added as solid KO_2. Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (*t*-BuOOH), sodium hypochlorite (NaOCl) were delivered from 30%, 70%, and 5% aqueous solutions, respectively. Hydroxyl radical (·OH) and *tert*-butoxy radical ($\cdot O^tBu$) were generated by Fenton reaction of 25 mM Fe^{2+} with 5 mM H_2O_2 or 5 mM *t*-BuOOH, respectively. Peroxynitrite solution was synthesized as reported.[3] The peroxynitrite concentration was estimated by using an extinction coefficient of 1670 ± 50 $cm^{-1}(mol/L)^{-1}$ at 302 nm.[4] Nitric oxide (·NO) was generated from SNP (Sodium Nitroferricyanide (III) Dihydrate).[5] Experiments employed 20 μM peroxynitrite, and 100 μM for all other ROS.

(a) Effect of pH on fluorescence response of probe 1 in the absence and the presence of ONOO⁻

We next evaluated the photophysical behavior of **1** and its reactivity with $ONOO^-$ in a series of buffers with pH values ranging from 3-9 (Fig. S3). The results show that although the emission properties of **1** are essentially pH-insensitive across a wide pH range, the fluorescent response of the probe toward $ONOO^-$ is pH-dependent. A maximum sensing response of **1** to 2 equiv of $ONOO^-$ is observed to take place at pHs > 6 (pK_a of ONOOH/$ONOO^-$ is 6.8), suggesting that the assay is compatible with most biological applications.

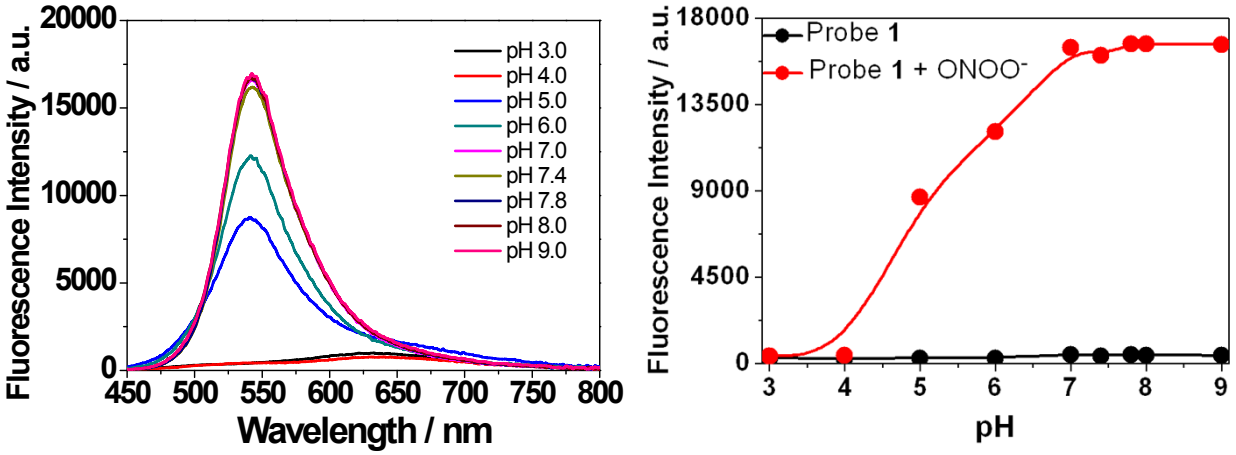


Fig. S3 (left) Emission spectra of probe **1** upon addition of $ONOO^-$ in different pH conditions (10% EtOH, 37 °C). (right) Relative fluorescence intensity of probe **1** without (black) and with (red) $ONOO^-$ in different pH conditions. λ_{ex} = 430 nm. Each spectrum was obtained 30 min after addition of $ONOO^-$. [**1**] = 10 μM. [$ONOO^-$] = 20 μM.

(b) $ONOO^-$ concentration-dependent fluorescence response of probe 1

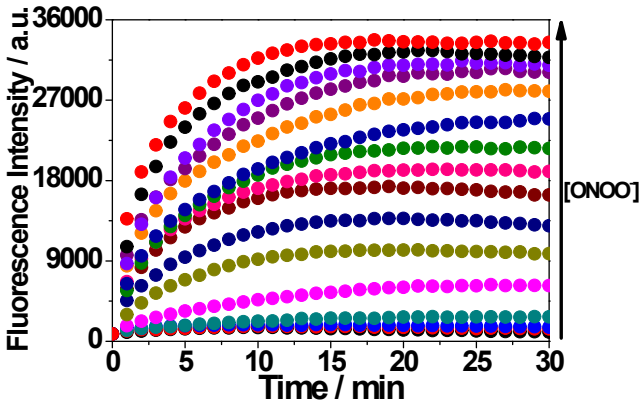


Fig. S4 Fluorescence response of probe **1** (10 μM) upon the reaction with different amounts of $ONOO^-$ (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20 μM) in phosphate buffer (10 mM, pH = 7.4, 37 °C) containing 10% EtOH as a cosolvent. The spectra were obtained 30 sec (0 - 30 min) after the addition of $ONOO^-$, and fluorescence intensity at 540 nm was measured with excitation at 430 nm.

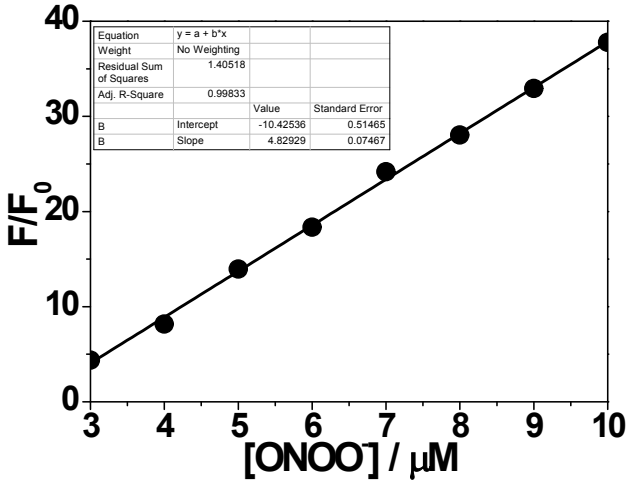


Fig. S5 The linear relationship between fluorescence intensity at 540 nm and $ONOO^-$ concentration (3 - 10 μM). Incubation time = 30 min. F_0 and F correspond to the fluorescence intensity of probe **1** in the absence and the presence of $ONOO^-$, respectively.

(c) Fluorescence response of probe 1 to reactive oxygen species (ROS)

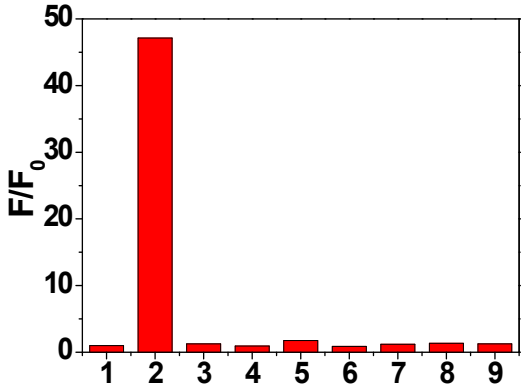


Fig. S6 Relative fluorescence responses of probe **1** (10 μM) to various analytes (20 μM $ONOO^-$, and 100 μM for others). All the data were obtained in phosphate buffer (10 mM, 10% EtOH, pH = 7.4) at 37 °C. The data were obtained after incubation with each analyte for 30 min. Excited at 430 nm. 1: only probe **1**, 2: $ONOO^-$, 3: OCl^-, 4: *t*-BuOOH, 5: H_2O_2, 6: $•O_2^-$, 7: •OH, 8: •O*t*Bu, 9: •NO

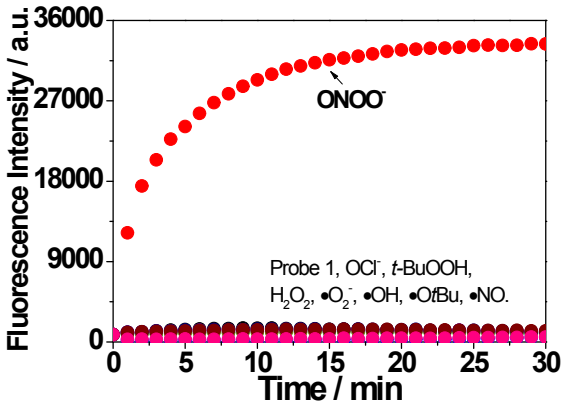


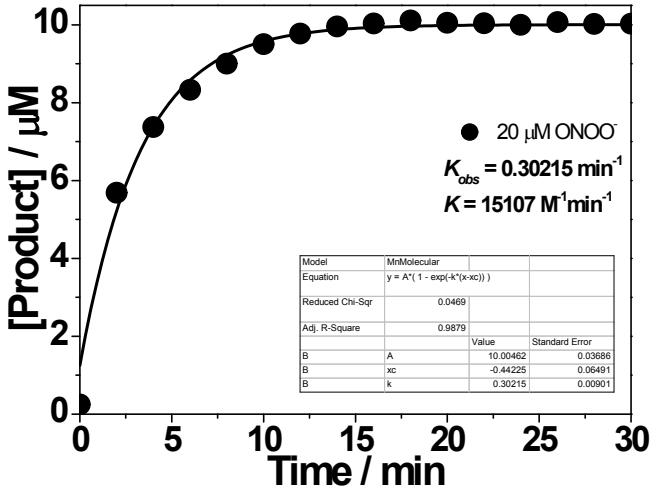
Fig. S7 Time-dependant fluorescence changes at 540 nm of probe **1** (10 μM) upon addition of each analyte (20 μM $ONOO^-$, and 100 μM for others). All the data were obtained in phosphate buffer (10 mM, 10% EtOH, pH = 7.4) at 37 °C. Excited at 430 nm.

(d) Kinetic studies

Rate constants were determined for the reaction between probe **1** (10 μM) and $ONOO^-$ (20 μM) in 10 mM phosphate buffer (pH 7.4) containing 10% ethanol as a cosolvent at 37 °C. Linear calibration of the fluorescence signal against the concentration of **3** produced from the reaction of probe **1** with $ONOO^-$ was carried under the assumption that probe **1** (10 μM) is completely converted to **3** in the presence of an excess of $ONOO^-$ (20 μM) and therefore the fluorescence signal at saturation corresponds to [**3**] = 10 μM. Fluorescence data were modeled (least squares curve fitting) against eq. 1 (see below) where the parameter *a* is added to account for delay between the addition of the reagents and the time at which the first measurement can be acquired due to experimental limitation. Rate constants were determined for the reaction between probe **1** (10 μM) and H_2O_2 (100 μM) in 10 mM phosphate buffer (pH 7.4) containing 10% ethanol as a cosolvent at 37 °C, using equation 2.

(a)

$$f(t) = 10\ \mu M\left[1 - exp(-k_{obs}(t + a))\right] \quad \text{eq.1}$$



(b) $\text{Ln}[(F_{max} - F_t)/F_{max}] = -k_{obs}t$ eq.2

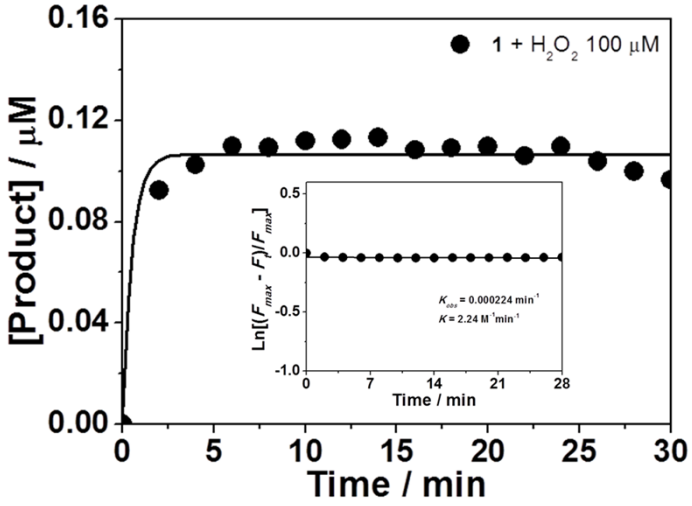


Fig. S8 Kinetics for the fluorescence response of probe **1** (10 μM) upon incubation with (a) $ONOO^-$ (20 μM) and (b) H_2O_2 (100 μM) in PBS buffer (10 mM, 10% EtOH, pH = 7.4, 37 °C). Fluorescence intensity at 540 nm was measured. Excited at 430 nm.

4. Identification of Reaction Product

The proposed detection scheme was confirmed by analyzing the crude reaction mixture of probe **1** with $ONOO^-$ using HPLC-MS. The probe **1** (10 μM) was dissolved in an ethanol-deionized water (v/v, 1:1), and then 2 equivalents of $ONOO^-$ were added into probe solution. The reaction mixture was analyzed by HPLC-MS with a linear gradient elution (eluent A/B = 20/80, A : deionized water, B : Methanol with 5 mM ammonium formate, flow rate 0.3 mL/min). The reaction mixture of probe **1** with $ONOO^-$ shows intense peak of $[\mathbf{3} + H]^+$ (*m/z* 374.1), consistent with that of compound **3**.

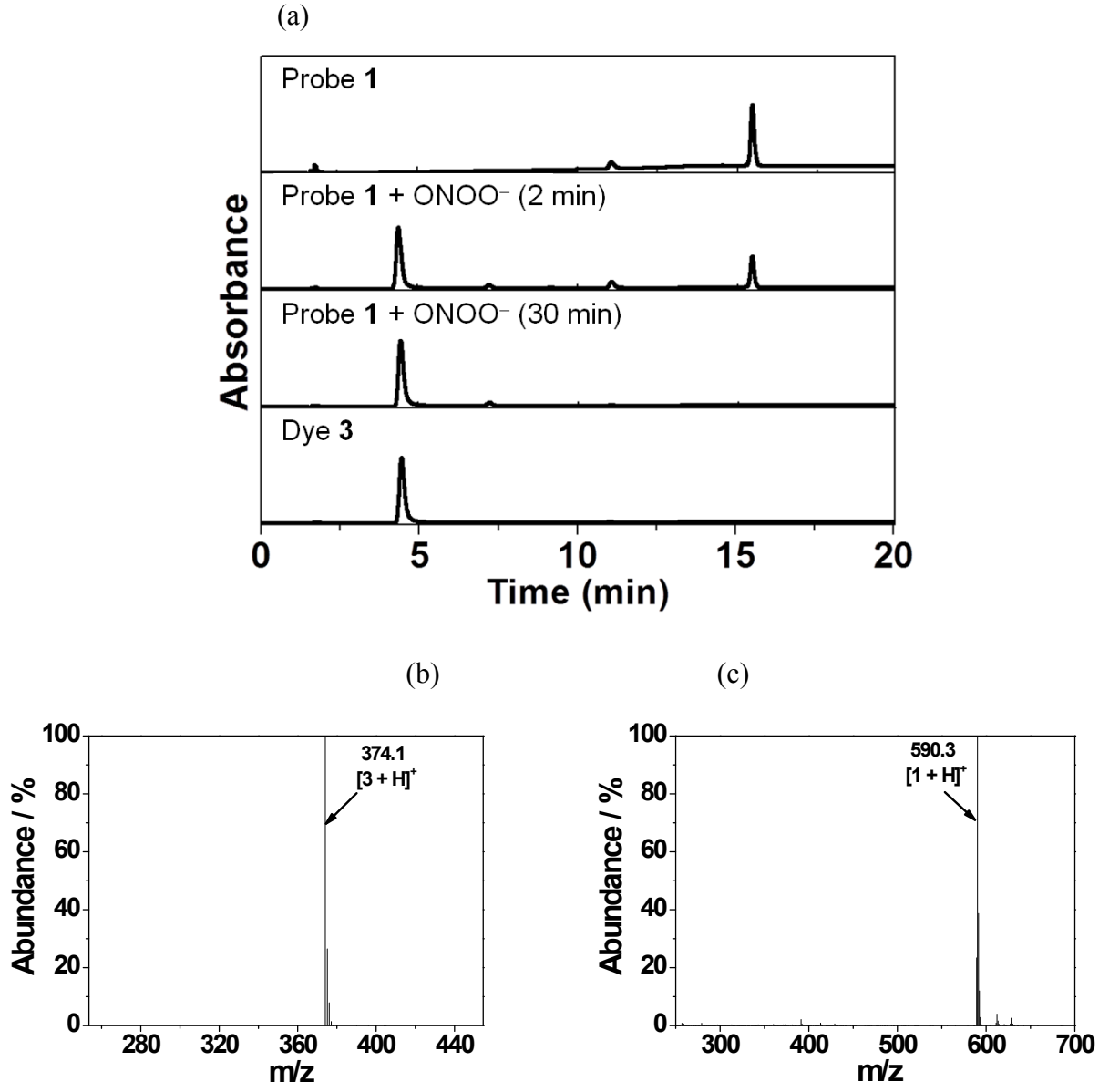


Fig. S9 (a) HPLC chromatograms of probe **1** without $ONOO^-$ treatment (top); after $ONOO^-$ treatment in ethanol-water (1: 1, v/v) for 2 min and 30 min at 25 °C, respectively (middle); and **3** only (bottom). ESI-MS spectra of the peak of retention time at 4.5 min (b) and 15.5 min (c). [**1**] = [**3**] = 10 μM, $[ONOO^-]$ = 20 μM.

To further verify the proposed sensing scheme, the separate reaction of probe **1** with 2 equiv $ONOO^-$ was carried out in an ethanol-deionized water (v/v, 1:1) solution at room temperature for 0.5 hour. The major reaction product was isolated and identified as **3** by studies of the ^1H NMR.

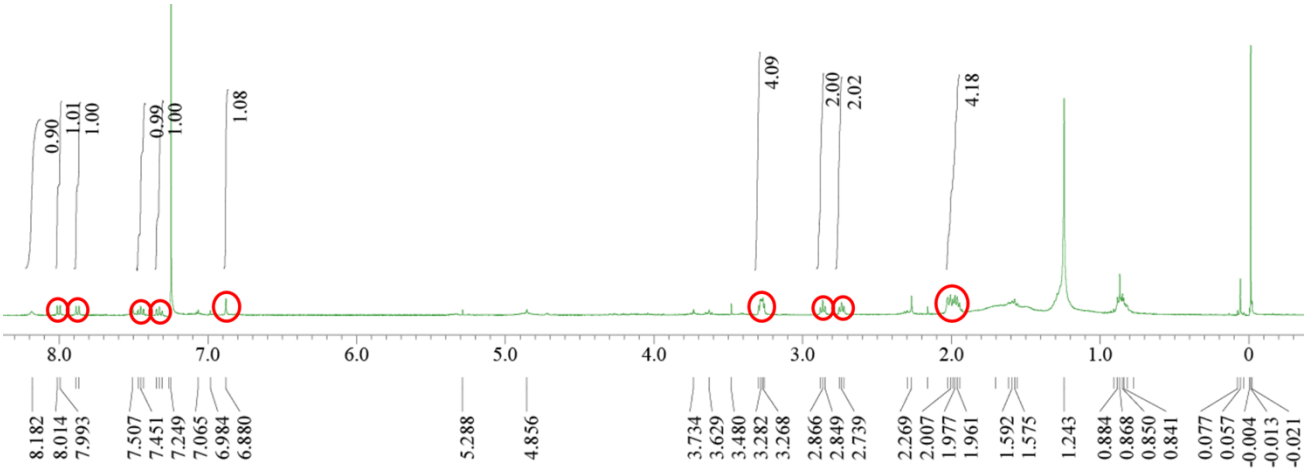


Fig. S10 ^1H-NMR spectrum of the isolated crude reaction product in $CDCl_3$.

5. Cell Studies

(a) Cell culture

The macrophoage cell line J774A.1 was purchased from American Type Culture Collection (ATCC) (Manassas, VA), and maintained in Dulbecco's modified Eagle's medium (DMEM, Life technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Life technologies), 1% antibiotic/antimycotic (Life technologies) at 37 °C under 5% CO_2 in a standard humidified incubator.

(b) Cell viability assay

J774A.1 macrophage cells were seeded in each well of 96-well plates at a density of 10,000 cells/well and incubated for 24 hours for cell attachment. The stock solution of probe **1** dissolved in DMSO (1.7 mM) was diluted with a cell culture medium containing 10% FBS to have a final concentration of 0, 10, 20, and 50 μM. Existing culture medium was replaced with 200 μL of the fresh one containing probe **1**, and the cells were incubated for 30 minutes. After washing the cells twice, the cells were further incubated for additional 24 hours. Then cell viability was measured using a cell counting kit-8 (Dojindo Laboratories). Cell viability was calculated as a percentage compared to untreated control cells (Figure S10). Data are expressed as a mean ± standard deviation. Statistical significance compared to the untreated control cells was calculated by determining *p* values by using the *t*-test. The result shows that no cytotoxic effect was observed up to the concentration of 50 μM probe **1**.

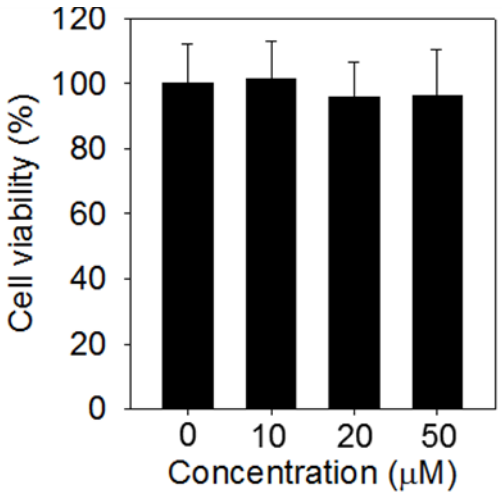


Fig. S11 Cell viability of J774A.1 cells treated with probe **1** at various concentrations (n = 4).

(c) Cell staining and confocal imaging

The cells were plated on a 8-well Lab-Tek chamber (Nalge Nunc International Corp., Rochester, NY) at a density of 100,000 cells per well, and incubated for 24 hours to allow for cell attachment. Untreated control cells were treated only with cell culture medium. Among the inactivated macrophage cells, first group was loaded with 10 μM probe **1** for 20 minutes. Second and third groups were loaded with 10 μM probe **1** for 20 minutes, washed, and treated with 50 μM H_2O_2 or NaOCl for 30 min, respectively, to check whether the probe **1** is affected by these reactive oxygen species. Cells in fourth group were co-incubated with 1 mM SIN-1 and 10 μM probe **1** for 20 minutes. To check fluorescence turn-on of probe **1** in the activated macrophages, macrophages were

activated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 4 hours, and then loaded with 10 μM probe **1** for 20 minutes. For inhibition assays, macrophages were activated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) in the presence of AG (1, 2, 5, 10 mM) or TEMPO (300 μM) for 4 hours, and then loaded with 10 μM probe **1** for 20 minutes. After washing the cells two times with culture media, phenol-red free media were added to each well and fluorescence images were acquired using a confocal laser scanning microscope (ZEISS LSM 510 META). (Ex. 405nm, Em. 529-614nm). Confocal images were analyzed by LSM image 5 browser and fluorescence intensity quantification in each cells (n > 50) was enabled by Axiovision. For quantification of fluorescence signal activation, we randomly took confocal microscopy images from three different positions of the each well. ROI (region of interest) was assigned to every single cell and recorded the mean intensity of the corresponding region. All the images were analyzed using AxioVision 4.3 Software (Zeiss). More than 50 cells per case were analyzed to obtain mean (SD) fluorescence intensity. Statistical significance was calculated by determining *p* values by using the *t*-test.

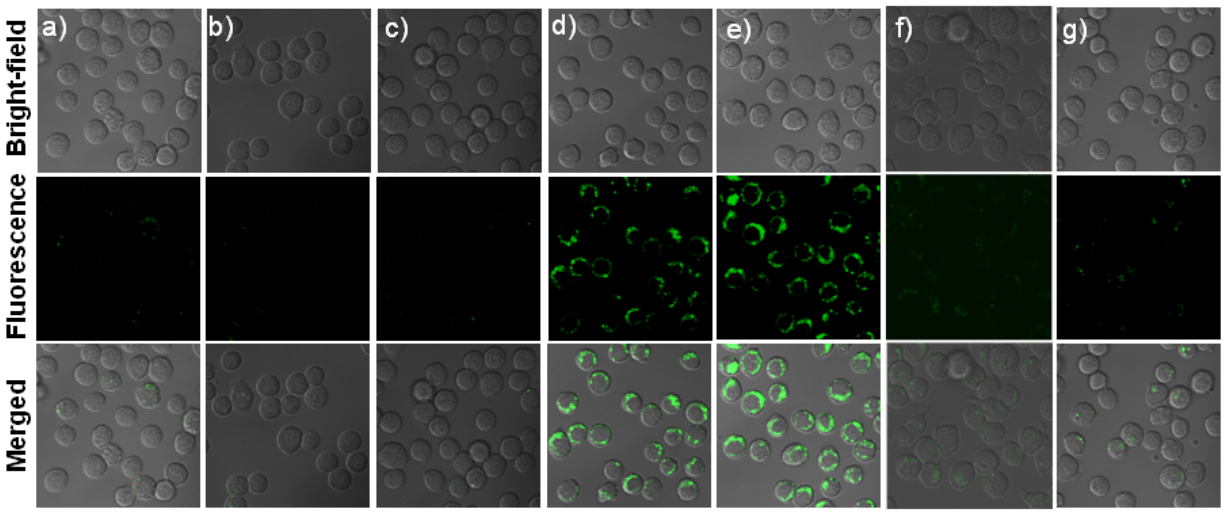


Fig. S12 The bright-field (top), fluorescence (middle), and merged images (bottom) of confocal fluorescence microscope images of **1** (10 μM)-loaded living macrophage cells (J774A.1) under different conditions. (a) Macrophages were incubated with **1** for 20 min at 37 °C and then imaged; (b) **1**-loaded macrophage cells were incubated with 50 μM H_2O_2 for 30 min; (c) **1**-loaded macrophage cells were incubated with 50 μM NaOCl for 30 min; (d) Macrophage cells were co-incubated with SIN-1 (1 mM) and **1** for 20 min; (e) Macrophage cells were stimulated with LPS (1 μg mL^{-1}) and IFN-γ (50 ng mL^{-1}) for 4 h, and incubated with **1** for 20 min at 37 °C; (f) NOS inhibitor, AG (5 mM) was co-incubated during LPS/IFN-γ stimulation for 4 h, and incubated with **1** for 20 min at 37 °C; (g) Superoxide scavenger, TEMPO (300 μM) was co-incubated during LPS/IFN-γ stimulation for 4 h; the other procedures were the same.

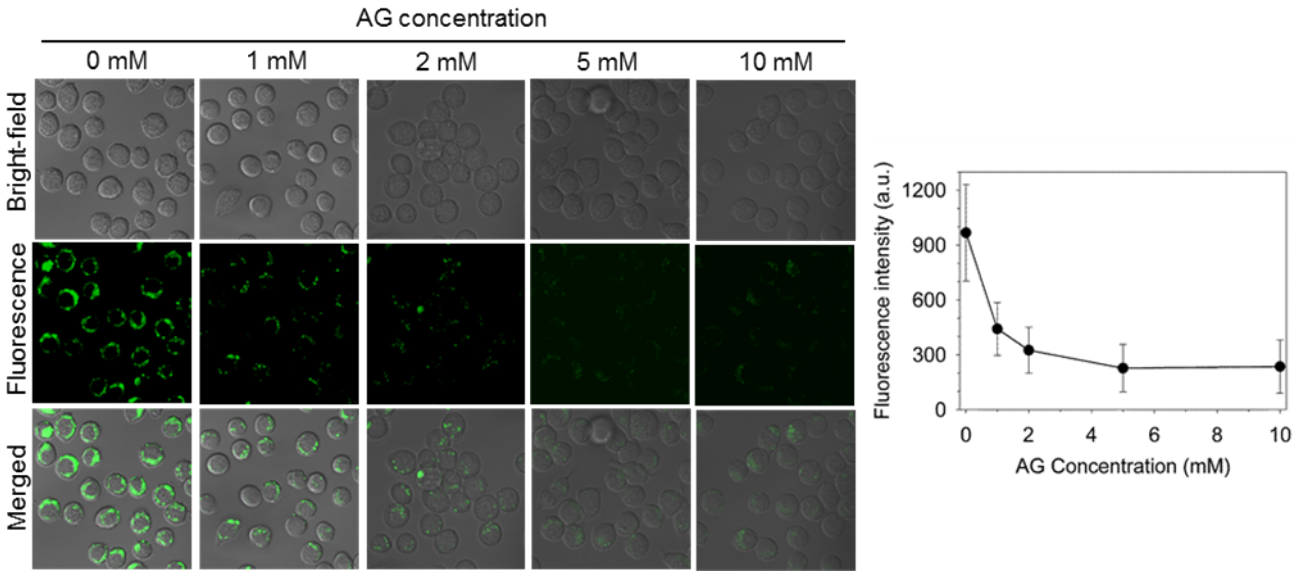
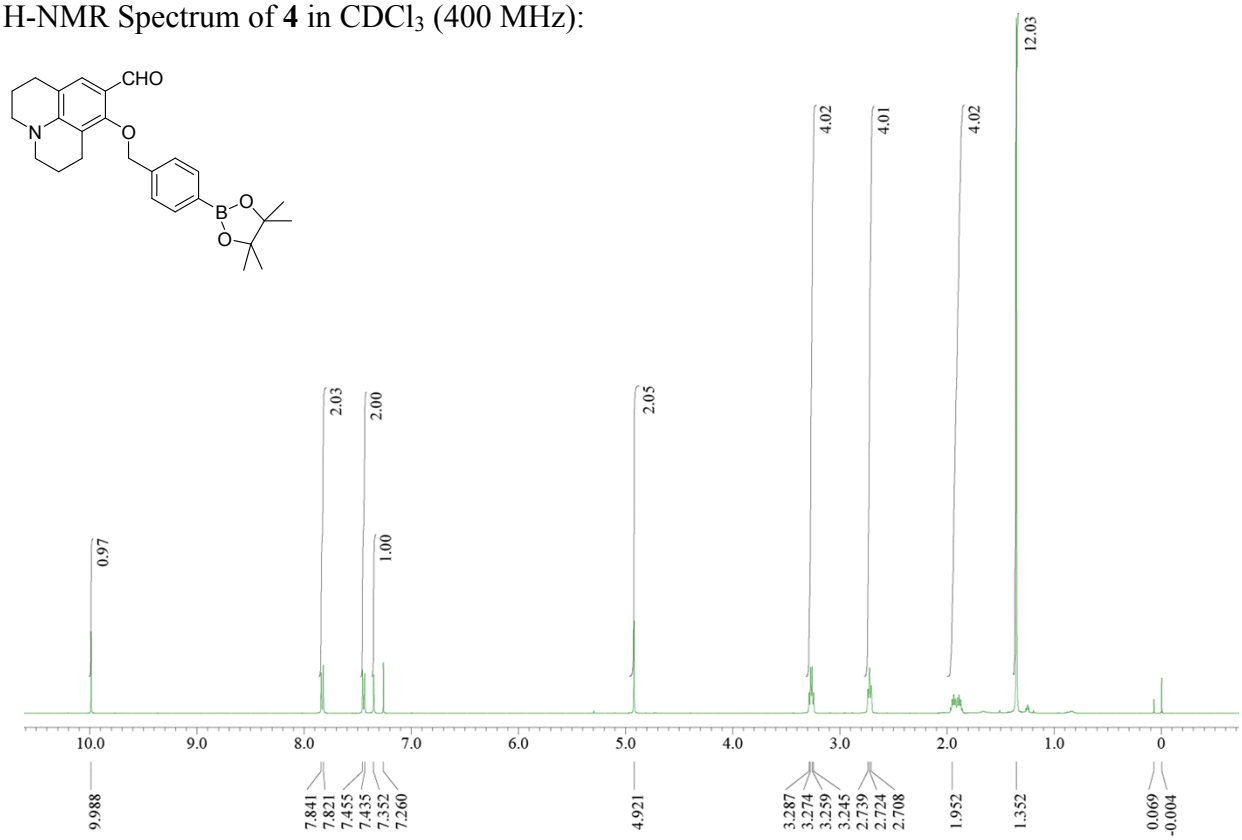


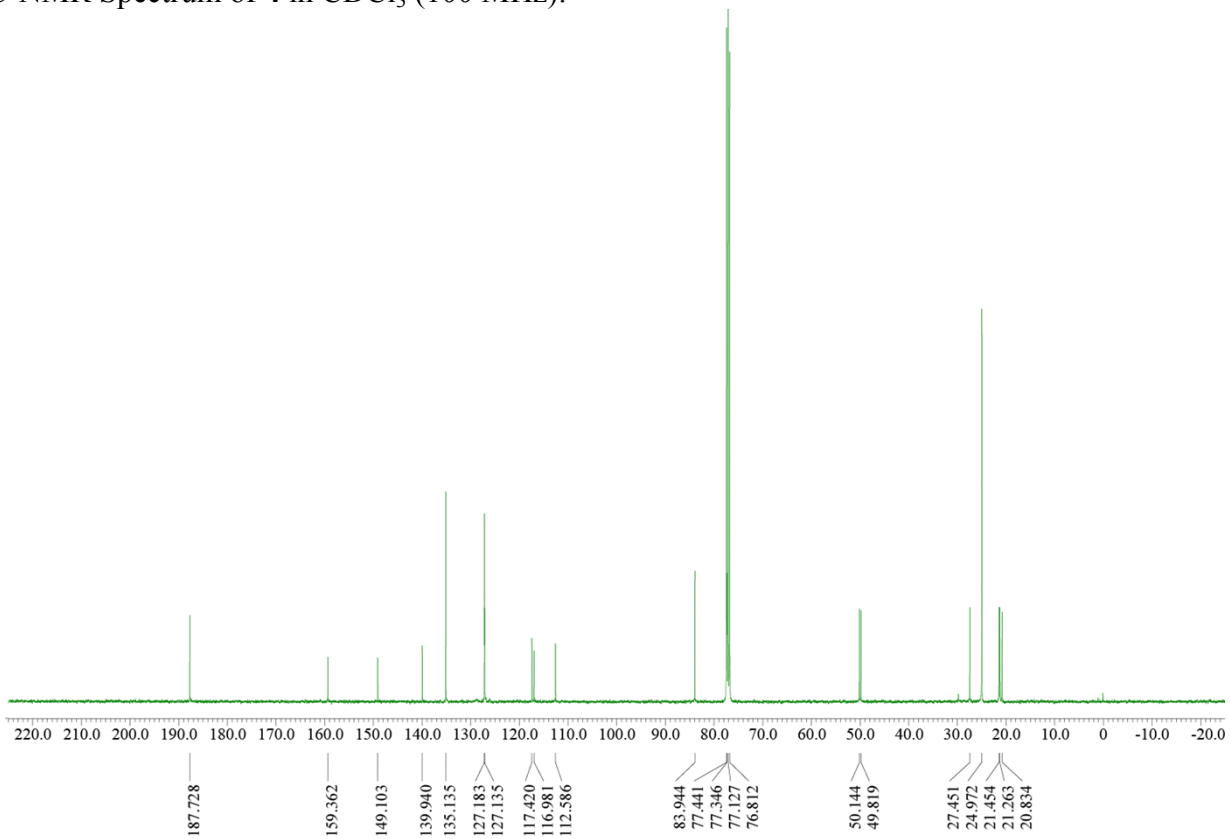
Fig. S13 (Left) Confocal fluorescence images of J774A.1 cells exposed to oxidative stress. Cells were incubated with AG (0, 1, 2, 5, 10 mM), LPS (1 μg/mL), and IFN-γ (50 ng/mL) and for 4 hours, and then loaded with probe **1**. (Right) Graph showing fluorescence inhibition depending on the concentration of AG (mean ± SD, n > 50).

6. ^{1}H-NMR and ^{13}C-NMR Spectra

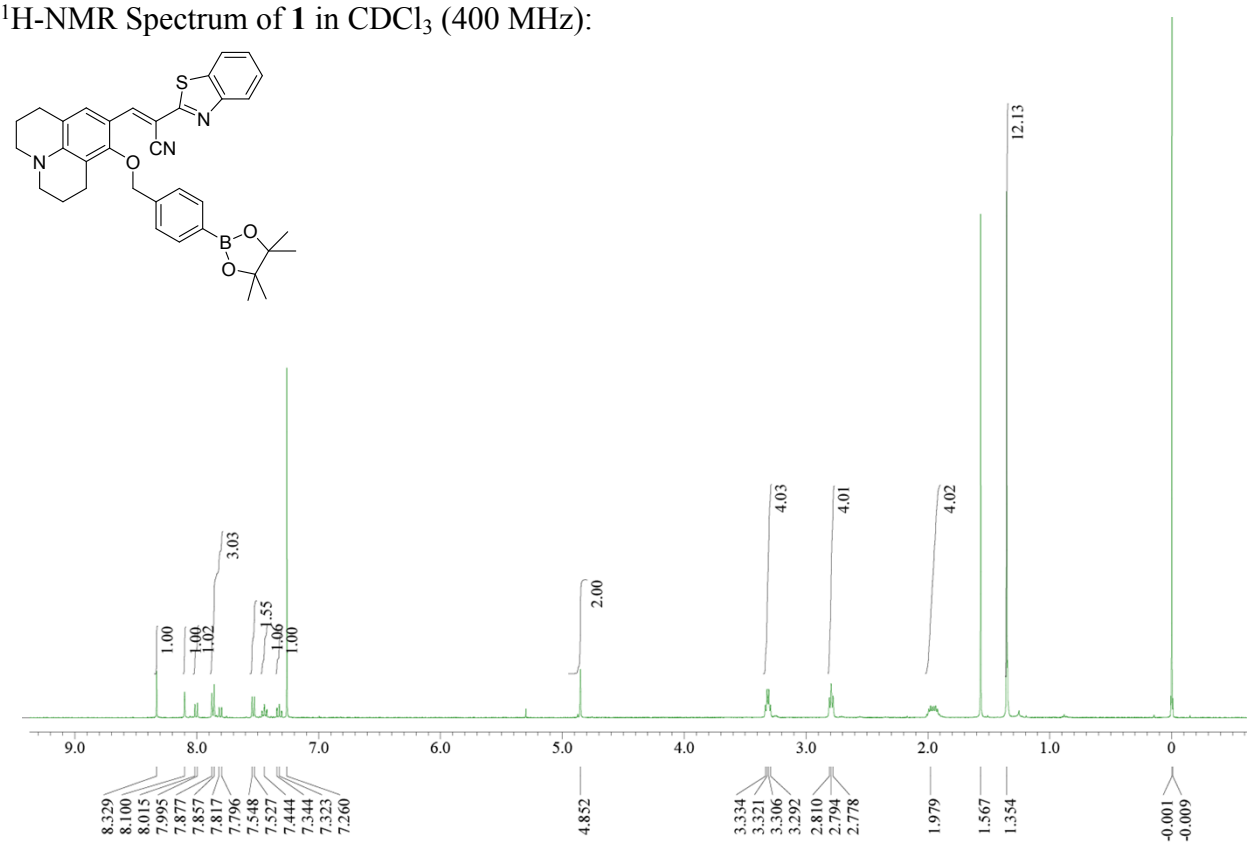
^{1}H-NMR Spectrum of **4** in $CDCl_3$ (400 MHz):



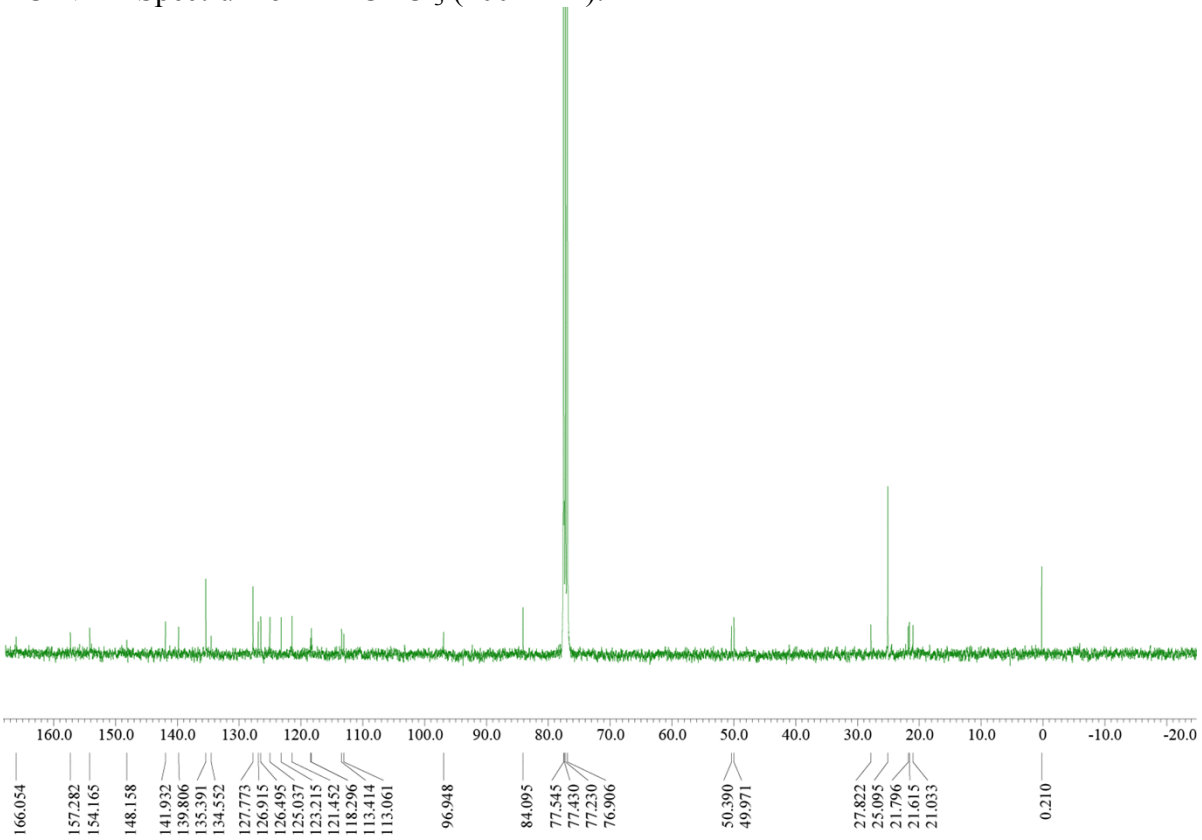
^{13}C-NMR Spectrum of **4** in $CDCl_3$ (100 MHz):



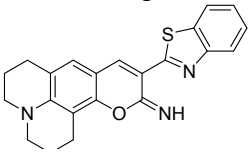
^{1}H-NMR Spectrum of **1** in $CDCl_3$ (400 MHz):

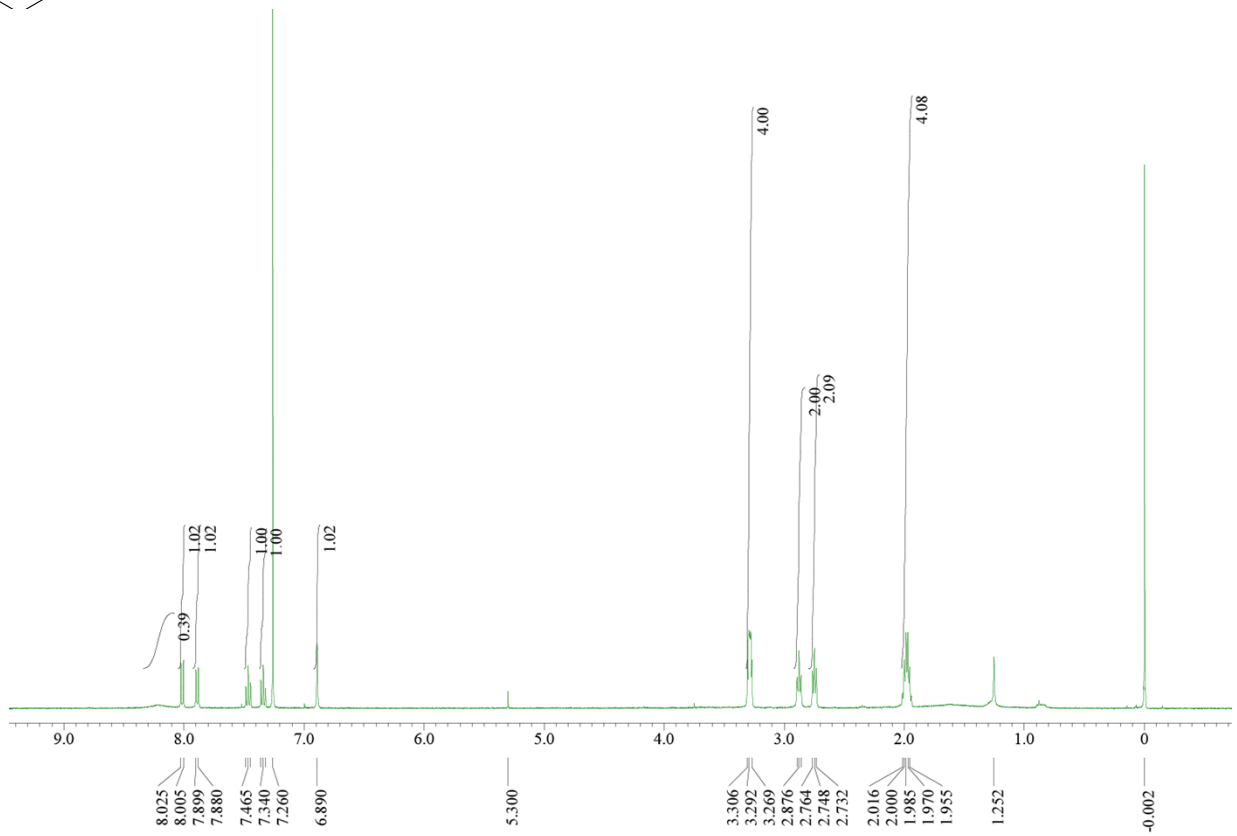


^{13}C-NMR Spectrum of **1** in $CDCl_3$ (100 MHz):



^{1}H-NMR Spectrum of **3** in $CDCl_3$ (400 MHz):





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