Supporting Information for

A Ratiometric Fluorescent Probe Based on a BODIPY-DCDHF Conjugate for the Detection of Hypochlorus Acid in Living Cells

Jeesook Park,‡[a] Hyunjin Kim,‡[b] Yongdoo Choi,*[b] and Youngmi Kim*[a]

[a] *Department of Chemistry, Dankook University, Institute of Nanosensor and Biotechnology, 126 Jukjeon-dong, Yongin-si, Gyeonggi-do, 448-701, Korea*
[b] *Molecular Imaging & Therapy Branch, National Cancer Center, 323 Ilsan-ro, Goyang-si, Gyeonggi-do 410-769, Korea*

youngmi@dankook.ac.kr
Tel: +82 31-8005-3156
Fax: +82 31-8005-3148

ydchoi@ncc.re.kr
Tel: +82 31-920-2512
Fax: +82 31-920-2529

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Experimentals

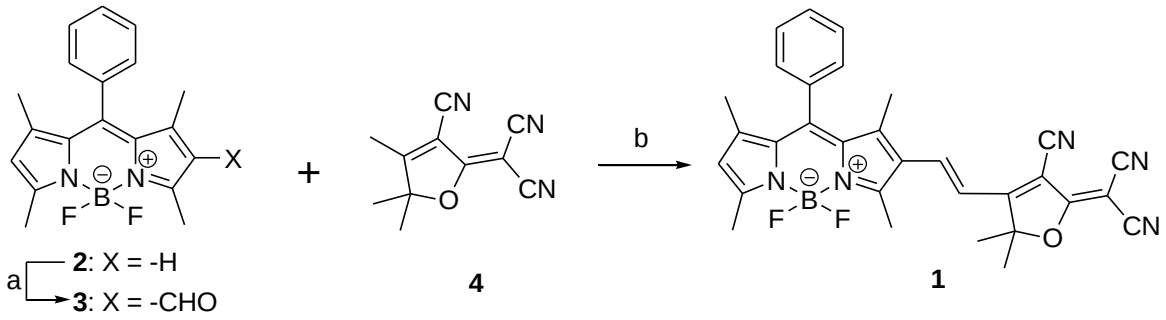
Materials

All reagents were of the highest commercial quality and used as received without further purification. All solvents were spectral grade unless otherwise noted. Anhydrous pyridine was obtained as a sure-seal bottle from Aldrich Co. Inc. (Milwaukee, WI). Silica gel (40 μm) was obtained from Merck Inc. Aqueous solutions were freshly prepared with deionized water from a water purification system (Human Corp. Korea). Compounds **3** and **4** were prepared according to the literature procedure.[1,2]

General methods, instrumentation and measurements

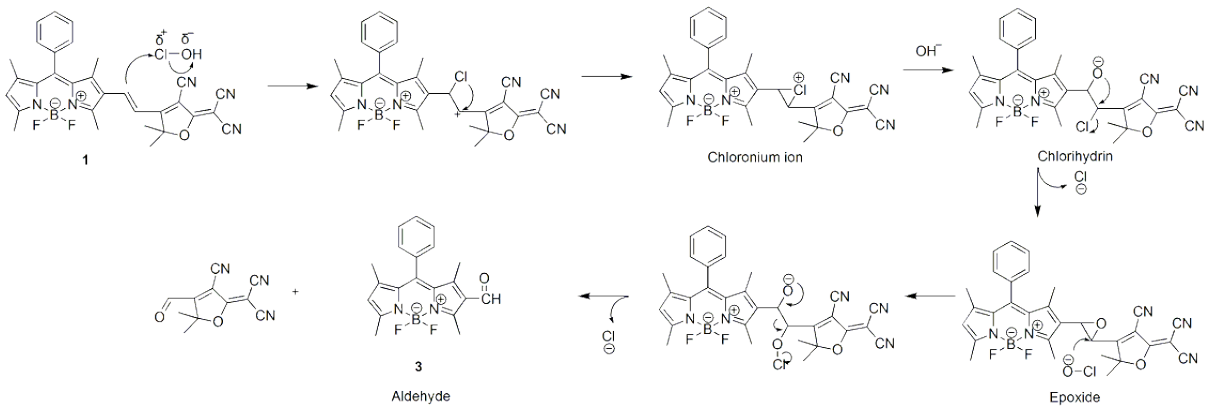
Synthetic manipulations that required an inert atmosphere (where noted) were carried out under argon using standard Schlenk techniques. NMR (^{1}H and ^{13}C) spectra were recorded on Bruker Advance 500 MHz spectrometers. The chemical shift data for each signal are given in units of δ (ppm) relative to tetramethylsilane (TMS) where δ (TMS) = 0, and referenced to the residual solvent resonances. Splitting patterns are denoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad). High-resolution electrospray ionization (ESI) mass spectrum was obtained at national center for inter-university research facilities. Absorption spectra were obtained on a Shimadzu UV-2501 spectrophotometer. Fluorescence measurements were recorded on a Hitachi F-7000 fluorescence spectrophotometer using 10 mm quartz cuvettes with a path length of 1 cm. The reaction with various analytes was measured by monitoring changes in fluorescence intensity upon addition of each analyte using a Synergy Mx Microplate Reader (BioTek, USA).

1. Synthesis of Probe 1



Scheme S1. Synthetic scheme of probe **1**. (a) $POCl_3$, DMF, $ClCH_2CH_2Cl$, 50 °C, 89%; (b) pyridine, acetic acid, 24 h, 40 °C, 14%.

To a 10 mL round-bottom flask with stiring bar was added formyl-BODIPY **3**[1] (46 mg, 0.131 mol), 4-methyl-DCDHF **4**[2] (27 mg, 0.135 mol), pyridine (1.5 mL), and acetic acid (several drops) at room temperature. The reaction mixture was warmed to 40 °C for 24 h. This solution turned from orange to violet. The reaction was stopped and cooled to room temperature. Following the removal of the solvent under reduced pressure, the crude product was purified by column chromatography on silica gel using progressively more polar 100:1 to 6:1 hexanes:acetone as the mobile phase to afford probe **1** as a violet solid. (10 mg, 14% yield); ^{1}H-NMR (500 MHz, $CDCl_3$, 25 ^{o}C, TMS): δ = 7.75 (d, 1 H), 7.48 (t, 3 H), 7.22 (t, 2 H), 6.45 (d, 1 H), 6.12 (s, 1 H), 2.67 (s, 3 H), 2.56 (s, 3 H), 1.63 (s, 6 H), 1.46 (s, 3 H), 1.37 ppm (s, 3 H); ^{13}C-NMR (125 MHz, $CDCl_3$, 25 ^{o}C, TMS): δ = 176.1, 175.0, 162.9, 155.2, 148.2, 140.2, 139.7, 134.1, 130.0, 129.8, 128.0, 124.8, 112.5, 112.4, 111.5, 111.3, 97.1, 95.7, 56.3, 32.1, 29.6, 26.8, 15.4, 15.2, 13.3 ppm; HR-MS (ESI): calcd. for $C_{31}H_{26}BF_2N_5O$ $[M]^+$ 533.2198, found 533.2275.



Scheme S2. A proposed oxidative cleavage reaction of probe **1** by HOCl/OCl^-.

2. Studies of Photophysical Properties

Table S1. Photophysical properties of probe **1** in various solvents

Compound	Solvent	$\lambda_{abs.\ max}$, nm	ε, $M^{-1}cm^{-1}$	$\lambda_{em.\ max}$, nm	Φ_{FL} [c]
1	Toluene	582	56000	622	0.07
1	CH_2Cl_2	584	46000	628	0.16
1	CH_3OH	577	67000	635	0.01
1	CH_3CH_2OH	581	47000	631	0.03
1	CH_3CN	569	72000	635	0.01
1	DMF	529	34000	638	0.008
1	DMSO	576	59000	647	0.02
1	Buffer[a]	583	67000	636	0.03
1	Buffer[b]	614	79000	629	0.13

Data were obtained in [a]PBS (10 mM, pH = 7.4)/Ethanol (1:1) and [b]PBS buffer (10 mM, pH = 7.4, 0.1% Triton X-100, 1% EtOH). Excited at 560 nm for **1** in various solvents except in DMF (excited at 500 nm). [c]Quantum yields vs. Rhodamine 6G in ethanol (Φ_F = 0.95)[3]

(a) Absorption and emission spectra of probe 1 in various solvents

The emission spectra of **1** were also affected by solvent polarity, implying a degree of internal charge transfer characteristics associated with these electronic transitions.

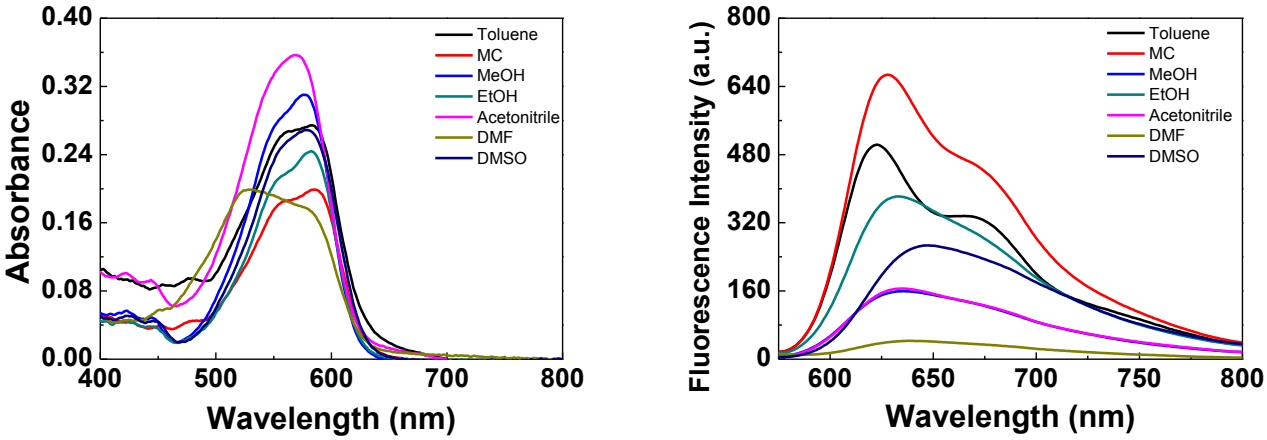


Figure S1. Absorption (left) and emission spectra (right) of probe **1** (5 μM) in various solvents. Excited at 560 nm for probe **1** in various organic solvents except in DMF (excited at 500 nm).

(b) Comparison of absorption and emission spectra of compounds 1 – 3

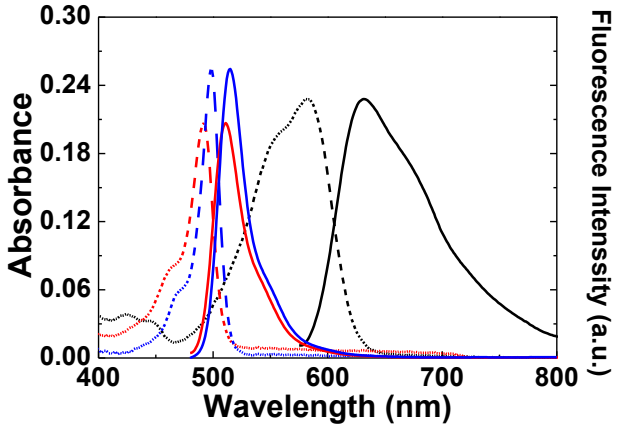


Figure S2. Absorption (dash-line) and emission spectra (solid-line) of probe **1** (black), **2** (blue) and **3** (red) in EtOH. Excited at 560 nm for **1** and 465 nm for **2** and **3**, respectively. [**1**] = [**2**] = [**3**] = 2.5 μM.

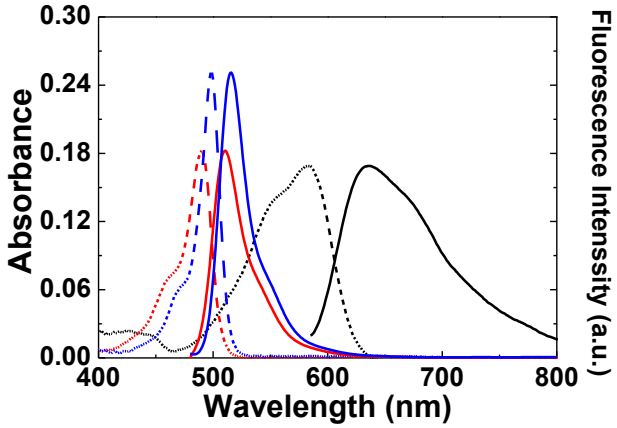


Figure S3. Absorption (dash-line) and emission spectra (solid-line) of probe **1** (black), **2** (blue) and **3** (red) in PBS buffer (10 mM, pH = 7.4, 25 ^{o}C) containing 50% EtOH as a cosolvent. Excited at 560 nm for **1** and 465 nm for **2** and **3**, respectively. [**1**] = [**2**] = [**3**] = 2.5 μM.

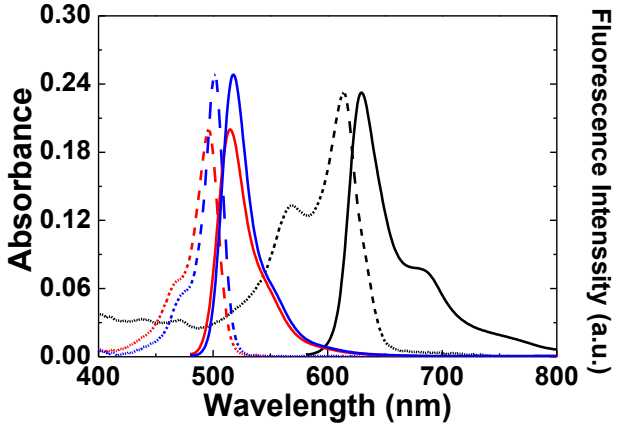


Figure S4. Absorption (dash-line) and emission spectra (solid-line) of probe **1** (black), **2** (blue) and **3** (red) in PBS buffer (10 mM, 0.1% Triton X-100, pH = 7.4, 25 ^{o}C) containing 1% EtOH as a cosolvent. Excited at 560 nm for **1** and 465 nm for **2** and **3**, respectively. [**1**] = [**2**] = [**3**] = 2.5 μM.

(c) Stability studies of probe 1

To investigate the stability of probe **1**, fluorescence spectra of probe **1** in aerated assay solution was recorded every one hour for 12 hour. The chemical stability of probe **1** was quantified by monitoring the fluorescence intensity at 520 nm as a function of incubation time.

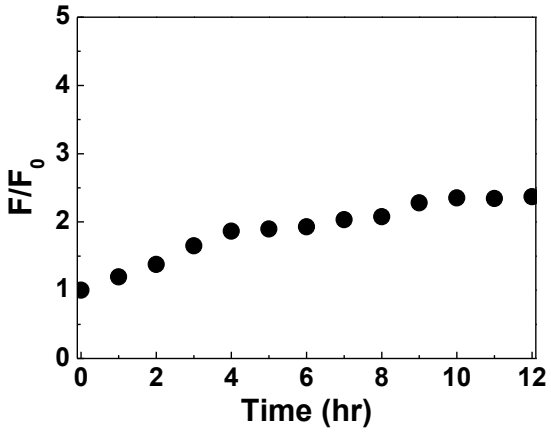


Figure S5. Chemical stability of probe **1** (2.5 μM) in PBS buffer (10 mM, 0.1% Triton X-100, 1% EtOH, pH = 7.4) at 25 ^{o}C. The emission spectra were obtained every 1 hr (0–12 hr), and the relative fluorescence intensity (F/F_0) at 520 nm was plotted as a function of time. Excited at 465 nm.

3. Fluorometric Assay Studies of Probe 1

(a) Fluorescence response of probe 1 toward HOCl in different assay media

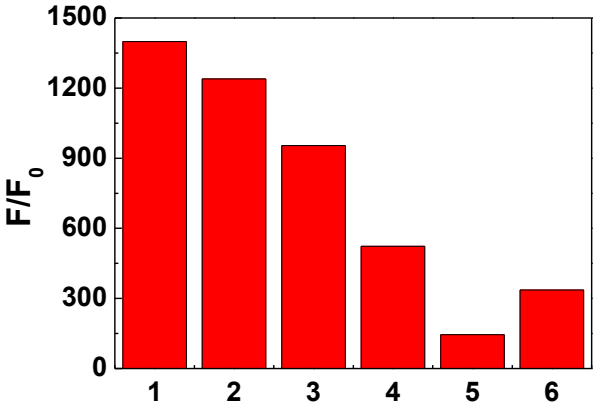


Figure S6. Relative fluorescence response of probe **1** (2.5 μM) upon addition of NaOCl (50 μM) in PBS (10 mM, pH 7.4, 1% EtOH, 25 ^{o}C) containing different amounts of Triton X-100. 1: 0.5 %, 2: 0.4%, 3: 0.3%, 4: 0.2%, 5: 0.1% Triton X-100, 6: PBS (10 mM, 50% EtOH, no Triton X-100). The relative fluorescence intensity (F/F_0) was measured at 520 nm immediately after addition of NaOCl. Excited at 465 nm. F_0 and F correspond to the fluorescence intensity of probe **1** in the absence and the presence of NaOCl, respectively.

(b) Effect of pH on fluorescence response of probe 1 in the absence and the presence of NaOCl

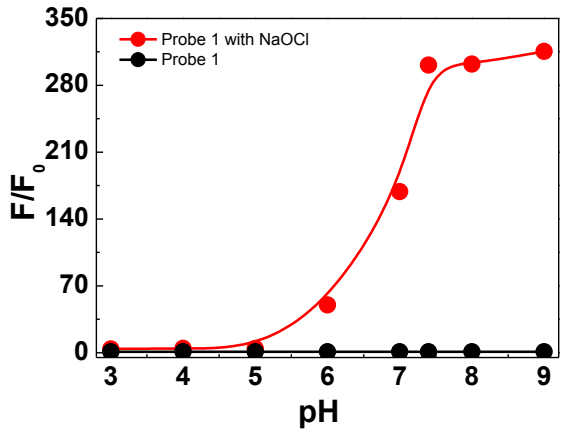


Figure S7. Relative fluorescence intensity of probe **1** without (black) and with (red) NaOCl (Incabation time = 15 min) in different pH conditions (0.1% Triton X-100, 1% EtOH, 25 ^{o}C). Excited at 465 nm. The relative fluorescence intensity (F/F_0) was measured at 520 nm. F_0 and F correspond to the fluorescence intensity of probe **1** in the absence and the presence of NaOCl, respectively. [**1**] = 2.5 μM. [NaOCl] = 25 μM.

(c) HOCl concentration-dependent fluorescence response of probe 1

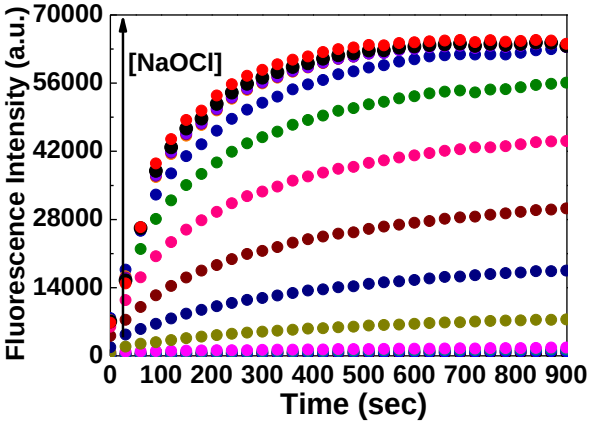


Figure S8. Fluorescence response of probe **1** (2.5 μM) upon incubation with different amounts of NaOCl (0, 0.25, 0.5, 1, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100 μM) in PBS buffer (10 mM, 0.1% Triton X-100, 1% EtOH, pH = 7.4, 25 ^{o}C). The spectra were obtained every 30 sec (0 – 15min) after the addition of NaOCl, and fluorescence intensity at 520 nm was measured with excitation at 465 nm.

(d) Fluorescence response of probe 1 to various analytes

A solution of probe **1** (0.25 mM in ethanol, 2 μL) was diluted with in PBS buffer (10 mM, 0.1% Triton X-100, 1% EtOH, pH = 7.4, 178 μL) at 25 ^{o}C. Each analyte was prepared as concentration of 0.5 mM in deionized water. Analytes were added to probe solution in 96-well flat bottom microtiter plates, and the reactions were monitored at 25 ^{o}C for 15 minutes. The fluorescence spectra were obtained with the excitation of 465 nm. Various reactive oxygen species (ROS) were prepared as follows: Superoxide ($\bullet O_2^-$) was added as solid KO_2. Hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (TBHP), sodium hypochlorite (NaOCl) were delivered from 30%, 70%, and 5% aqueous solutions, respectively. Hydroxyl radical (•OH) and *tert*-butoxy radical (•O*t*Bu) were generated by Fenton reaction of 1 mM Fe^{II} with 200 μM H_2O_2 or 200 μM TBHP, respectively. Peroxynitrite solution was synthesized as reported.[4] The peroxynitrite concentration was estimated by using an extinction coefficient of 1670 ± 50 $cm^{-1}(mol/L)^{-1}$ at 302 nm.[5] Various nucleophilic analytes (NaOH, NaCN, tetrabutylammonium fluoride, propyl amine, Glutathione, Cysteine, Na_2S, and NaSH) were used to investigate sensory response of probe **1**. Experiments employed 25 μM NaOCl, 25 μM peroxynitrite, and 50 μM for all other ROS.

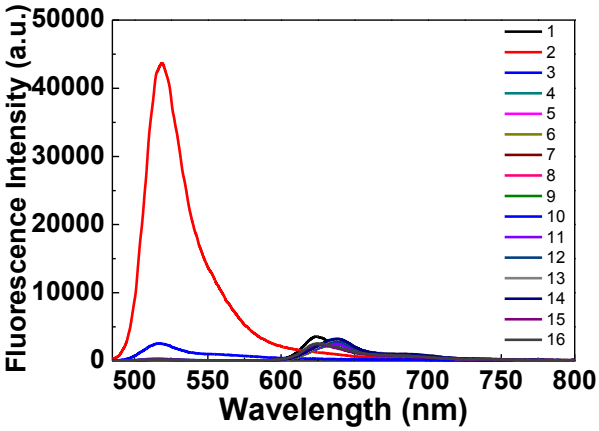


Figure S9. Fluorescence emission spectra of **1** (2.5 μM) to various analytes (25 μM NaOCl, 25 μM peroxynitrate, and 50 μM for others). Excited at 465 nm. All the data were obtained in PBS buffer (10 mM, 0.1% Triton X-100, 1% EtOH, pH = 7.4) at 25 ^{o}C. The emission spectra of **1** were obtained after incubation with each analyte for 15 min. 1: blank, 2: OCl^-, 3: $ONOO^-$, 4: $\bullet O_2^-$, 5: •OH, 6: •O*t*Bu, 7: H_2O_2, 8: TBHP, 9: OH^-, 10: CN^-, 11: F^-, 12: propyl amine, 13: glutathione, 14: cysteine, 15: S^{2-}, 16: SH^-.

(e) Control experiment: fluorescence response of formyl-BODIPY 3 toward NaOCl

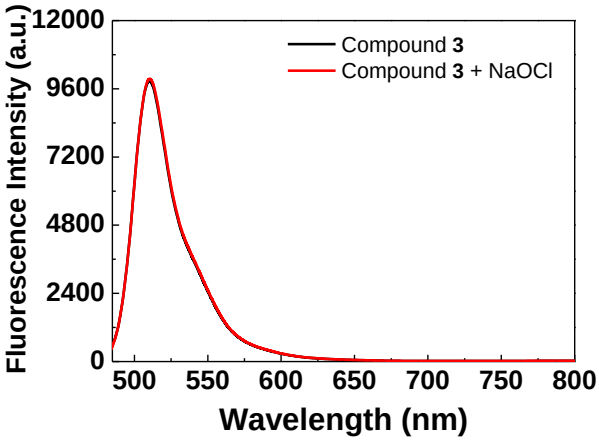


Figure S10. Fluorescence emission spectra of compound **3** in the absence (black) and presence (red) of NaOCl. The data were obtained in PBS buffer (10 mM, 0.1% Triton X-100, 1% EtOH, pH = 7.4, 25 ^{o}C). Excited at 465 nm. Incubation time = 30 min. [**1**] = 2.5 μM. [NaOCl] = 50 μM.

(e) Control experiment: fluorescence response of probe 1 toward a variety of chloramines

we have determined the reactivity of probe **1** toward a variety of chloramines, potential reactive species. Chloramines were prepared by adding equimolar amounts of NaOCl dropwise to each of respective amino acids (L-cysteine, L-lysine L-serine, Glutathione, taurine, β-alanine, glycine, L-methionine) at pH 7.4. Each preparation of chloramine was monitored by measuring its UV absorption spectra (190-350 nm) to insure that the reactions proceeded to completion (no leftover HOCl/OCl^{-}). Each chloramine was prepared immediately before use. Unlike the remarkable fluorescence response of probe **1** with HOCl/OCl^{-}, fluorescence emission changes were not observed upon reaction of probe **1** with any of the chloramines.

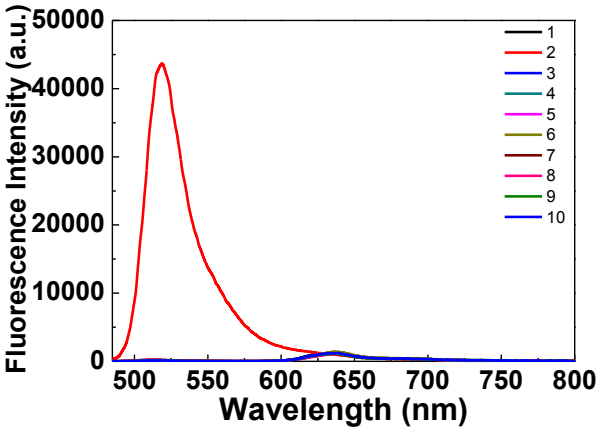


Figure S11. Fluorescence emission spectra of **1** (2.5 μM) to various analytes (25 μM NaOCl and chloramines). Excited at 465 nm. All the data were obtained in PBS buffer (10 mM, 0.1% Triton X-100, 1% EtOH, pH = 7.4) at 25 ^{o}C. The emission spectra of **1** were obtained after incubation with each analyte for 15 min. 1: blank, 2: OCl^{-}, 3: L-Cysteine, 4: L-Serine, 5: Taurine, 6: Glutathione, 7: Beta-Alanine, 8: Glycine, 9: L-Lysine, 10: L-Methionine.

4. Kinetic Studies

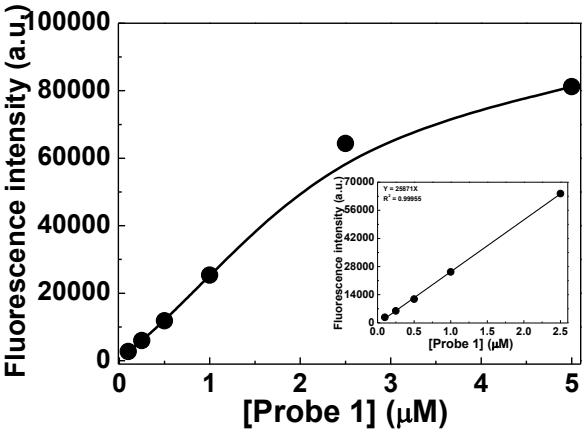


Figure S12. Standard fluorescence curve of probe **1** upon incubation with excess NaOCl (100 μM) in PBS buffer (10 mM, 0.1% Triton X-100, 1% EtOH, pH = 7.4, 25 °C). Fluorescence intensity at 520 nm was measured. [**1**] = 0~5 μM. Inset shows linear increase of fluorescence intensity at low concentrations of probe **1**. Excited at 465 nm.

Determination of kinetic constant: Pseudo-first Order Kinetics

Rate constants were determined for the reaction between probe **1** and NaOCl in PBS buffer (10 mM, 0.1% Triton X-100, pH = 7.4, 1% EtOH, 25 °C). Linear calibration of the fluorescence signal against the concentration of fomyl-BODIPY **3** produced from the reaction of probe **1** with NaOCl was carried under the assumption that probe **1** (2.5 μM) is completely converted to fomyl-BODIPY **3** in the presence of a large excess of NaOCl (50, 100 μM) and therefore the fluorescence signal at saturation corresponds to [**3**] = 2.5 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. The pseudo-first order rate constants (k_{obs}) obtained 1.13 min^{-1} for 100 μM NaOCl and 0.56 min^{-1} for 50 μM NaOCl. These correspond to second order rate constant (k) of 11298M^{-1} min^{-1} for 100 μM NaOCl and 11268 M^{-1} min^{-1} for 50 μM NaOCl after division by the concentration of analyte.

$$f(t) = 2.5\ \mu M[1 - \exp(-k_{obs}(t + a))] \quad \text{eq.1}$$

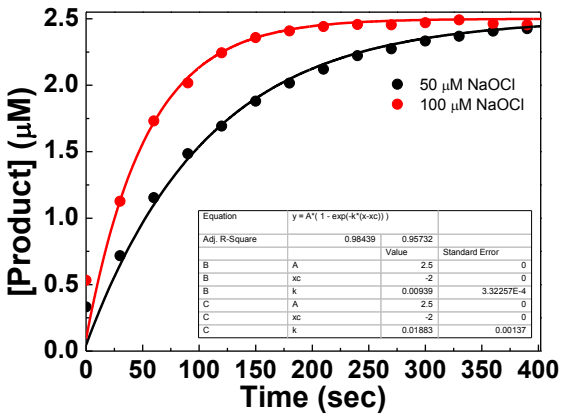


Figure S13. Pseudo-first order kinetics for the fluorescence response of probe **1** (2.5 μM) upon incubation with excess NaOCl (50, 100 μM) in PBS buffer (10 mM, 0.1% Triton X-100, pH = 7.4, 1% EtOH, 25 °C). Fluorescence intensity at 520 nm was measured. Excited at 465 nm.

5. Analysis of Reaction Product by NMR and HPLC-MS

To verify the proposed sensing scheme, the separate reaction of probe **1** with 4 equiv NaOCl was carried out in an ethanol-deionized water (v/v, 1:1) solution at room temperature for 1 hour. The major reaction product was isolated and purified through a silica gel column (hexane/EtOAc=5:1) to afford a dark purple solid. This isolated product was identified as formyl-BODIPY **3** by studies of the ^{1}H NMR.

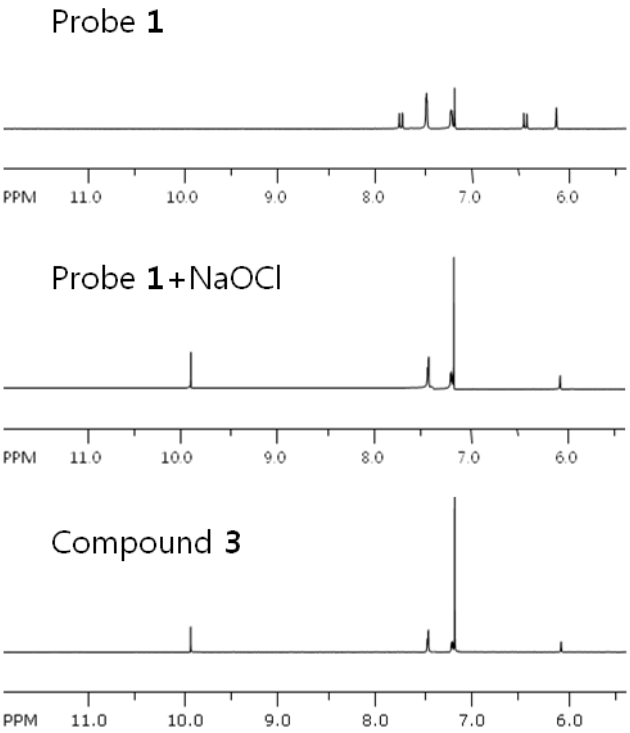


Figure S14. Partial ^{1}H-NMR Spectra of probe **1** (top) and the isolated reaction product (middle) and formyl-BODIPY **3** (bottom) in $CDCl_3$.

In addition, the proposed detection scheme was confirmed by analyzing the crude reaction mixture of probe **1** with NaOCl using HPLC-MS. The probe **1** (2.5 μM) was dissolved in an ethanol-deionized water (v/v, 1:1), and then 4 equivalents of NaOCl 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 NaOCl shows intense peak of $[\mathbf{3}+H]^+$ (*m/z* 353.2), consistent with that of formyl-BODIPY (**3**).

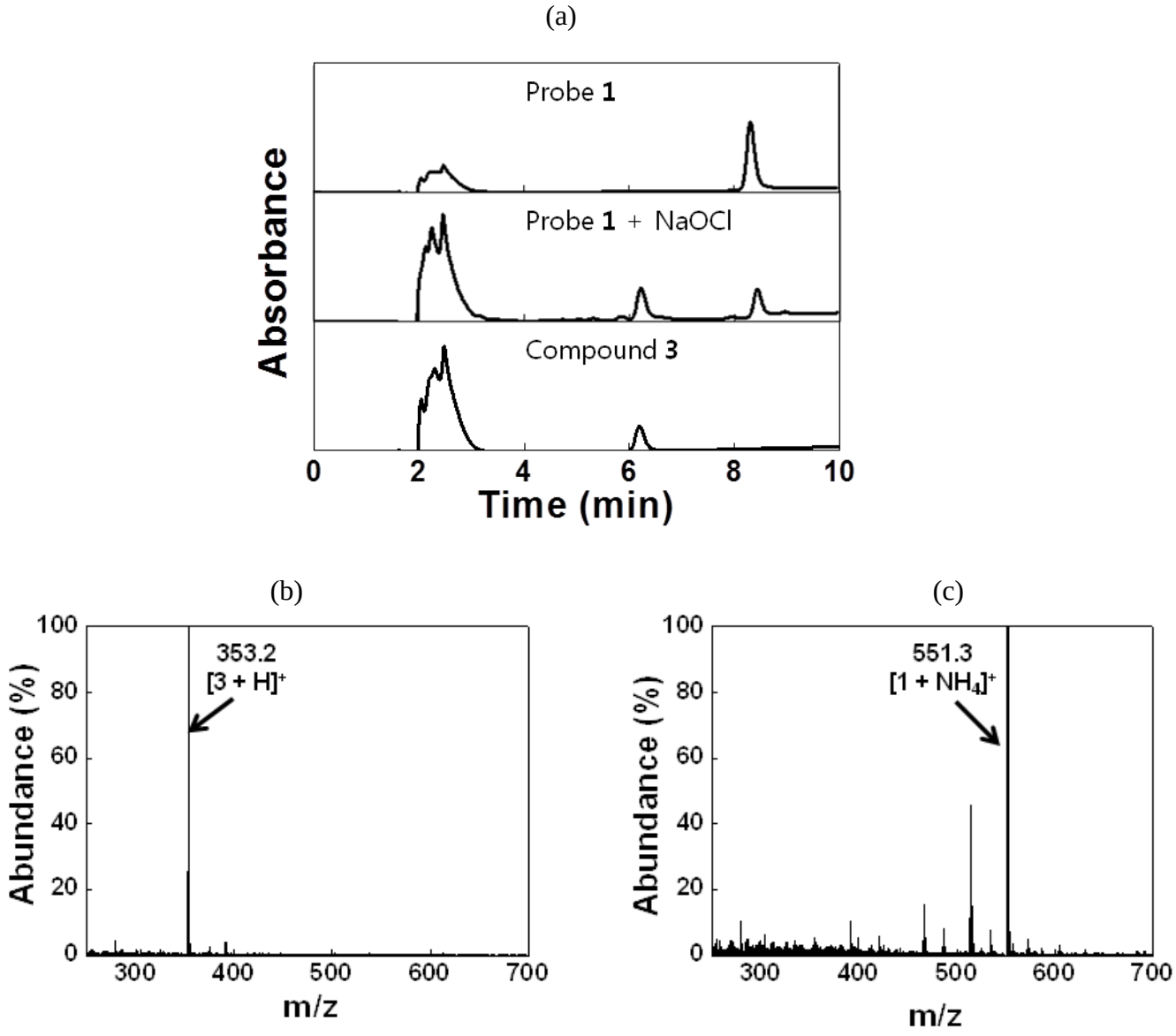


Figure S15. (a) HPLC chromatograms of probe **1** only (top), immediately after addition of NaOCl (middle), and formyl-BODIPY **3** only (bottom). (b) ESI-MS spectra of the peak of retention time at 6.2 min (b) and 8.4 min (c). [**1**] = 2.5 μM, [NaOCl] = 10 μM.

6. Cell studies

(a) Cell culture

RAW264.7 macrophage cell lines were obtained from the American Type Culture Collection (Rockville, MD), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% antibiotic-antimycotic in a humidified incubator containing 5% CO_2 at 37 °C. Myeloperoxidase(MPO) inhibitor, 4-aminobenzoic acid hydrazide (4-ABAH), was obtained from Aldrich Co. Inc.

(b) Cell viability test

RAW264.7 macrophage cells were seeded in each well of 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h for cell attachment. The stock solution of probe **1** dissolved in DMSO (1.88 mM) was diluted with a cell culture medium containing 10% FBS to have a final concentration of 0, 2.5, 5, 10, and 15 μM, respectively. Existing culture medium was replaced with 100 μL of the fresh one containing probe **1**, and the cells were incubated for 1 h. After washing the cells twice, a fresh cell culture medium was added, and further incubated for an additional 24 h. The cell viability of macrophage cells was measured using a cell counting kit-8 (Dojindo Laboratories). Cell viability was calculated as a percentage compared to untreated control cells. 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.

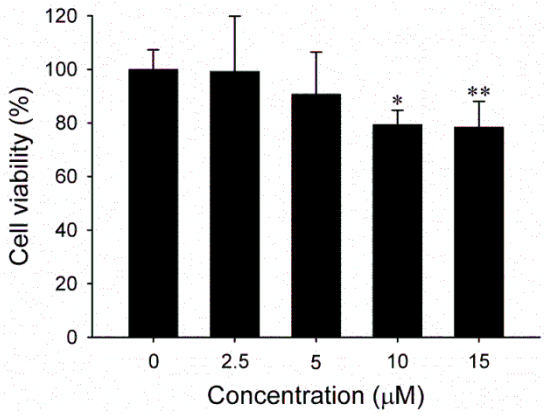


Figure S16. Cell viability of RAW264.7 macrophage cells treated with probe **1** for 1 hour at different concentrations (n =4). (* $P < 0.05$, ** $P < 0.01$).

(c) Confocal fluorescence imaging of exogenous HOCl in macrophage cells

RAW264.7 macrophage cells were plated at a density of 1×10^5 cells/well in a LabTek-II chambered cover glass (Nalge Nunc International Corp., Rochester) and incubated for 24 h for cell attachment. The stock solution of probe **1** in DMSO was diluted with cell culture medium to have a final concentration of 1 μM probe **1**. HOCl was diluted with PBS solution at different concentrations (10, 30, and 50 μM, respectively). Existing cell culture medium was exchanged with cell culture medium containing probe **1**, and incubated for 30 min. The cells were then washed 3 times with pre-warmed cell culture medium, and treated with PBS solution containing HOCl for 30 min. Confocal fluorescence images (Ex. 488 nm, Em. 497-550 for green channel and 625-700 for red channel) were obtained using a confocal laser scanning microscope (40×, Carl Zeiss LSM 510 META, Germany). All the data were acquired using identical settings in order to ensure reproducibility. Ratiometric images were obtained using LSM510 software (Ion Conc. Process).

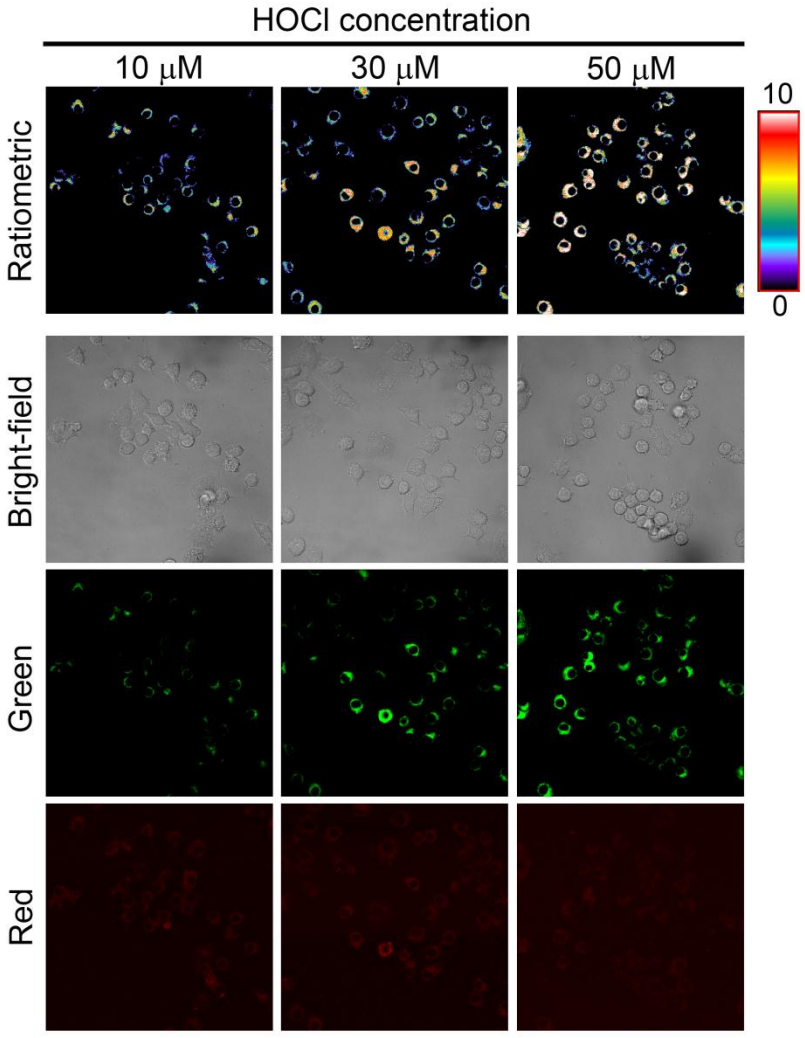


Figure S17. Confocal fluorescence images of RAW264.7 macrophages incubated with probe **1** and treated with various concentrations of HOCl. Macrophages were incubated with 1 μM probe **1** for 30 min at 37 °C and then subjected to different treatments; (left to right) 10 μM, 30 μM, 50 μM HOCl for 30 min at 37 °C. Fluorescence image; (top to bottom) ratiometric images ($I_{497\text{-}550}/I_{625\text{-}700}$), brightfield images, fluorescence images from green channel (497-550 nm) and red channel (625-700 nm). Magnification 40×. Digital zoom 2×.

(d) Confocal fluorescence imaging of endogenous HOCl in activated macrophage cells during phagocytic immune response

Zymosan A Bioparticles was used to stimulate macrophage cells in this study. Opsonized zymosan was prepared according to the instructions supplied by company (Molecular probes, Invitrogen). To opsonize Bioparticles, opsonizing reagent (0.5 mL) was mixed with 0.53 mL of Bioparticles (20 mg /mL), and incubation for 1 h at 37 °C. After reconstitution, the opsonized Bioparticles were washed 3 times with DPBS (centrifugation: 800 × g, 15 min). Reconstituted zymosan A Bioparticles were diluted 50 times with PBS and stored at 4 °C. The opsonized zymosan particles (20 μL) was mixed with cell culture medium (480 μL) for cell experiments.
RAW264.7 macrophage cells were plated at a density of 1×10^5 cells/well in a LabTek-II chambered cover glass (Nalge Nunc International Corp., Rochester) and incubated for 24 h for cell attachment. The stock solution of probe **1** in DMSO was diluted with cell culture medium to have a final concentration of 5 μM. Existing cell culture medium was exchanged with cell culture medium containing probe **1**, and incubated for 30 min. The cells were then washed 3 times with pre-warmed cell culture medium. To activate macrophage cells, the cells were incubated cell culture medium containing the opsonized zymosan particles, and thereafter confocal fluorescence images (Ex. 488 nm, Em. 497-550 for green channel and 625-700 for red channel) were obtained every 30 sec with a confocal laser scanning microscope (80×, Carl Zeiss LSM 510 META, Germany). Ratiometric images were obtained using LSM510 software (Ion Conc. Process).

(e) MPO-inhibition study

To check whether generation of green fluorescence is due to the conversion of H_2O_2 into HOCl by the action of myeloperoxidase (MPO) in the activated macrophage cells during phagocytosis, inhibition assay of MPO was performed. Control cells were incubated only with probe **1 (**5 μM**)** for 30 min, washed two times with cell culture media, and thereafter confocal fluorescence images (Ex. 488 nm, Em. 497~550 nm) were obtained using a confocal laser scanning microscope (80×, Carl Zeiss LSM 510 META, Germany). For activating macrophage cells, the cells were incubated with cell culture medium containing probe **1** for 30 min, washed two times with cell culture media, treated with the opsomized zymosan particles for 5 min, and then confocal fluorescence images were obtained (Ex. 488 nm, Em. 497-550). For inhibition study, the cells were preincubated with MPO inhibitor 4-ABHA (100 μM) for 1 h, treated with 5 μM probe **1** for 30 min, washed two times with cell culture media, and further treated with the opsomized zymosan particles for 5 min. Confocal fluorescence images were obtained (Ex. 488 nm, Em. 497-550). All the data were acquired using identical settings in order to ensure reproducibility. For quantification of fluorescence signal activation, ROI (region of interest) was assigned to every single cell and the mean intensity of the corresponding region was recorded. Fluorescence intensity of each group was defined as the arithmetic mean of the single cell-numerical values (excluding two highest and two lowest values, $n \geq 30$). Mean values for the case and control group are represented by relative fluorescence intensity (Fold Ratio). All the images were analyzed using AxioVision 4.3 Software (Zeiss). Statistical significance was calculated by determining *p* values by using the t-test.

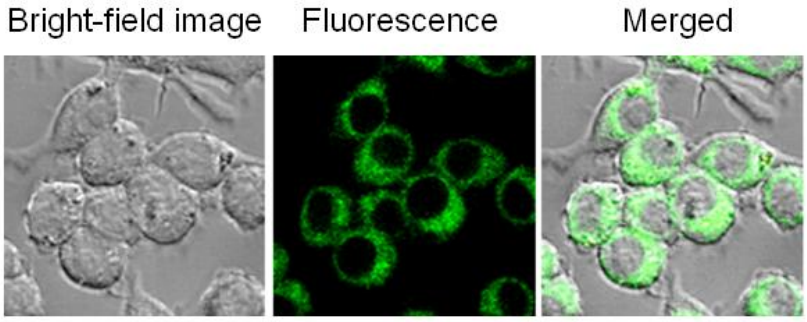


Figure S18. Magnified images of endogenously-generated HOCl in activated RAW264.7 during phagocytic immune response. Macrophages were incubated with probe **1** (5 μM) for 30 min, treated with the opsonized zymosan particles, and then fluorescence images were obtained at 300 sec. Merged image clearly shows that fluorescence signals are well distributed within the cytoplasm region. This is magnified image of Fig. 4l.

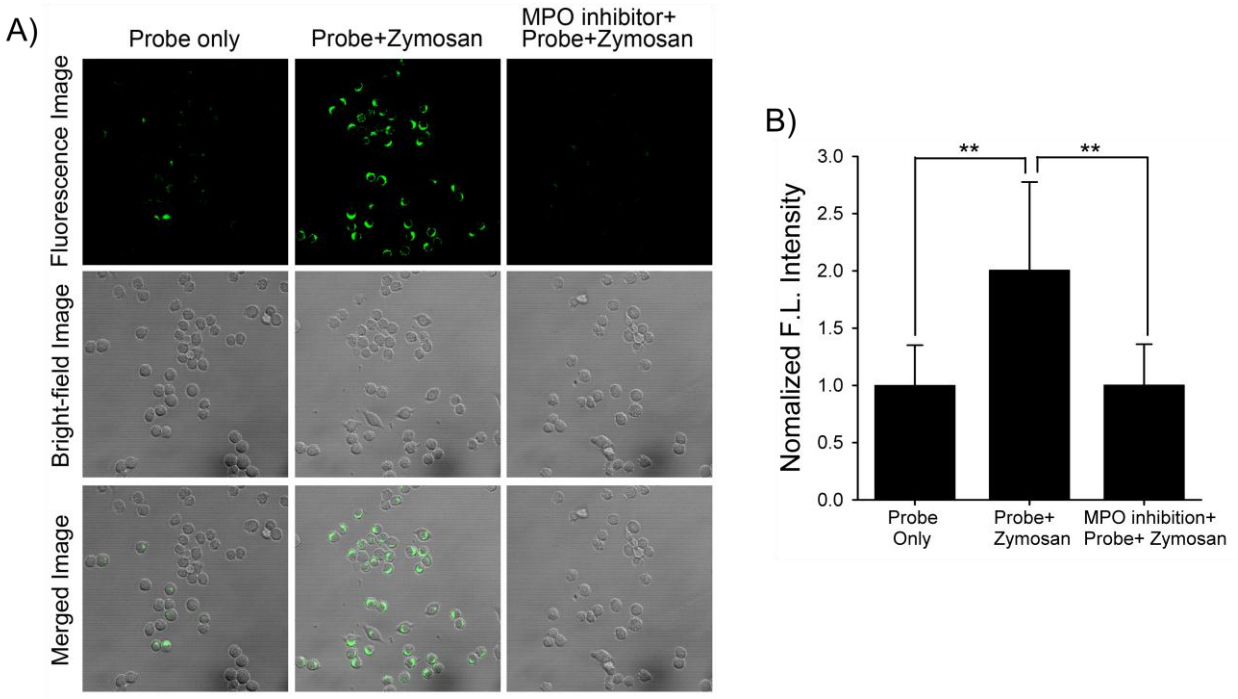
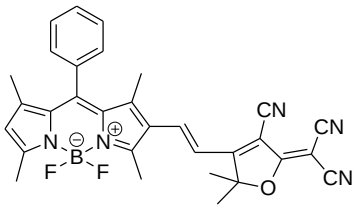
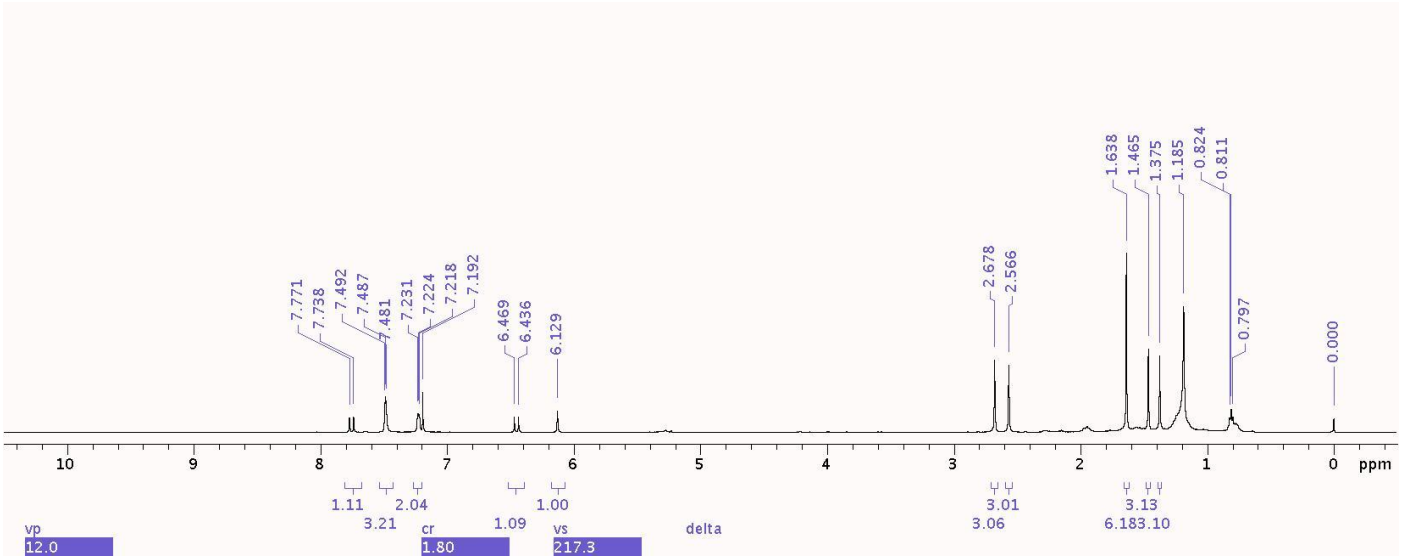


Figure S19. (a) Relative **c**onfocal fluorescence images of living macrophages (RAW264.7) under different conditions with probe **1**. (left) Macrophages were incubated with 5 μM probe **1** for 0.5 h at 37 ^{o}C and then imaged. (middle) Macrophage cells loaded with probe **1** were stimulated with zymosan for 300 sec, and then imaged. (right) Macrophage cells were pre-treated with MPO inhibitor, 4-ABAH (final concentration=100 μM), for 1 h, incubated with 5 μM probe **1** for 0.5 h, and then stimulated with zymosan for 300 sec. (top: fluorescence images, middle: bright-field images, bottom: merged images, Ex=488 nm, Em=497–550 nm). (b) Quantitative analysis of the fluorescence intensity by zymosan-stimulated HOCl and MPO-inhibition. Data were normalized to probe only control (n > 30). ** P < 0.01

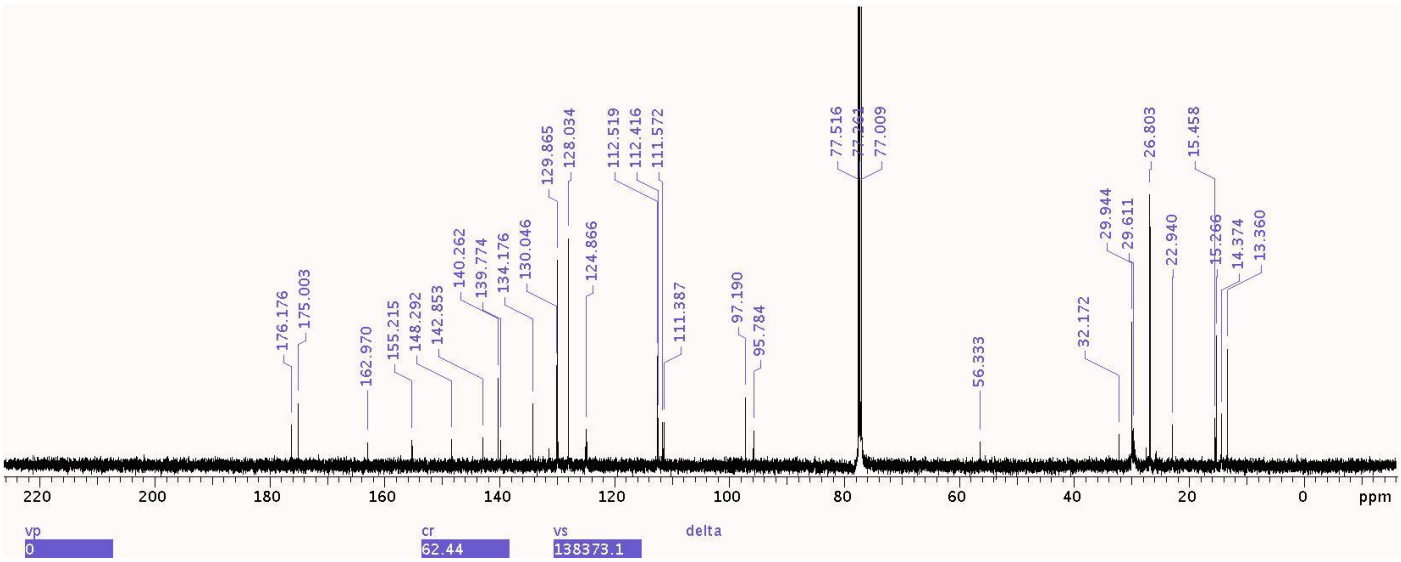
7. ^{1}H-NMR and ^{13}C-NMR Spectra

^{1}H-NMR Spectrum of **1** in $CDCl_3$ (500 MHz):

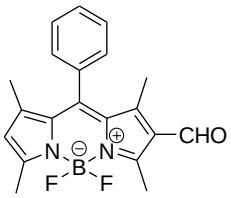


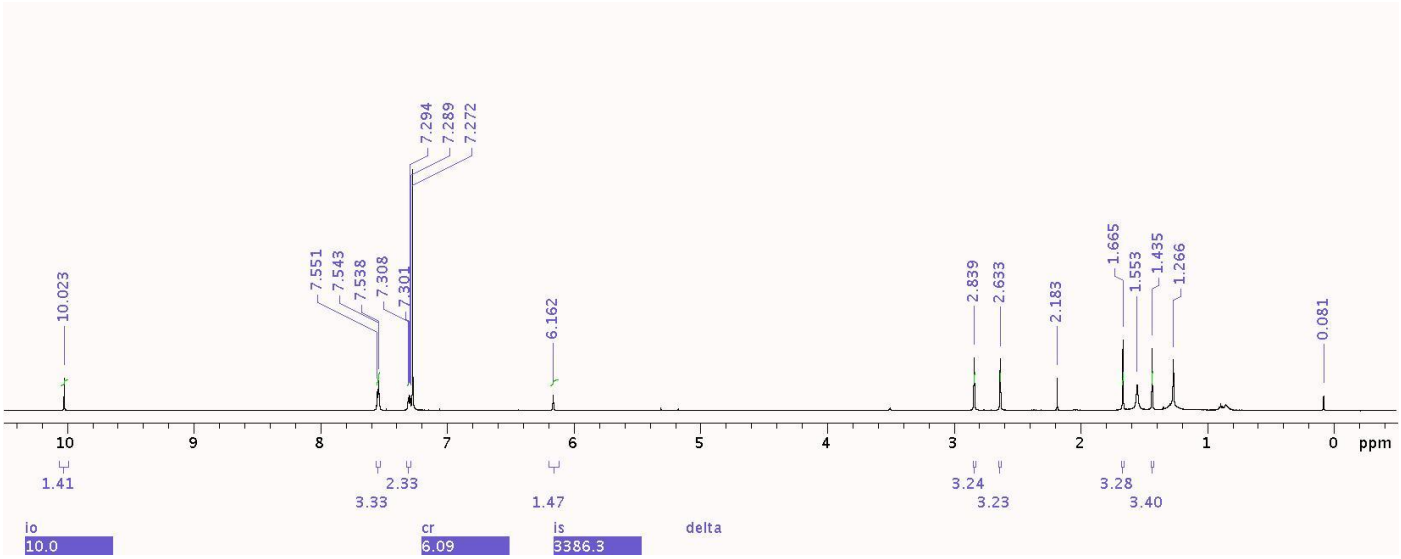


^{13}C-NMR Spectrum of **1** in $CDCl_3$ (125 MHz):

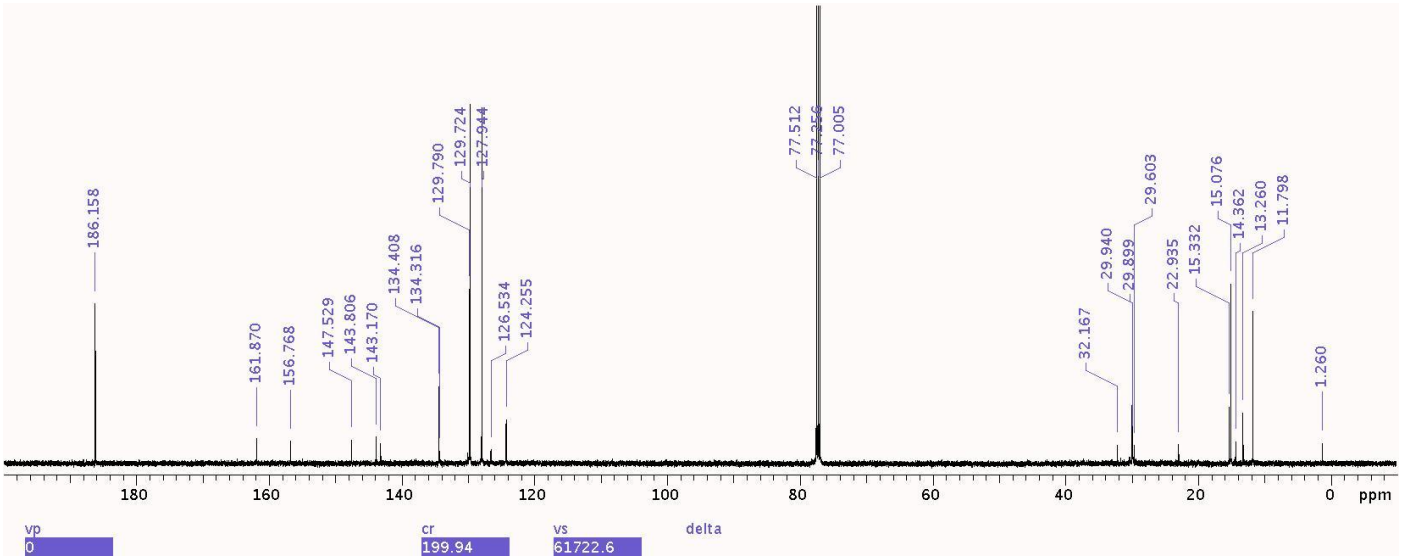


^{1}H-NMR Spectrum of **3** in $CDCl_3$ (500 MHz):





^{13}C-NMR Spectrum of **3** in $CDCl_3$ (125 MHz):



8. References

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