Supporting Information

Design of a Ratiometric Two-photon Probe for Imaging of Hypochlorous Acid (HClO) in Wounded Tissues

Zhiqiang Mao,[a‡] Miantai Ye,[a‡] Wei Hu,[b] Xiaoxue Ye,[a] Yanying Wang,[a] Huijuan Zhang,[a] Chunya Li*[a] and Zhihong Liu*[b]

[a] Key Laboratory of Analytical Chemistry of the State Ethnic Affairs Commission, College of Chemistry and Materials Science, South-Central University for Nationalities, Wuhan, 430074, China. E-mail:lichychem@163.com

[b] Key Laboratory of Analytical Chemistry for Biology and Medicine (Ministry of Education), College of Chemistry and Molecular Sciences, Wuhan University, Wuhan, Hubei 430072, China. E-mail: zhhliu@whu.edu.cn.

Table of Content

1. Materials and apparatus

Lipopolysaccharide (LPS) and Phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich. Other regents were from Aladdin Chemical Reagent and Sinopharm Chemical Reagent Co. Lt. and used without further purification. All reactions were performed under argon atmosphere unless otherwise stated. Anhydrous solvents for organic synthesis were prepared by standard methods. All aqueous solutions were prepared in ultrapure water with a resistivity of 18.25 MΩ•cm (purified by the Milli-Q system supplied by Millipore). Two-photon excited fluorescence data were measured by exciting with a mode-locked Ti: sapphire femtosecond pulsed laser (Chameleon Ultra II, Coherent Inc.) with a pulse width of 140 fs and repetition rate of 80 MHz. NMR spectra were recorded in DMSO-d_6, with tetraethylsilane (TMS) as internal reference, on a Bruker Advance III NMR Spectrometer (400 MHz). Mass spetra were determined on Waters Micromass GCT Premier. The two-photon excited fluorescence intensity was recorded on a DCS200PC Photon Counting (Beijing Zolix Instruments Co., Ltd.) with single-photon sensitivity through an Omni-λ5008 monochromator (Beijing Zolix Instruments Co., Ltd.). One-photon excited fluorescence was measured on a F-7000 fluorescence spectrophotometer (Hitachi). Absorption measurements were conducted on a UV2550 UV-vis spectrophotometer (Shimadzu Scientific Instruments Inc.). Two-photon microscopy was performed on a Zeiss LSM 780 multiphoton laser scanning confocal microscope (Carl Zeiss, Germany).

2. **Experimental detail**

Spectroscopic Measurements The fluorescence quantum yield was determined with quinine sulfate (Φ=0.55, 0.05 M H_2SO_4) as the reference with a literature method. [1] NaClO stock solution was prepared by dilution of commercial NaClO solution in ultrapure water (≥18.25MΩ•cm) and the concentration of the ClO^- stock solution was determined by measuring the absorbance at 209 nm with a molar extinction coefficient of 350 $M^{-1}cm^{-1}$.

Measurement of Two-photon Cross Section The two-photon cross section (δ) was determined by using femtosecond (fs) fluorescence measurement technique as described.[2] Probe (5.0×10^{-6} M) was dissolved in 10 mM PBS buffer (pH 7.4), and the two-photon induced fluorescence intensity was measured at 700-900 nm by using rhodamine B as the reference, whose two-photon property

has been well characterized in the literature. The intensities of the two-photon induced fluorescence spectra of the reference and sample at the same excitation wavelength were determined. The 2P absorption cross section was calculated by using $\delta=\delta_r[S_s\Phi_r\phi_r c_r]/(S_r\Phi_s\phi_s c_s)$, where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the two-photon excited fluorescence was denoted as *S*. Φ is the fluorescence quantum yield, and ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the 2P absorption cross section of the reference molecule.

Experimental Calculation of Limit of Detection (LOD) The limit of detection was calculated based on the method reported in the previous literature.[3] The fluorescence emission spectrum of **QClO** was measured by eleven times and the standard deviation of blank measurement was achieved. The fluorescence ratio ($I_{562nm}/I_{492\ nm}$) was plotted as a concentration of HClO. The limit of detection was calculated by using detection limit $3\sigma/k$: Where σ is the standard deviation of blank measurement, *k* is the slope between the fluorescence intensity versus HClO concentration.

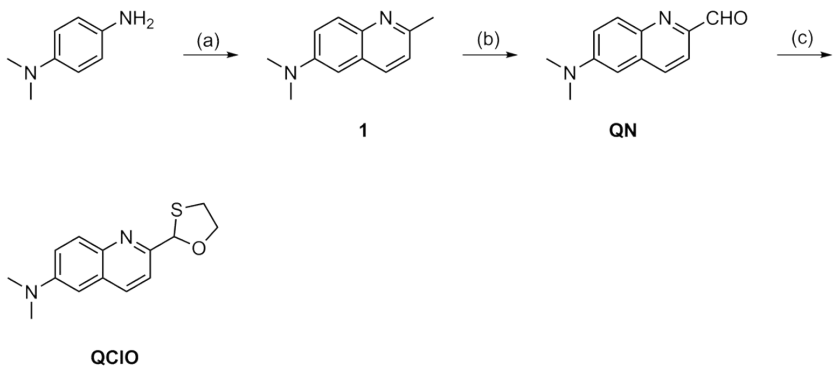
Cytotoxicity Assay The cytotoxicity was evaluated by MTT assay. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in 96-well microplates at 37 °C under 5% CO_2 for 12 h. The medium was next replaced by fresh medium containing various concentrations of QClO (0-30 μM). Each concentration was tested in three replicates. Cells were rinsed twice with phosphate buffer saline 24 h later and incubated with 0.5 mg/mL MTT reagent for 4h at 37 °C. 150 μL DMSO was then added to dissolve formazan. The absorbance at 490 nm was measured in a microplate reader. Cell viability (%) was calculated according to following equation: Viability = (mean Abs. of treated wells/mean Abs. of control wells) ×100%.

Cell Culture and Imaging HeLa cells were cultured with DMEM supplemented with 10% (v/v) newborn calf serum (Gibco), 100 $U{\cdot}mL^{-1}$ penicillin, and 100 $\mu g{\cdot}mL^{-1}$ streptomycin in a humidified atmosphere with 5/95 (v/v) of CO_2/air at 37 °C. One day before imaging, cells were detached with a treatment of 0.2% (w/v) trypsin-EDTA solution (Gibco) and suspended in culture media. The cell suspension was then transferred to confocal dishes to grow with adherence. For

probe loading, the growth medium was replaced with 5.0 μM **QClO** in culture media and incubated at 37 °C under 5% CO_2 for 30 min. Next, the cells were washed with serum-free DMEM for three times. Various concentration HClO solution was added to the dishes and incubated at 37 °C under 5% CO_2 for 30 min. Raw 264.7 cells were maintained with DMEM supplemented with 10% (v/v) newborn calf serum (Gibco), 100 U•mL^{-1} penicillin, and 100 μg•mL^{-1} streptomycin in a humidified atmosphere with 5/95 (v/v) of CO_2/air at 37 °C. For confocal imaging, RAW 264.7 cells at 80% confluence were harvested by scraping and transferred to confocal dishes to grow with adherence. For endogenous HClO production, RAW 264.7 cells were pretreated with 1.0 μg/mL LPS and 1.0 μg/mL PMA for 1 hour. Then, RAW 264.7 cells were incubated with 5.0 μM **QClO** in PBS at 37 °C for 30 min and washed with PBS three times for imaging. Two-photon excited fluorescence images were obtained by Zeiss LSM 780 multiphoton laser scanning confocal microscope.

Tissue imaging After the Kunming mice (~35 g) anesthesia, a wound (1 cm×3 mm) on left rear leg of the mice was artificially caused by a scalpel. Then, 150 μL of 1.0 mM **QClO** was intramuscularly injected to the wound margin at days 0, 1 and 4. 1 h later, the mice were anesthetized, the skin of the wounded tissues were harvested and embedded in tissue-freezing medium, frozen and consecutively sectioned into slices. Then, the slices were washed with PBS three times and imaged by two-photon microscope. Animal care and handing procedures were reviewed and approved by Animal Care and Use Committee of Wuhan University.

3. Synthesis and Characterization



Scheme S1. Synthesis of **QClO**. Reagents and conditions: (a) crotonaldehyde, 6 M HCl, reflux; (2) SeO_2, 1, 4-dioxane, reflux; (c) 2-mercaptoethanol, methanesulfonic acid, dichloromethane, 50 °C.

Synthesis of compound 1. *N, N*, 2-trimethylquinolin-6-amine was prepared by literature method.[4] A mixture of 3.0g (22.02 mmol) of *N, N*-dimethylbenzene-1, 4-diamine and HCl (6M, 100 mL) was heated to 100°C. Crotonaldehyde (2.65 mL, 66.05 mmol) was added slowly and the reaction was refluxed 7h. After cooling to room temperature, the mixture was poured into water and adjusted to alkaline by using aqueous ammonia. After evaporation, the residue was purified by column chromatography using petroleum ether-EtOAc 1:1 (v/v) as the eluent to obtain compound **1** (2.0g, 66.7%).

Synthesis of QN. 2.0 g (10.74mmol) of compound **1** (*N, N*, 2-trimethylquinolin-6-amine) was added to a solution of 1, 4-dioxane (20mL) and the reacton was heated to 60°C. SeO_2 was added under reflux and stirring at 80 ° C for 4h. The mixture was filtered and washed thoroughly with dichloromethane, and the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography (petroleum ether: dichloromethane, 1:4) to obtain compound **QN** (0.85g, 42.5%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.13 (d, J = 0.8 Hz, 1H), 8.06 (d, J = 9.4 Hz, 1H), 8.01 (d, J = 8.5 Hz, 1H), 7.91 (d, J = 8.5 Hz, 1H), 7.42 (dd, J = 9.4, 2.8 Hz, 1H), 6.79 (d, J = 2.9 Hz, 1H), 3.16 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 193.51, 150.17, 148.82, 141.68, 134.25, 132.24, 131.27, 119.73, 118.14, 103.82, 40.42. EI-HRMS Calcd. For $C_{12}H_{12}N_2O$ [M^+]=200.0950. Found 200.0946.

Synthesis of QClO. 0.10 g (0.53 mmol) of 6-(dimethylamino)quinoline-2-carbaldehyde was added to a solution of 2-mercaptoethanol (55.58mg, 0.64 mmol) and methanesulfonic acid (378.90mg, 3.94mmol) in 10 mL of dichloromethane under N_2 atmosphere. The mixture was reflux at 50 °C for 4h. The crude product was purified by silica gel chromatography (ethyl acetate: petroleum ether, 1:6) to yield light yellow powder (42 mg, 42%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (d, J = 8.0 Hz, 1H), 7.95 (d, J = 8.0 Hz, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.35 (dd, J = 4.0, 2.8 Hz, 1H), 6.80 (d, J = 4 Hz, 1H), 6.29 (s, 1H), 4.65 (ddd, J = 9.0, 6.0, 2.8 Hz, 1H), 4.06 (td, J = 9.1, 5.9 Hz, 1H), 3.26 (m, 2H), 3.08 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.53, 147.77, 134.48, 128.48, 128.43, 118.54, 117.46, 103.85, 86.65, 71.66, 39.66, 32.84, 29.95. EI-HRMS Calcd. For $C_{14}H_{16}N_2OS$ [M^+]=260.0983. Found 260.0989.

4. Spectroscopic properties

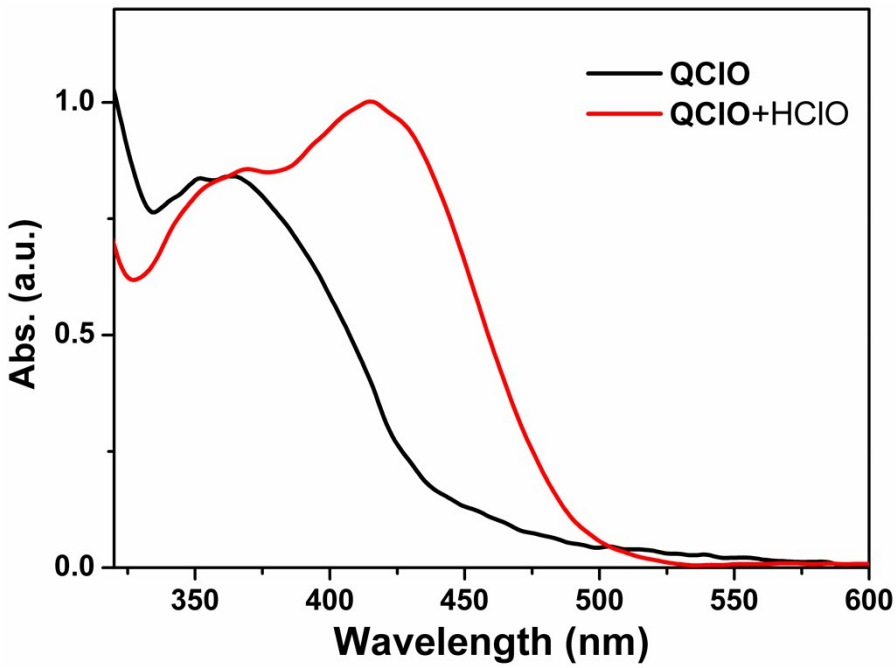


Fig. S1 UV-vis absorption spectra of **QClO** with excess HClO (black line) and without excess HClO (red line) in 10 mM PBS buffer (pH=7.4, containing 5% DMF).

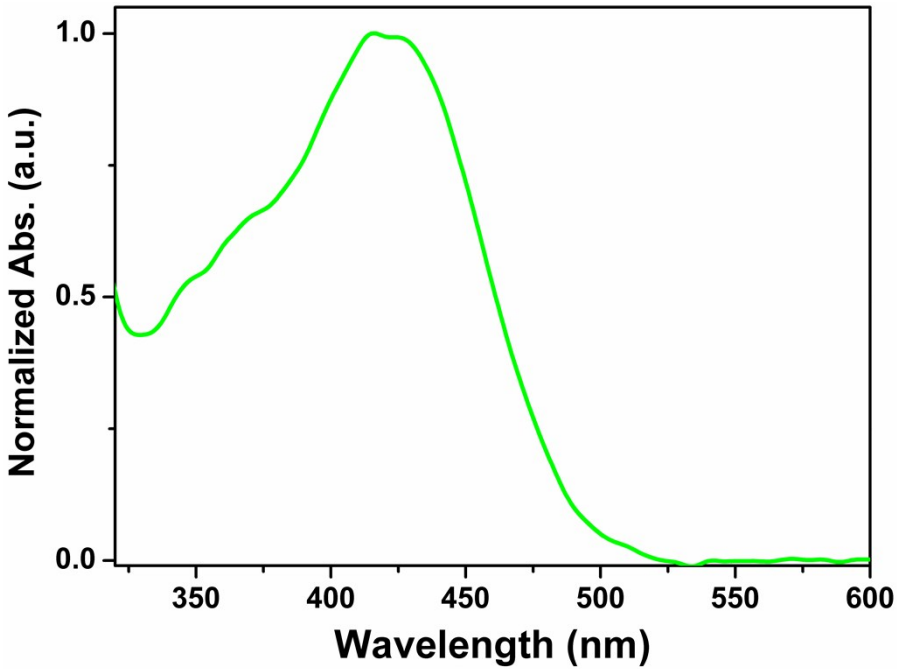


Fig. S2 Normalized UV-vis absorption spectra of **QN** in 10 mM PBS buffer (pH=7.4, containing 5% DMF).

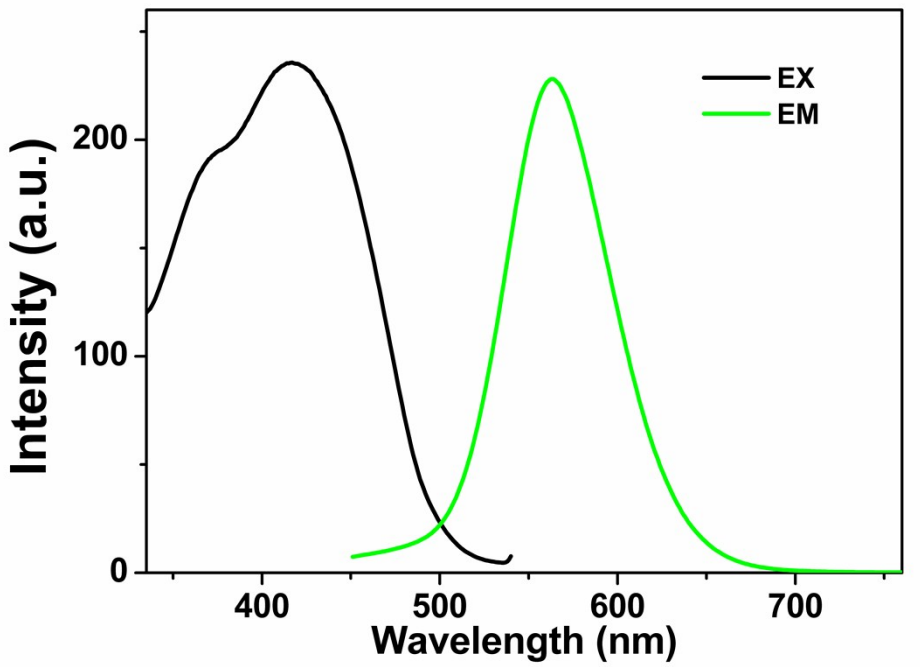
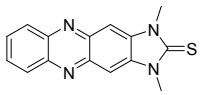
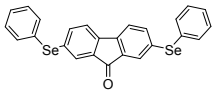
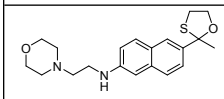
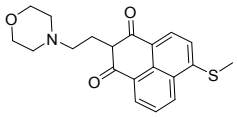
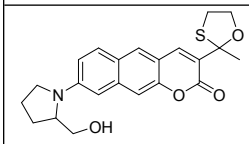
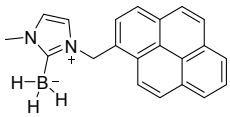
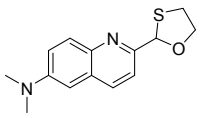


Fig. S3 Excitation and emission spectra of the compound **QN** in 10 mM PBS buffer (pH=7.4, containing 5% DMF).

Table S1. Properties of the reported two-photon probes for HClO.

Probes	$\lambda_{ex}/\lambda_{em}$ (nm)	Detection range (μM)	Limit of detection (LOD)	Reaction time	TP action cross section (buffer)	Imaging application
 PIS	378/505	0-10	0.071 μM	ca. 10 min	**PIS**: n.d. [a] **PIS**+HClO: 0.72GM(800 nm)	Cell imaging & hippocampal slice imaging[5]
 FO-PSe	415/520	5-100	0.35 μM	instantaneous	**FO-PSe**: --[b] **FO-PSe**+HClO: 78 GM (800 nm)	Cell & mice model & zebrafish[6]
 **Lyso-TP**	356/500	0-0.200	16.6 nM	seconds	**Lyso-TP:** n.d. **Lyso-TP**+HClO: 74 GM (750 nm)	Cell & tissue imaging[7]
 **L1**	405/505	3-150	0.674 μM	2.5 min	**L1**:-- **L1**+HClO:--	Cell imaging[8]
 **rTP-HOCl 1**	424/633; 487/598	0-10	34.8 nM	seconds	**rTP-HOCl 1:** 41 GM (900 nm) **rTP-HOCl 1**+HClO: 53 GM (900 nm)	Cell & brain tissue imaging[9]
 **1•BH3**	365/467	2.9-11.2	--	$t_{1/2}$=1.5 s	**1·BH3: -- 1·BH3**+HClO**:--**	Cell & hippocampal Slice imaging[10]
 **QClO**	360/492 414/563	0.8-12.5	89 nM	< 60 s	**QClO:** 25 GM (810 nm) **QClO+HClO:** 37 GM (820 nm)	Cell & tissue imaging & mouse model (This work)

[a] Not detectable. [b] Not mentioned.

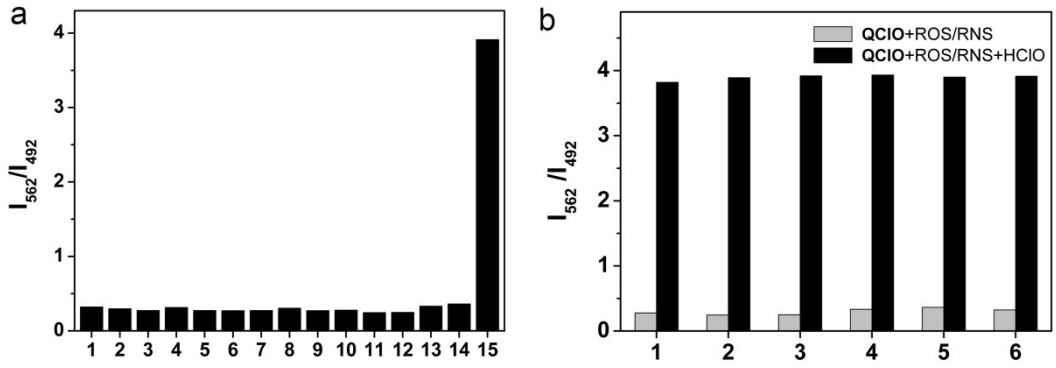


Fig. S4 (a) Response of 5.0 μM **QClO** to HClO and various potential interfering species in PBS buffer, including **1** blank, 100 μM metal ions **2-6** (Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}), 1.0 mM biothiols **7-9** (Cys, Hcy, GSH), 50 μM ROS **10-12** (H_2O_2, O_2^-, ·OH) and 50 μM RNS **13-14** ($ONOO^-$, NO) and 25 μM HClO **15**. (b) Response of **QClO** to various ROS/RNS (grey bar), and ROS/RNS +25 μM HClO (black bar), **1** blank, **2-6** 50 μM ROS/RNS (H_2O_2, O_2^-, ·OH, $ONOO^-$, NO).

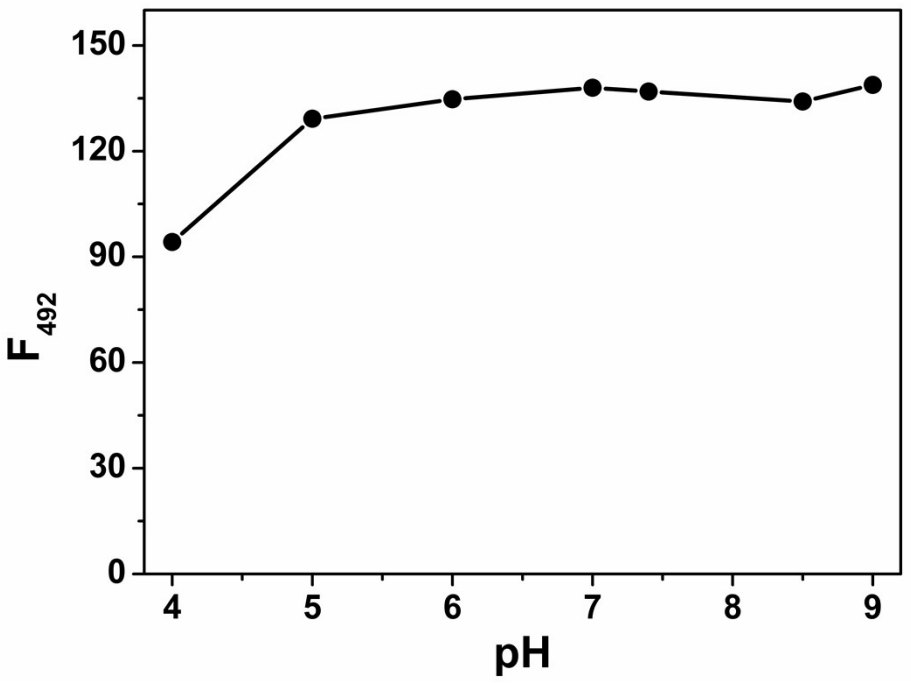


Fig. S5 Fluorescence intensities at 492 nm of **QClO** in different pH buffer (4.0-9.0).

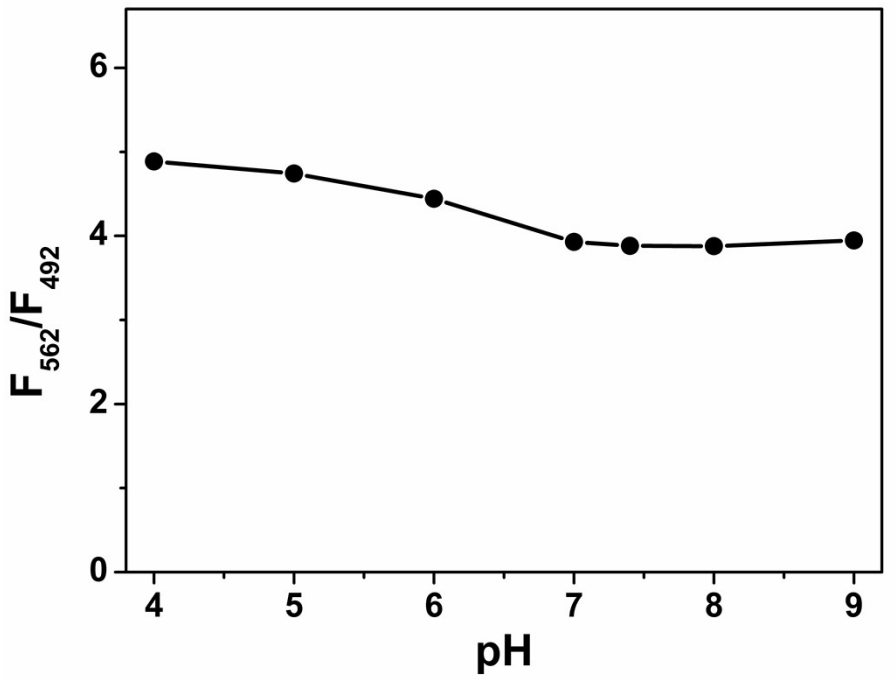


Fig. S6 Fluorescence intensity ratios of **QClO** with excess HClO in 10 mM PBS buffer with different pH (4.0-9.0).

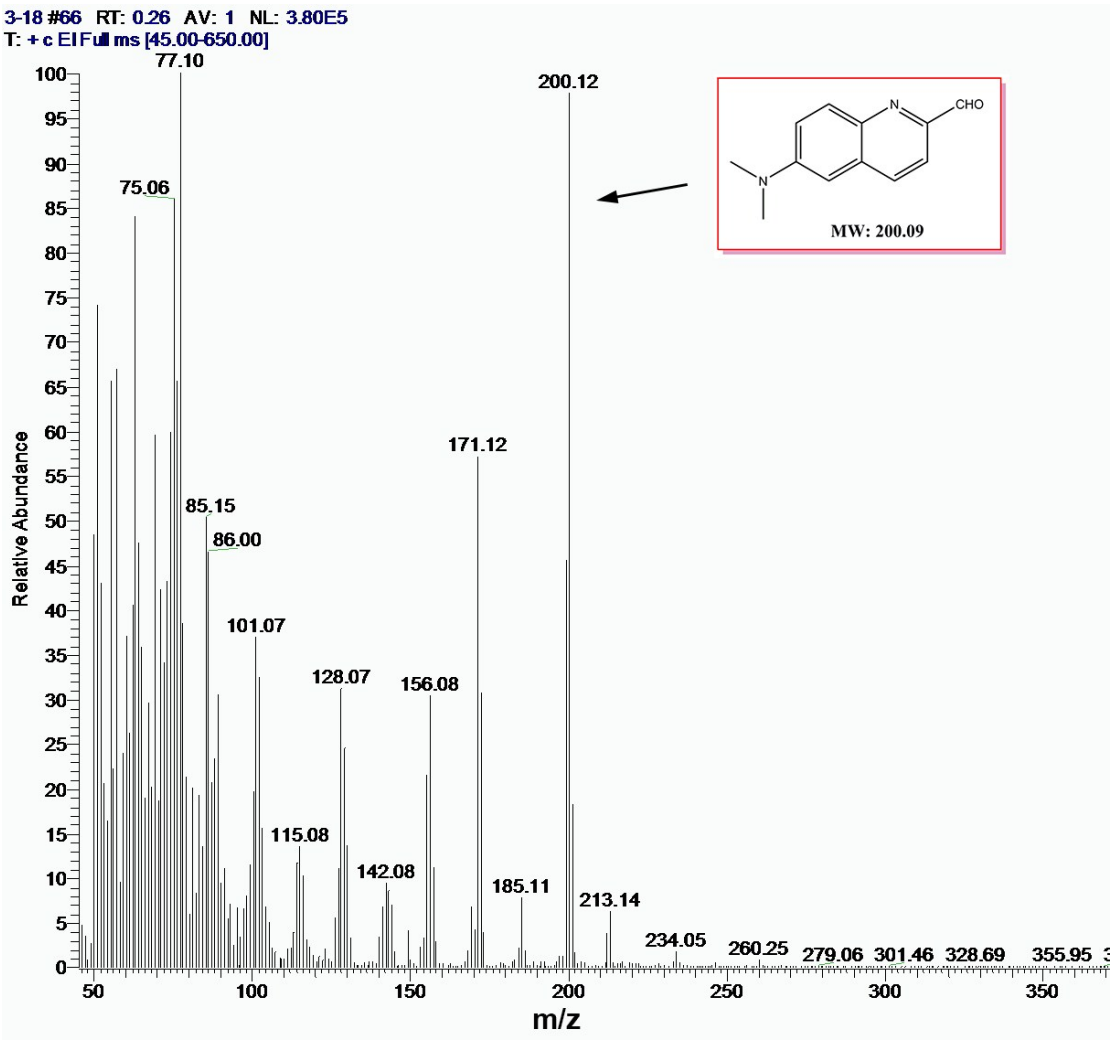
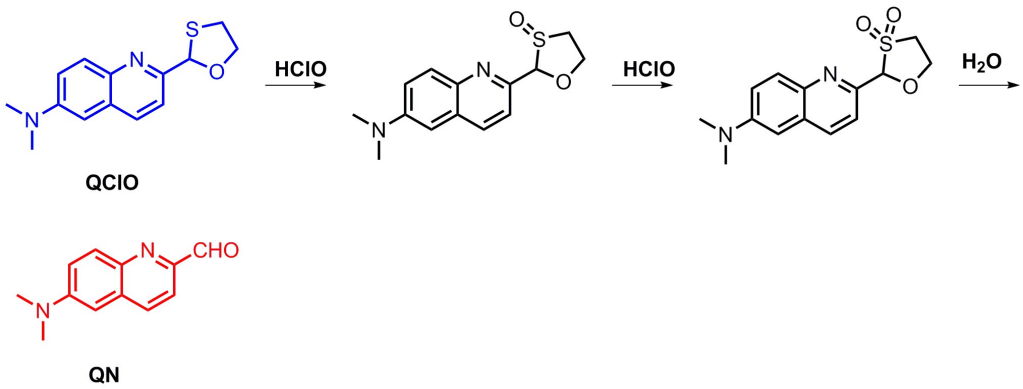


Fig. S7 EI-MS of the reaction product of **QClO** and excess HClO in PBS buffer.



Scheme S2. Proposed sensing mechanism of **QClO** in response to HClO.

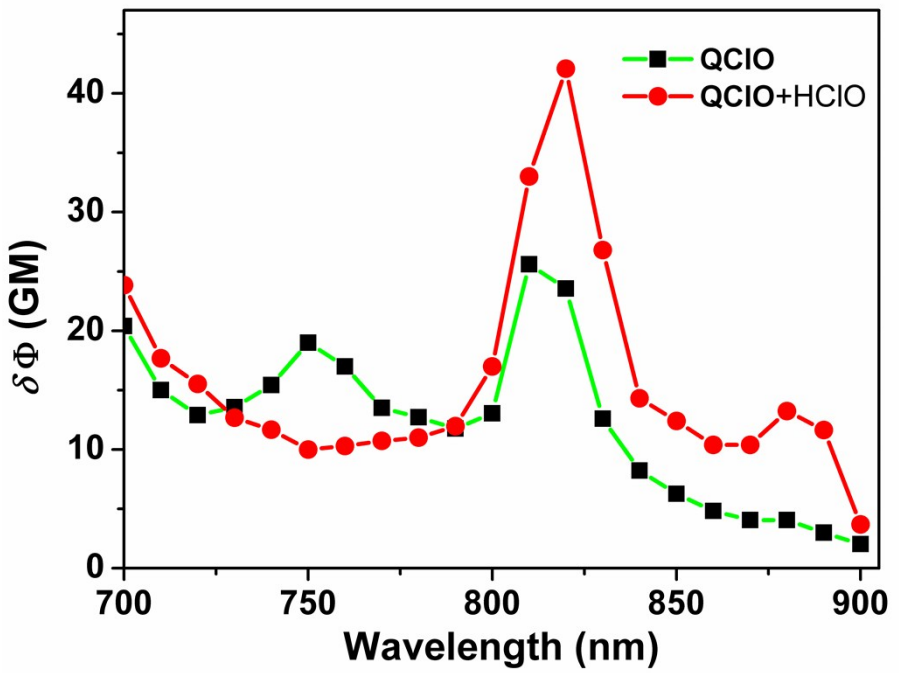


Fig. S8 Two-photon spectra of **QClO** in the absence and presence of excess HClO in 10 mM PBS buffer (pH=7.4, containing 5% DMF).

5. Cytotoxicity

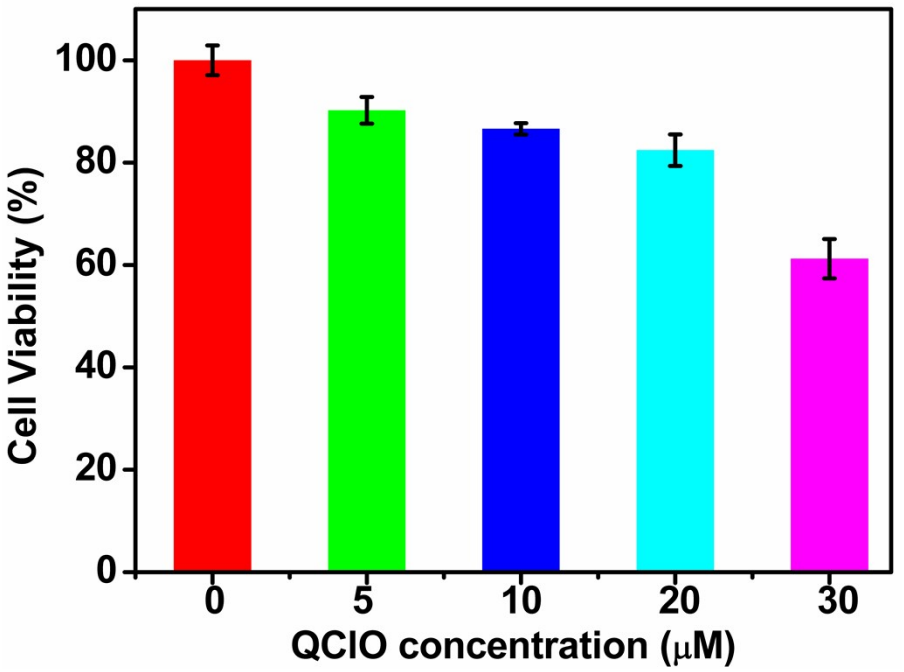


Fig. S9 Viability of HeLa cells incubated with various concentrations (0-30 μM) of **QClO** measured by MTT assay.

6. Tissue imaging under two photon microscopy

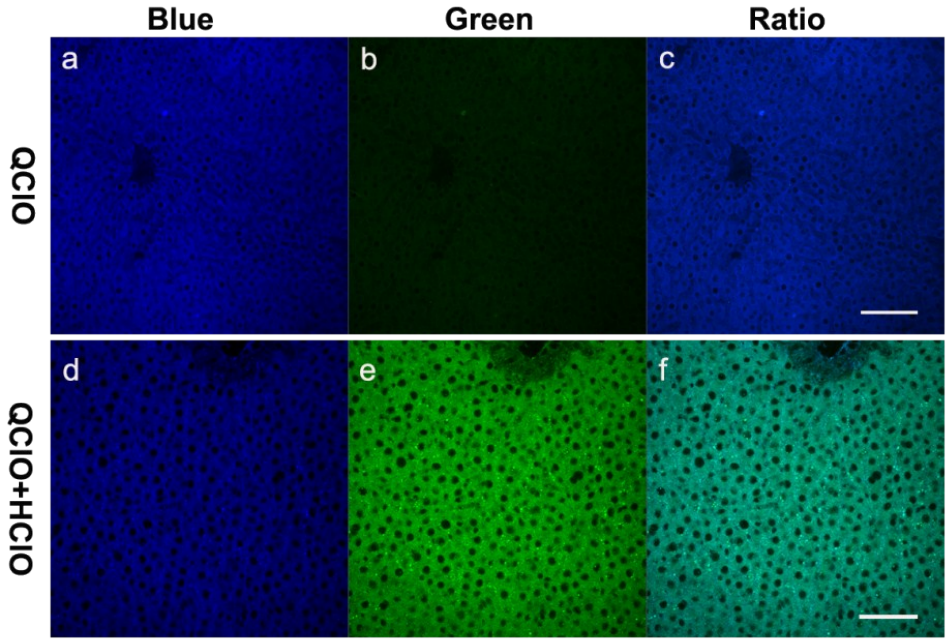


Fig. S10 TP images of rat liver tissues stained with 20 μM **QClO** for 1h, and then incubated with 20 μM NaClO for 1 h. The TP fluorescence was collected at blue channel (400-500 nm) and green channel (550-600 nm) upon the excitation of 820 nm. Scale bar: 100 μm.

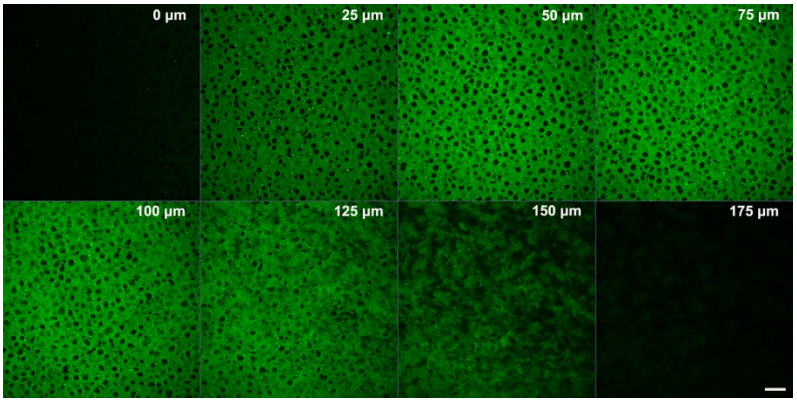


Fig. S11 *Z*-direction TP images for 20 μM **QClO**-stained rat liver tissue incubated with 20 μM NaClO solution for 1h. The TP fluorescence was collected at green channel (550-600 nm) upon the excitation of 820 nm. Scale bar: 50 μm

7. NMR and MS Data

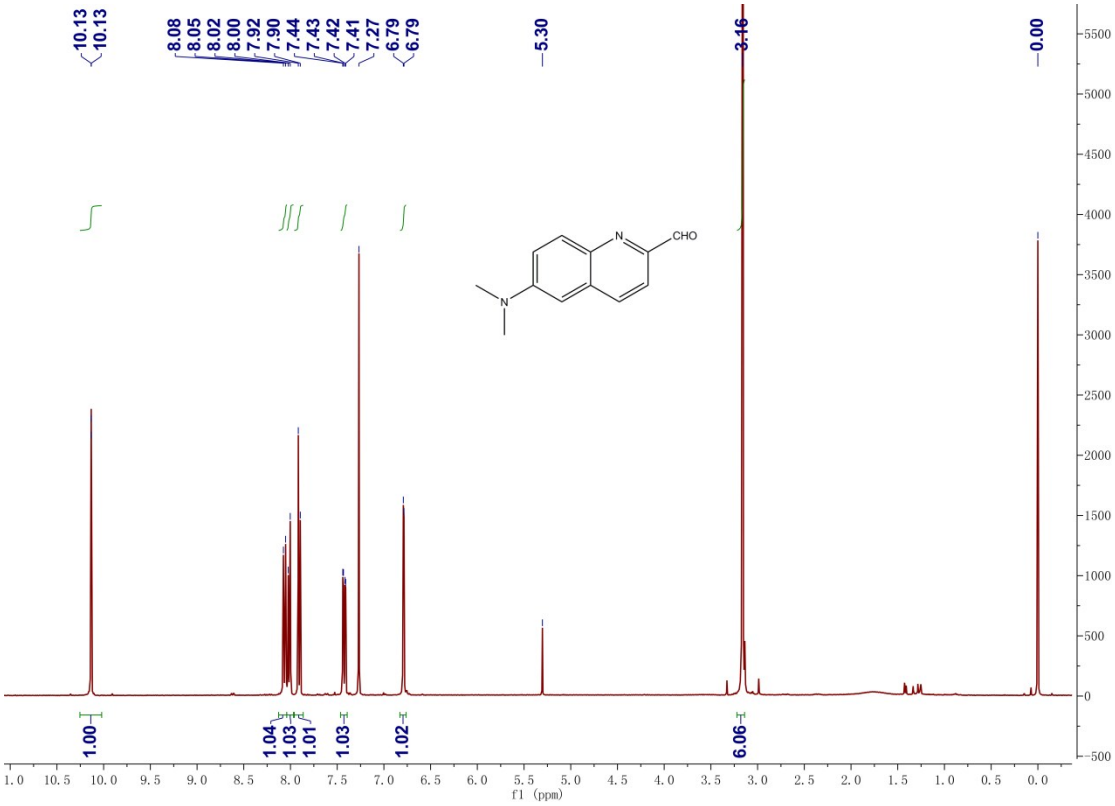


Fig. S12 ^{1}H NMR spectrum of **QN** ($CDCl_3$, 298K, 400 MHz).

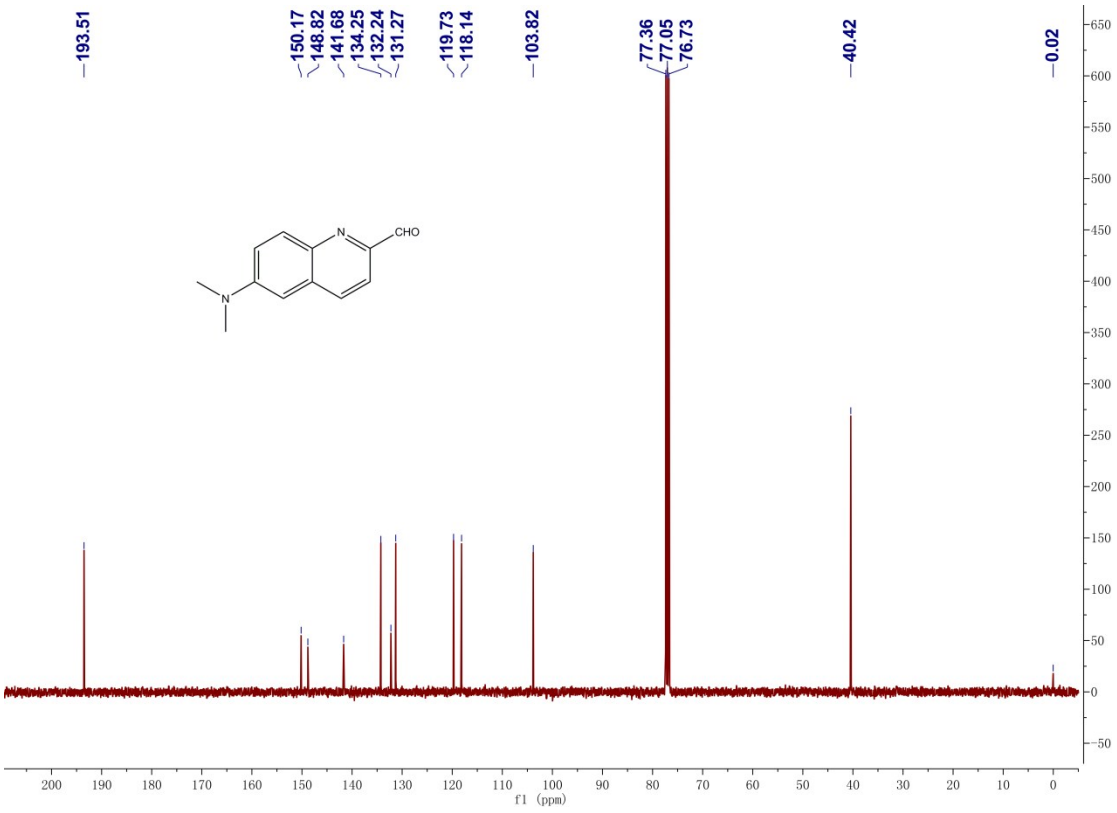


Fig. S13 ^{13}C NMR spectrum of **QN** ($CDCl_3$, 298K, 101 MHz).

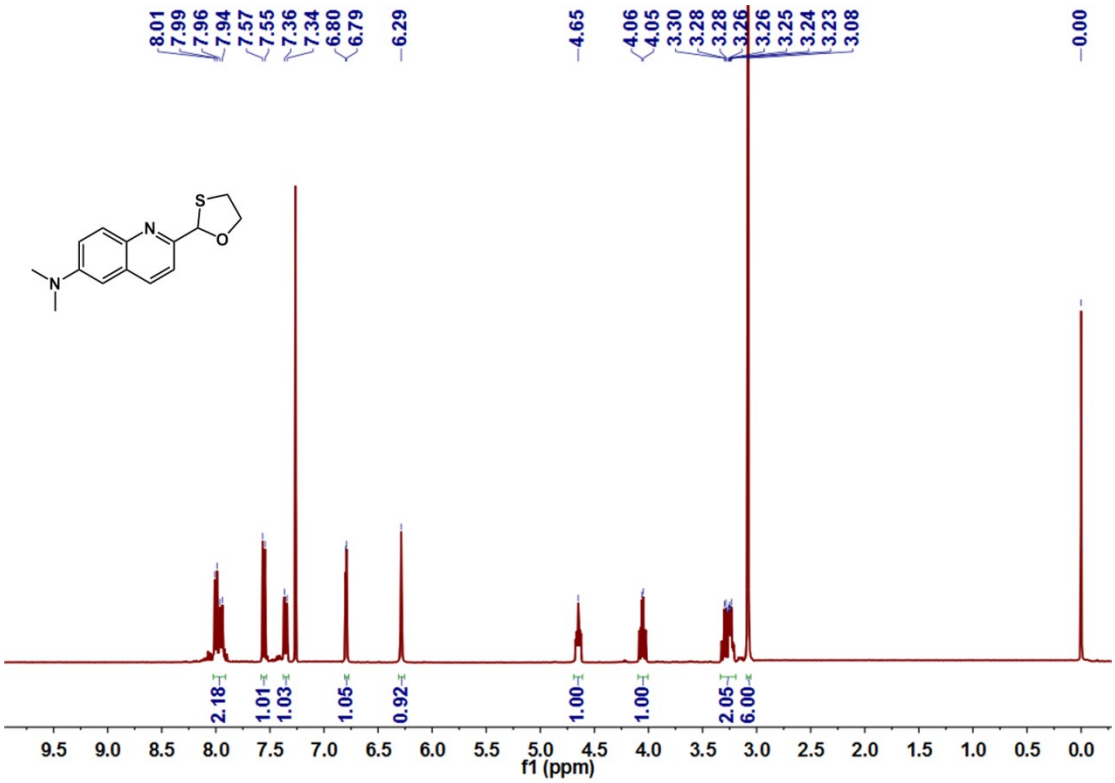


Fig. S14 ^{1}H NMR spectrum of **QClO** (DMSO-*d*$_6$, 298K, 400 MHz).

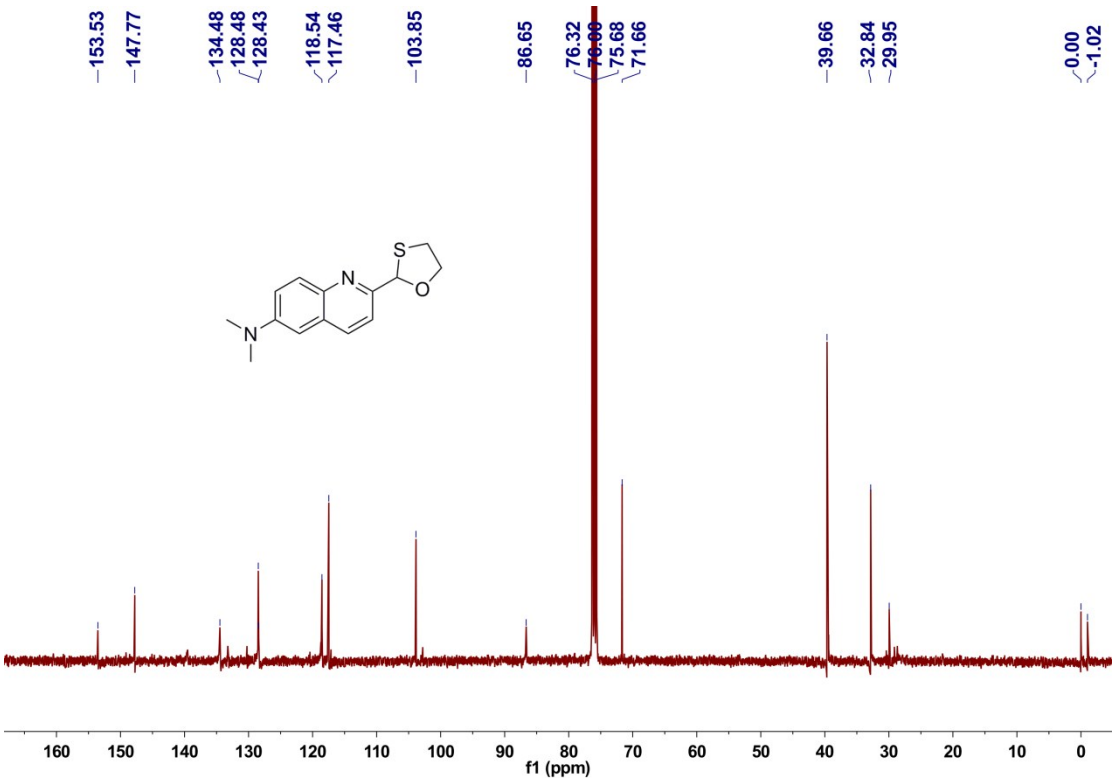
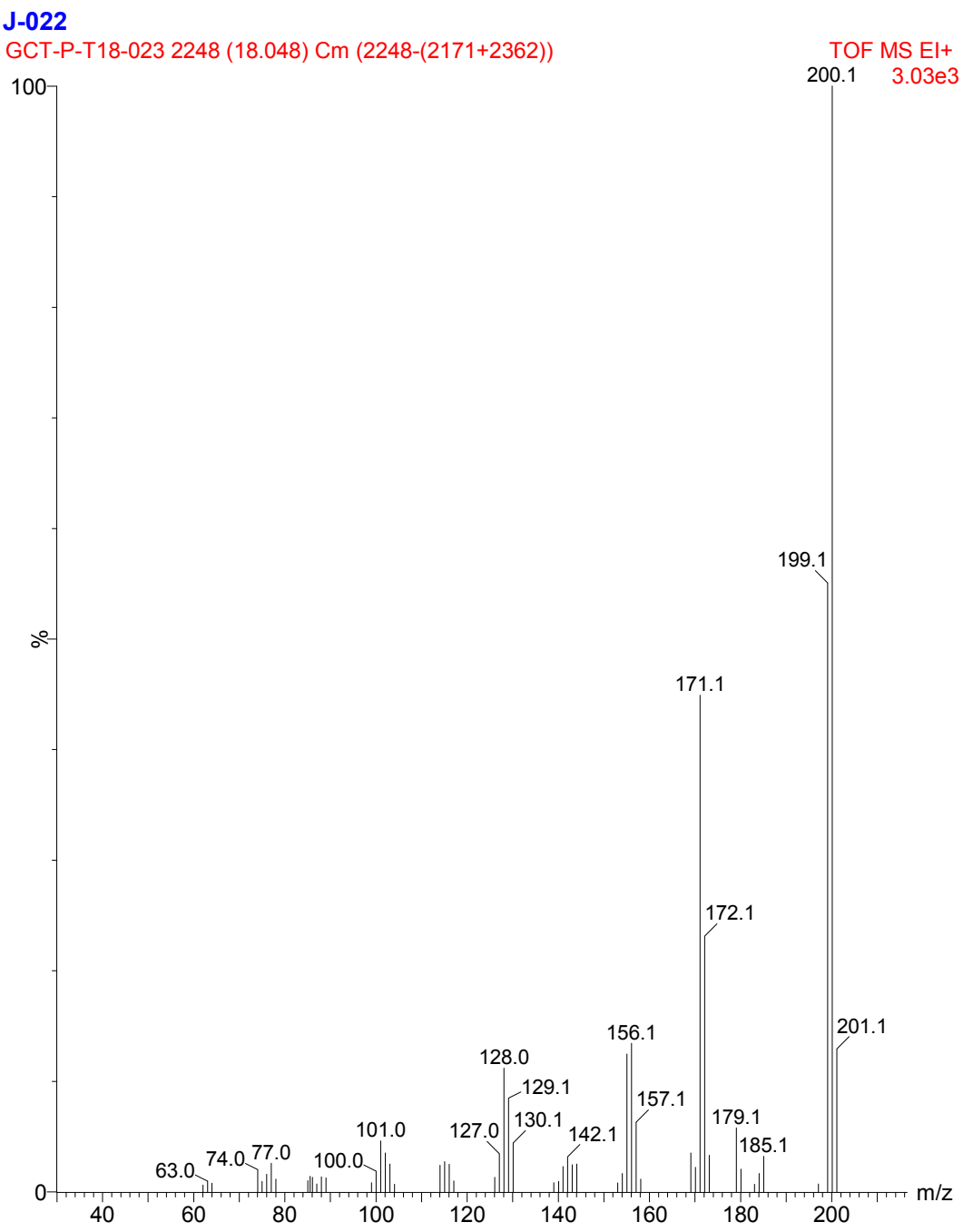
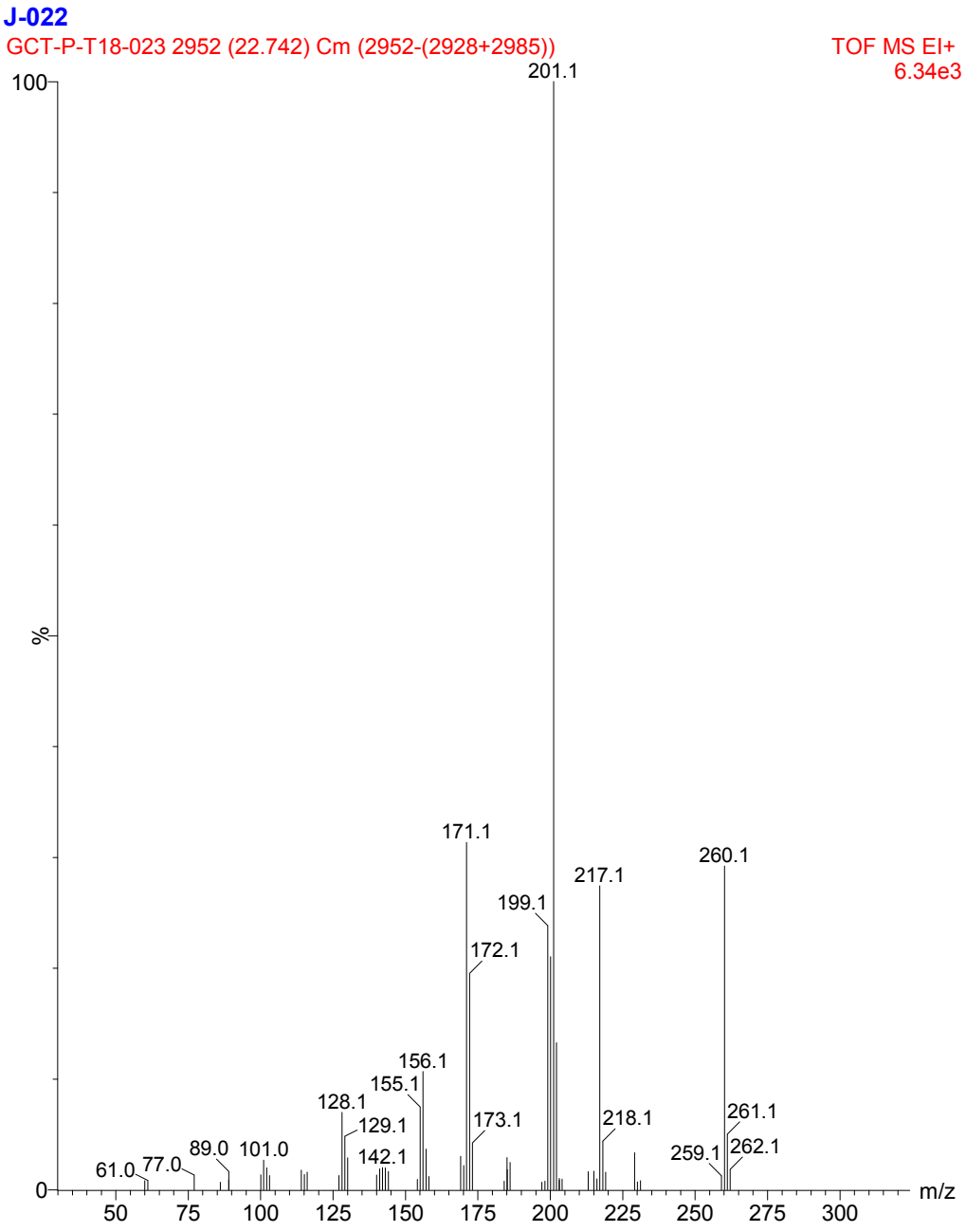


Fig. S15 ^{13}CNMR spectrum of **QClO** (DMSO-*d*$_6$, 298K, 101 MHz).



Mass	Calc. Mass	mDa	PPM	DBE	Formula
200.0946	200.0950	-0.4	-2	8.0	$C_{12}H_{12}N_2OS$

Fig. S16 EI-HRMS of the compound **QN**.



Mass	Calc. Mass	mDa	PPM	DBE	Formula
260.0989	260.0983	0.6	2.3	8.0	**$C_{14}H_{16}N_2OS$**
	260.0995	-0.6	-2.3	4.0	$C_{11}H_{17}N_2O_2FS$
	260.1001	-1.2	-4.6	13.0	$C_{19}H_{13}F$
	260.0984	0.5	1.9	1.0	$C_8H_{15}N_2O_4F_3$

Fig. S17 EI-HRMS of the compound **QClO**.

8. Reference

1. J. N. Demas and G. A. Crosby, *J. Phys. Chem*., 1971, **75**, 991-1024.
2. H. J. Kim, J. H. Han, M. K. Kim, C. S. Lim, H. M. Kim and B. R. Cho, *Angew. Chem., Int. Ed.,* 2010*,* **49***,* 6786-6789.
3. Z. Mao, L. Hu, X. Dong, C. Zhong, B. F. Liu and Z. Liu, *Anal. Chem*., 2014, **86**, 6548-6554.
4. M. Petit, C. Tran, T. Roger, T. Gallavardin, H. Dhimane, F. Palma-Cerda, M. Blanchard-Desce, F. C. Acher, D. Ogden, and P. I. Dalko, *Org. Lett*., 2012, **14**, 6366-6369.
5. Q. Xu, C. H. Heo, G. Kim, H. W. Lee, H. M. Kim and J. Yoon, *Angew. Chem. Int. Ed*., 2015, **54**, 4890-4894.
6. W. Zhang, W. Liu, P. Li, J. kang, J. Wang, H. Wang and B. Tang, *Chem. Commun*., 2015, **51**, 10150-10153.
7. L. Yuan, L. Wang, B. K. Agrawalla, S. J. Park, H. Zhu, B. Sivaraman, J. Peng, Q. H. Xu and Y. T. Chang, *J. Am. Chem. Soc*., 2015, **137**, 5930-5938.
8. B. Zhang, X. Yang, R. Zhang, Y. Liu, X. Ren, M. Xian, Y. Ye and Y. Zhao, *Anal. Chem*., 2017, **89**, 10384-10390.
9. Y. W. Jun, S. Sarkar, S. Singha, Y. J. Reo, H. R. Kim, J. J. Kim, Y. T. Chang and K. H. Ahn, *Chem. Commun*., 2017, **53**, 10800-10803.
10. Y. L. Pak, S. J. Park, D. Wu, B. Cheon, H. M. Kim, J. Bouffard and J. Yoon, *Angew. Chem. Int. Ed*., 2018, **57**, 1567-1571.