Electronic Supplementary Information for

HKOCl-3: A Fluorescent Hypochlorous Acid Probe for Live-cell and *In Vivo* Imaging and Quantitative Application in Flow Cytometry and 96-Well Microplate Assay

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1. General methods

Xanthine, xanthine oxidase, SNP (sodium nitroferricyanide(III) dihydrate), 2,2'-azobis(2-amidinopropane)dihydrochloride, and hydrogen peroxide solution were purchased from Sigma-Aldrich. Peroxynitrite was synthesized as reported.[1] Peroxynitrite solution was split into small aliquots and frozen at less than –18 °C. All other chemicals used were of analytical grade and were purchased from Acros or Sigma-Aldrich. Potassium phosphate buffer was prepared by mixing aqueous solution of $KHPO_4$ (1 M, 1.98 mL) and KH_2PO_4 (1 M, 8.02 mL) (final pH 7.4) followed by dilution with deionized water to 100 mL in a volumetric flask. NMR spectra were recorded in deuteriochloroform unless otherwise stated, with tetramethylsilane (TMS) as internal reference at ambient temperature, mainly on a Bruker Avance DPX 300 Fourier Transform Spectrometer operating at 300 MHz for ^1H and at 75.47 MHz for ^{13}C and Bruker Avance DPX 400 Fourier Transform Spectrometer operating at 400 MHz for ^1H and at 100.6 MHz for ^{13}C. Mass spectra were recorded with a Thermo Scientific DFS High Resolution Magnetic Sector mass spectrometer for both low resolution and high resolution mass analysis.

The fluorescent probe was dissolved in DMF to make a 10 mM stock solution, which was then diluted to 10 μM testing solution in 0.1 mM potassium phosphate buffer at pH 7.4. The absorption and fluorescence spectra of the testing solution were recorded under a CARY 50 Bio UV-Visible spectrophotometer and a Hitachi F-7000 fluorescence spectrophotometer, respectively. For fluorescence measurement, slit widths were set at 2.5 nm for both excitation and emission spectra, and the photomultiplier voltage was 700 V.

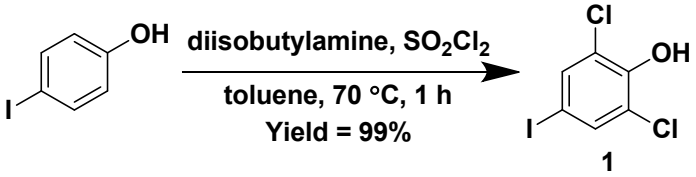
Aliquots of analyte solutions were slowly added to probe testing solution (5 mL) with vigorous stirring at room temperature in the dark. The volume changes after addition of analyte solutions were less than 1%. The fluorescence intensities of the testing solutions were recorded after 30 min.

To determine the quantum yields, probe or fluorophore stock solutions were prepared by accurately weighing and dissolving the samples in CH_3CN (HPLC grade). Testing solutions for measuring UV absorbance and fluorescence were prepared by further dilution of the stock solutions with 50 mM potassium phosphate buffer at pH 8.0. The quantum yields of the probes or fluorophores were estimated by comparison of the integrated area of the corrected emission spectrum of the sample with that of a reference solution, i.e., a solution of fluorescein in 0.1 M NaOH solution ($\Phi = 0.95$). The quantum yield of a sample was related to that of the reference, and determined by the equation

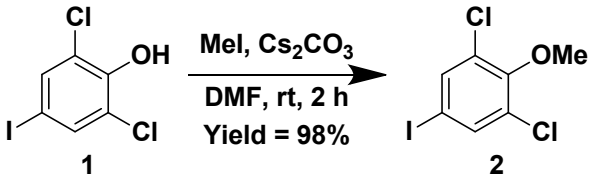
$$\Phi_{\text{sample}} = \left(\frac{A_{\text{reference}}}{A_{\text{sample}}}\right)\left(\frac{F_{\text{sample}}}{F_{\text{reference}}}\right)\Phi_{\text{reference}} \qquad (1)$$

wherein Φ is the fluorescence quantum yield, A is the absorbance at the excitation wavelength, F is the area under the emission curve. The concentration of the reference was adjusted to match the absorbance of the test sample at the wavelength of excitation so that the absorbance ratio is equal to 1.

2. Preparation of HKOCl-3

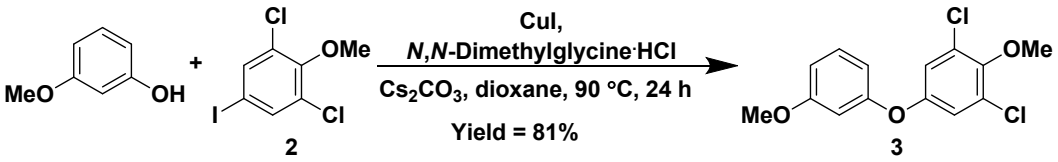


To a solution of 4-iodophenol (5.50 g, 25 mmol) in dry toluene (90 mL) at room temperature was added diisobutylamine (35 μL, 0.2 mmol) under argon atmosphere. The resulting solution was warmed up to 70 °C. Then, SO_2Cl_2 (6.05 mL, 75 mmol) was slowly added via a syringe (while gas escaped to balloon). The reaction mixture was stirred at 70 °C for 1 h and allowed to cool to room temperature. Then, the reaction mixture was diluted with Et_2O and washed sequentially with saturated $NaHCO_3$ solution and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The target compound **1** [34074-22-1] was isolated as a white solid (7.20 g; 99%) by flash chromatography on silica gel, by using EtOAc:hexane (1:99) as an eluent. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (s, 2H), 5.81 (brs, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 148.0, 136.4, 122.1, 80.5.

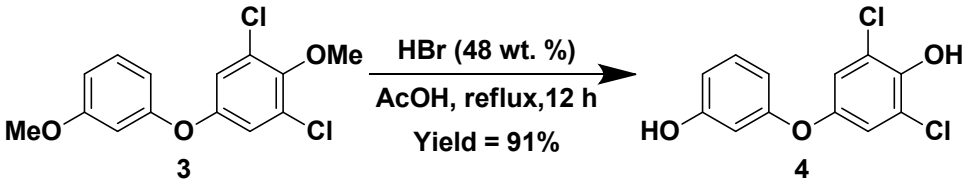


To a mixture of **1** (1.66 g, 5.75 mmol) and Cs_2CO_3 (2.25 g, 6.90 mmol) in anhydrous DMF (20 mL) at room temperature was added iodomethane (0.43 mL, 6.90 mmol) slowly under argon atmosphere. The resulting mixture was stirred at room temperature for 2 h. Then, the reaction mixture was diluted with ethyl acetate and washed sequentially with 1 N HCl, water and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The target compound **2** [71742-42-2] was isolated as a white solid (1.71 g; 98%) by flash chromatography on

silica gel, by using Et_2O:hexane (1:99) as an eluent. ^1H NMR (400 MHz, $CDCl_3$) δ 7.57 (s, 2H), 3.86 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.4, 137.1, 130.3, 86.3, 60.7.

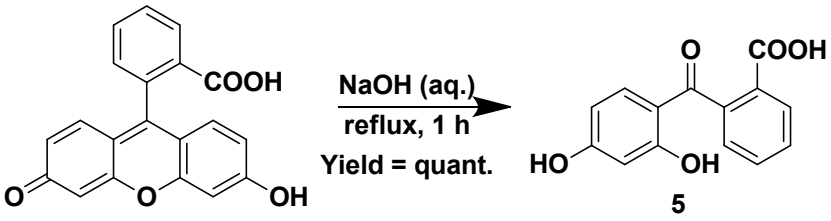


A mixture of **2** (470 mg, 1.55 mmol), 3-methoxyphenol (289 mg, 2.33 mmol), CuI (30 mg, 0.155 mmol), *N*,*N*-dimethylglycine hydrochloride (65 mg, 0.465 mmol), Cs_2CO_3 (1.01 g, 3.10 mmol) and anhydrous 1,4-dioxane (10 mL) in a sealed flask was heated to 90 °C and stirred for 24 h under argon atmosphere. The reaction mixture was allowed to cool to room temperature, diluted with ethyl acetate, and washed sequentially with 1 N HCl, water and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The target compound **3** was isolated as a colorless oil (376 mg; 81%) by flash chromatography on silica gel, by using EtOAc:hexane (1:19) as an eluent. ^1H NMR (300 MHz, $CDCl_3$) δ 7.25 (t, J = 8.1 Hz, 1H), 6.95 (s, 2H), 6.74–6.68 (m, 1H), 6.60–6.53 (m, 2H), 3.88 (s, 3H), 3.79 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.2, 157.4, 153.5, 148.3, 130.6, 129.9, 119.1, 111.4, 110.0, 105.6, 61.0, 55.6; LRMS (EI, 20 eV) *m/z* (%) 298 (M^+; 100), 283 (92); HRMS (EI): calcd for $C_{14}H_{12}O_3Cl_2$ (M^+): 298.0163, found: 298.0159.

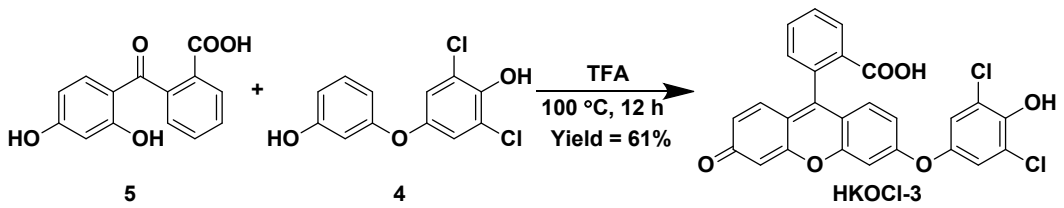


The solution of **3** (218 mg, 0.73 mmol) in acetic acid (7 mL) and HBr (48 wt. % in H_2O, 7 mL) was heated to reflux and stirred for 12 h under argon atmosphere. The reaction mixture was allowed to cool to room temperature, diluted with ethyl acetate, and washed sequentially with 1 N HCl, water and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The target compound **4**

was isolated as a white sticky solid (180 mg; 91%) by flash chromatography on silica gel, by using EtOAc:hexane (3:17) as an eluent. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.15 (t, *J* = 8.1 Hz, 1H), 6.96 (s, 2H), 6.60 (dd, *J* = 8.1, 2.3 Hz, 1H), 6.51 (dd, *J* = 8.1, 2.2 Hz, 1H), 6.47 (t, *J* = 2.3 Hz, 1H), 6.31 (brs, 1H), 6.07 (brs, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.3, 157.0, 149.7, 144.4, 130.7, 121.5, 119.6, 111.0, 110.6, 105.9; LRMS (EI, 20 eV) *m/z* (%) 270 (M^+; 100), 272 (66); HRMS (EI): calcd for $C_{12}H_8O_3Cl_2$ (M^+): 269.9850, found: 269.9845.



The solution of fluorescein (3.32 g, 10 mmol) in aqueous NaOH solution (12.5 M, 12 mL) was heated to reflux and stirred for 1 h under argon atmosphere. The reaction mixture was allowed to cool to room temperature, and carefully acidified with conc. HCl at 0 °C until large amount of precipitate was formed. Then, the target compound **5** [2513-33-9] was filtered, washed with water and dried in air for 24 h to obtain a brown solid (2.58 g; quant.). ^{1}H NMR (300 MHz, CD_3OD) δ 8.08 (d, *J* = 7.6 Hz, 1H), 7.64 (t, *J* = 7.4 Hz, 1H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.32 (d, *J* = 7.4 Hz, 1H), 6.93 (d, *J* = 8.8 Hz, 1H), 6.35 (d, *J* = 2.2 Hz, 1H), 6.22 (dd, *J* = 8.8, 2.3 Hz, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ 202.3, 168.2, 165.7, 141.2, 135.7, 132.9, 131.2, 130.1, 129.8, 128.0, 114.3, 108.8, 103.3.



The solution of **4** (17 mg, 0.066 mmol) and **5** (18 mg, 0.066 mmol) in TFA (2 mL) in a sealed flask was heated to 100 °C and stirred for 12 h under argon atmosphere. The reaction mixture was allowed to cool to room temperature and azeotroped with

toluene for 3 times. The target compound **HKOCl-3** was isolated as a yellow sticky solid (20 mg; 61%) by flash chromatography on silica gel, by using EtOAc:hexane (3:7) as an eluent. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.02 (d, J = 7.6 Hz, 1H), 7.73 (t, J = 7.5 Hz, 1H), 7.66 (t, J = 7.5 Hz, 1H), 7.22 (d, J = 7.6 Hz, 1H), 7.03 (s, 2H), 6.79 (d, J = 2.0 Hz, 1H), 6.75–6.69 (m, 2H), 6.66 (dd, J = 8.7, 2.0 Hz, 1H), 6.61 (d, J = 8.7 Hz, 1H), 6.56 (dd, J = 8.7, 1.9 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.1, 159.4, 159.3, 152.9, 152.5, 152.3, 147.8, 146.2, 135.3, 129.9, 129.5, 129.0, 126.6, 124.9, 124.0, 122.5, 120.4, 113.7, 113.5, 112.6, 109.6, 105.1, 102.7, 83.8; LRMS (EI) *m/z* (%) 492 (M^+; 1), 271 (100); HRMS (EI): calcd for $C_{26}H_{14}O_6Cl_2$ (M^+): 492.0167, found: 492.0155.

3. Absorption spectra of HKOCl-3 before and after treatment of HOCl

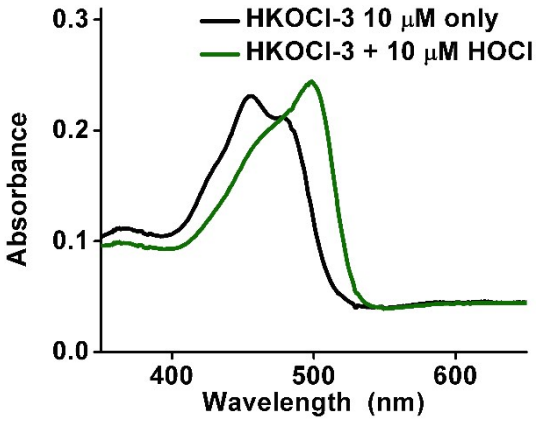


Figure S1. Absorption spectra of **HKOCl-3** (10 μM) in 0.1 M potassium phosphate buffer (0.1% DMF, pH 7.4) before and after treatment with 1 equiv HOCl.

4. Chemostability of HKOCl-3 toward HOCl

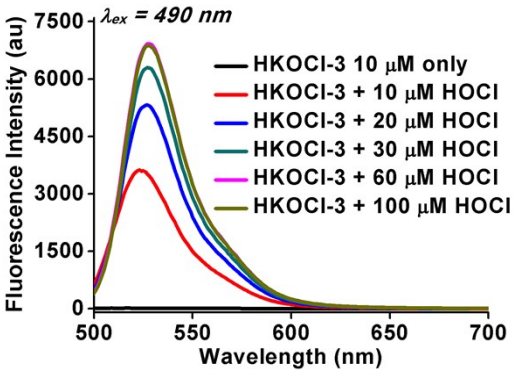


Figure S2. Fluorescence emission spectra of the fluorescent probe **HKOCl-3** upon treatment of various amounts of HOCl. The probe **HKOCl-3** was dissolved in 0.1 M potassium phosphate buffer at pH 7.4 to a final concentration of 10 μM (containing 0.1% DMF). Different amounts of HOCl (10–100 μM) were added to the testing solutions of **HKOCl-3** (10 μM). The fluorescence spectra were recorded at 30 min with an excitation at 490 nm.

5. Time course of the reaction between HKOCl-3 and HOCl

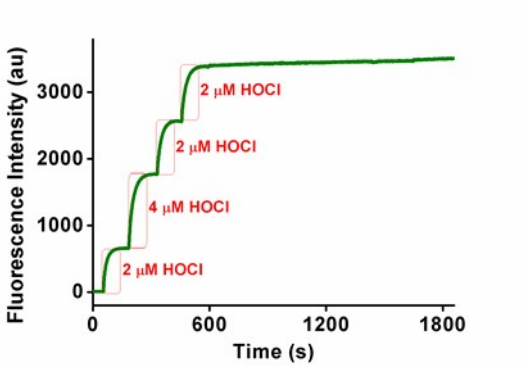


Figure S3. Time course of fluorescence intensity of fluorescent probe **HKOCl-3** at 527 nm after treatment of HOCl (1 equiv in total). The probe **HKOCl-3** was dissolved in 0.1 M potassium phosphate buffer at pH 7.4 to a final concentration of 10 μM (containing 0.1% DMF). The fluorescence intensity was monitored at an emission wavelength of 527 nm with an excitation at 490 nm.

6. Stability of HKOCl-3 toward pH changes

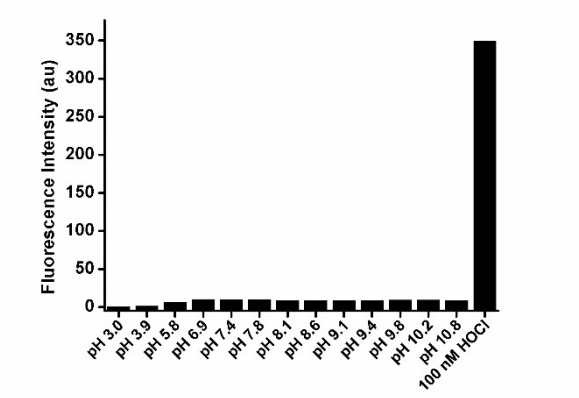


Figure S4. The effect of pH changes on fluorescence intensity of **HKOCl-3**. Fluorescence intensity of **HKOCl-3** (10 μM) in 0.1 M potassium phosphate buffer at various pH (3–10.8) at 25 °C was recorded after 30-min incubation. As a positive control, fluorescence response of **HKOCl-3** (10 μM) toward HOCl (100 nM) was measured at 527 nm with an excitation at 490 nm.

7. The effect of pH changes on the HOCl detection with HKOCl-3

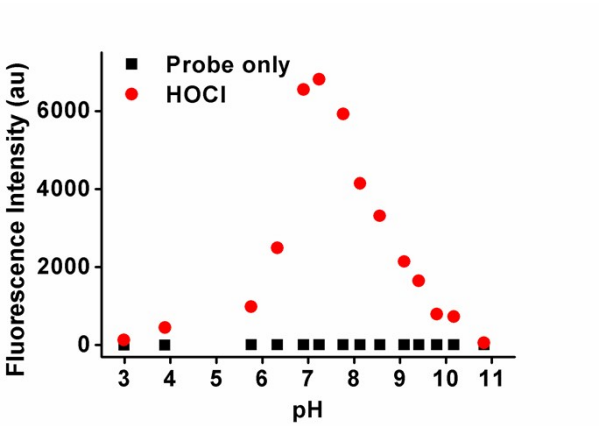


Figure S5. The effect of pH changes on the HOCl detection with **HKOCl-3**. Fluorescence intensity of **HKOCl-3** (10 μM) in the absence or presence of HOCl (100 μM) in 0.1 M potassium phosphate buffer at various pH (3–10.8) at 25 °C was recorded after 30 min. The fluorescence intensity was measured at 527 nm with an

excitation at 490 nm.

8. Competition experiments of HKOCl-3

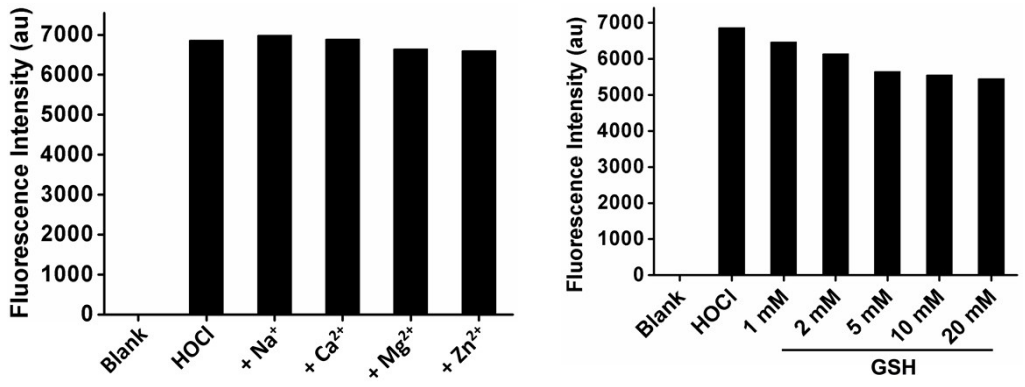
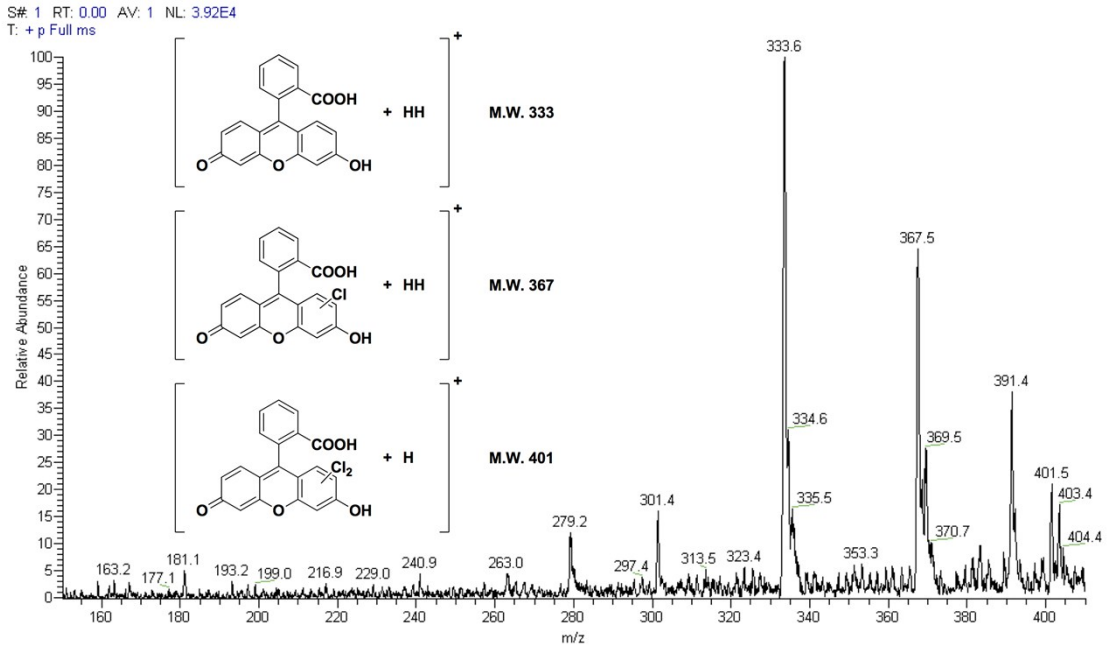


Figure S6. Fluorescence intensity of **HKOCl-3** (10 μM in 0.1 M potassium phosphate buffer at pH 7.4) co-incubated with HOCl (100 μM) and other potential competing species including (*left*) Na^+ (100 μM), Ca^{2+} (100 μM), Mg^{2+} (100 μM), Zn^{2+} (100 μM) and (*right*) GSH (1–20 mM) at 25 °C was recorded after 30 min. The fluorescence intensity was measured at 527 nm with an excitation at 490 nm.

9. Detection of reaction product by ESI-MS (electrospray ionization mass spectroscopy)

The probe **HKOCl-3** were dissolved in DMF and then diluted to 10 μM with 0.1 M potassium phosphate buffer at pH 7.4. Then, 1 equiv or 10 equiv of sodium hypochlorite was injected into each probe solution by means of a pipette in the dark. After 30 min, the reaction mixture was extracted with ethyl acetate. The combined organic layers were analyzed by ESI-MS.

(1) **HKOCl-3** in the presence of 1 equiv of sodium hypochlorite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COOH
+ HH
M.W. 333
COOH
Cl
+ HH
M.W. 367
COOH
Cl2
+ H
M.W. 401
Relative Abundance
m/z
333.6
367.5
391.4
334.6
369.5
401.5
403.4
335.5
370.7
404.4
301.4
279.2
353.3
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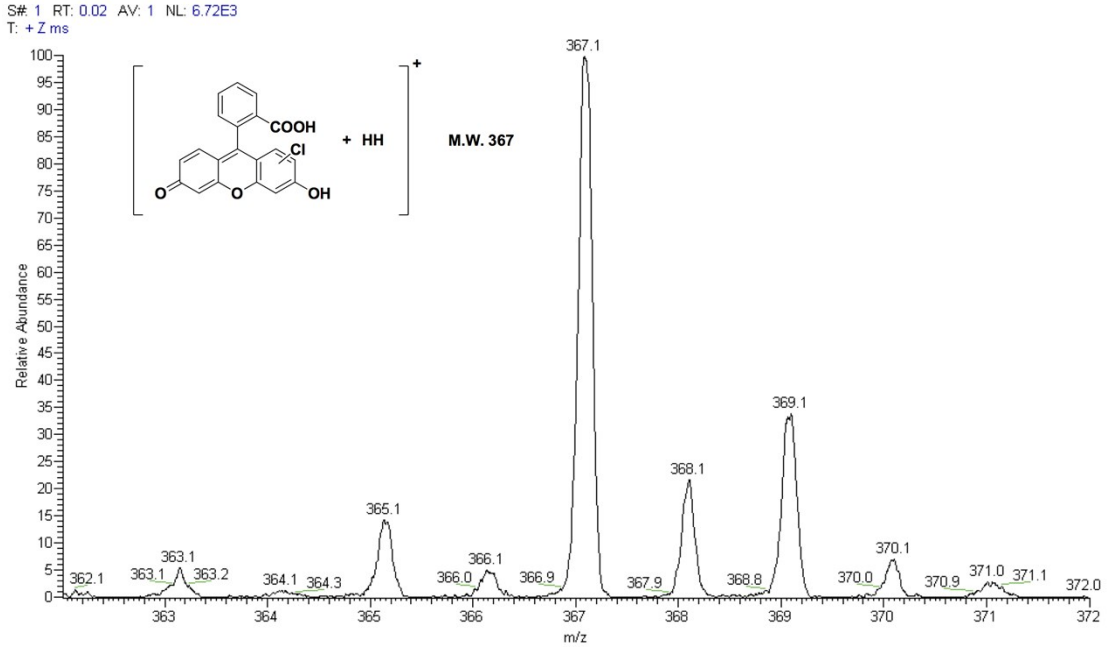
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Cl
+ HH
M.W. 367
Relative Abundance
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370.1
363.1
366.1
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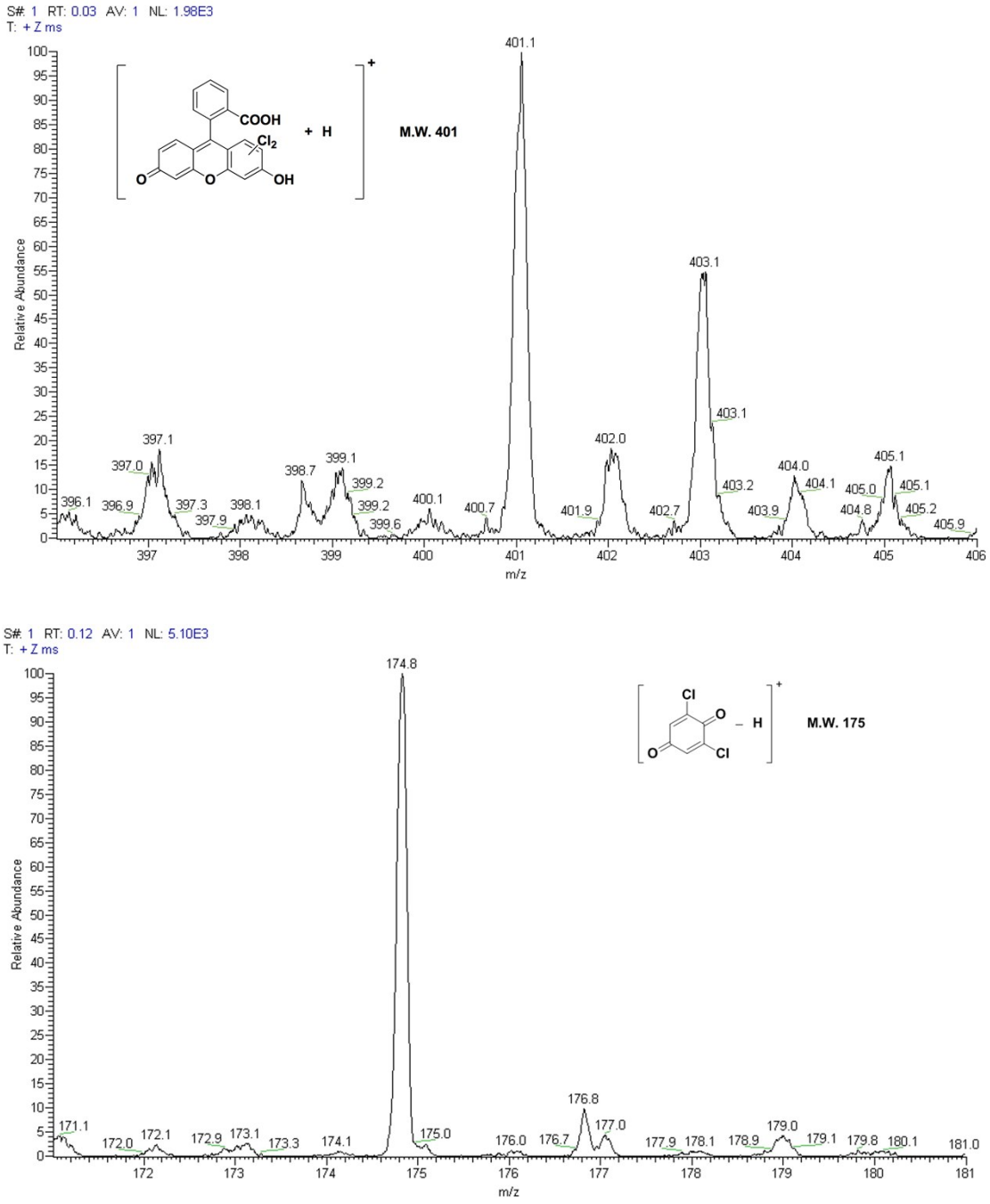
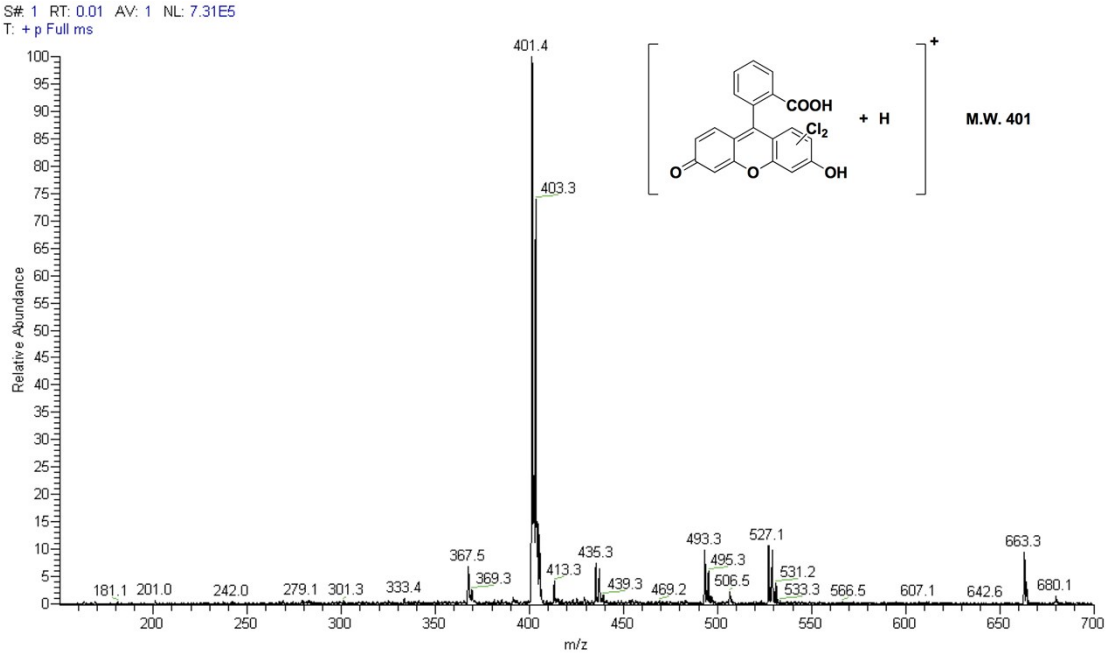
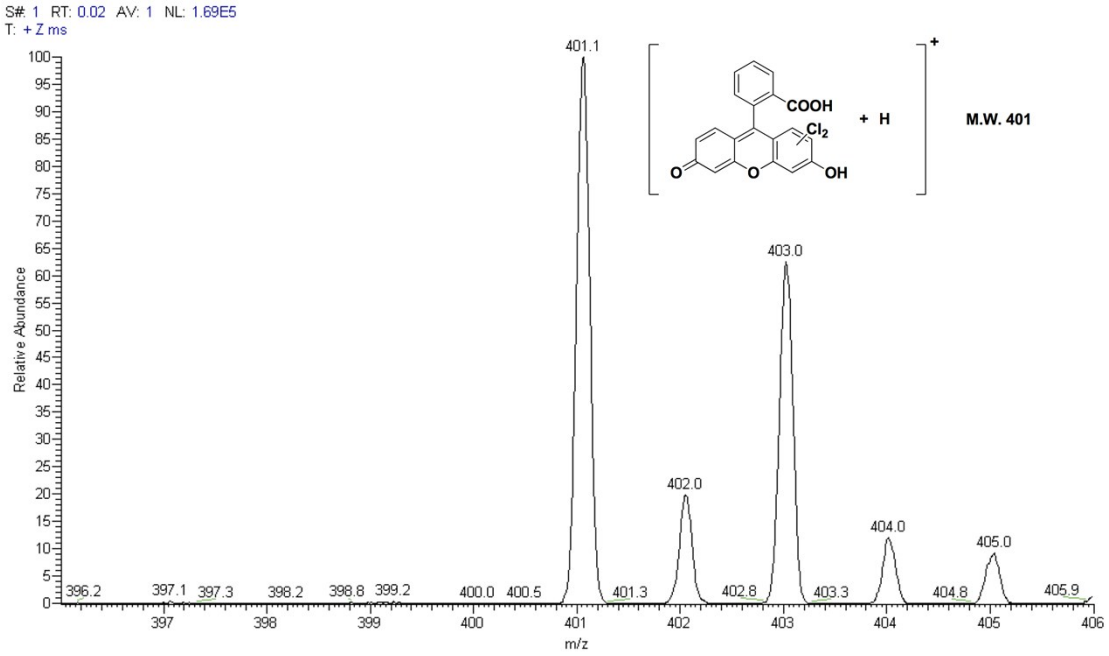


(2) **HKOCl-3** in the presence of 10 equiv of sodium hypochlorite:





10. Preparation of analyte solutions

ROO•: Alkylperoxyl radical was generated from 2,2'-azobis(2-amidinopropane) dihydrochloride (10 mM), which was added into the testing solutions directly.

$^1O_2$: Singlet oxygen was generated from 3,3'-(naphthalene-1,4-diyl)dipropionic acid (10 mM).

H_2O_2: H_2O_2 solution (10 mM) was added directly.

$^{\bullet}$NO: Nitric oxide was generated from SNP (sodium nitroferricyanide(III) dihydrate) (10 mM).

$O_2^{\bullet-}$: Superoxide was generated from xanthine/xanthine oxidase system. Xanthine oxidase (0.1 U/mL) was added before xanthine (30 mM).

HOCl: NaOCl solution (10 mM) was added directly.

$^{\bullet}$OH: Hydroxyl radical was generated by Fenton reaction. To generate $^{\bullet}$OH, ferrous chloride was added in the presence of 10 equiv of H_2O_2. The concentration of $^{\bullet}$OH was equal to the Fe(II) concentration (10 mM).

$ONOO^-$: Peroxynitrite solution was synthesized according to literature report.[1] Briefly, a mixture of sodium nitrite (0.6 M) and hydrogen peroxide (0.7 M) was acidified with hydrochloric acid (0.6 M), and sodium hydroxide (1.5 M) was added within 1–2 s to make the solution alkaline. Excess hydrogen peroxide was removed by passing the solution through a short column of manganese dioxide. The resulting solution was split into small aliquots and stored at lower than –18 °C. The aliquots were thawed immediately before use, and the concentration of peroxynitrite was determined by measuring the absorption of the solution at 302 nm. The extinction coefficient of peroxynitrite solution in 0.1 M NaOH is 1670 M^{-1} cm^{-1} at 302 nm.

11. Mammalian cell culture and isolation of primary human neutrophils

RAW264.7 cells, a mouse monocytic macrophage line, were acquired from ATCC (American Type Culture Collection) and maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin/streptomycin, at 37 °C with 5% CO_2. The growth medium was renewed every two to three days. At 80% confluence, the cells were detached by scraping, washed with fresh medium and spun down (500 rpm in Eppendorf microfuge) for cell counting. For confocal imaging, cells were typically seeded at a density of 2×10^4 cells/mL in 35-mm confocal dish (Mat-Tek: MA, USA). BV-2

mouse microglia were obtained as a gift from Department of Pediatrics, University of Hong Kong, and maintained in the same manner as RAW264.7 cells. THP-1 human monocytes were acquired as a gift from Prof. Lijian Jin, School of Dentistry, The University of Hong Kong, and maintained in RPMI (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1% penicillin/streptomycin, and 55 μM *β*-mercaptoethanol, at 37 °C in 5% CO_2. Prior to drug treatment, THP-1 monocytes were differentiated into mature macrophages with low-dose PMA (5 ng/mL) as previously described.[2]

Isolation of primary human neutrophils (or polymorphonuclear leukocytes, PMNs) was carried out by the gradient density centrifugation method with Histopaque (Sigma) solutions 1077 and 1119 in 12-mL polypropylene centrifuge tubes, as described previously by Freitas *et al*.[3] Briefly, 3 mL of Histopaque 1077 was carefully layered on top of 3 mL of Histopaque 1119 in a 12-mL polypropylene tube. Then, 6 mL of collected blood was added as a topmost layer. After centrifugation at 870× *g* for 30 min at 20 °C, neutrophils positioned immediately on top of erythrocytes were carefully collected by means of a Pasteur pipette. Neutrophil pellet was retrieved and doubled in volume by using PBS sans Ca^{2+} or Mg^{2+}. After centrifugation at 870× *g* for 5 min at 4 °C, cell pellet was resuspended as a mixture of 1.25 ml in PBS, without Ca^{2+} or Mg^{2+}, and 5.25 ml of sterile distilled water was added to lyse any remaining erythrocytes. Following gentle inversion for 1.30 min, 2.2 mL of 3% NaCl was added. After further centrifugation at 870× *g* for 5 min at 4 °C, neutrophil pellet was resuspended in PBS containing Ca^{2+} and Mg^{2+}. Isolated neutrophils were kept on ice until use. Neutrophils from one volunteer were used per experiment.

12. Drug treatment

For acute HOCl induction (30 min), PMA or zymosan was added at specified doses to HBSS (Hank's balanced salt solution, supplemented with 0.6 mM L-arginine, 0.01% chloramphenicol) and co-incubated with our fluorescent probe until imaging. Enzyme

inhibitors (such as NOX inhibitor DPI, and MPO inhibitor ABAH) and HOCl scavenger (such as taurine) were added along with PMA or zymosan during HOCl induction.

13. Probe incubation and confocal imaging settings

Fluorescent probe **HKOCl-3** stock solutions (1 or 2 mM) were prepared in anhydrous DMF. Before probe incubation, cells were washed with PBS and added with probe (1 or 2 μM final concentration) in 1.5-mL HBSS supplemented with 0.1% chloramphenicol (a bacteriostatic) and 0.6 mM L-arginine. Cells were typically incubated for 30 min at 37 ^{o}C with 5% CO_2. During imaging, the dish was mounted onto a live cell imaging support module (Axiovision). Single-photosection images were acquired with a Zeiss LSM 510 confocal microscope, by using the following settings: Ex 488 nm, Em 500–530 nm (band-pass).

14. Cytotoxicity assay of HKOCl-3

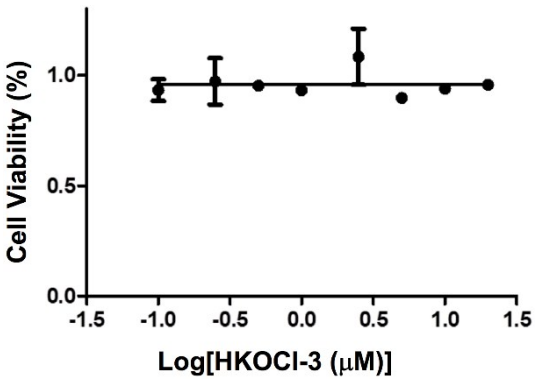


Figure S7. Cytotoxicity of **HKOCl-3** in RAW264.7 cells. RAW264.7 cells were allowed to incubate with increasing concentrations of **HKOCl-3** overnight. The probes showed negligible or no cytotoxicity after 24-h incubation. Data represent mean ± s.e.m. for Cell-Titer Glo assays performed in triplicates.

To assess potential toxicity of our probe **HKOCl-3**, RAW264.7 cells were seeded at

2×10^5 cells/mL in 100 μL per well in a 96-well microplate (Corning), in DMEM supplemented with 10% heat-inactivated FBS (fetal bovine serum) and 1% penicillin/streptomycin. Cells were allowed to attach for overnight. Probe stock solutions of different concentrations in DMF were diluted to their working concentrations (**HKOCl-3**: 0.1–20 μM) in fresh medium and added to the cells (100 μL per well) in triplicates. After 24-h probe incubation, cells were loaded with 50-μL Cell-Titer Glo Reagent (Promega) and subjected to gentle shaking for cell lysis at room temperature (10 min). The microplate was mounted onto a DTX 880 multimode plate reader (Molecular Devices) for luminescence detection, by using cellular ATP contents as a measure of cell viability. Data were collected for three separate serial dilutions and averaged. Cells viability was calculated according to the following equation:

Cell viability (%) = $100 \times A_{\text{with probe}} / A_{\text{control}}$, where A = luminescence intensity.

15. Validation of HKOCl-3 for imaging endogenous HOCl with excellent selectivity and sensitivity

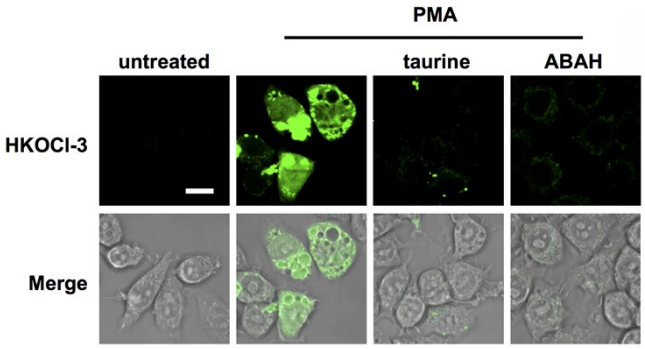


Figure S8. RAW264.7 cells were co-incubated with **HKOCl-3** (1 μM) with or without PMA (200 ng/mL) and taurine (10 mM) or ABAH (50 μM), before confocal imaging. Merge: fluorescence and phase images merged. Scale bar = 10 μm.

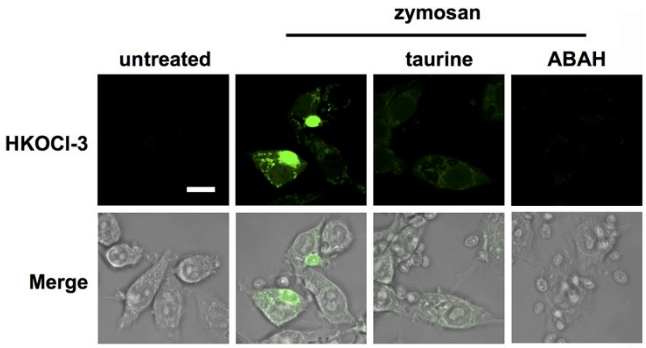


Figure S9. RAW264.7 cells were co-incubated with **HKOCl-3** (1 μM) with or without zymosan (50 μg/mL) and taurine (10 mM) or ABAH (50 μM), before confocal imaging. Merge: fluorescence and phase images merged. Scale bar = 10 μm.

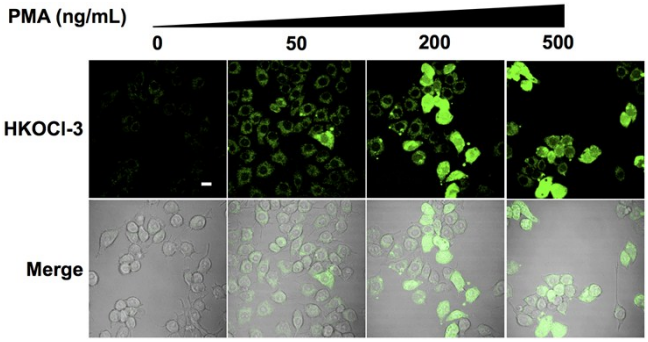


Figure S10. RAW264.7 cells were co-incubated with **HKOCl-3** (1 μM) and a dose gradient of PMA (0–500 ng/mL) for 30 min, before confocal imaging. Merge: fluorescence and phase images merged. Scale bar = 10 μm.

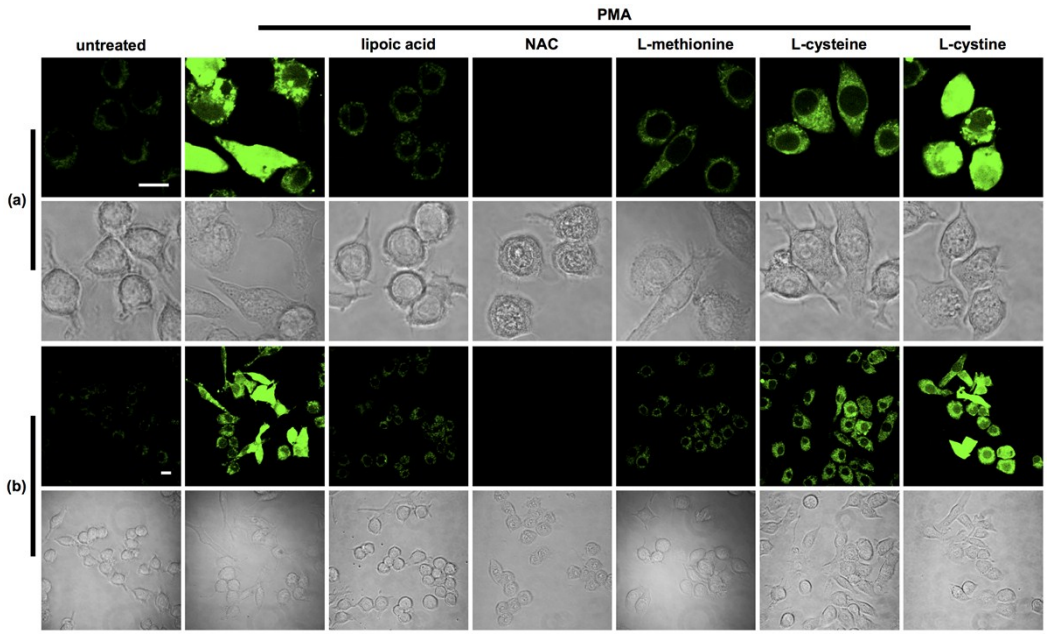


Figure S11. Scavengers confirm selective detection of endogenous HOCl in confocal imaging with **HKOCl-3**. RAW264.7 cells were co-incubated with **HKOCl-3** (1 μM), PMA (200 ng/mL), in the absence or presence of HOCl scavengers : α-lipoic acid (6 mM), NAC (6 mM), L-methionine (6 mM), L-cysteine (6 mM), and L-cystine (200 μM) for 30 min, before confocal imaging at high magnification (**a**; 315×) and lower magnification (**b**; 63×). Scale bars represent 10 μm.

16. 96-Well microplate fluorometric measurement

RAW264.7 mouse macrophages were plated in 96-well microplates (Costar 3603; Corning), at a density of 5×10^5 cells/mL (100 μL/well in triplicates) one day before assay. The medium used was DMEM (Dulbecco's modified Eagle medium; high glucose) supplemented with 10% heat-inactivated FBS (fetal bovine serum) and 1% penicillin/streptomycin. For fluorometric measurement, all reagents and probe solutions were freshly dissolved in HBSS buffer (supplemented with 0.6 mM L-arginine and 0.01% chloramphenicol). Before drug addition, cells were washed with HBSS (100 μL/well). Then, 100 μL of 2 μM **HKOCl-3** together with 500 ng/mL PMA and scavengers in supplemented HBSS was added. At the end of 30-min co-incubation, drug gradients were removed by aspiration. Cells were briefly washed

with HBSS, and re-loaded with probe-free HBSS containing 500 ng/mL PMA and scavengers (100 μL/well) before fluorescence reading on a DTX880 multimode detector (Beckman Coulter) with the following settings: excitation at 485 nm and emission at 535 nm. Data are presented, without subtracting background fluorescence, as mean ± s.e.m. for fluorometric measurement with replicates (n = 4) in at least three independent experiments.

17. Flow cytometry analysis

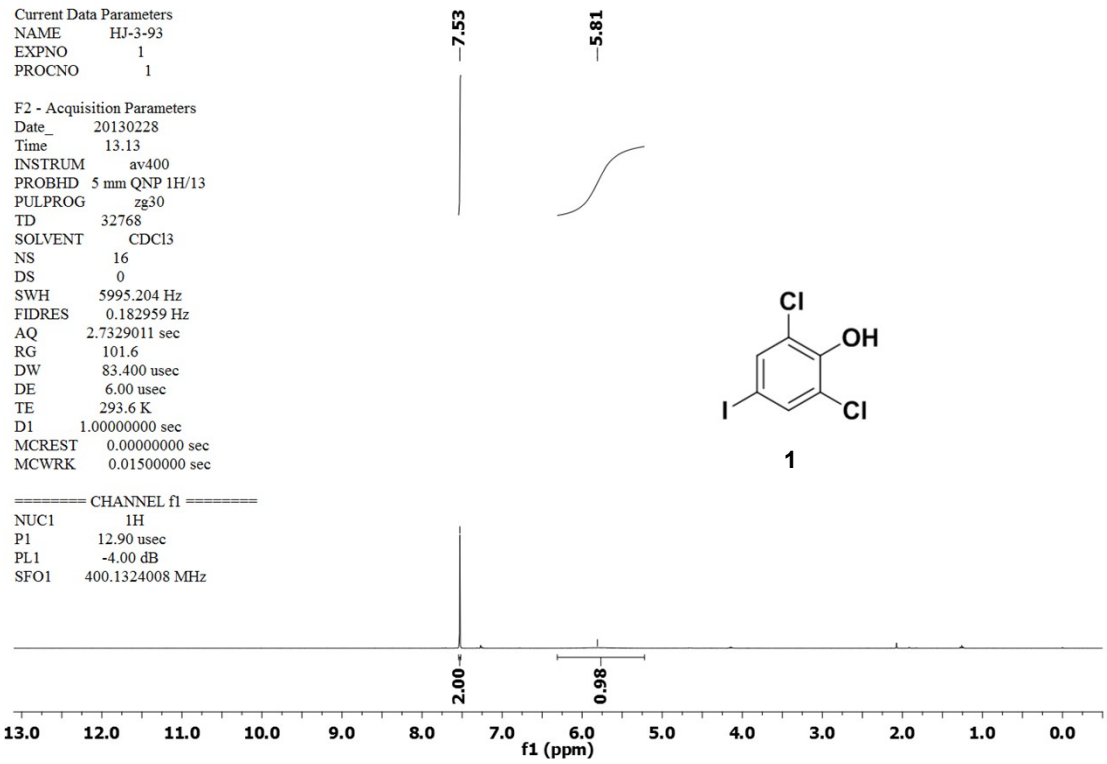
RAW264.7 cells were seeded into a 150-mm culture dish (Corning), cultured overnight for attachment and recovery, and grown to a required cell density (70-80% confluence or about 1.5×10^7 cells). For harvest, cells were scraped off gently and collected into a clean 50-mL Falcon tube. Cells were spun down (500 rpm, room temperature, 3 min). After discarding the supernatant, 5 mL of warm HBSS was added gently to re-suspend the cell pellet. Then, cells were spun down (500 rpm, room temperature, 2 min). After this wash step, 2 mL of HBSS mix (HBSS supplemented with 0.6 mM L-arginine and 0.01% chloramphenicol) to re-suspend the cell pellet into single cells, followed by addition of another 6 mL HBSS mix. For acute HOCl detection, cells were co-incubated with 2 μM probe **HKOCl-3** in the absence or presence of PMA (500 ng/mL) in HBSS (supplemented with 0.6 mM L-arginine and 0.01% chloramphenicol) at 37 °C for 30 min. The NOX inhibitor DPI (100 nM) was added to inhibit the HOCl generation along with 500 ng/mL PMA for test of selectivity. Finally, HOCl levels were evaluated by measuring fluorescence intensity in FITC channel by using a flow cytometer (BD LSR Fortessa Analyzer).

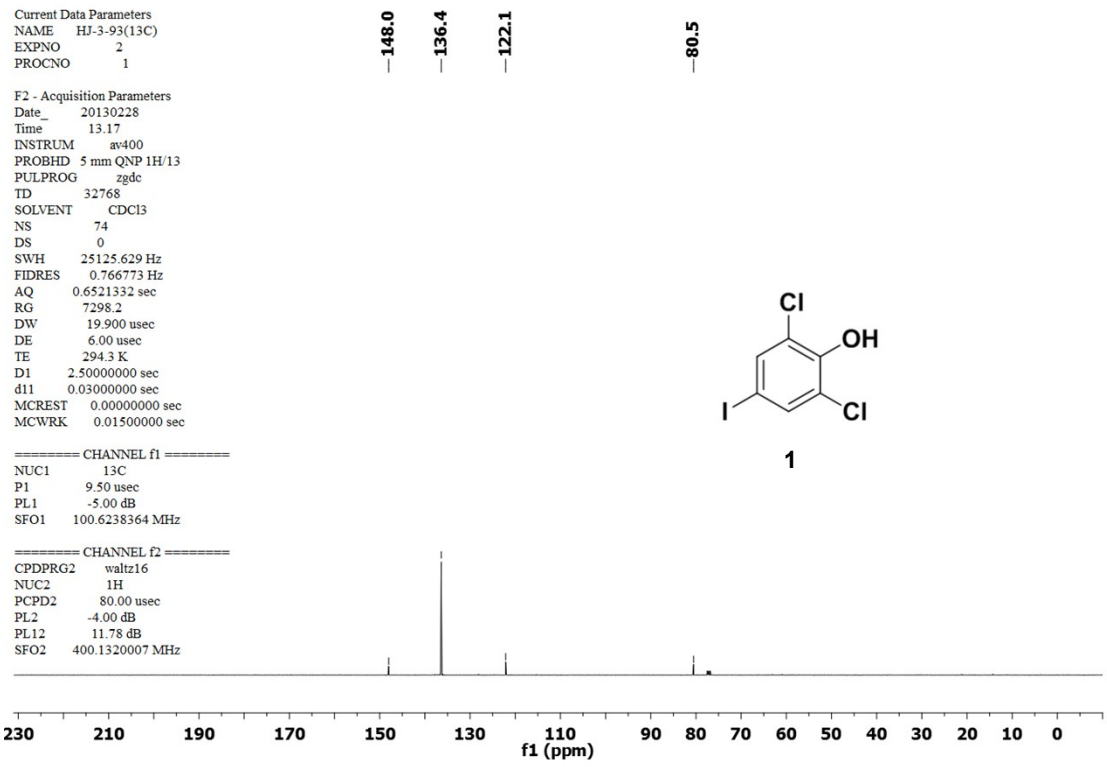
18. Zebrafish culture and imaging

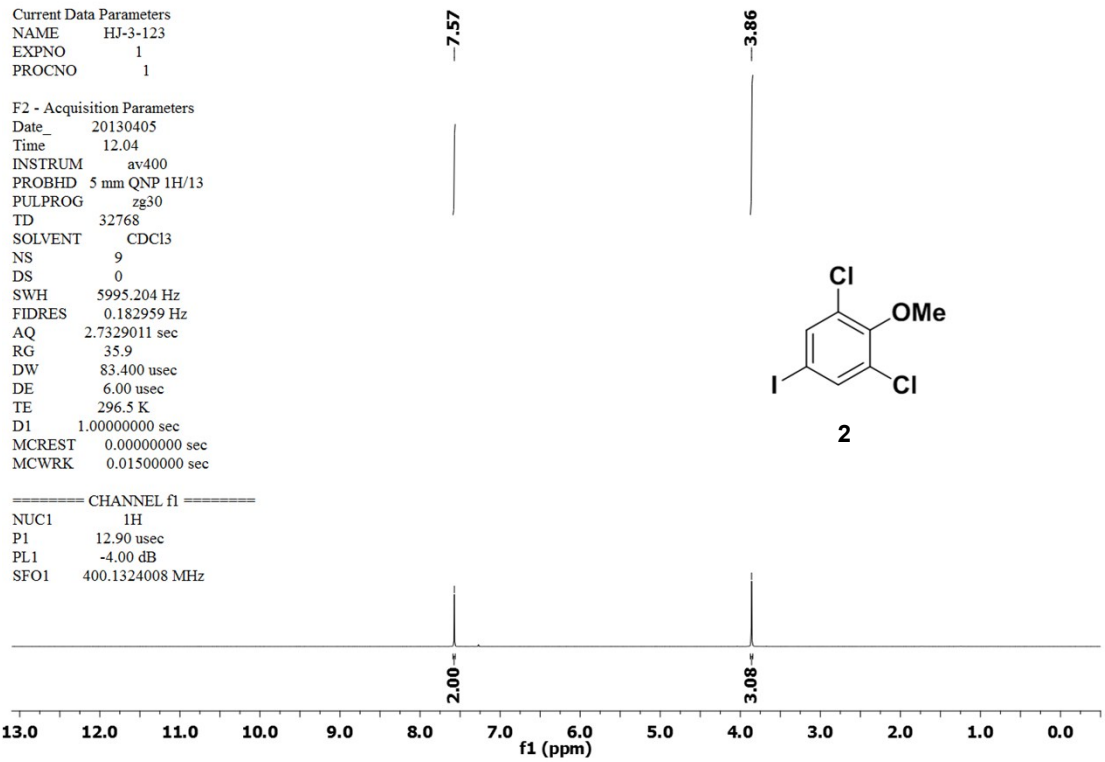
Zebrafish were maintained at 28 °C as described previously.[4] Wild-type zebrafish were obtained from local fish farm. Embryos were obtained by natural spawning and

were maintained in E3 zebrafish water at 28.5 °C and staged according to Kimmel *et al*.[5] The study was conducted according to regulations by the Committee of the Use of Laboratory and Research Animals at The University of Hong Kong. On different post-fertilization days (pfd), zebrafish embryos were incubated with **HKOCl-3** (10 μM) for 30 min, followed by low-magnification imaging with a regular fluorescence microscope. The images were acquired with an Axio Observer.Z1 (Carl Zeiss) fluorescence microscope.

19. NMR Spectra



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PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 9
DS 0
SWH 5995.204 Hz
FIDRES 0.182959 Hz
AQ 2.7329011 sec
RG 35.9
DW 83.400 usec
DE 6.00 usec
TE 296.5 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
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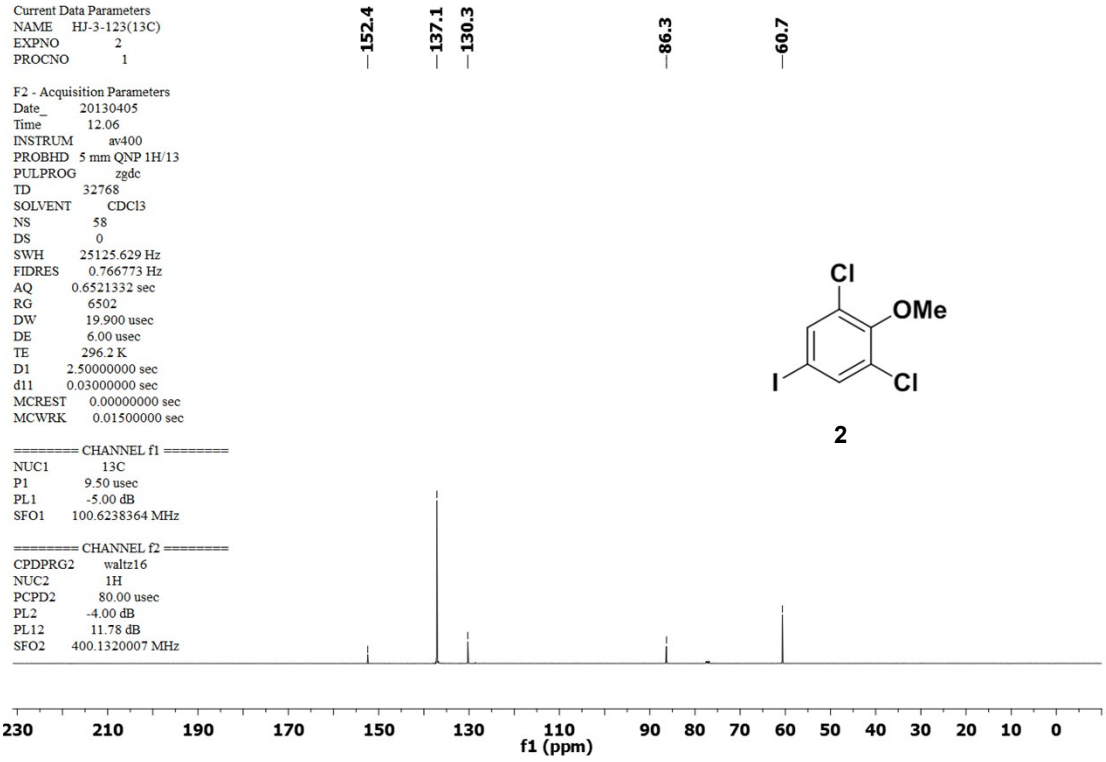
NUC1 1H
P1 12.90 usec
PL1 -4.00 dB
SFO1 400.1324008 MHz
7.57
3.86
2.00
3.08
Cl
OMe
I
Cl
2
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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PULPROG zgdc
TD 32768
SOLVENT CDCl3
NS 58
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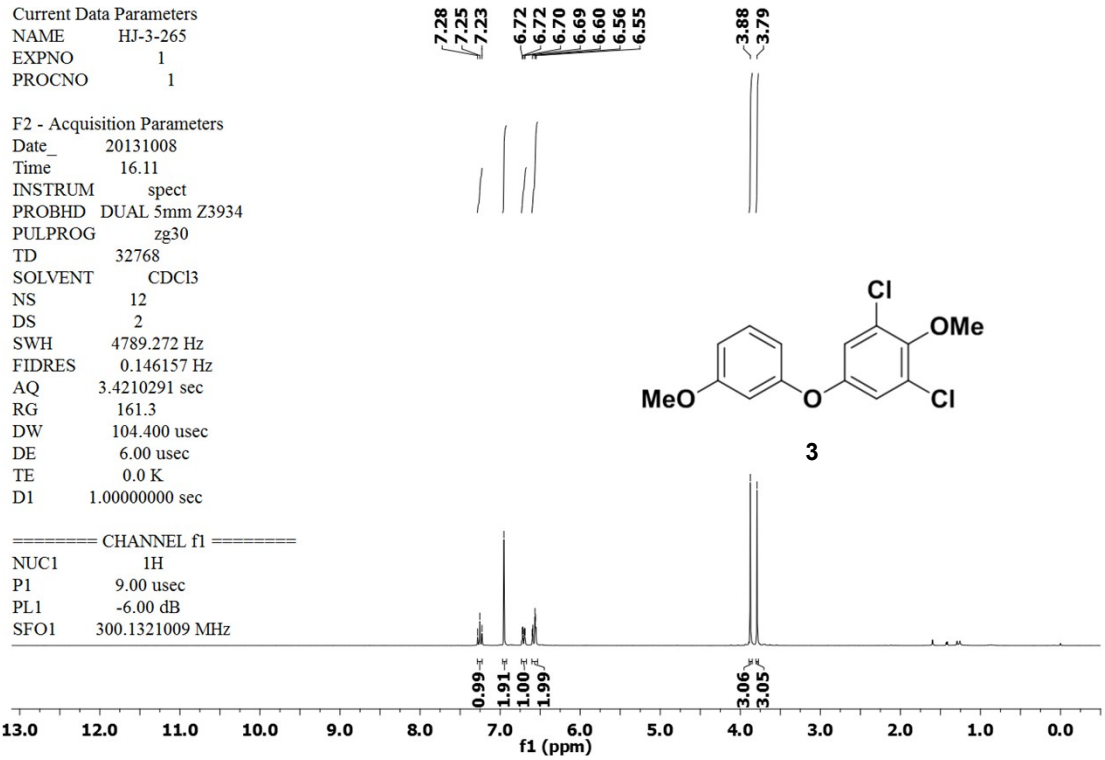
SWH 25125.629 Hz
FIDRES 0.766773 Hz
AQ 0.6521332 sec
RG 6502
DW 19.900 usec
DE 6.00 usec
TE 296.2 K
D1 2.50000000 sec
d11 0.03000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
======== CHANNEL f1 ========
NUC1 13C
P1 9.50 usec
PL1 -5.00 dB
SFO1 100.6238364 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -4.00 dB
PL12 11.78 dB
SFO2 400.1320007 MHz
152.4
137.1
130.3
86.3
60.7
Cl
OMe
I
Cl
2
230
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)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PROCNO 1
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PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 12
DS 2
SWH 4789.272 Hz
FIDRES 0.146157 Hz
AQ 3.4210291 sec
RG 161.3
DW 104.400 usec
DE 6.00 usec
TE 0.0 K
D1 1.00000000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 9.00 usec
PL1 -6.00 dB
SFO1 300.1321009 MHz
7.28
7.25
7.23
6.72
6.72
6.70
6.69
6.60
6.56
6.55
3.88
3.79
0.99
1.91
1.00
1.99
3.06
3.05
Cl
OMe
MeO
O
Cl
3
13.0
12.0
11.0
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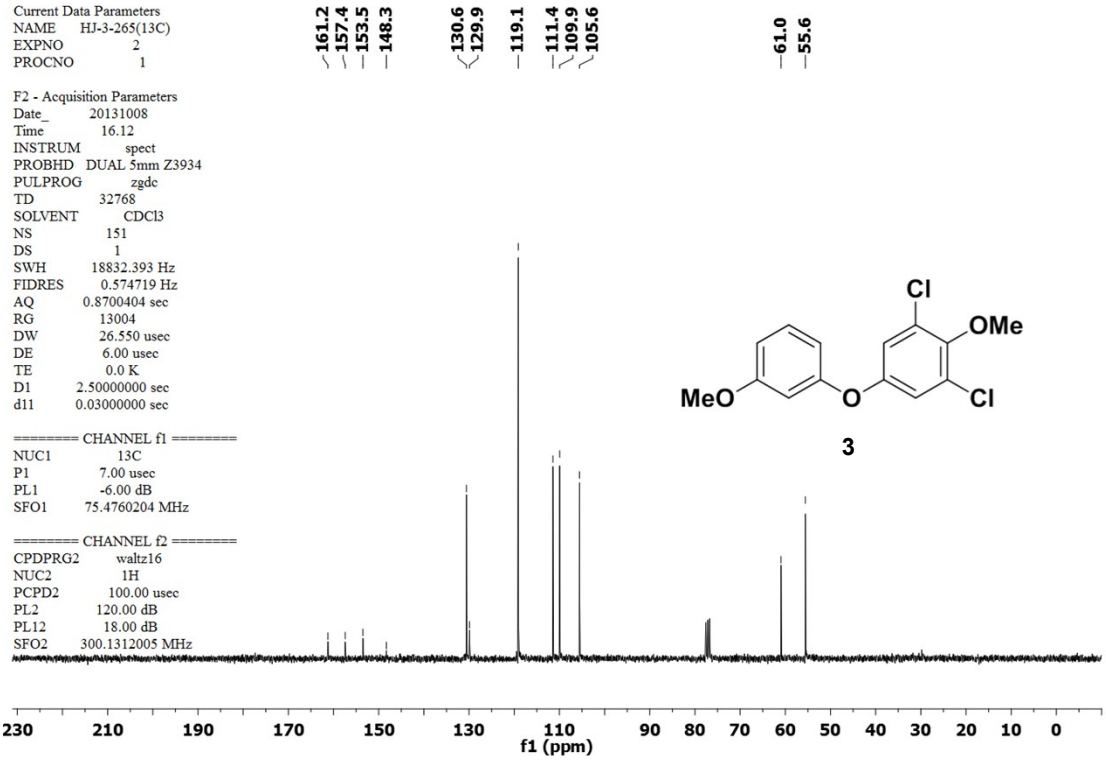
2.0
1.0
0.0
f1 (ppm)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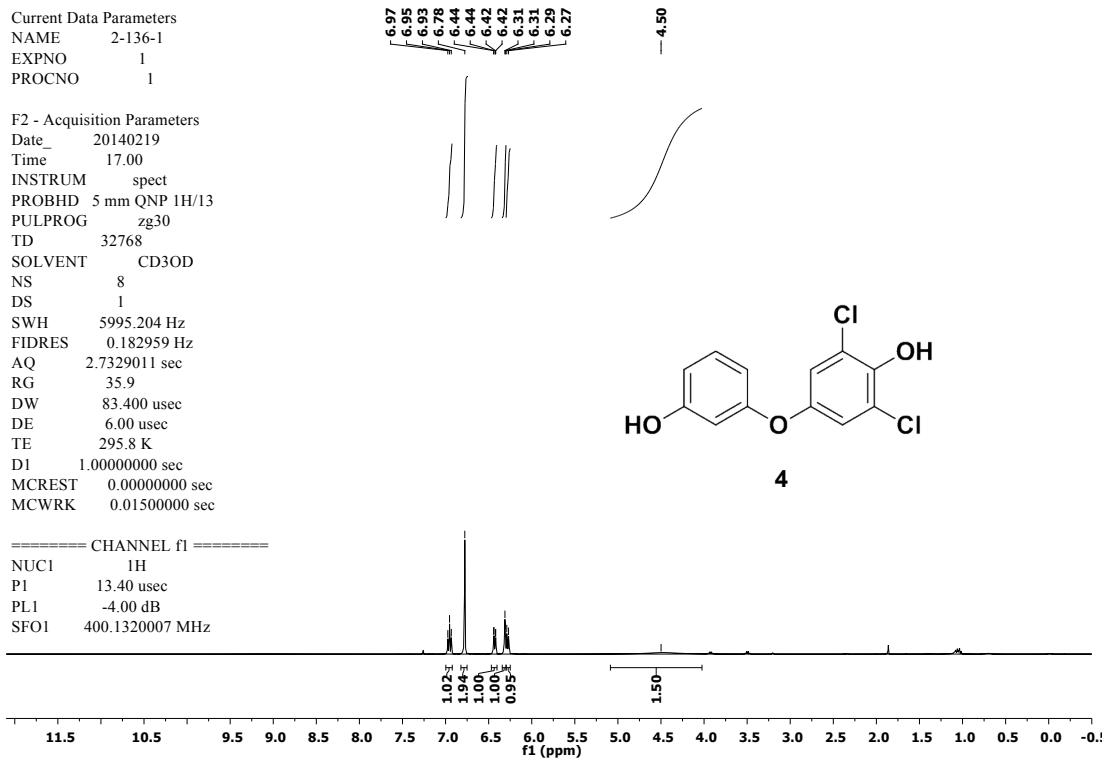
TD 32768
SOLVENT CDCl3
NS 151
DS 1
SWH 18832.393 Hz
FIDRES 0.574719 Hz
AQ 0.8700404 sec
RG 13004
DW 26.550 usec
DE 6.00 usec
TE 0.0 K
D1 2.50000000 sec
d11 0.03000000 sec
======== CHANNEL f1 ========
NUC1 13C
P1 7.00 usec
PL1 -6.00 dB
SFO1 75.4760204 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 120.00 dB
PL12 18.00 dB
SFO2 300.1312005 MHz
161.2
157.4
153.5
148.3
130.6
129.9
119.1
111.4
109.9
105.6
61.0
55.6
Cl
OMe
MeO
O
Cl
3
230
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)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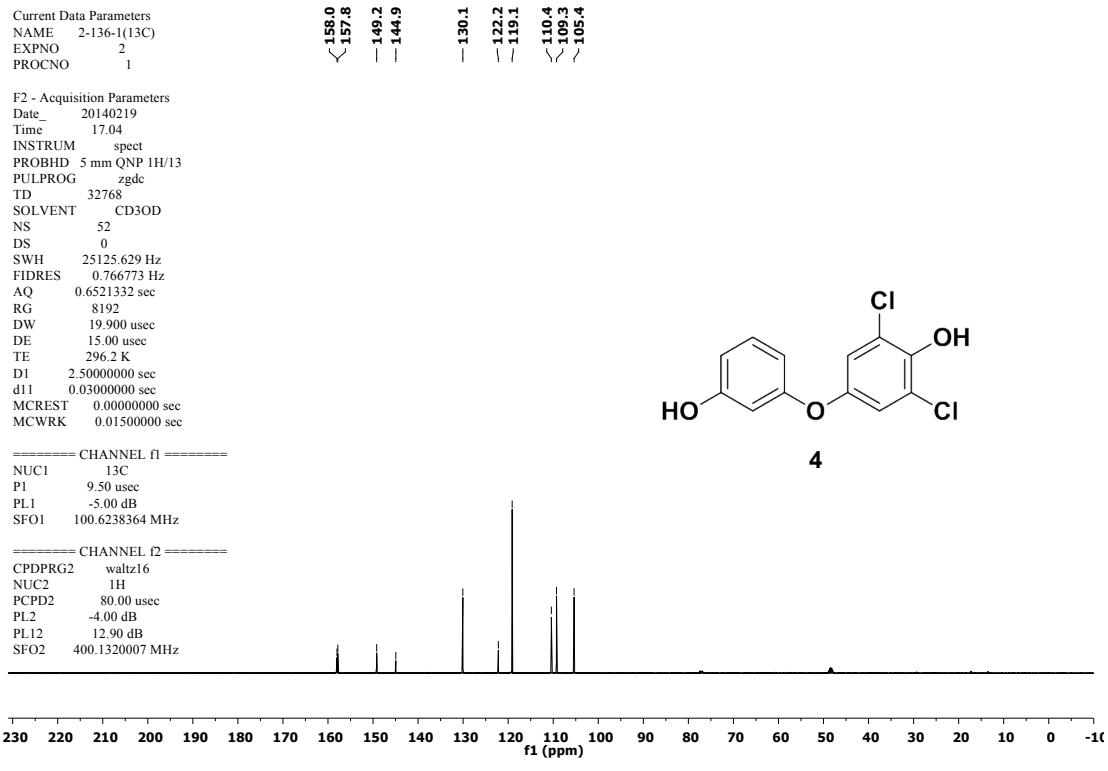
PULPROG zg30
TD 32768
SOLVENT CD3OD
NS 8
DS 1
SWH 5995.204 Hz
FIDRES 0.182959 Hz
AQ 2.7329011 sec
RG 35.9
DW 83.400 usec
DE 6.00 usec
TE 295.8 K
D1 1.00000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 13.40 usec
PL1 -4.00 dB
SFO1 400.1320007 MHz
6.97
6.95
6.93
6.78
6.44
6.44
6.42
6.42
6.31
6.31
6.29
6.27
4.50
1.02
1.94
1.00
1.00
0.95
1.50
Cl
OH
HO
O
Cl
4
f1 (ppm)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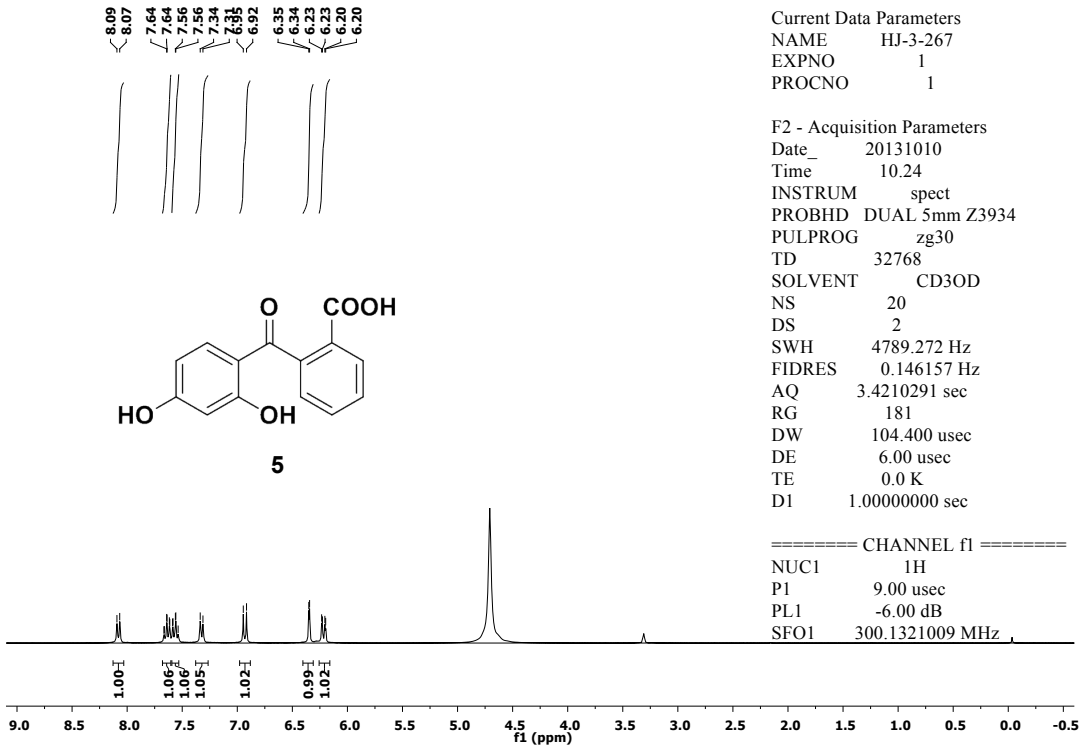
PULPROG zgdc
TD 32768
SOLVENT CD3OD
NS 52
DS 0
SWH 25125.629 Hz
FIDRES 0.766773 Hz
AQ 0.6521332 sec
RG 8192
DW 19.900 usec
DE 15.00 usec
TE 296.2 K
D1 2.50000000 sec
d11 0.03000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec
======== CHANNEL f1 ========
NUC1 13C
P1 9.50 usec
PL1 -5.00 dB
SFO1 100.6238364 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -4.00 dB
PL12 12.90 dB
SFO2 400.1320007 MHz
158.0
157.8
149.2
144.9
130.1
122.2
119.1
110.4
109.3
105.4
Cl
OH
HO
O
Cl
4
f1 (ppm)



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PROCNO 1

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PULPROG zg30
TD 32768
SOLVENT CD3OD
NS 20
DS 2
SWH 4789.272 Hz
FIDRES 0.146157 Hz
AQ 3.4210291 sec
RG 181
DW 104.400 usec
DE 6.00 usec
TE 0.0 K
D1 1.00000000 sec

======== CHANNEL f1 ========
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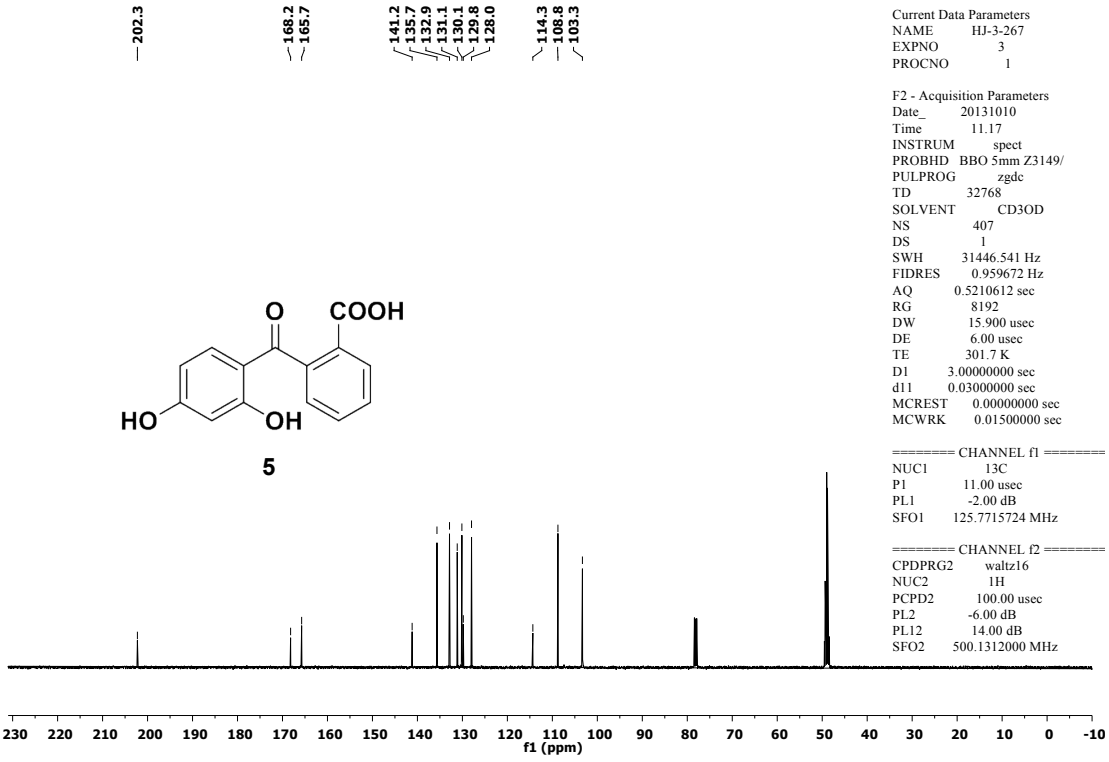
P1 9.00 usec
PL1 -6.00 dB
SFO1 300.1321009 MHz



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PROCNO 1

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PULPROG zgdc
TD 32768
SOLVENT CD3OD
NS 407
DS 1
SWH 31446.541 Hz
FIDRES 0.959672 Hz
AQ 0.5210612 sec
RG 8192
DW 15.900 usec
DE 6.00 usec
TE 301.7 K
D1 3.00000000 sec
d11 0.03000000 sec
MCREST 0.00000000 sec
MCWRK 0.01500000 sec

======== CHANNEL f1 ========
NUC1 13C
P1 11.00 usec
PL1 -2.00 dB
SFO1 125.7715724 MHz

======== CHANNEL f2 ========
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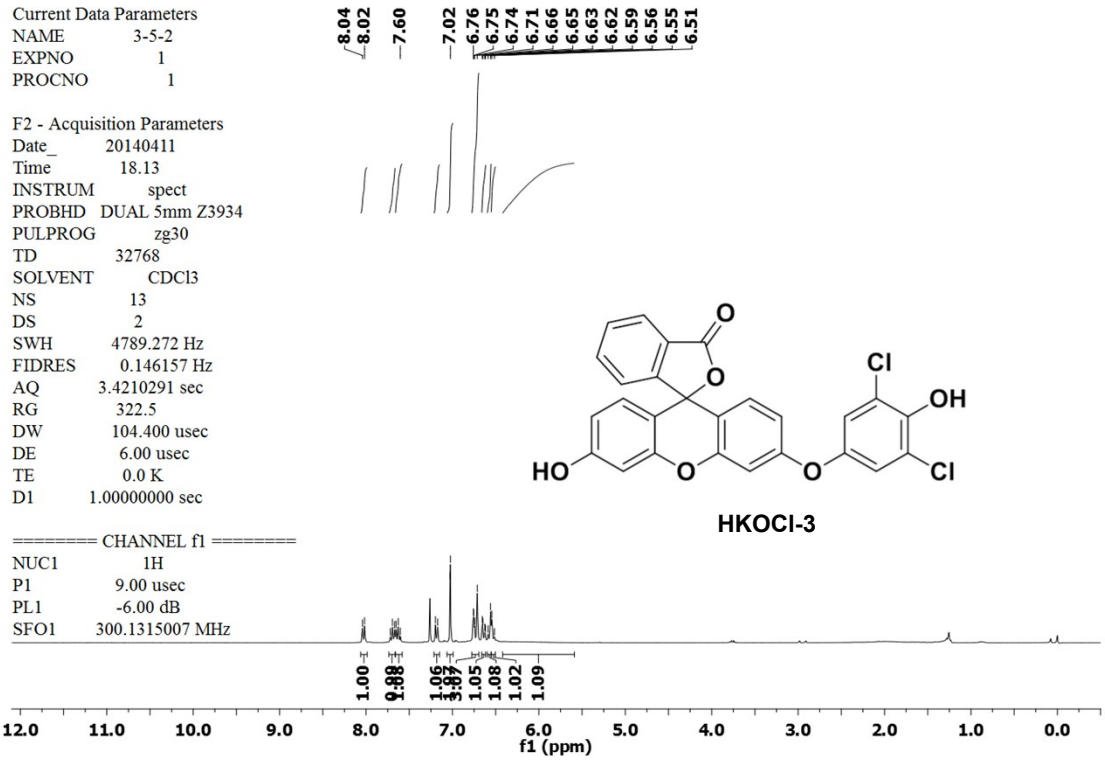
NUC2 1H
PCPD2 100.00 usec
PL2 -6.00 dB
PL12 14.00 dB
SFO2 500.1312000 MHz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PROCNO 1
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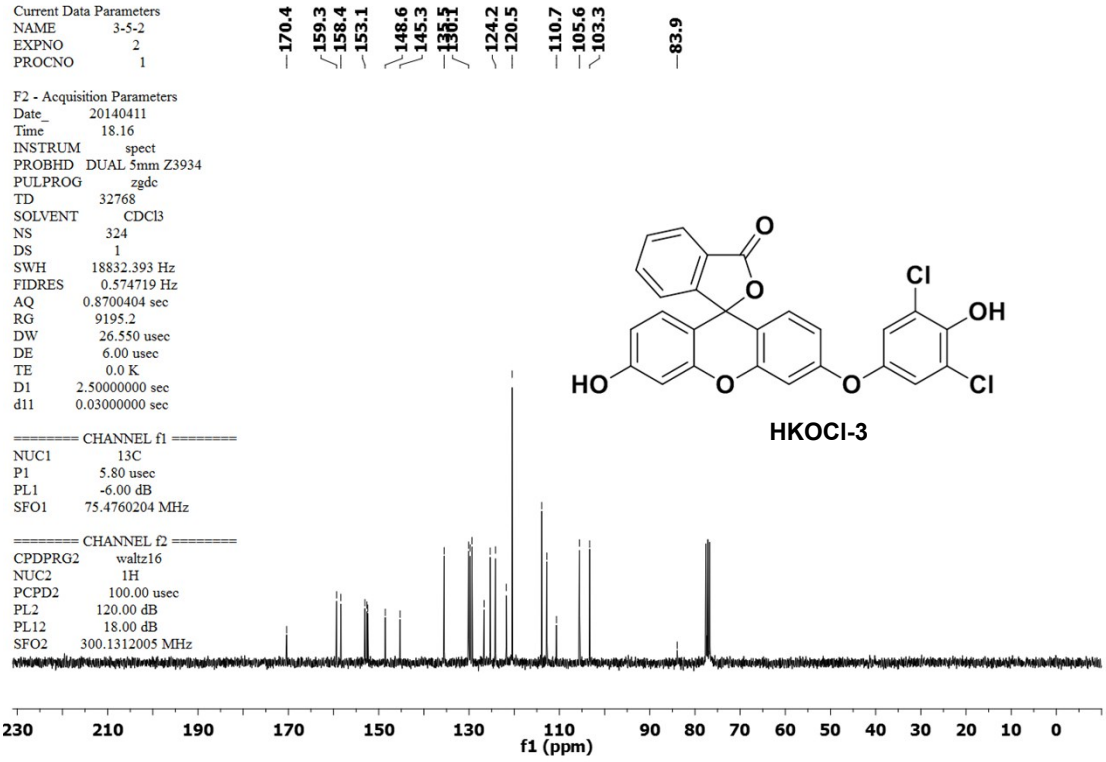
PROBHD DUAL 5mm Z3934
PULPROG zg30
TD 32768
SOLVENT CDCl3
NS 13
DS 2
SWH 4789.272 Hz
FIDRES 0.146157 Hz
AQ 3.4210291 sec
RG 322.5
DW 104.400 usec
DE 6.00 usec
TE 0.0 K
D1 1.00000000 sec
======== CHANNEL f1 ========
NUC1 1H
P1 9.00 usec
PL1 -6.00 dB
SFO1 300.1315007 MHz
8.04
8.02
7.60
7.02
6.76
6.75
6.74
6.71
6.66
6.65
6.63
6.62
6.59
6.56
6.55
6.51
1.00
1.06
1.05
1.08
1.02
1.09
HO
Cl
OH
Cl
HKOCl-3
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
f1 (ppm)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PROCNO 1
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TD 32768
SOLVENT CDCl3
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FIDRES 0.574719 Hz
AQ 0.8700404 sec
RG 9195.2
DW 26.550 usec
DE 6.00 usec
TE 0.0 K
D1 2.50000000 sec
d11 0.03000000 sec
======== CHANNEL f1 ========
NUC1 13C
P1 5.80 usec
PL1 -6.00 dB
SFO1 75.4760204 MHz
======== CHANNEL f2 ========
CPDPRG2 waltz16
NUC2 1H
PCPD2 100.00 usec
PL2 120.00 dB
PL12 18.00 dB
SFO2 300.1312005 MHz
170.4
159.3
158.4
153.1
148.6
145.3
135.5
130.1
124.2
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103.3
83.9
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OH
Cl
HKOCl-3
230
210
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

20. Tables

Table S1. Fluorescence responses of probe **HKOCl-3** toward various ROS/RNS

Entry	Fluorescence Intensity (au)
Blank (probe only; 10 μM)	**9.8**
H_2O_2 (100 μM)	42.3
$^1O_2$ (100 μM)	39.6
ROO$^\bullet$ (100 μM)	40.3
TBHP (100 μM)	37.0
$^\bullet$NO (100 μM)	37.1
$O_2^{\bullet-}$ (100 μM)	40.1
$^\bullet$OH (100 μM)	33.1
$ONOO^-$ (100 μM)	42.5
HOCl (100 μM)	**3524**

Table S2. Comparison of performance of recently published fluorescent probes for HOCl imaging[a]

Probe	Sensitivity[b]	Selectivity[c]
HySOx[6]	> 50-fold (2.5 equiv)	~10-fold over •OH
MMSiR[7]	> 50-fold (1.0 equiv)	>20-fold
Rhodamine B thiospirolactone[8]	> 50-fold (0.4 equiv)	>20-fold
R19-S[9]	> 50-fold (1.0 equiv)	>20-fold
FBS[10]	> 50-fold (10 equiv)	>20-fold
$[Ru(byp)_2(DNPS\text{-}bpy)]\text{-}[PF_6]_2$[11]	~190-fold (5.0 equiv)	>20-fold
$[Ru(byp)_2(AN\text{-}bpy)]\text{-}[PF_6]_2$[12]	~110-fold (5.0 equiv)	>20-fold
Cou-Rho-HOCl[13]	I_{594}/I_{473} > 50-fold (20 equiv)	~13-fold over •OH
HKOCl-1[14]	~1079-fold (1.0 equiv)	~19-fold over $ONOO^-$
HKOCl-2b[15]	~908-fold (2.0 equiv)	>20-fold
Flu-1[16]	~61-fold (20 equiv)	>20-fold
Bodipy-OX[17]	~48-fold (40 equiv)	>20-fold
BClO[18]	~100-fold (5.0 equiv)	>20-fold
SeCy7[19]	~19.4-fold (2.0 equiv)	~4.8-fold over $^1O_2$
HCSe[20]	> 50-fold (1.0 equiv)	>20-fold
CM1[21]	> 50-fold (7.0 equiv)	>20-fold
Lyso-NI-Se[22]	~22-fold (1.6 equiv)	~5.4-fold over $ONOO^-$
ThioRB-FITC-MSN[23]	I_{586}/I_{526} ~7-fold[d]	--
PZ-Py[24]	~40.5-fold (10 equiv)	>20-fold
Ir2[25]	~12.1-fold (200 equiv)	~8-fold
Rh-Py[26]	~380-fold (10 equiv)	>20-fold
TP-HOCl[27]	~679-fold (20 equiv)	>20-fold
HCH[28]	~14.1-fold (25 equiv)	~11-fold over H_2O_2
Probe 1[29]	~50-fold (1.0 equiv)	>20-fold
HKOCl-3[30]	~358-fold (1.0 equiv)	~83-fold

[a] The scope of comparison includes only works published between 2007 and 2015 that involved cell or tissue imaging of HOCl.

[b] Sensitivity was evaluated by fluorescence enhancement for a turn-on probe or emission ratio changes for a ratiometric probe upon treatment of reported amount (equiv) of HOCl. In cases where no actual values were provided in the references, meta-analysis was performed based on the original published figures to give an estimated range, such as >50-fold.

[c] Selectivity was evaluated by fluorescence enhancement of a turn-on probe or emission ratio changes for a ratiometric probe upon the treatment of HOCl over other analytes. Meta-analysis was done based on figures originally presented in the references. In cases where fluorescence enhancement of competing analytes was less than 5% of HOCl's enhancement, a probe is regarded as very selective toward HOCl in imaging, and marked as >20-fold. The major competing analyte is indicated under the selectivity column, if its response toward probe seems significant.

[d] ThioRB-FITC-MSN is based on nanoparticles. Calculations were not possible for the amount (equivalents) of HOCl added.

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