Development of Targetable Two-Photon Fluorescent Probes to Image Hypochlorous Acid in Mitochondria and Lysosome in Live Cell and Inflamed Mouse Model

Lin Yuan[1,2‡], Lu Wang[1‡], Bikram Keshari Agrawalla[1], Sung-Jin Park[3], Hai Zhu[1], Balasubramaniam Sivaraman[3], Juanjuan Peng[3], Qing-Hua Xu[1], Young-Tae Chang[1,3]*

[1]Department of Chemistry, National University of Singapore, Singapore 117543

[2]State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, PR China

[3]Laboratory of Bioimaging Probe Development, Singapore Bioimaging Consortium, Singapore 117543.

Supporting Information

Table of content

1. Supplemental Figures and Tables

Table S1. Properties of respresentive fluorescent HOCl probes

Probes[i]	$\lambda_{ab}/\lambda_{em}$ nm[a] and FEF[b]	Sensing moieties[c]	Detection limit/nM[d]	Reaction time[e]	Solution[f] and worked pH range[g]	Imaging application[h]
N-benzoyl rhodamine B–hydrazide[1]	554/578; --	dibenzoylhydrazine	27	30 min	$Na_2B_4O_7$/NaOH buffer (pH 12); pH range: pH > 9.0	--
HySOx[2]	555/575; --	thioether	--	within seconds	PBS (pH 7.4, 0.10 to 20% DMF); pH range: 4.0–9.0	Endogenous / porcine neutrophil
MMSiR[3]	652/670; --	thioether	--	within seconds	PBS (pH 7.4); pH range: 4.0-9.0	Endogenous / model mouse
Rhodamine B thiospirolactone[4]	555/579; --	internal thioester	300	within 5 min	PBS (pH 7.4, 10% DMSO); pH range: 3.0-8.0	Endogenous / neutrophils and fresh kidney sections
R19-S[5]	--/550; --	internal thioester	--	--	KH_2PO_4 buffer/CH_3CN (pH 5.5, 99:1); --	Endogenous / neutrophils and intestinal epithelia
FBS[6]	~498/523;	internal thioester and boronate	200	within 1 min	KH_2PO_4 buffer (pH 7.4); pH range: ≥ 5.5	Endogenous / intestinal epithelia of *Drosophila*.
$[Ru(bpy)_2(DNPS\text{-}bpy)]\text{-}(PF_6)_2$[7]	450/626; ~190-fold	thioether	53.5	--	PBS (pH 7.4); pH range: 4.5–10.5	Endogenous / RAW 264.7 cells
$Ru(bpy)_2(AN\text{-}bpy)](PF_6)_2$[8]	456/612; ~110-fold	ether	33	within 10 min	borate buffer (pH 7.4); pH range: 3.0–10.0	Endogenous / porcine neutrophils
Cou-Rho-HOCl[9]	410/473-594	thiosemicarbazide	52	within minuties	PBS/DMF (pH 7.4, 1:1); pH range: 3.0–11.0	Endogenous / RAW 264.7 cells
HKOCl-1[10]	--/541; ~1079-fold	p-methoxyphenol.	--	--	PBS (pH 7.5) ; pH range: 5.0-8.0	Endogenous / RAW 264.7 macrophages
HKOCl-2a[11]	--/525; ~765-fold	p-methoxyphenol.	42	within 15 min	PBS (pH 7.4) --	--
HKOCl-2b[11]	--/523; ~900-fold	p-methoxyphenol.	18	within 15 min	PBS (pH 7.4); pH range of 4-10	Endogenous / macrophages
HKOCl-2c[11]	--/525; ~274-fold	p-methoxyphenol.	37	within 15 min	PBS (pH 7.4) --	--
Phenanthroimidazole- oxime[12]	--/509-439; ~9.8-fold (ratio)	oxime	--	within seconds	PBS/DMF (pH 9.0, 1:4); pH range: > 8.5	--
Flu-1[13]	--/530; ~61-fold	oxime	--	within seconds	HEPES/DMSO (pH 7.05, 1:9); pH range: 7.0–10.0	Exogenous / HeLa cell
Bodipy-OX[14]	502/529; ~48-fold	oxime	500	within seconds	PBS/DMF (pH 7.2, 4:1) pH range: 4.0–9.0	Exogenous / MCF10A cell
$Ir(ppy)_2(L_1)](PF_6)$[15]	346/578; ~31-fold	oxime	≈ 13 (1 ppm)	--	DMF/HEPES (pH 7.2, 4 : 1) pH range: 2.0–13.0	--
BClO[16]	500/505; ~100-fold	pyrrole	0.56	within 1 s	PBS/EtOH (pH 7.4, 1:9); pH range: 4.0–9.0	Endogenous / MCF-7 cells
SeCy7[17]	--/786; ~19.4-fold	selenide	310	within dozens of seconds	PBS buffer (pH 7.4); pH range: 4.0-10.0	Endogenous / model mouse

HCSe[18]	--/526; --	selenide	7.98	within 4 minuties	MPBS /CH_3CN (pH7.4, 99/1); pH range: 5.5-8.0	Endogenous / RAW264.7 cells
CM1[19]	405/480; ~63-fold	selenide	10	within seconds	PBS (pH=7.4); pH range: 4.0-9.0	Endogenous / RAW264.7 cells
Lyso-NI-Se[20]	450/540 ~22-fold	selenide	18.5	within seconds	CH_3CN/H2O (pH 5.0, 1:1); pH range: 2.5-8.5	Exogenous / lysosome of RAW 264.7 cell
ThioRB-FITC-MSN[21]	490/526,586 ~7-fold (ratio)	internal thioester	--	within seconds	Na_2HPO_4/citrate buffer (pH 5.0); pH range: 4.5-8.0	Exogenous / lysosome of RAW 264.7 cell
PZ–Py[22]	400/562 ~40.5-fold	thioether	17.9	within seconds	PBS (pH 7.3) pH range: 4.0-10.0	Endogenous / mitochondria of RAW 264.7 cells and nude mice
Ir2[23]	405/565 ~12.8-fold	diaminomaleonitrile-functionalized Schiff base	--	within seconds	DMF/PBS buffer (pH 7.4, 3:7); pH range: 4.0-10.0	Exogenous / mitochondria of RAW 264.7 cells and Hela cells
Rh-Py[24]	544/577 ~380-fold	dibenzoylhydrazine	24	within seconds	PBS (pH 7.4) pH range: 4.0-10.0	Exogenous / mitochondria of HeLa cells and nude mice
TP-HOCl 1[25]	375/500 ~670-fold	1,3-oxathiolane	16.6	within seconds	PBS/EtOH (1:1, PH 7.4) pH range: 4.0-9.0	--
MITO-TP[25]	375/500 ~634-fold	1,3-oxathiolane	17.2	within seconds	PBS (PH 7.4) --	Endogenous / mitochondria of RAW 264.7 cells and inflamed tissues
LYSO-TP[25]	375/500 ~610-fold	1,3-oxathiolane	19.6	within seconds	PBS (PH 5.0) --	Endogenous / lysosome of RAW 264.7 cells and inflamed tissues

[a] Absorption and emission wavelength. [b] The fluorescence enhanced factor or emission ratio changes before and after interaction with HOCl. [c] The recognition moieties that respond to HOCl. [d] The reported detection limit of corresponding probes. [e] The reaction time for responding to HOCl. [f] The solution and pH used for spectrum measurement. [g] The worked pH range for responding to HOCl. [h] Imaging of endogenous or exogenous HOCl. [i] In recent years, some fluorescent probes with high selectivity and sensitivity, fast response, and wide range of working pH have been developed for the detection of HOCl in biological system. The design strategies for developing HOCl probes are based on specific reactions between HOCl and recognition sites, which changes fluorescence intensity significantly before and after reaction. Based on the reference, the recognition moieties include p-methoxyphenol[10,11], dibenzoylhydrazine,[1] oxime,[12-15] selenide,[17-20] thiol (tetrahydrothiophene/thioether[2-3,7,22], internal thioester[4-6,21] and thiosemicarbazide[9]) and other groups.[8,16] Most of the probes can respond to HOCl with good sensitivity (usually dozens of nM) in a very short reaction time (usually from seconds to minutes). Some probes were successfully applied in real-time detection of endogenous HOCl insides cells and a small number of probes were used to image HOCl in inflamed tissues of mice. Among these probes, several organelles (mitochondria[22-24] and lysosome[20-21]) targetable probes have been reported since 2013. The endogenous mitochondrial HOCl was successfully detected by PZ–Py,[22] which was developed by Wang's group. However, the detection of endogenous HOCl in lysosome has not been reported in existing papers, even two lysosomal probes were developed. In addition, no report exists describing a TPM (two-photon microscopy) based probe for direct imaging of endogenous HOCl produced in both live cells and tissues. Therefore, considering greater tissue penetration depths, higher spatial resolution, and lower photo-toxicity of TPM, organelles targetable two-photon probes can be a useful tool to elucidate the functions of HOCl in biological system.

Table S2. Photophysical parameters of fluorophores.

Dye	$\lambda_{max\ OP}$ (nm)[a]	$\varepsilon_{max\ SP}$ ($10^4 cm^{-1}mol^{-1}$)[b]	Φ [c]	$\lambda_{max\ TP}$ (nm)[d]	$\Phi\sigma_{max}$ [GM][e]
1	356	1.26	0.40	750	82
TP-HOCl 1	356	1.23	<0.001	750	n.d.[f]
7	356	1.24	0.41	750	83
MITO-TP	356	1.22	<0.001	750	n.d.[f]
4	356	1.25	0.35	750	74
LYTO-TP	356	1.23	<0.001	750	n.d.[f]
8	356	1.24	<0.001	750	n.d.[f]
9	356	1.27	0.38	750	79

[a] Maximum one photon absorbance wavelength of fluorophores in MeOH. [b] The molar extinction coefficients at λ_{max}. [c] Fluorescence quantum yield. [d] Maximum two photon-excitation wavelength. [e] The peak of two-photon-action cross-section (GM: 10^{-50} cm^4 s $photon^{-1}$). [f] n.d.: not determined. The two-photon emission intensity was too weak to measure the cross-section accurately.

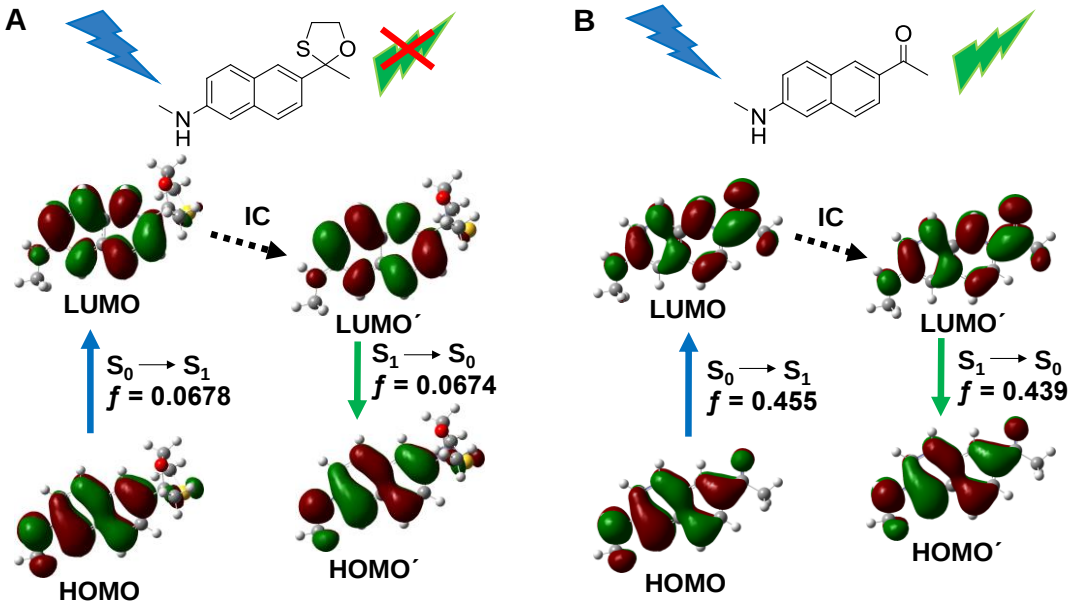


Figure S1. The frontier molecular orbitals (MOs) of **TP-HOCl 1** (A) and compound **1** (B) involved in the vertical excitation (i.e. UV-vis absorption, the left columns) and emission (right columns) shown in solid line. Methanol (CPCM model) was employed as the solvent for the DFT calculations. The vertical excitation related calculations are based on the optimized ground state (S_0 state), the emission related calculations were based on the optimized excited state (S_1 state), at the B3LYP/6-31G (d)/level using Gaussian 09W. IC stands for internal conversion (dotted lines).

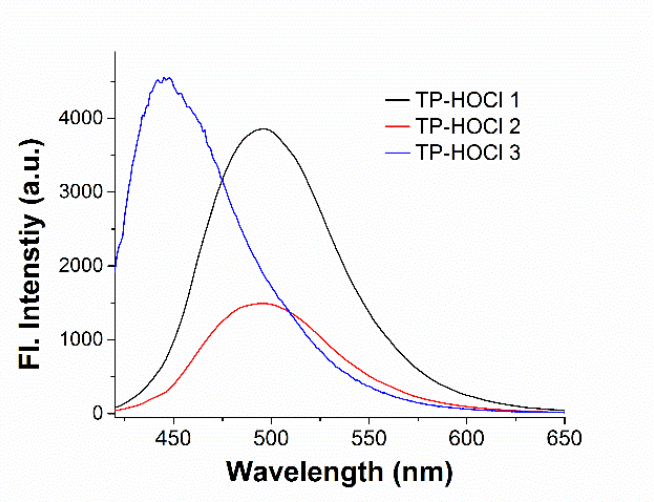


Figure S2. The emission spectra of three probes (5μM) after adding 50 μM HOCl. Excitation wavelength: 375 nm.

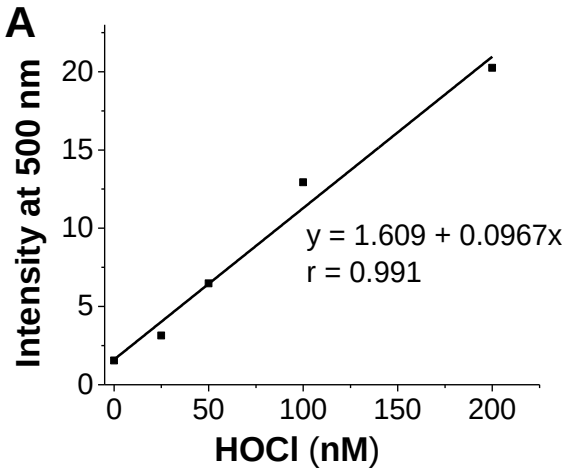


Figure S3. The linear fluorescence response of **TP-HOCl 1** to the concentration of HOCl at the nanomolar concentration range (0-200 nM).

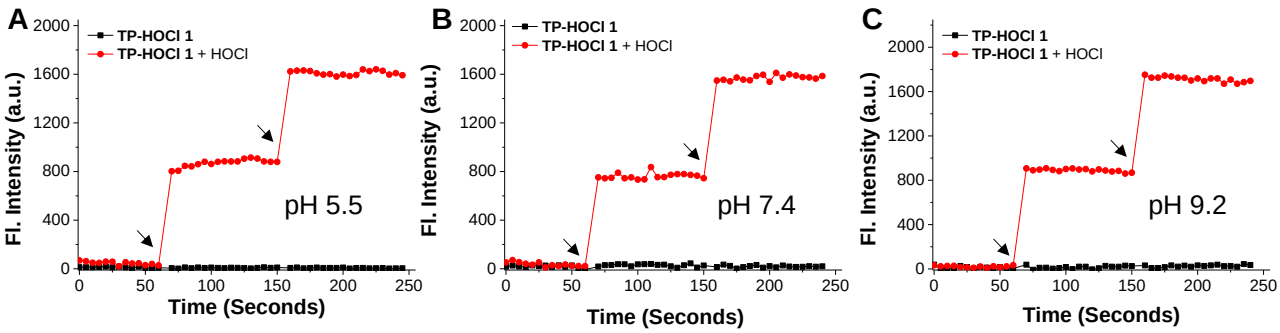


Figure S4. **TP-HOCl 1** can respond to HOCl within seconds at pH 5.5 (A), 7.4 (B) and 9.2 (B). Arrows indicate the addition of 25 μM HOCl.

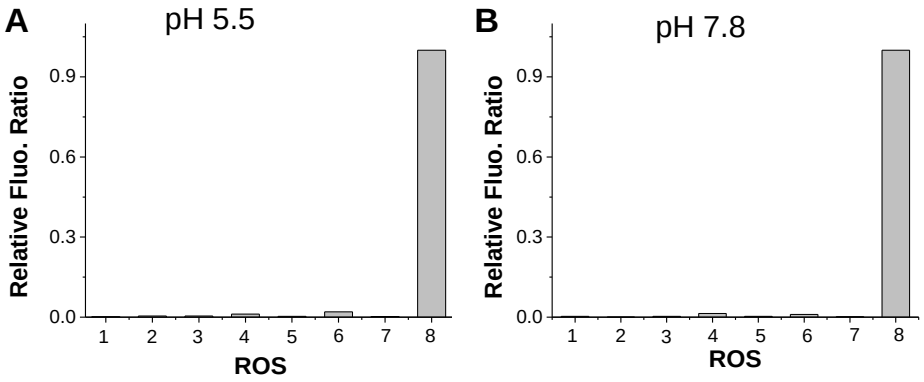


Figure S5. Fluorescence response of **TP-HOCl 1** (5 μM) towards various ROS (50 μM for HOCl (8) and 250 μM for other ROS/RNS (1-7)) at pH 5.5 (A) and 7.8 (B). 1) H_2O_2; 2) •OH; 3) O^{2-}; 4) NO•; 5) *t*-BuOOH; 6) *t*-BuOO•; 7) $ONOO^-$; 8) HOCl.

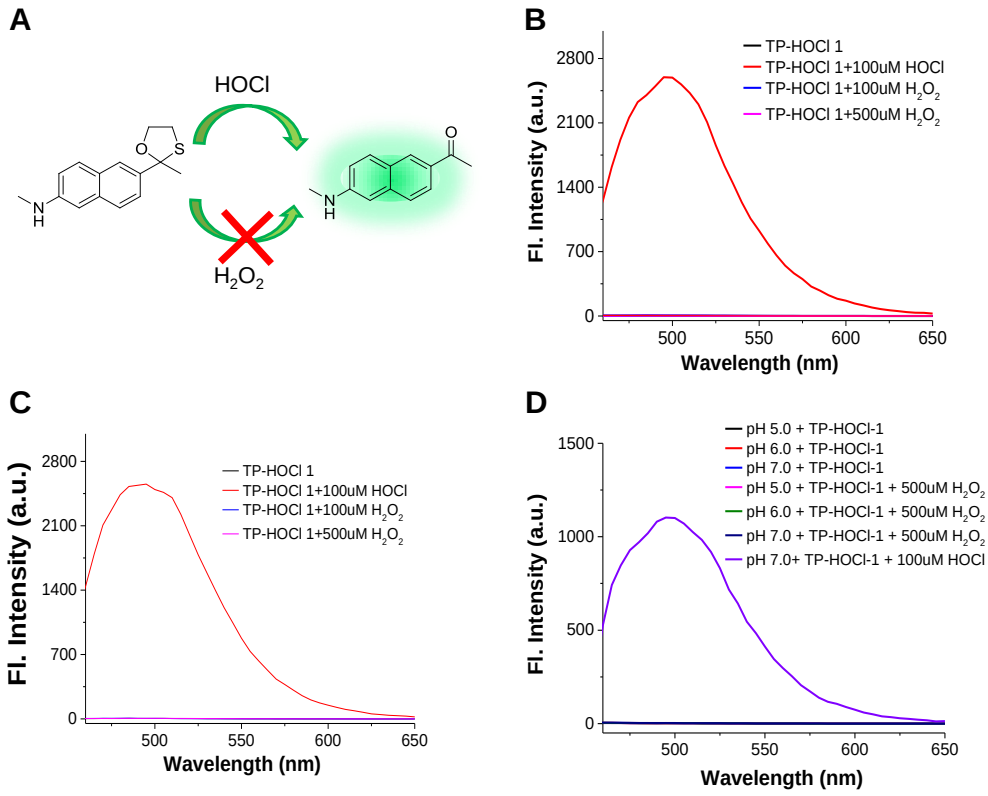


Figure S6. The fluorescence spectra of **TP-HOCl 1** (5 μM) after adding HOCl (100 μM) and H_2O_2 (100 and 500 μM) in EtOH (B) and PBS/MeOH (1 : 1) solution (C). D) **TP-HOCl 1** kept stable in the presence of H_2O_2 (500 μM) in PBS (pH 5.0, 6.0, 7.0).

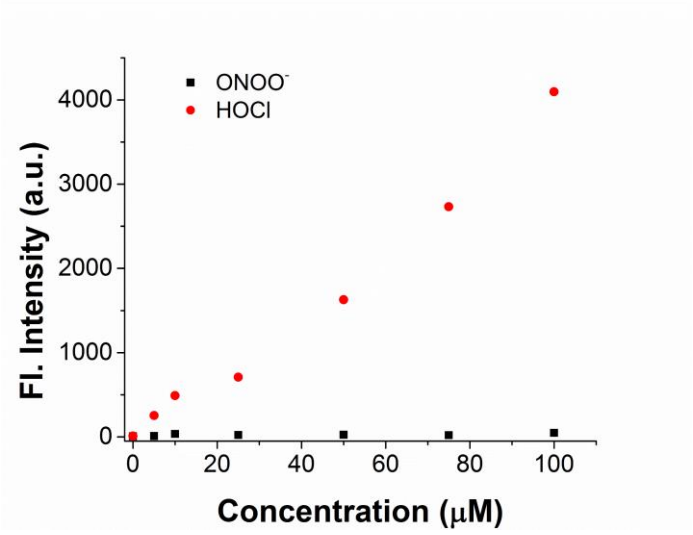


Figure S7. Fluorescence intensity of **TP-HOCl 1** (5 μM) before and after adding HOCl (●) or $ONOO^-$ (■) at different concentrations (0 to 100 μM).

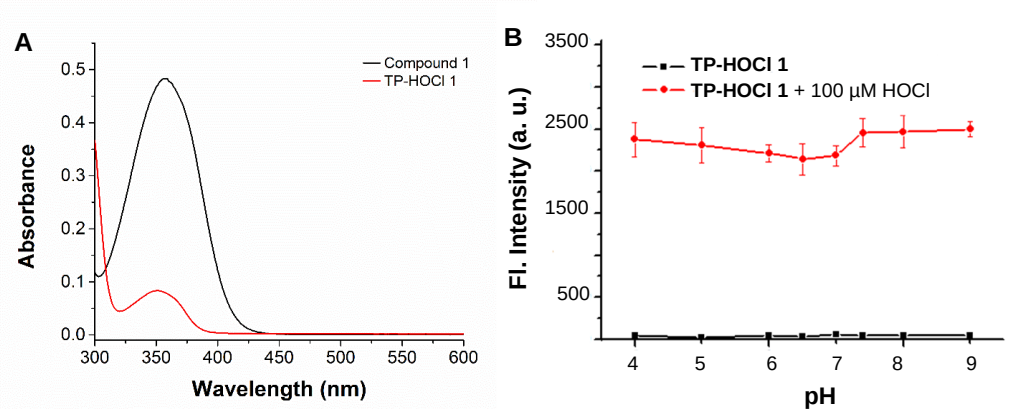


Figure S8. A) The absorption spectra of **TP-HOCl 1** and de-protected compound **1** in MeOH. B) The fluorescence spectra of **TP-HOCl 1** before and after addition of HOCl in PBS buffer (pH 4 to 9), data acquired at 20 seconds after addition of HOCl (λ_{ex} = 375 nm).

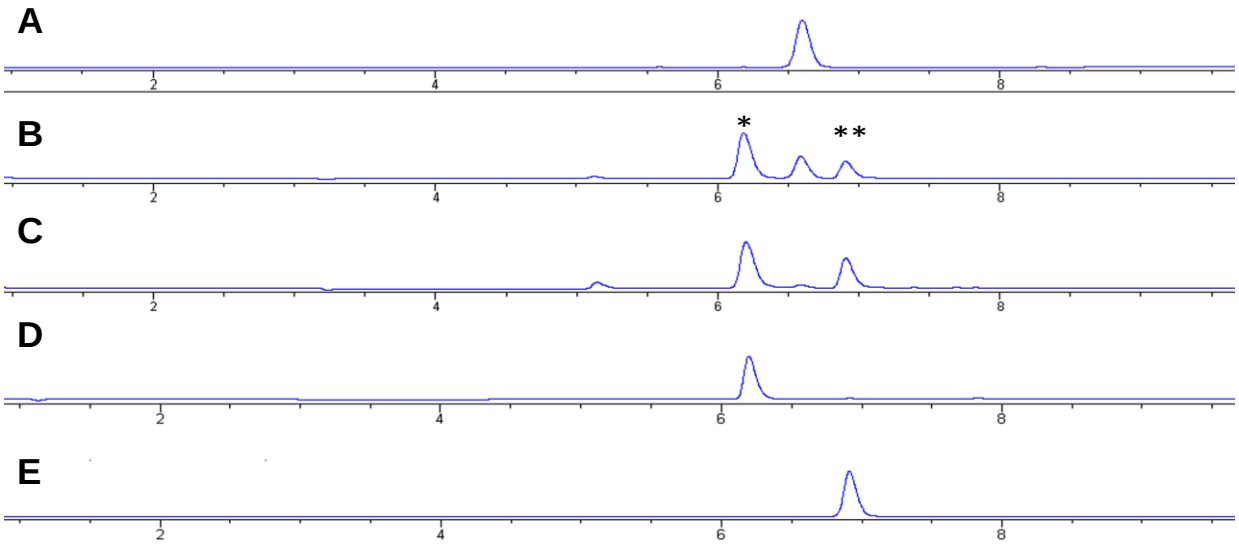


Figure S9. Reversed-phase HPLC chromatograms with absorption (350 nm) detection. The * and ** indicate compound **1** and chlorinated fluorophore (compound **9**), respectively. A) Reversed-phase HPLC chromatograms of 100 μM **TP-HOCl 1**. B) Reversed-phase HPLC chromatograms of 100 μM **TP-HOCl 1** in the presence of 100 μM HOCl for 1 min. C) Reversed-phase HPLC chromatograms of 100 μM probe **TP-HOCl 1** in the presence of 200 μM HOCl for 1 min. D) Reversed-phase HPLC chromatograms of 50 μM compound **1**. E) Reversed-phase HPLC chromatograms of 50 μM chlorinated fluorophore (compound **9**).

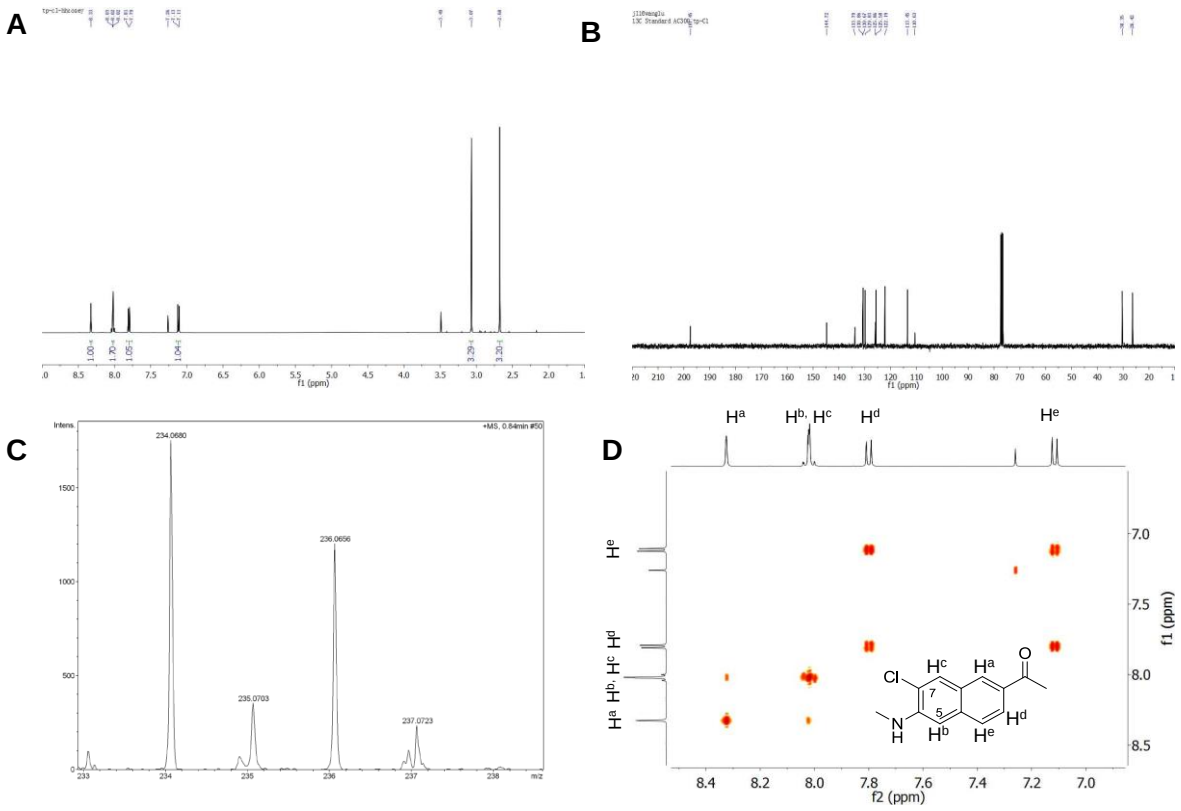


Figure S10. ^{1}H NMR, ^{13}C NMR, HRMS and H-H COSY spectrum of compound **9.** Assignments and proton connectivity of compound **9** were based on vicinal and long-distance *J*-coupling in COSY experiments. All the connections between adjacent protons were observed. The peak at 8.325 ppm was assigned to H^a, which has a long distance *J*-coupling with H^c (δ = 8.023 (d, *J* = 1.5 Hz, 1H)). The single peak at 8.018 ppm was assigned to H^b. The correlated proton resonances at 7.800 ppm and 7.115 ppm were assigned to H^d and H^e respectively.

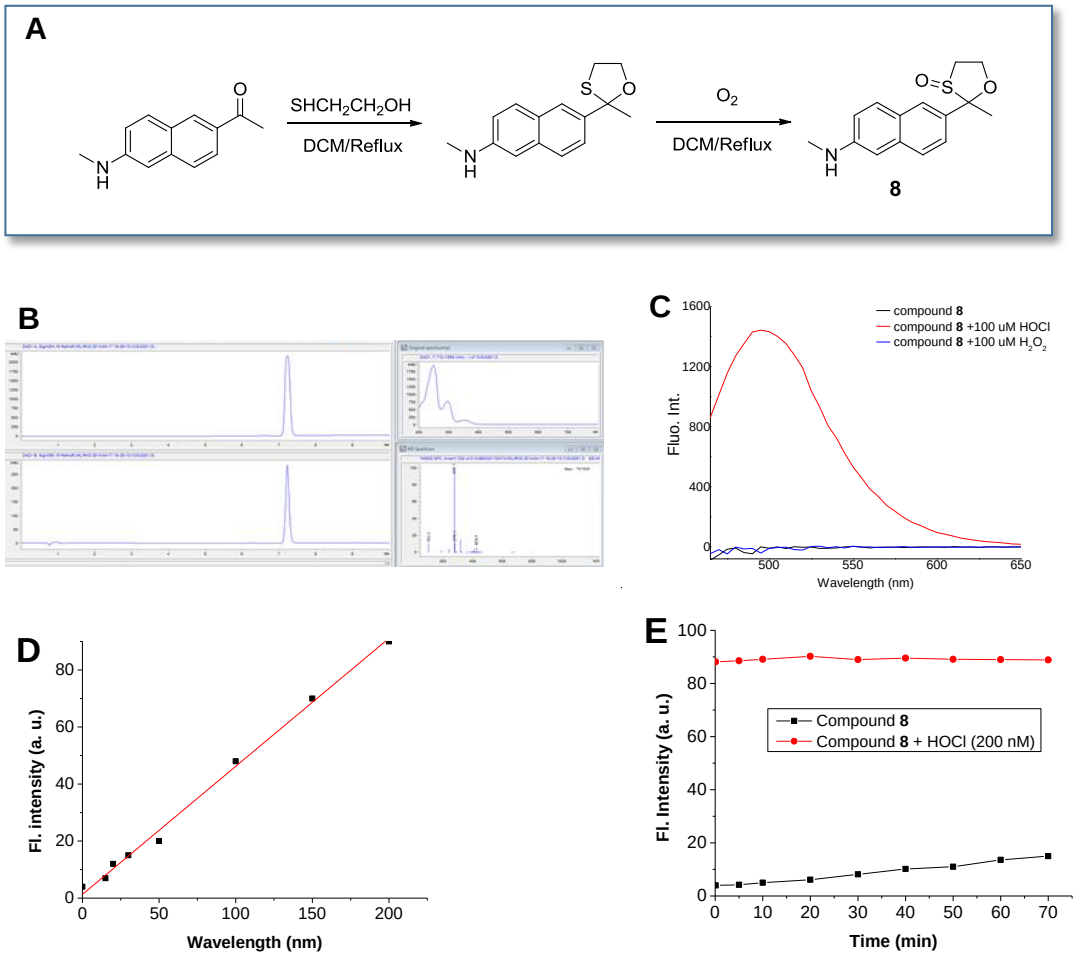


Figure S11. A) Synthetic route toward intermediate **8**. B) The purity and molecular mass of compound **8** was confirmed by Liquid chromatography–mass spectrometry (LC-MS). C) The fluorescence enhancement of compound **8** responding to HOCl rather than H_2O_2 at 100 μM. Probe concentration: 10 μM. Excitation wavelength: 375 nm. D) The fluorescence response of compound **8** to HOCl (15 nM – 200 nM) in PBS/EtOH (1:1, pH 7.4), with the detection limit about 10.3 nM for HOCl. E) Fluorescence intensity of compound **8** and compound **8** in the presence of HOCl (200 nM) with time changes.

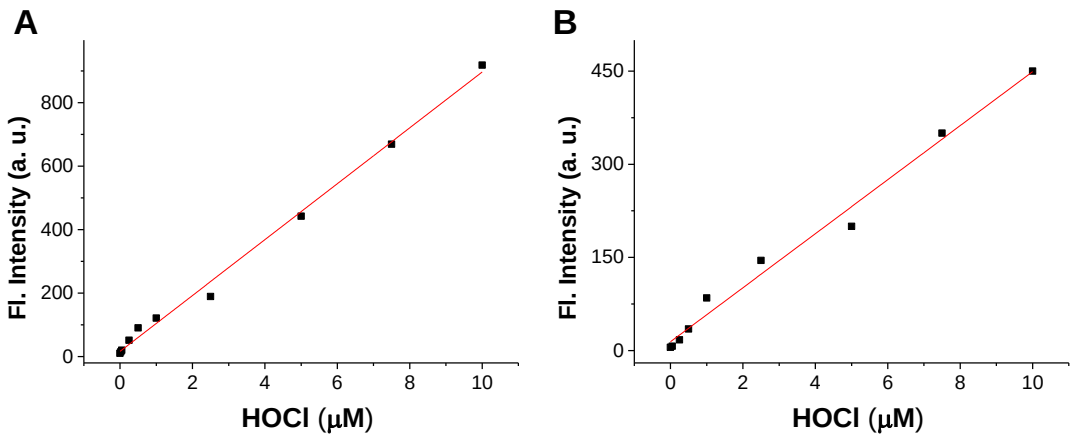


Figure S12. A) The fluorescence response of **MITO-TP** to HOCl (25 nM – 10 μM) in pH 7.4 PBS buffer (1% DMSO). B) The fluorescence respond of **LYSO-TP** to HOCl (50 nM - 10 μM) in pH 5.0 PBS buffer (1% DMSO). Excitation wavelength: 375 nm.

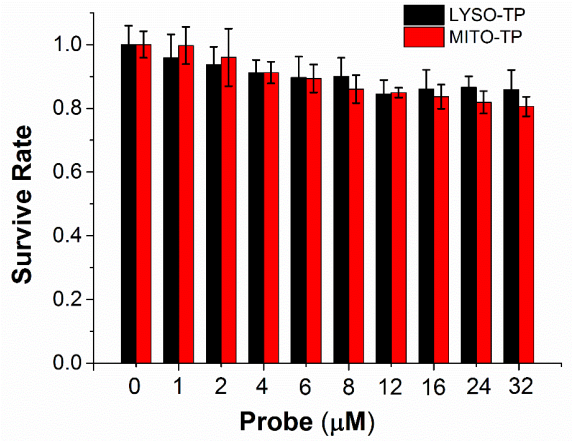


Figure S13. Cytotoxic effect of **MITO-TP** and **LYSO-TP**. HeLa cells were incubated with each concentration of probes for 12 h. Cell viability was assayed with MTT test. Results are expressed as mean ± standard deviation of 5 independent experiments.

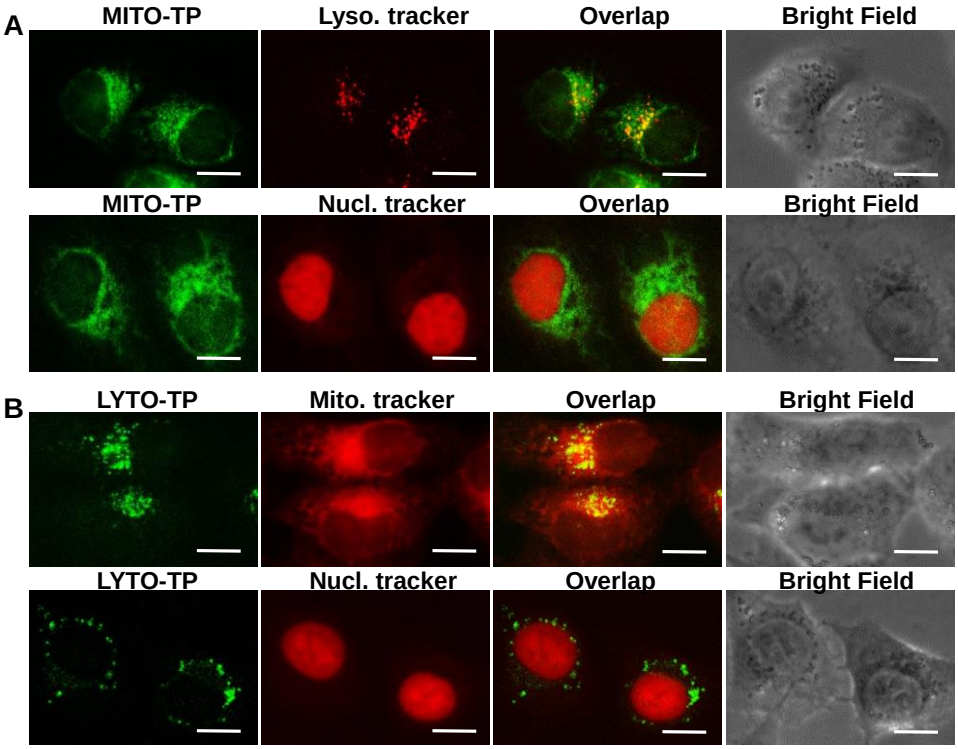


Figure S14. Intracellular localization of **MITO-TP** (A) and **LYSO-TP** (B) in HeLa cells. A) Images of HeLa cells pre-treated respectively with 10 μM **MITO-TP** for 20 min and subsequently 1 μM Lyso-Tracker Red (or 1 μM red cell permeable DNA dye Vybrant® DyeCycle™ Ruby) for 10 min. Then 50 μM HOCl was treated for another 5 min. B) Images of HeLa cells pre-treated respectively with 10 μM **LYSO-TP** for 20 min and subsequently 1 μM Mito-Tracker Red (or 1 μM red cell permeable DNA dye Vybrant® DyeCycle™ Ruby) for 10 min. Then 50 μM HOCl was treated for another 5 min. Green: probe fluorescence; red: Mito-Tracker, Lyso-Tracker and Nucl-Tracker fluorescence (Vybrant® DyeCycle™ Ruby); yellow: merged signal. Scale bar: 10 μM.

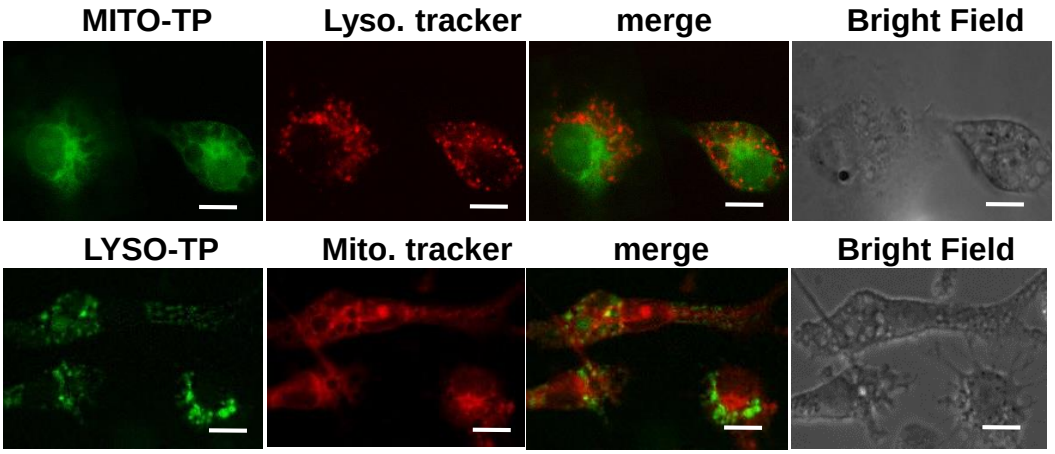


Figure S15. Intracellular distribution of **MITO-TP** and **LYSO-TP** in LPS/IFN-γ-stimulated macrophage cells. RAW 264.7 cells was pre-treated with LPS (120 ng/mL) / IFN-γ (20 ng/mL) for 24 h, then treated with 10 μM **MITO-TP** (or 10 μM **LYSO-TP**) for 20 min and subsequently 1 μM Lyso-Tracker Red (or 1 μM Mito-Tracker Red) for another 10 min. Green: probe fluorescence; red: Mito-Tracker and Lyso-Tracker fluorescence; yellow: merged signal. Scale bar: 10 μM.

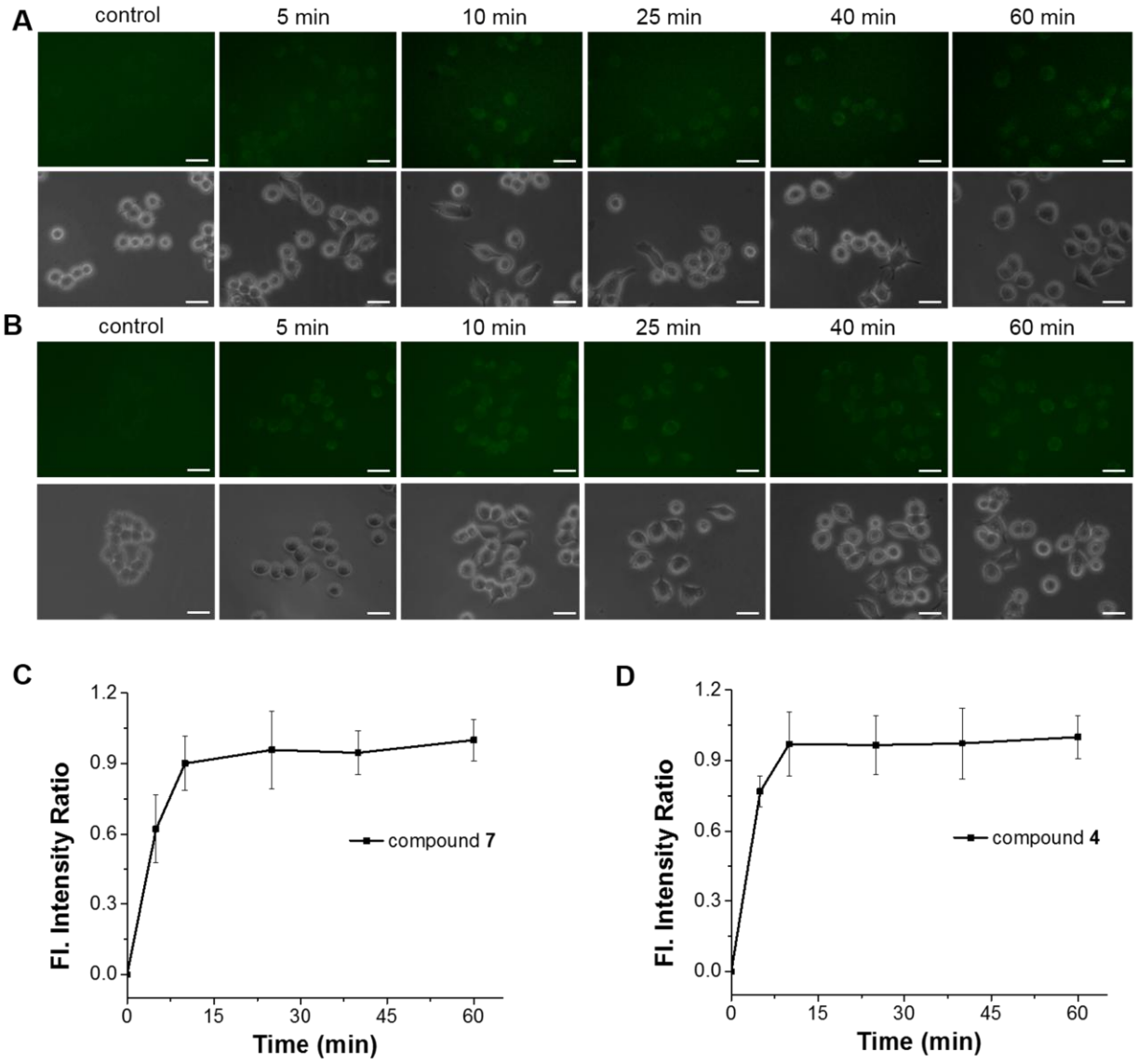


Figure S16. Fluorescent imaging of RAW cells incubated with compound **7** and **4** (precursor of **MITO-TP** and **LYSO-TP**) for different times. A) Compound **7** (10 μM) was incubated with Raw cells for different times and washed by pH 7.4 PBS prior to image. B) Compound **4** (10 μM) was incubated with Raw cells for different times and washed by pH 7.4 PBS prior to image. C, D). Time course of fluorescent intensity ratio of RAW cells after incubation with compound **7** and **4**. The intensities were normalized to that at 60 min. Bright field images were shown below. Error bars are ± SD. n = 3. Scale bar: 30 μM.

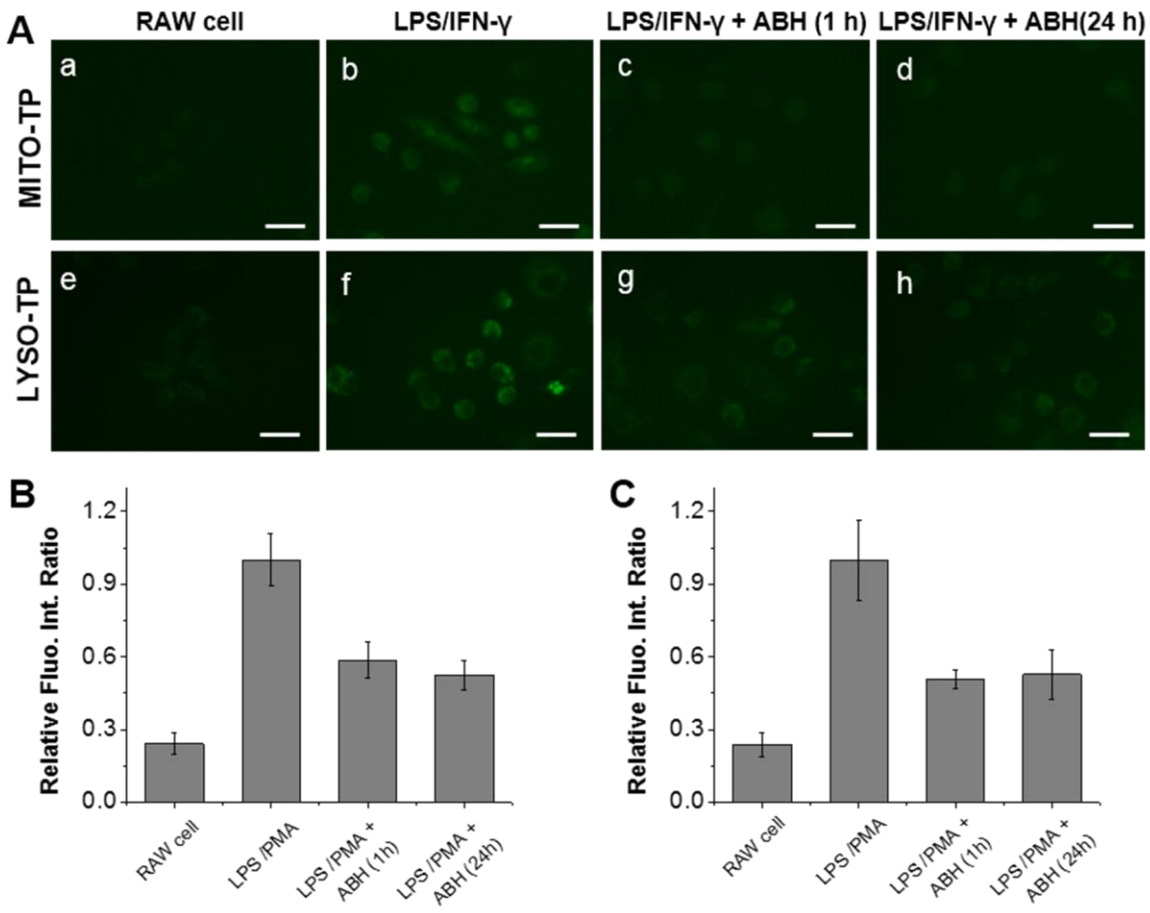


Figure S17. Detection of endogenous HOCl in live RAW 264.7 macrophage cells via **MITO-TP** and **LYSO-TP**. A) Representative fluorescent images of macrophage cells. a, e) **MITO-TP** or **LYSO-TP** (10 μM) was incubated with macrophages cells for 30 min. b, f) Cells were stimulated with LPS (120 ng/mL) / IFN-γ (20 ng/mL) for 24 h and then incubated with **MITO-TP** or **LYSO-TP** (10 μM) for 30 min, washed by PBS buffer prior to imaging. c, g) Cells were stimulated with LPS (120 ng/mL) / IFN-γ (20 ng/mL) for 24 h and then treated with 200 μM ABH for 1 h, subsequently incubated with **MITO-TP** or **LYSO-TP** (10 μM) for 30 min, washed by PBS buffer prior to imaging. d, h) Cells were stimulated with LPS (120 ng/mL) / IFN-γ (20 ng/mL) and ABH (200 μM) for 24 h, subsequently incubated with **MITO-TP** or **LYSO-TP** (10 μM) for 30 min, washed by PBS buffer prior to imaging. B, C) Quantification of the fluorescence signals from a-h. Data were normalized to the fluorescence intensity from LPS/IFN-γ stimulated macrophage cells (b or f). Error bars are ± SD. n = 3. Scale bar: 30 μm.

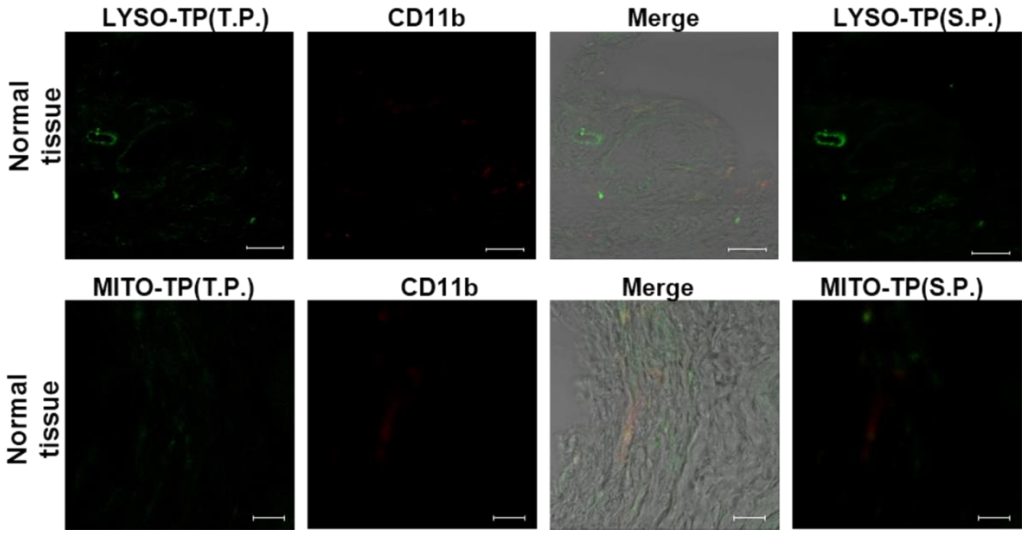


Figure S18. Normal tissue imaging with **LYSO-TP** (or **MITO-TP**) and antibody CD11b. Probe fluorescence was shown in green, antibody CD11b fluorescence was shown in red. Scale bar: 30 μM.

2. Materials and General Experimental Methods

All chemical reagents for the probe synthesis were obtained from Sigma Aldrich, Alfa Aesar, MERCK or Acros, and used without further purification unless otherwise specified. Column chromatography was carried out on Merck Silica Gel 60 (0.040-0.064 mm, 230–400 mesh). Synthetic reactions and analytical characterization were monitored by HPLC-MS (Agilient-1200 series) with a DAD detector and a single quadrupole mass spectrometer (6130 series) with an ESI probe. NMR spectra (^{1}H-300 or 500 MHz and ^{13}C-75 or 125 MHz) were recorded on Bruker Avance 300 or 500 NMR spectrometers. The high resolution electron spray ionization (HR-ESI) mass spectra were obtained on a Bruker micrOTOFQII. Spectroscopic and quantum yield data were measured on spectroscopic measurements, performed on a fluorometer and UV/VIS instrument, Spectra Max M2 by Molecular Device (The slit width: 1 nm). pH Value was determined by a Mettler Toledo S220 SEVENCOMPACT pH meter (Columbus, OH). Fluorescence microscopic images were obtained from a fluorescence Ti microscope (Nikon) inverted microscope with epifluorescence and phase contrast optics using 20× objective lenses. The green fluorescence was collected by using an Ex 370 nm/40, Em 500 nm/40 filter. Two-Photon tissue images were acquired

in Leica TCS SP5 X Confocal Microscope.

3. Synthesis and Characterization

Preparation of TP-HOCl 1, **TP-HOCl 2** and **TP-HOCl 3**. Compound **1** (10 mg, 0.05 mmol), methanesulfonic acid (20 μL) and 2-mercaptoethanol (5.2 mg, 0.06 mmol) (or Ethanedithiol (4.7 mg, 0.06 mmol) or hydroxylamine (4.2 mg, 0.06 mmol)) were mixed in 3 mL dichloromethane solution and refluxed for 3 h under nitrogen protection. The reaction was monitored by HPLC-MS and a product with reduced polarity was observed. Then the mixture was purified by preparative TLC with a solvent system (CH_2Cl_2: hexane = 1:1). Light yellow solid product was obtained.

TP-HOCl 1. Yield: 7.7 mg (59.6%). ^{1}H NMR (300 MHz, CD_2Cl_2) δ 7.82 (d, J = 1.7 Hz, 1H), 7.66 (d, J = 3.1 Hz, 1H), 7.63 (d, J = 2.9 Hz, 1H), 7.50 (dd, J = 8.6, 1.9 Hz, 1H), 6.93 (dd, J = 8.8, 2.4 Hz, 1H), 6.82 (d, J = 2.2 Hz, 1H), 4.40 (ddd, J = 9.1, 6.3, 5.0 Hz, 1H), 4.21 – 4.09 (m, 1H), 3.28 (dt, J = 10.0, 6.6 Hz, 1H), 3.20 – 3.09 (m, 1H), 2.95 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (75 MHz, CD_2Cl_2) δ 147.21, 139.75, 134.38, 128.86, 126.51, 125.78, 124.28, 122.76, 118.02, 103.21, 95.64, 70.73, 34.39, 31.74, 30.44. ESI-MS $C_{15}H_{18}NOS^+$ [M + H^+], found 260.1109, calculated 260.1104.

TP-HOCl 2. Yield: 7.1 mg (51.4 %). ^{1}H NMR (300 MHz, CD_2Cl_2) δ 8.00 (d, J = 2.0 Hz, 1H), 7.85 – 7.73 (m, 1H), 7.64 (dd, J = 8.7, 5.2 Hz, 2H), 6.93 (dd, J = 8.8, 2.4 Hz, 1H), 6.81 (d, J = 2.2 Hz, 1H), 3.63 – 3.37 (m, 4H), 2.96 (d, J = 4.6 Hz, 3H), 2.28 (d, J = 4.5 Hz, 3H). ^{13}C NMR (75 MHz, CD_2Cl_2) δ 147.45, 138.43, 134.19, 128.89, 126.44, 126.17, 125.64, 124.46, 117.92, 103.12, 68.47, 40.50, 33.33, 30.41. ESI-MS: $C_{15}H_{18}NS_2^+$ [M+H^+], found 276.0882, calculated 276.0875.

Preparation of compound 3. Compound **3** was synthesized according the literature procedure.[25]

Preparation of compound 4. Compound **3** (186.1 mg, 1.0 mmol), 2-morpholinoethanamine (65.1 mg, 5.0 mmol) and $Na_2S_2O_5$ (380.2 mg, 2.0 mmol) were dissolved in 4 mL water and stirred at 160 ˚C under microwave (80 mW) for 5 h. After reaction, the solvent was removed under vacuum. The solid product was purified by column chromatography using EtOAc : hexane (1 : 1) solution. Yield: 172 mg, 57.7%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.34 (s, 1H), 7.96 (dd, J = 8.6, 1.6 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.65 (d, J = 8.7 Hz, 1H), 7.01 (dd, J = 8.8, 2.1 Hz, 1H), 6.81 (d, J = 2.0 Hz, 1H), 3.86 – 3.74 (m, 4H), 3.35 (t, J = 5.8 Hz, 2H), 2.80 – 2.73 (m, 2H), 2.70 (s, 3H), 2.58 (d, J = 4.1 Hz, 4H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.94, 148.38, 138.19, 130.97, 130.91, 130.53, 126.08, 126.05,

124.92, 118.82, 103.72, 66.99, 56.85, 53.39, 39.50, 26.57. ESI-MS: $C_{18}H_{23}N_2O_2^+$ [M+H$^+$], found 299.1765, calculated 299.1754.

Preparation of compound 5. The mixture of compound **3 (**71.0 mg, 0.35 mmol), propane-1,3-diamine (77.5 mg, 1.05 mmol) and $Na_2S_2O_5$ (136.1 mg, 0.70 mmol) in 4.0 mL water and was stirred at 160 ˚C under microwave (80 mW) for 5 h. Then the solvent was removed under vacuum and the solid product was purified by preparative HPLC (method: 0 min, 5 % CH_3CN 7 min, 35% CH_3CN; 9 min 95% CH_3CN; 10 min 5% CH_3CN). The water and acetonitrite mixture was removed under vacuum. Yield: 49.7 mg (53.8%). ^{1}H NMR (500 MHz, DMSO) δ 8.46 (s, 1H), 8.35 (s, 1H), 7.78 (t, *J* = 9.1 Hz, 2H), 7.58 (d, *J* = 8.6 Hz, 1H), 7.03 (d, *J* = 8.8 Hz, 1H), 6.76 (s, 1H), 3.23 (t, *J* = 6.6 Hz, 2H), 2.90 (t, *J* = 7.2 Hz, 2H), 2.58 (s, 3H), 1.96 – 1.77 (m, 2H). ^{13}C NMR (75 MHz, DMSO) δ 198.21, 149.69, 138.93, 131.30, 131.25, 130.82, 126.23, 126.15, 124.88, 119.53, 102.93, 37.93, 27.29, 26.40. ESI-MS: $C_{15}H_{19}N_2O^+$ [M+H$^+$], found 243.1496, calculated 243.1492.

Preparation of compound 7. Compound **5** (121.2 mg, 0.50 mmol), Triethylamine (5 μL) and 2-bromoacetyl chloride (86.4 mg, 0.55 mmol) were dissolved in 6 mL dichloromethane and stirred at room temperature for 50 min. The compound **6** was obtained and used to next step without purification. Then 393 mg triphenylphosphine (1.5 mmol) was added into the mixture and stirred at room temperature for 16 h. The light yellow solid product **7** was obtained after purification by column chromatography with CH_2Cl_2 / MeOH (100 : 8) mixture solution. Yield: 223.4 mg (82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.70 (s, 1H), 8.22 (s, 1H), 7.85 (dd, *J* = 8.7, 1.8 Hz, 1H), 7.80 – 7.67 (m, 9H), 7.63 – 7.55 (m, 7H), 7.53 (d, *J* = 8.8 Hz, 1H), 7.03 (dd, *J* = 8.8, 2.2 Hz, 1H), 6.65 (d, *J* = 2.0 Hz, 1H), 4.89 (d, *J* = 14.2 Hz, 2H), 3.23 (dd, *J* = 11.9, 5.9 Hz, 2H), 3.15 (t, *J* = 6.2 Hz, 2H), 2.62 (s, 3H), 1.74 (dt, *J* = 12.4, 6.3 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 197.78, 162.35, 162.29, 148.51, 138.13, 134.97, 134.93, 133.97, 133.83, 130.51, 130.39, 130.19, 130.16, 129.98, 125.65, 125.42, 124.45, 119.06, 118.81, 117.64, 102.70, 40.30, 37.42, 27.06, 26.33. ESI-MS: $C_{35}H_{34}N_2O_2P^+$ [M$^+$], found 545.2360, calculated 545.2352.

Preparation of MITO-TP and LYSO-TP. Compound **4** (29.8 mg, 0.1 mmol) (or compound **7** (54.5 mg, 0.1 mmole)), 2-mercaptoethanol (10.4 mg, 0.12 mmol) and methanesulfonic acid (20 μL) were mixed in 4 mL dichloromethane solution and refluxed for 3 h under nitrogen protection. The reaction was monitored by HPLC-MS and the product with reduced polarity was observed. Then the mixture was purified by preparative TLC with a solvent system (CH_2Cl_2 / Methanol = 100 : 8). Light yellow product was obtained.

LYSO-TP. Yield: 21.8 mg (61.1%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.79 (d, J = 1.7 Hz, 1H), 7.62 (d, J = 9.0 Hz, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.47 (dd, J = 8.6, 1.9 Hz, 1H), 6.94 (dd, J = 8.7, 2.3 Hz, 1H), 6.78 (d, J = 2.1 Hz, 1H), 4.38 (ddd, J = 9.1, 6.4, 4.7 Hz, 1H), 4.09 (ddd, J = 9.1, 7.3, 6.2 Hz, 1H), 3.81 – 3.71 (m, 4H), 3.33 – 3.27 (m, 2H), 3.27 – 3.20 (m, 1H), 3.14 – 3.06 (m, 1H), 2.77 – 2.68 (m, 2H), 2.59 – 2.49 (m, 4H), 1.98 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 146.17, 139.73, 134.35, 129.15, 126.67, 126.01, 124.31, 122.97, 118.30, 104.15, 95.98, 70.60, 66.75, 56.89, 53.25, 39.76, 34.41, 32.07. ESI-MS: $C_{20}H_{27}N_2O_2S^+$ [M+H$^+$], found 359.1800, calculated 359.1788.

MITO-TP. Yield: 39.4 mg (65.2%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.89 (t, J = 5.5 Hz, 1H), 7.81 – 7.66 (m, 10H), 7.58 (ddd, J = 10.8, 5.4, 2.5 Hz, 7H), 7.51 (s, 1H), 7.41 (dd, J = 8.6, 1.9 Hz, 1H), 6.95 (dd, J = 8.8, 2.2 Hz, 1H), 6.68 (d, J = 2.1 Hz, 1H), 4.91 (d, J = 14.2 Hz, 2H), 4.35 (ddd, J = 9.1, 6.4, 4.7 Hz, 1H), 4.06 (ddd, J = 9.1, 7.2, 6.2 Hz, 1H), 3.17 – 3.27 (m, 3H), 3.15 – 3.01 (m, 3H), 1.95 (d, J = 6.8 Hz, 3H), 1.79 – 1.68 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.43, 162.36, 146.16, 139.13, 134.92, 134.88, 134.43, 133.99, 133.85, 130.14, 129.97, 128.92, 126.29, 125.89, 124.04, 122.94, 118.92, 118.62, 117.75, 103.62, 96.04, 77.42, 77.00, 76.57, 70.55, 41.35, 40.75, 37.50, 34.36, 32.03, 27.36. ESI-MS: $C_{37}H_{38}N_2O_2PS^+$ [M$^+$], found 605.2393, calculated 605.2386.

Preparation of compound 8. Compound **1** (10 mg, 0.05 mmol), 2-mercaptoethanol (5.2 mg, 0.06 mmol) and methanesulfonic acid (20 μL) were mixed in 3 mL dichloromethane (CH_2Cl_2) solution and refluxed for 8 h in air. The solvent was removed under vacuum and the mixture was purified by preparative TLC with a solvent system (CH_2Cl_2 / hexane = 1 : 1). Light yellow product was obtained. Yield: 6.6 mg, 48.2% ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.96 (t, J = 10.8 Hz, 1H), 7.75 (dd, J = 8.7, 2.1 Hz, 1H), 7.60 (dd, J = 8.7, 3.4 Hz, 2H), 6.88 (dd, J = 8.8, 2.4 Hz, 1H), 6.77 (d, J = 2.2 Hz, 1H), 3.55 – 3.37 (m, 4H), 2.93 (s, 3H), 2.23 (d, J = 5.2 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 147.25, 138.52, 134.20, 129.09, 126.33, 125.95, 124.58, 117.92, 103.57, 68.80, 40.33, 33.40, 30.71. ESI-MS: $C_{15}H_{18}NO_2S^+$ [M + H$^+$], found 276.1058, calculated 276.1053.

Preparation of compound 9. 20 mg (0.1 mmole) compound **1** was mixed with 100 μL NaOCl (4%, 0.11 mmol) in MeOH / H_2O (1 : 1) solution and stirred at room temperature for 2 h. After reaction, 20 mL ice water was added and extracted with dichloromethane (3 x 40 mL). The organic layer was dried by anhydrous Na_2SO_4 and yellow solid product was obtained, which was purified by preparative TLC with dichloromethane and hexane (2 : 1) mixture solvent. Yield: 19.3 mg, 83%. ^{1}H NMR (500 MHz, CD_2Cl_2) δ 8.325 (s, 1H), 8.023 (d, J = 1.5 Hz, 1H), 8.018 (s, 1H), 7.800 (d, J = 8.9 Hz, 1H), 7.115 (d, J = 8.9 Hz, 1H), 3.07 (s, 3H), 2.68 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ

197.45, 144.72, 133.79, 130.86, 130.67, 129.83, 125.86, 125.58, 122.19, 113.45, 110.63, 30.35, 26.42. ESI-MS: $C_{13}H_{13}ClNO^+$ [M + H^+], found 234.0680, calculated 234.0680.

4. Photo-spectroscopic Studies

Measurement of photo-physical properties. All absorption and emission spectra were recorded on a Spectra Max M2 by Molecular Device at 25 °C. The slit width of both excitation and emission is 1 nm. Stock solutions of the probes in DMSO (10 mM) were used to prepare the working solutions in PBS (0.1M, pH 7.4) / EtOH (1 : 1) or PBS (1% DMSO, pH 7.4 or 5.0) buffer solution with a final concentration of 5.0 μM. Absorbance and emission spectra were recorded immediately after addition of different concentration of HOCl.

Measurements of two-photon fluorescence spectra. Two-photon fluorescence spectra were measured by using a Ti: sapphire oscillator (Avesta Ti-Sapphire TiF-100M) as the excitation source, which gives output of 750 nm laser pulses with pulse duration of 80 fs and repetition rate of 84.5 MHz. The laser beam was filtered through a 750/10 nm bandpass filter (Semrock LD01-785/10-25) to reduce their interference to the measured emission. The sample was excited by a tightly focused laser beam with fluorescence collected at a 90° angle to minimize the scattering. The emission signal was directed into a CCD (Princeton Instruments, Pixis 100B) coupled monochromator (Acton, Spectra Pro 2300i) with an optical fiber.

***In vitro* Myeloperoxidase (MPO)/H_2O_2/Cl^- system.** 1 U/mL MPO (Sigma) was dissolved in PBS (pH=7.4, 37 ˚C) and the probe **TP-HOCl 1** (5 ìM) was further added into the solution. After addition of H_2O_2 (20 μM), the fluorescence intensities were recorded immediately in a Spectra Max M2 micro-plate reader. Besides, in inhibition experiment, 10 μM 4-aminobenzoic acid hydrazide (ABH) was added to inhibit MPO before addition of H_2O_2. Furthermore, ABH (4-Aminobenzoic acid hydrazide, 10 μM) or Cys (cysteine, 200 μM) was added at 1 and 2 min respectively.

5. Analytical HPLC analysis

To examine the sensing mechanism of **TP-HOCl 1** responding to HOCl, high pressure liquid chromatography (HPLC) – Mass spectra (MS) were conducted after the incubation of HOCl at different concentration. The signals were recorded at 350 nm and 500 nm as a function of retention time. H_2O (eluent A) / acetonitrile (eluent B) with a linear gradient elution profile: 0 min, 95% A; 1 min, 95% A; 7 min, 5% A; 8.5 min, 5% A; 9 min, 95% A; 10 min, 95% A was used as the mobile

phase. The temperature of the column was maintained at 25 ºC and the flow rate of the mobile phase was 1.0 mL/min.

6. *In Vitro* Fluorescence Microscopic Studies

Cell culture. Murine RAW 264.7 cells lines and HeLa cells lines (American Type Culture collection) were grown as mono-layers in 75-cm^2 flasks containing DMEM (BSF) supplemented with 10% FBS (PAA), 1% penicillin streptomycin glutamine (GIBCO) at 37ºC in a humidified incubator of 5% CO_2. Cells were carefully harvested and split when they reached 80% confluence to maintain exponential growth.

Fluorescence microscopic imaging. To avoid the artifacts that occur during fixation procedures, all the experiments were conducted in live cells. The cell experiment can be divided into four groups. The first group is that RAW 264.7 macrophage cells were incubated with 15 μM **MITO-TP** (or **LYSO-TP**) for 20 min. Then the cells were washed by PBS buffer before imaging. In the second group, RAW 264.7 macrophage cells were incubated with 15 μM **MITO-TP** (or **LYSO-TP**) for 20 min, washed by PBS buffer and subsequently incubated with Lipopolysaccharide (LPS, 1 μg/mL) and Phorbol Myristate Acetate (PMA, 1 μg/mL) for 1 h prior to imaging. In the third and fourth the group RAW 264.7 macrophage cells were incubated with 15 μM **MITO-TP** (or **LYSO-TP**) for 20 min, washed by PBS buffer and subsequently incubated with Lipopolysaccharide (LPS, 1 μg/mL) / Phorbol Myristate Acetate (PMA, 1 μg/mL) and 4-aminobenzoic acid hydrazide (ABH, 200 μM) or N-acetylcysteine (NAC, 2 mM) respectively for 1 h prior to imaging.

Determining the subcellular location of probes. Live RAW 264.7 macrophage cells cultured in 35-mm glass bottom culture dishes were incubated with LPS (120 ng/mL) and IFN –γ (20 ng/mL) for 24 h and 10 μM probes (**MITO-TP** and **LYSO-TP**) was further added. After 20 min treatment at 37 ºC, MitoTracker (1.0 μM) and LysoTracker (1.0 μM) were added for another 10 min. Then the cells were washed by PBS prior to imaging. For further confirming the intracellular co-localization, HeLa cells were used to co-localize with trackers. The cells were treated with probes (10 μM) for 20 min and subsequently with Trackers for 10 min, then with 50 μM HOCl for another 5 min. Then the cells were washed by PBS prior to imaging.

Cell cytotoxicity in MTT assay. Cells were plated in 96-well flat-bottomed plates at 1×10^5 cells per

well and allowed to grow 12 h prior to exposure to **MITO-TP** and **LYSO-TP**. Then the MTT reagent was added for 4 h at 37 ^{o}C and DMSO (100 μL/well) was further incubated with cells for 15 min after removing the medium. The absorbance at 570 nm and 690 nm (background signal) was recorded in a Spectra Max M2 microplate reader. The following formula was used to calculate the viability of cell growth: Cell viability (%) = (mean of A value of treatment group / mean of A value of control) × 100.

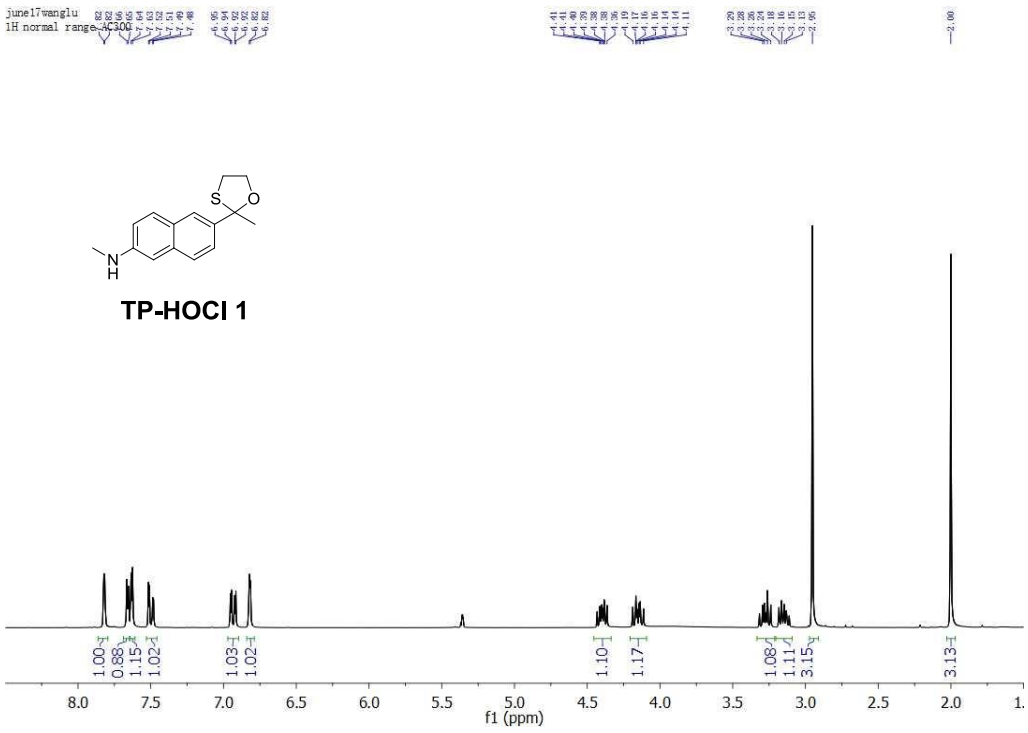
7. Two-photon Tissue Imaging Studies

LPS-induced tissue inflammation. After anesthesia, 100 μL of 1 mg/mL LPS (Sigma) was injected on right paws of mice (6-8 week, C57BL6/J). After 1 day, **MITO-TP** and **LYSO-TP** (200 μL, 1 mM) were injected through tail vein. After 1 h, the skin of the inflamed (right paw) and normal (left paw) tissues were harvested and embedded in tissue-freezing medium (Triangle Biomedical Sciences), frozen and consecutively cryo-sectioned into 10 μm or 300 μm segments.

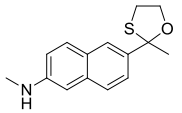
Immunohistofluorescence. The 10 μm segments were carefully rinsed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Then the tissues were treated with 1% bovine serum albumin in PBS for 1 h. For confirmation of HOCl probes stain pattern, rat anti mouse CD11b antibody (Abcam, dilution factor 1:300) was applied and was visualized by cy5-conjugated goat anti rat secondary antibody (Life Technologies^{TM}, dilution factor 1:300). All imagings were taken by Leica TCS SPS X two photon microscope. The excitation wavelength is 750 nm and the collection wavelength range is from 450-520 nm. For the detection of cy5, the excitation wavelength is 630 nm and the collection wavelength range is from 650-670 nm.

Two-photon inflamed tissue imaging. The 300 μm segments were carefully rinsed with PBS and covered with coverslip. Leica TCS SPS X microscope was used to take two photon imaging. The excitation wavelength is 750 nm and the collection wavelength range is from 450-520 nm. 3D construction was performed in Leica TCS SPS X software.

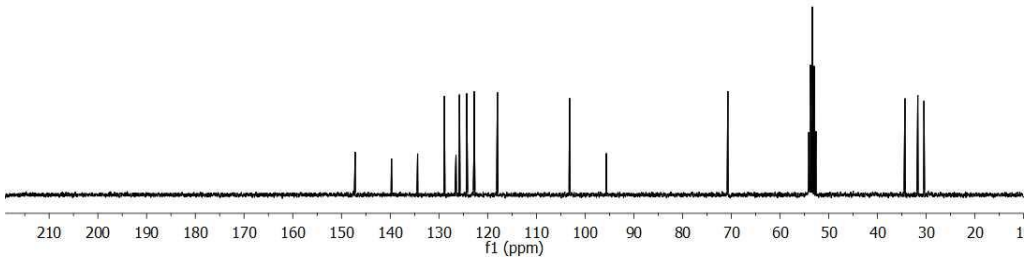
8. Supplemental Spectra

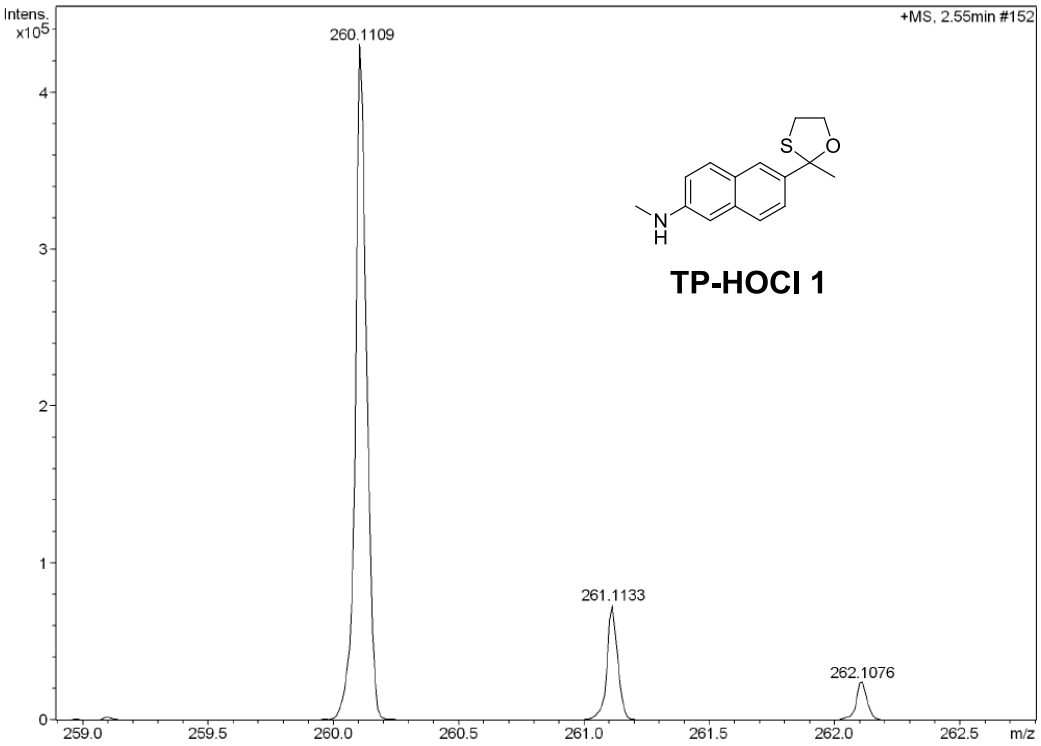


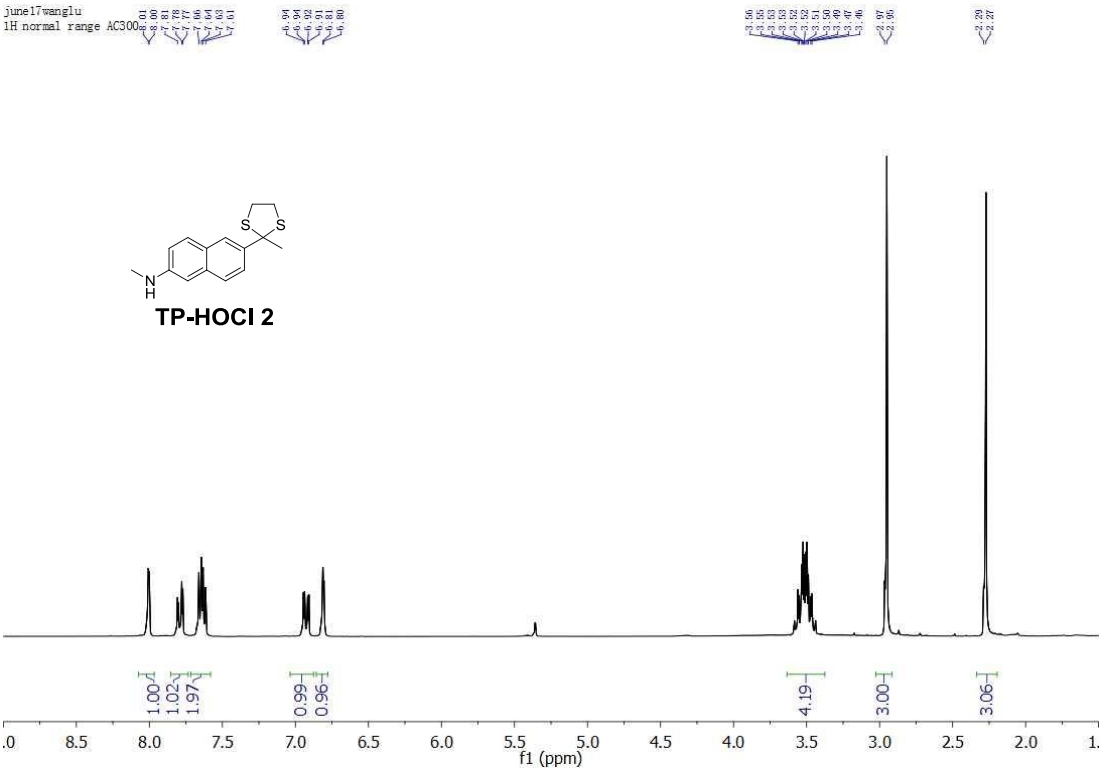

june17wanglu
13C Standard AC300
147.21
139.75
134.38
128.86
128.51
125.78
124.28
122.76
118.02
103.21
85.64
70.73
34.99
31.74
28.44

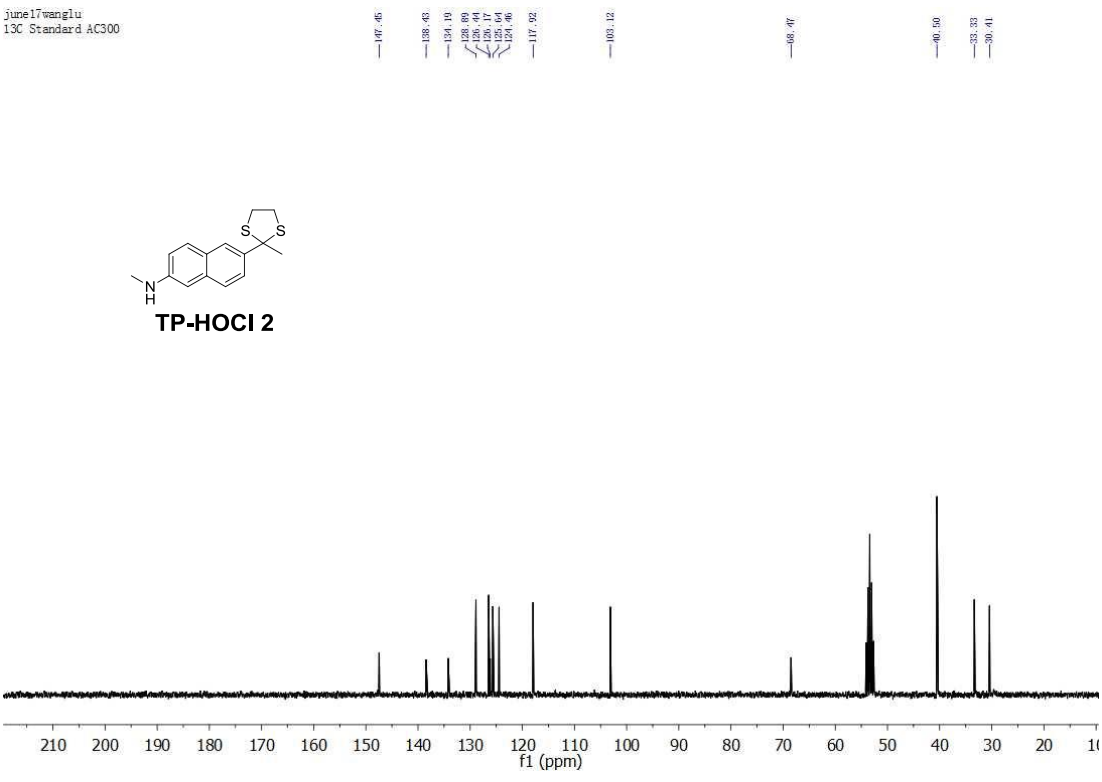

S
O
N
H

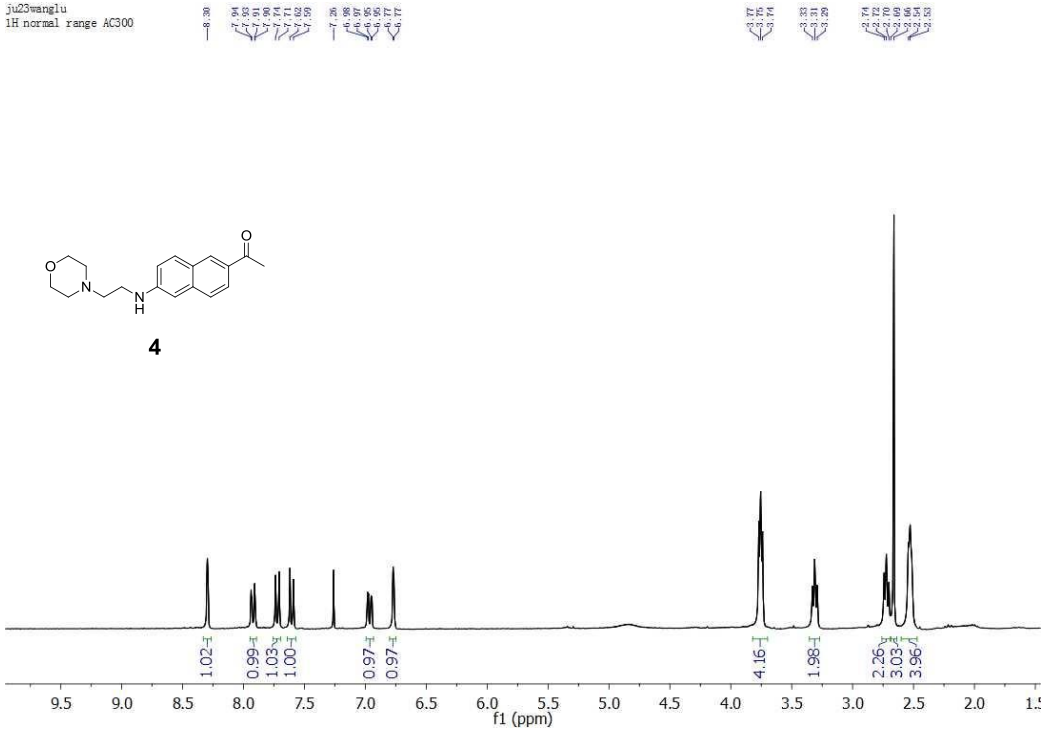
TP-HOCl 1

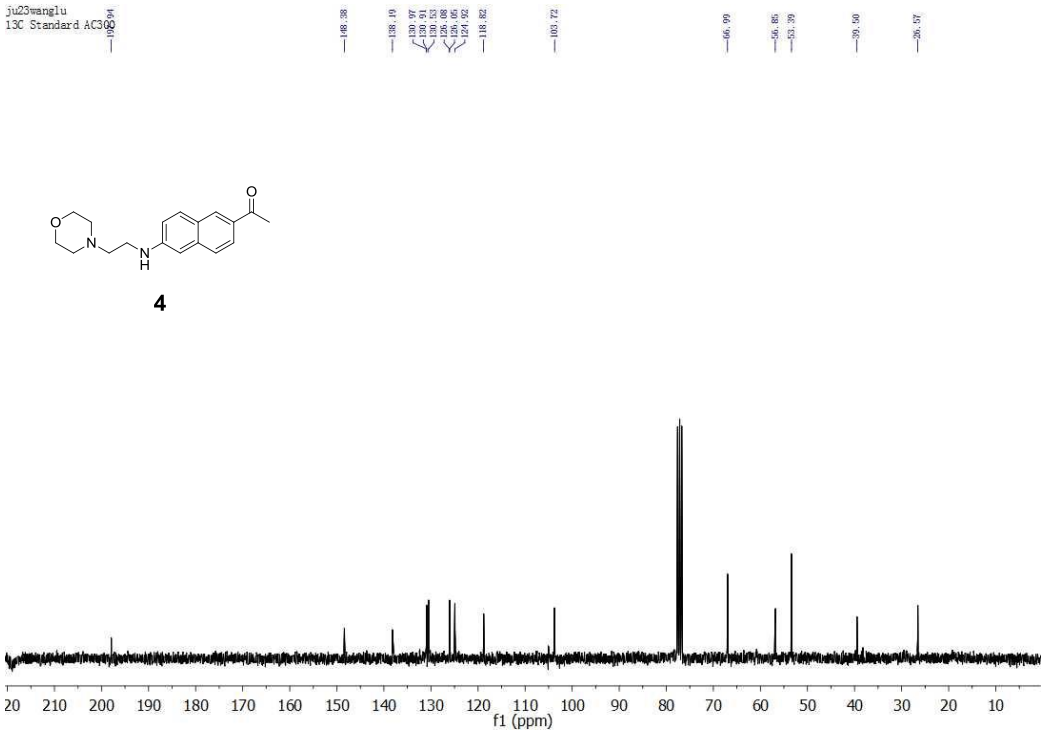

210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
1
f1 (ppm)


Intens.
x10^5
+MS, 2.55min #152
260.1109
261.1133
262.1076
S
O
N
H
TP-HOCl 1
0
1
2
3
4
259.0
259.5
260.0
260.5
261.0
261.5
262.0
262.5
m/z

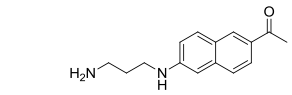

june17wanglu
1H normal range AC300
TP-HOCl 2
1.00
1.02
1.97
0.99
0.96
4.19
3.00
3.06
f1 (ppm)


june17wanglu
13C Standard AC300
TP-HOCl 2
f1 (ppm)

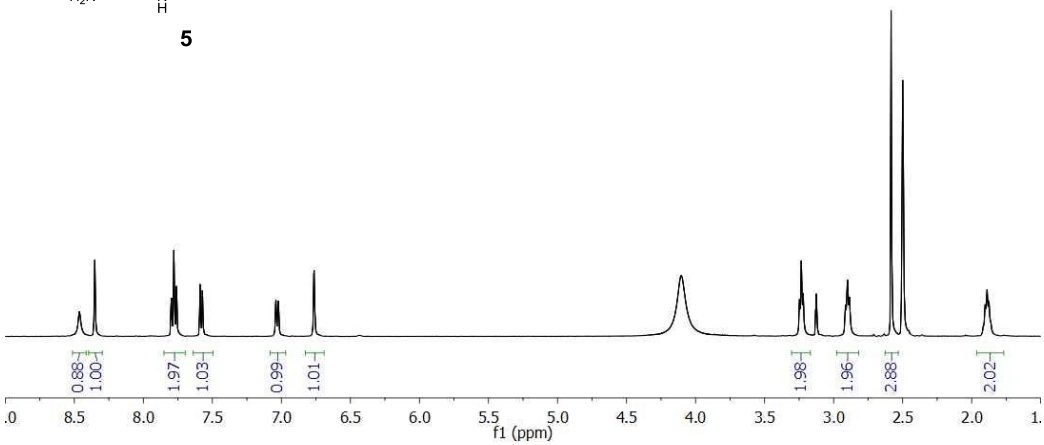

ju23wanglu
1H normal range AC300
4
1.02
0.99
1.03
1.00
0.97
0.97
4.16
1.98
2.26
3.03
3.96
f1 (ppm)


ju23wanglu
13C Standard AC300
4
f1 (ppm)

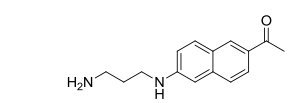
jl14wanglu
1H AMX500
WL-NH2



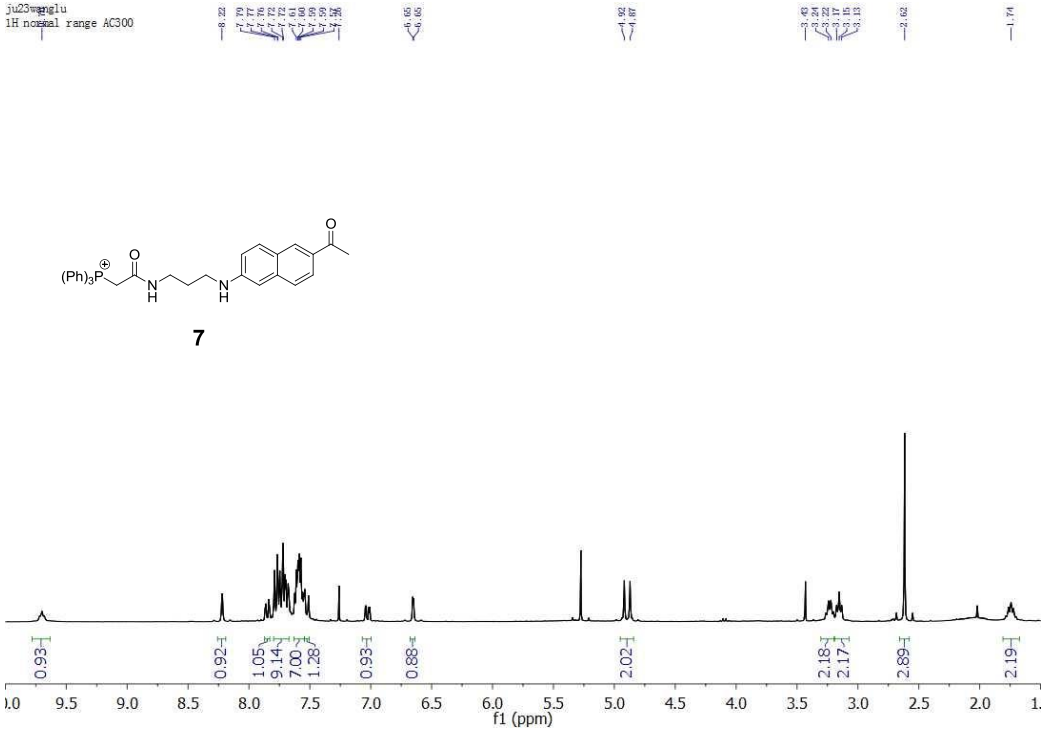
5

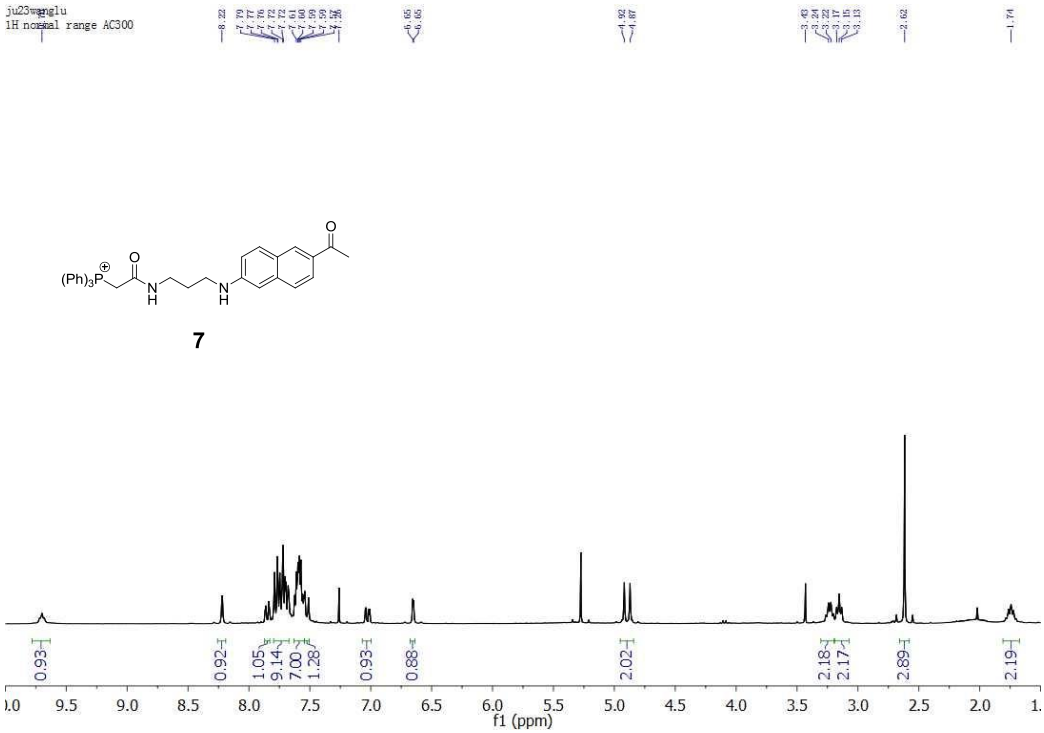


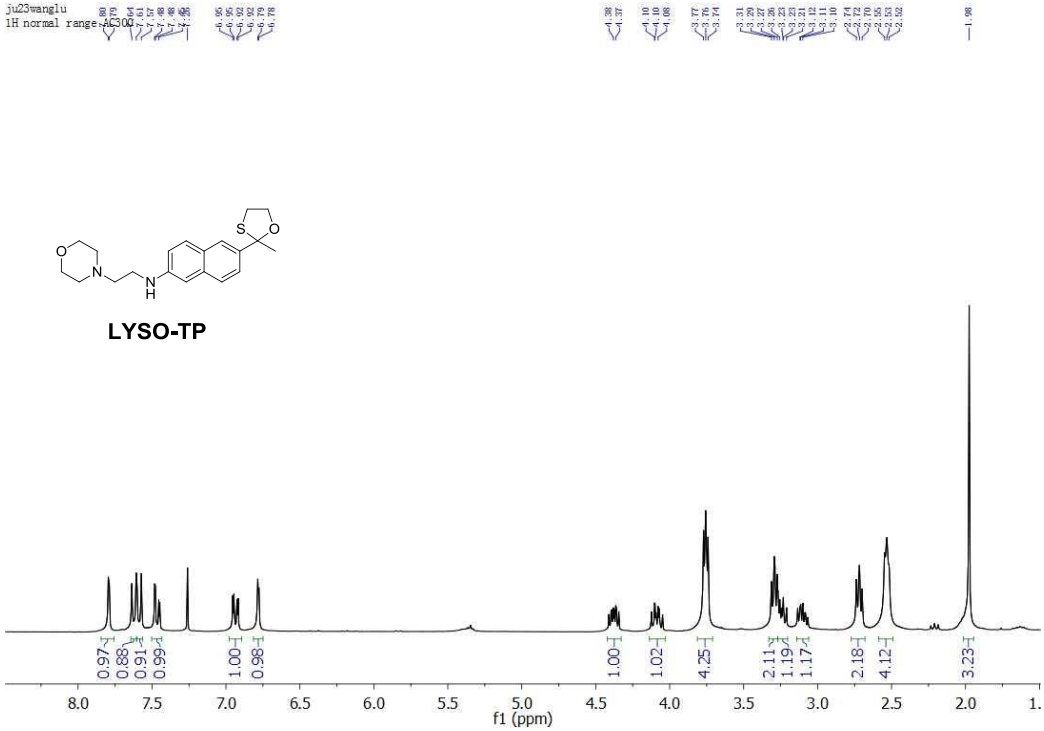
jl14wanglu
13C Standard AC30



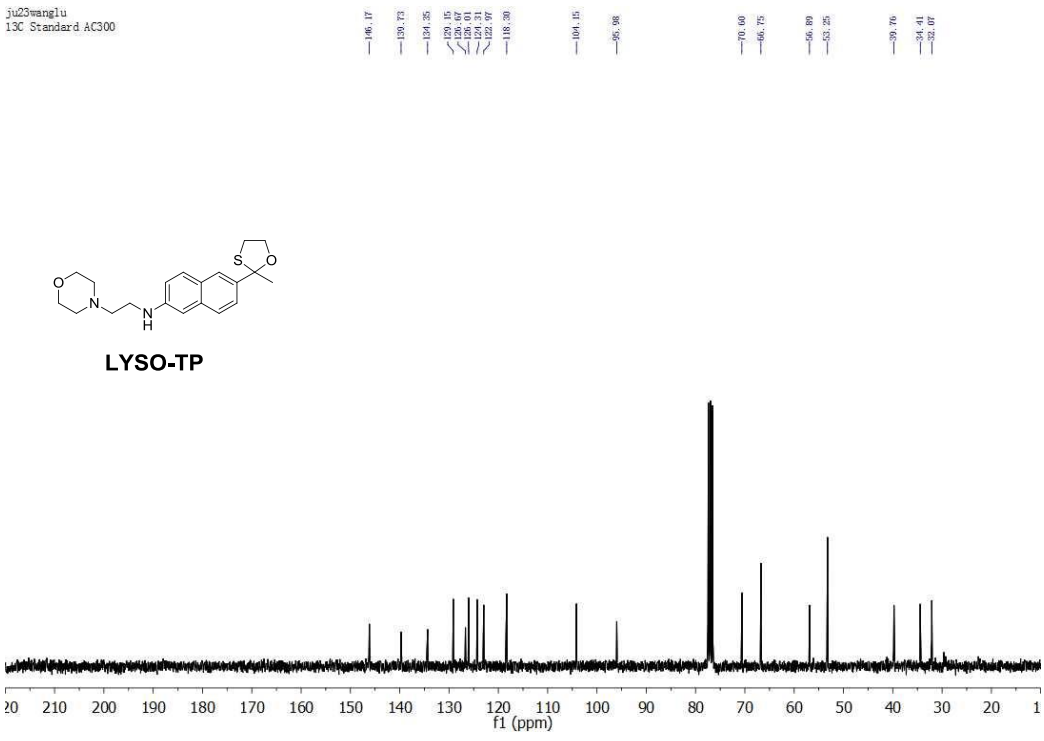
5


ju23wangl u
1H normal range AC300
(Ph)3P
O
N
H
O
7
0.93
0.92
1.05
9.14
7.00
1.28
0.93
0.88
2.02
2.18
2.17
2.89
2.19
f1 (ppm)

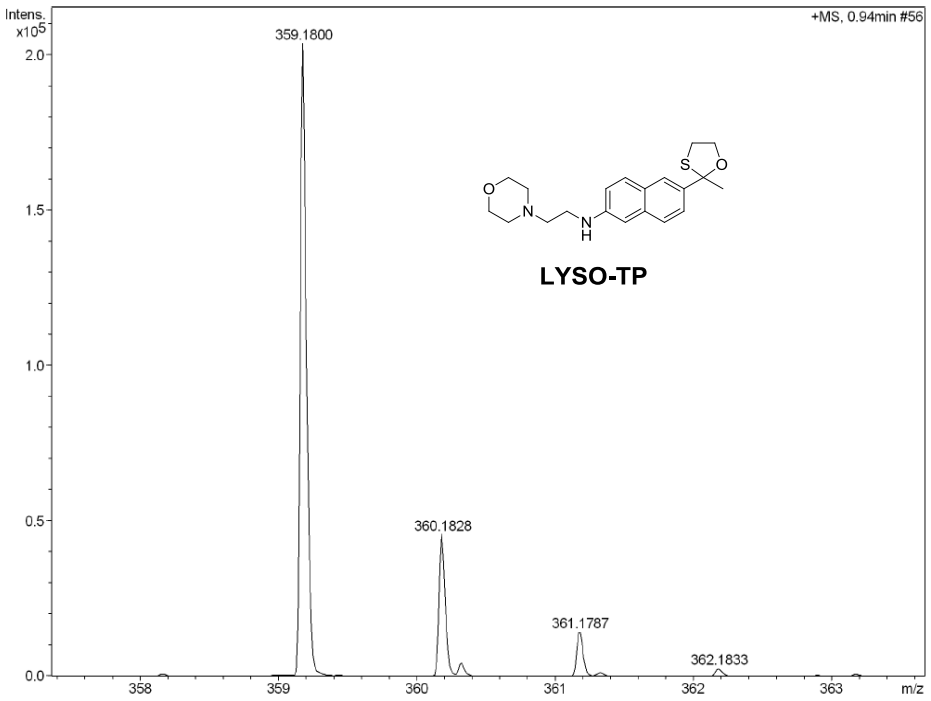

ju23wangl u
1H normal range AC300
(Ph)3P
O
N
H
O
7
0.93
0.92
1.05
9.14
7.00
1.28
0.93
0.88
2.02
2.18
2.17
2.89
2.19
f1 (ppm)



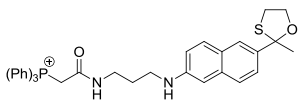
LYSO-TP



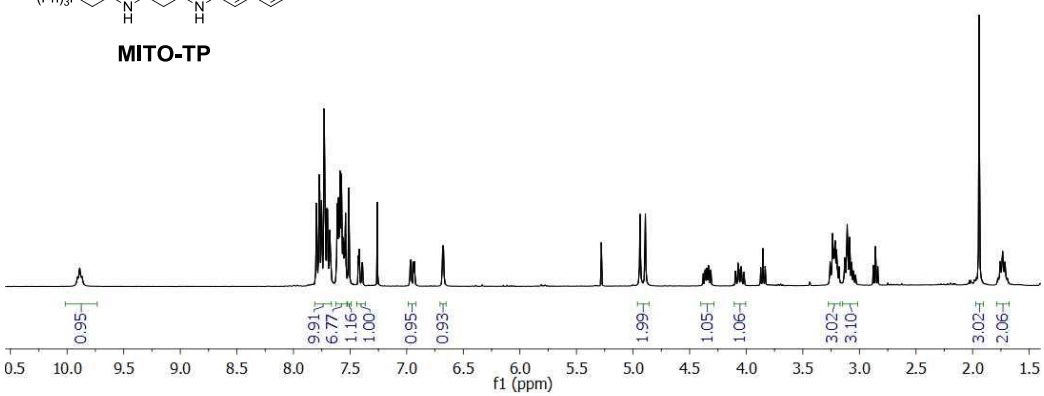
LYSO-TP

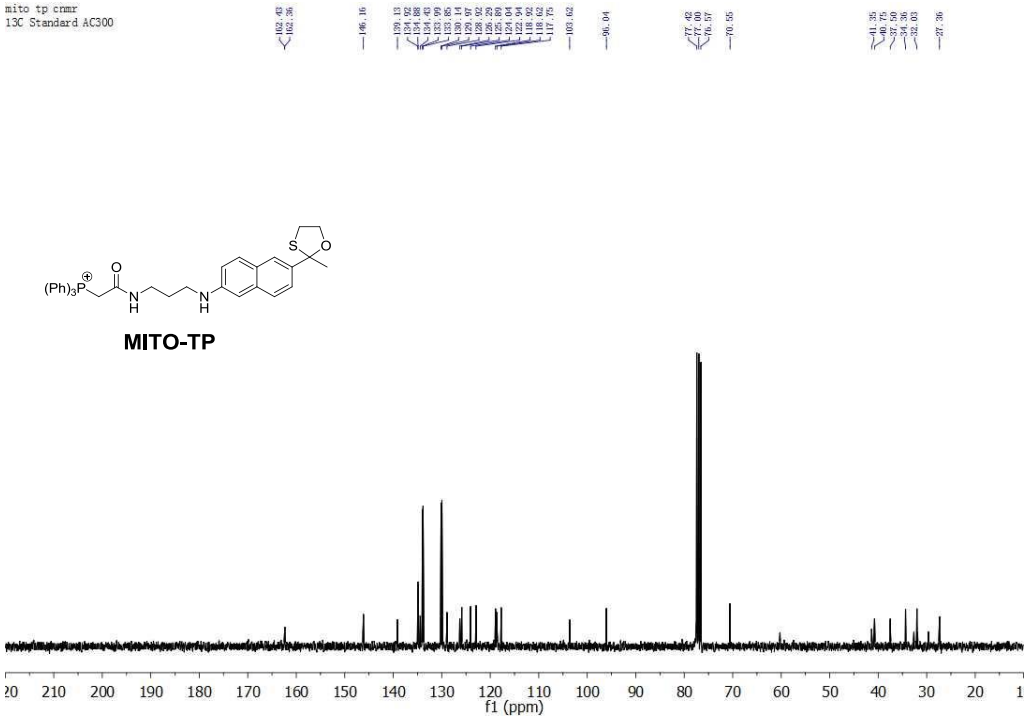


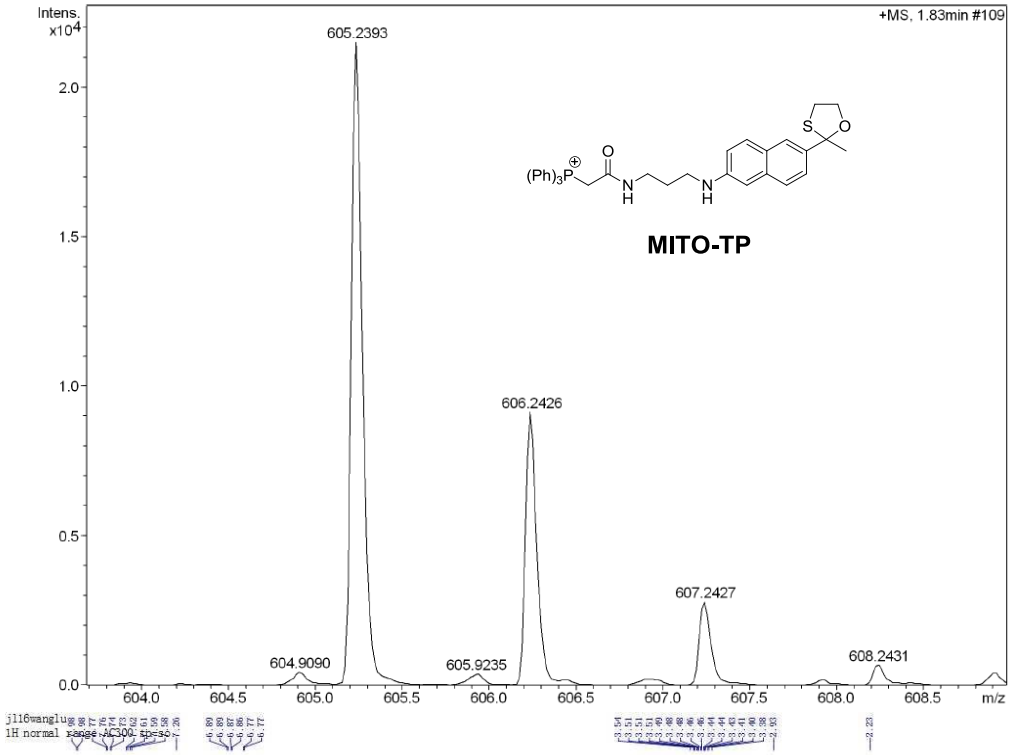
LYSO-TP

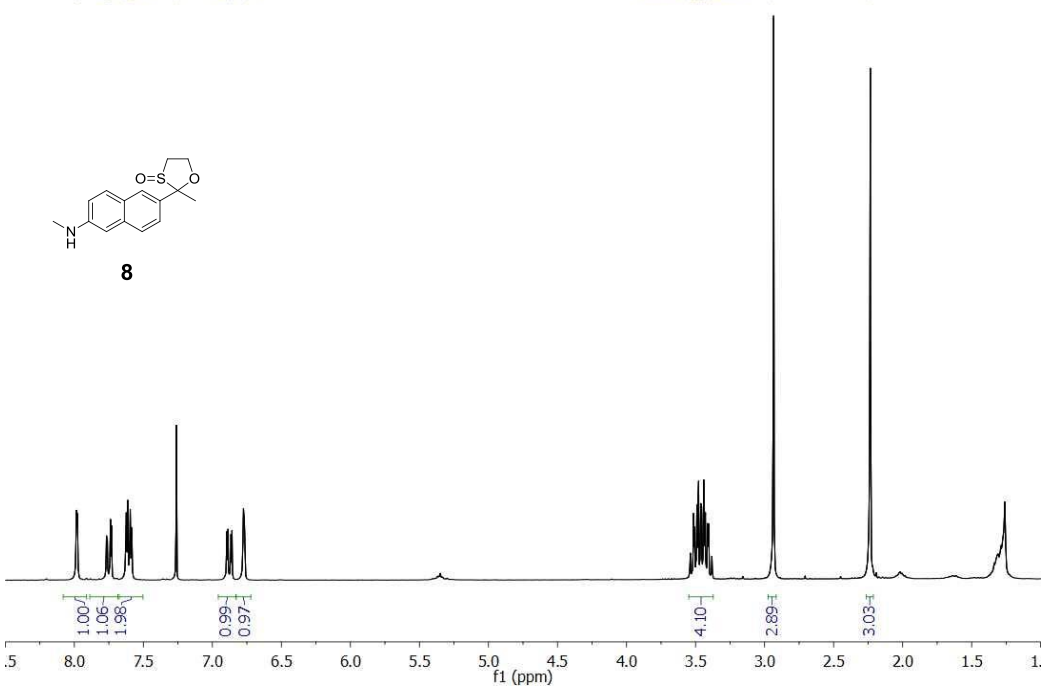


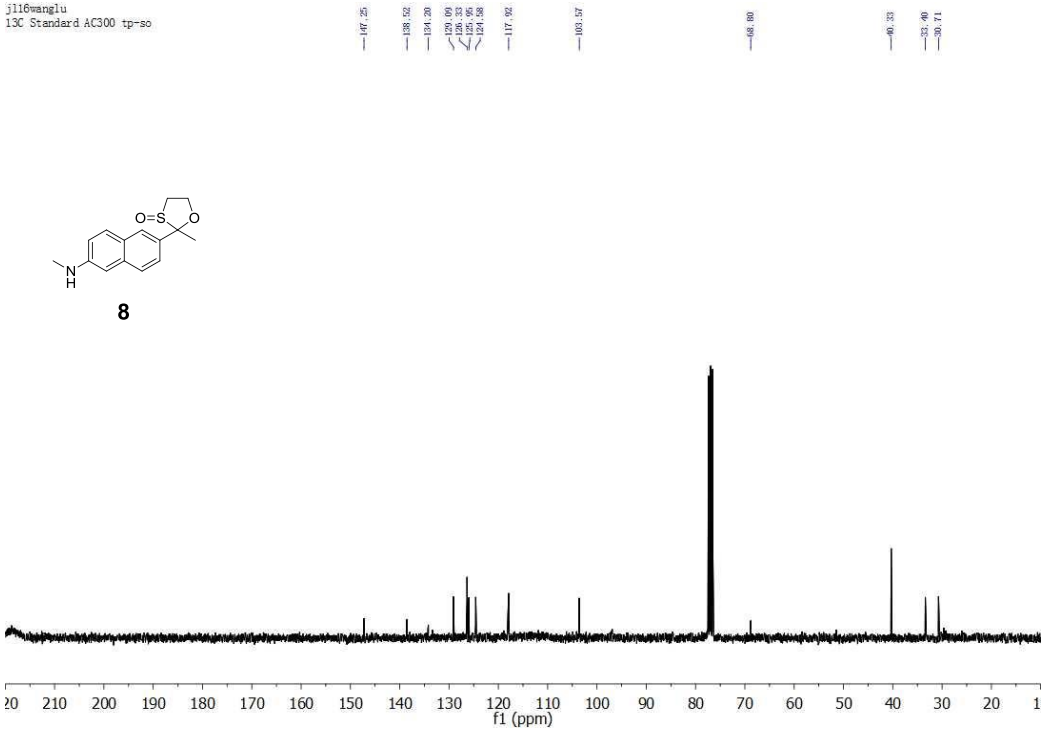
MITO-TP




mito tp cnmr
13C Standard AC300
MITO-TP
f1 (ppm)


Intens.
x10^4
+MS, 1.83min #109
605.2393
606.2426
607.2427
604.9090
605.9235
608.2431
m/z
MITO-TP


8
f1 (ppm)



9. Reference:

1. Chen, X.; Wang, X.; Wang, S.; Shi, W.; Wang, K.; Ma, H. *Chem. Eur. J.* **2008**, *14*, 4719.

2. Kenmoku, S.; Urano, Y.; Kojima, H.; Nagano, T . *J. Am. Chem. Soc.* **2007,** *129*, 7313.

3. Koide, Y.; Urano, Y.; Hanaoka, K.; Terai, T.; Nagano, T. *J. Am. Chem. Soc.* **2011**, *133*, 5680.

4. Zhan, X.-Q.; Yan, J.-H.; Su, J.-H.; Wang, Y.-C.; He, J.; Wang, S.-Y.; Zheng, H.; Xu, J.-G. *Sensor. Actuat. B: Chem.* **2010**, *150*, 774.

5. Chen, X.; Lee, K. A.; Ha, E. M.; Lee, K. M.; Seo, Y. Y.; Choi, H. K.; Kim, H. N.; Kim, M. J.; Cho, C. S.; Lee, S. Y.; Lee, W. J.; Yoon, J. *Chem. Commun.* **2011**, *47*, 4373.

6. Xu, Q.; Lee, K. A.; Lee, S.; Lee, K. M.; Lee, W. J.; Yoon, J. *J. Am. Chem. Soc.* **2013**, *135*, 9944.

7. Zhang, R.; Ye, Z.; Song, B.; Dai, Z.; An, X.; Yuan, J. *Inorg. Chem.* **2013**, *52*, 10325.

8. Ye, Z.; Zhang, R.; Song, B.; Dai, Z.; Jin, D.; Goldys, E. M.; Yuan, J. *Dalton Trans.* **2014**, *43*, 8414.

9. Yuan, L.; Lin, W.; Xie, Y.; Chen, B.; Song, J. *Chem. Eur. J.* **2012**, *18*, 2700.

10. Sun, Z. N.; Liu, F. Q.; Chen, Y.; Tam, P. K.; Yang, D. *Org. Lett.* **2008**, *10*, 2171.

11. Hu, J. J.; Wong, N. K.; Gu, Q.; Bai, X.; Ye, S.; Yang, D. *Org. Lett.* **2014**, *16*, 3544.

12. Lin, W.; Long, L.; Chen, B.; Tan, W. *Chem. Eur. J.* **2009**, *15*, 2305.

13. Cheng, X.; Jia, H.; Long, T.; Feng, J.; Qin, J.; Li, Z. *Chem. Commun.* **2011**, *47*, 11978.

14. Emrullahoglu, M.; Ucuncu, M.; Karakus, E. *Chem. Commun.* **2013**, *49*, 7836.

15. Zhao, N.; Wu, Y. H.; Wang, R. M.; Shi, L. X.; Chen, Z. N. *Analyst* **2011**, *136*, 2277.

16. Zhu, H.; Fan, J.; Wang, J.; Mu, H.; Peng, X. *J. Am. Chem. Soc.* **2014**, *136*, 12820.

17. Cheng, G.; Fan, J.; Sun, W.; Cao, J.; Hu, C.; Peng, X. *Chem. Commun.* **2014**, *50*, 1018.

18. Liu, S. R.; Wu, S. P. *Org. Lett.* **2013**, *15*, 878.

19. Li, G.; Zhu, D.; Liu, Q.; Xue, L.; Jiang, H. *Org. Lett.* **2013**, *15*, 2002.
20. Qu, Z.; Ding, J.; Zhao, M.; Li, P. *J. Photoch. Photobio. A* **2015**, *299*, 1.
21. Wu, X. J.; Li, Z.; Yang, L.; Han, J. H.; Han, S. F. *Chem. Sci.* **2013**, *4*, 460.
22. Xiao, H.; Xin, K.; Dou, H.; Yin, G.; Quan, Y.; Wang, R. *Chem. Commun.* **2015**, *51*, 1442.
23. Li, G.; Lin, Q.; Ji, L.; Chao, H. *J. Mater. Chem. B* **2014**, *2*, 7918.
24. Hou, J.-T.; Wu, M.-Y.; Li, K.; Yang, J.; Yu, K.-K.; Xie, Y.-M.; Yu, X.-Q. *Chem. Commun.* **2014,** *50*, 8640.
25. This work.