Supplementary Material (ESI) for Chemical Communications

Specific and Sensitive Method for Detection of Hypochlorous Acid for the Imaging of Microbe-Induced HOCl Production

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Methods

Quantification of Fluorescence.

Fluorescence emission spectra were obtained using a RF-5301/PC spectrofluorophotometer (Shimadzu). For the selectivity assays, the test solution was excited at 515 nm for **R19** fluorophore and at 565 nm for **R101** fluorophore. The slits were 1.5 nm/1.5 nm for **R19-S** and **R19-Se**, and 3 nm/3 nm for **R101-S**.

Generation of ROS/RNS.

Various ROS and RNS, including H_2O_2, $ROO^{\bullet}$, $NO^{\bullet}$, $^{\bullet}O_2^-$, $^{\bullet}OH$, HOCl, $^1O_2$ and $ONOO^-$ were prepared according to the following methods. H_2O_2 (final 200 μM) was added to 0.1 M KH_2PO_4 buffer (pH 5.5) and the mixture was then stirred for 30 min at 25 ^oC. $ROO^{\bullet}$ that was generated from 2,2'-Azobis(2-amidinopropane)dihydrochloride (200 μM) was added to 0.1 M KH_2PO_4 buffer (pH 5.5) and the mixture was then stirred at 25 ^oC for 30 min. Similarly, $NO^{\bullet}$ was generated from SNP (Sodium Nitroferricyanide (III) Dihydrate). SNP (final 200 μM) in 0.1 M KH_2PO_4 buffer (pH 5.5) was stirred for 30 min at 25 ^oC. Next, $\bullet O_2^-$ was generated by mixing xanthine and xanthine oxidase. Specifically, 0.1 unit xanthine oxidase was added first and then allowed to dissolve, after xanthine (final 200 μM) was added and the mixtures were stirred at 25 ^oC for 30 min.(Absorbance at 293 nm assays showed xanthine oxidase keep the high activity even if in low pH (5.5), see Figure S16). $^{\bullet}OH$ was generated by the addition of ferrous perchlorate and H_2O_2. Specifically, ferrous perchlorate (1 mM) and H_2O_2 (1 mM) were added to 0.1 M KH_2PO_4 buffer (pH 5.5) at room temperature and the mixture was then stirred for 30 min at 25 ^oC. HOCl was prepared from NaClO at room temperature in KH_2PO_4 buffer (pH 5.5) at 25 ^oC. $^1O_2$ generated from the metal-catalyzed decomposition of *tert*-butyl hydroperoxide.[1] Specifically, ferrous ammonium sulfate (1 mM) and *tert*-butyl hydroperoxide (1 mM) was incubated for 200 sec. in KH_2PO_4 buffer (pH 5.5) at 25 ^oC. $ONOO^-$ was obtained commercially from Enzo Life Sciences, and diluted by 0.1 M KH_2PO_4 buffer (pH 5.5) to 100 μM at 25 ^oC.

Concentration titration of HOCl.

In a typical experiment, test solution was prepared in a test cell by placing 30 μL of the probe stock solution (1 mM) and diluting the solution to 3 mL with 0.1 M KH_2PO_4 buffer (pH 5.5). Fluorescent spectra were obtained by increasing addition of NaClO stock solution in the same cell. The incubation time for every titration was 1 min. The test solution was excited at 515 nm for **R19** fluorophore and at 565 nm for **R101** fluorophore, and the excitation and emission slit widths were 1.5 nm/1.5 nm for **R19-S** and **R19-Se**, and 3 nm/3 nm for **R101-S**.

Product R19 from the reaction of R19-S and NaClO.

Two milliliters of NaClO solution with a concentration of 100 mM were added to a solution of **R19-S** (50 mg, 0.11 mmol) in CH_2Cl_2(5 mL)/CH_3CN(10 mL)/H_2O (5 mL). The mixture was then stirred for 2 h at room temperature. After the solvent was evaporated under reduced pressure, the crude product was purified using a silica-gel column (CH_2Cl_2/CH_3OH, 100:20) to give 27 mg of **R19**. ^{1}H NMR (DMSO-d_6, 250 MHz) δ (ppm): 7.93 (d, 1H, J = 7.5 Hz), 7.77-7.62 (m, 2H), 7.15 (d, 1H, J = 7.5 Hz), 6.31 (s, 2H), 6.19 (s, 2H), 5.29 (t, 2H, J = 5.0 Hz), 3.17-3.12 (q, 4H, J = 7.5 Hz), 1.87 (s, 6H), 1.19 (t, 6H, J = 7.2 Hz). FAB-MS m/z = 415.20, $[R19 + H]^+$, cal. for $C_{26}H_{27}N_2O_3$ = 415.20; m/z = 437.18, $[R19 + Na]^+$, cal. for $C_{26}H_{26}N_2NaO_3$ = 437.18.

Measurement of myeloperoxidase-mediated HOCl.

A constant amount (0.01 Unit) of human leukocyte myeloperoxidase (Sigma) was incubated with reaction mixture (1 mL) containing H_2O_2 (100 μM) and NaCl (100 mM) in 0.2 M Na_2HPO_4/0.1 M citric acid (pH 4.6). To see the chloride-dependent HOCl production, NaCl was omitted from the reaction mixture. After incubation at 37 ^{o}C for 10 min, the fluorescence response of **R19-S** (10 μM) was measured (excitation 515 nm, emission 545 nm).

Isolation of human polymorphonuclear neutrophils.

Polymorphonuclear neutrophils were purified from the blood obtained from healthy volunteers as

described previously.[2] The cells were incubated in fetal bovine serum (FBS) in RPMI-1640 medium.

HOCl detection in human polymorphonuclear neutrophils.

Human polymorphonuclear neutrophils (2×10^5) were treated with PMA (1.2 μM) or 10^6 *Saccharomyces cerevisiae* zymosan particles (Sigma) in the presence of **R19-S** (10 μM). Zymosan particles were previously serum-opsonized as described previously.[3] After 20 minutes of incubation, live fluorescent images were taken under a LSM510 Meta Confocal Microscope (Carl Zeiss, Germany).

Intestinal HOCl detection in live animals.

To induce HOCl, the adult female *Drosophila* were subjected to oral ingestion with 5% sucrose solution containing bacterial extract (1 mg/mL) in the absence of **R19-S** for 30 min and subsequently in the presence of **R19-S** (10 μM) for 90 min. Midguts were dissected and fixed in 4% formaldehyde. Microbe-induced HOCl production was visualized by using a LSM510 Meta Confocal Microscope (Carl Zeiss, Germany).

Animals.

Drosophila was maintained on standard cornmeal-agar medium at 25 °C and 60% relative humidity. The following fly lines, which have been described previously, were also used in this study: *UAS-DUOX-RNAi*;[4] *UAS-DUOX*;[4] *norpA*;[5] and *Da-GAL4*.[6] Standard *Drosophila* genetic techniques were used to generate transgenic flies and knock down flies of different genetic backgrounds.

Statistic analysis.

All statistical analyses were performed with Student's t-test.

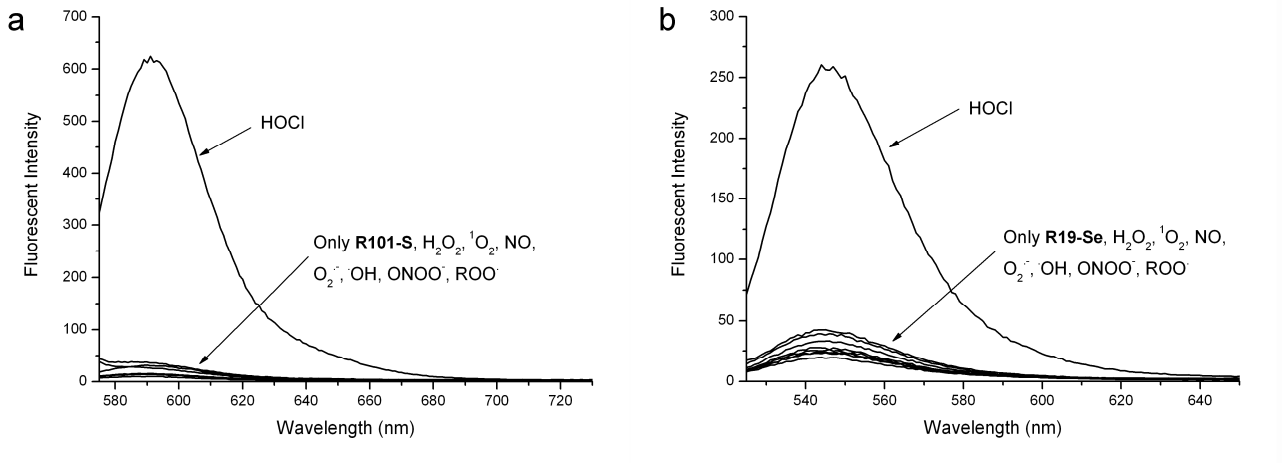


Figure S1. The selectivity assays of **R101-S** in KH_2PO_4 buffer (0.1 M, pH 5.5, 1%DMF) and **R19-Se** in KH_2PO_4 buffer (0.1 M, pH 5.5, 1%CH_3CN) for HOCl (excitation at 565 nm for **R101-S** and excitation at 515 nm for **R19-Se**). (a) Fluorescence changes of **R101-S** (10 μM) at 585 nm in response to treatment with HOCl (10 μM), H_2O_2 (200 μM), ROO• (200 μM AAPH), •OH (1 mM Fe^{2+} + 1 mM H_2O_2), NO• (200 μM SNP), $\bullet O_2^-$ (200 μM xanthine + 0.1 unit xanthine oxidase), $^1O_2$ (1mM of Fe^{2+} + 1mM *tert*-butyl hydroxide) and $ONOO^-$ (100 μM peroxynitrite). (b) Fluorescence changes of **R19-Se** (10 μM) at 550 nm in response to treatment with HOCl (10 μM), H_2O_2 (200 μM), ROO• (200 μM AAPH), •OH (1 mM Fe^{2+} + 1 mM H_2O_2), NO• (200 μM SNP), $\bullet O_2^-$ (200 μM xanthine + 0.1 unit xanthine oxidase), $^1O_2$ (1mM of Fe^{2+} + 1mM *tert*-butyl hydroxide) and $ONOO^-$ (100 μM peroxynitrite). AAPH: 2,2'-Azobis(2-amidinopropane)dihydrochloride; SNP: Sodium nitroferricyanide (III) dihydrate.

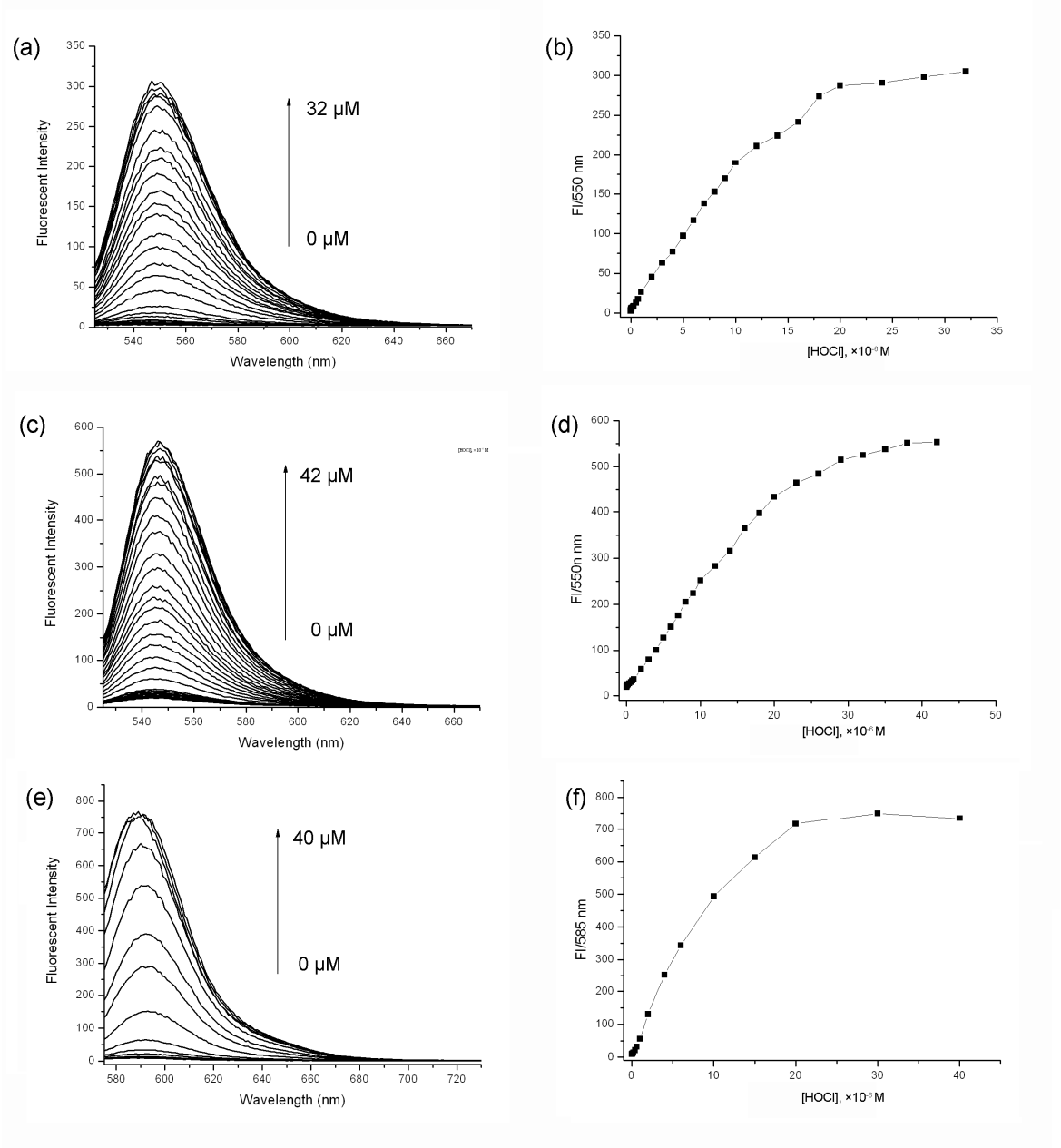


Figure S2. (a) Fluorescent titrations of **R19-S** (10 μM) in response to the addition of HOCl (slit: 1.5 nm/1.5 nm). (b) The change in the fluorescence intensity of **R19-S** (10 μM) at 550 nm against varied concentrations of HOCl from 0 to 32 μM (slit: 1.5 nm/1.5 nm). (c) Fluorescent titrations of **R19-Se** (10 μM) in response to the addition of HOCl (slit: 1.5 nm/1.5 nm). (d) The change in the fluorescence intensity of **R19-Se** (10 μM) at 545 nm against varied concentrations of HOCl from 0 to 42 μM (slit: 1.5 nm/1.5 nm). (e) Fluorescent titrations of **R101-S** (10 μM) in response to the addition of HOCl (slit: 3 nm/3 nm). (f) The change in the fluorescence intensity of **R101-S** (10 μM) at 585 nm against varied concentrations of HOCl from 0 to 40 μM (slit: 3 nm/3 nm).

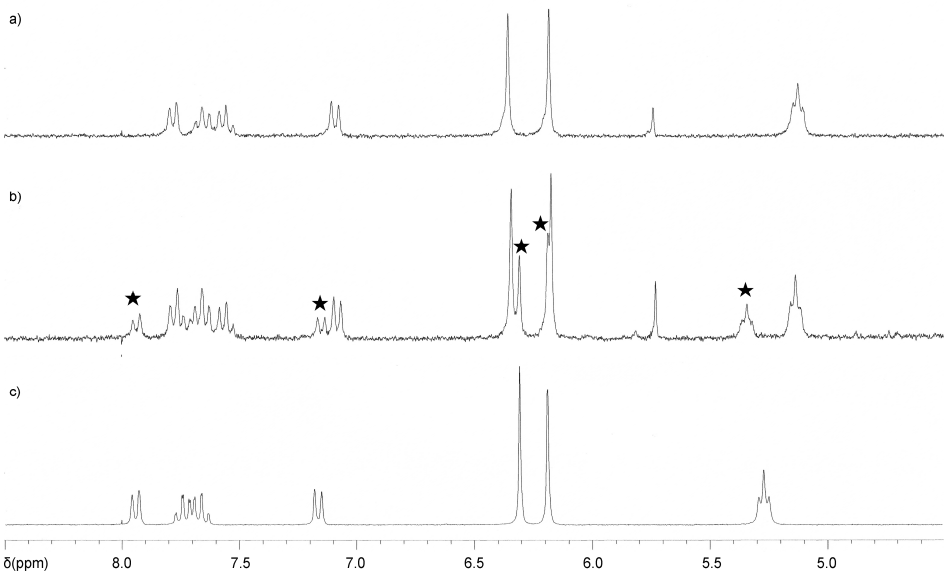


Figure S3. [1]HNMR spectra of **R19-S**, **R19 and R19-S** with NaClO in d_6-DMSO. (a) [1]HNMR spectrum of 4 mM **R19-S** in the absence of NaClO. (b) [1]HNMR spectrum obtained of 4 mM **R19-S** in the presence of 10 eq. NaClO. The signals marked with ★ are for the protons from the production of **R19**.(c) [1]HNMR spectrum of **R19**.

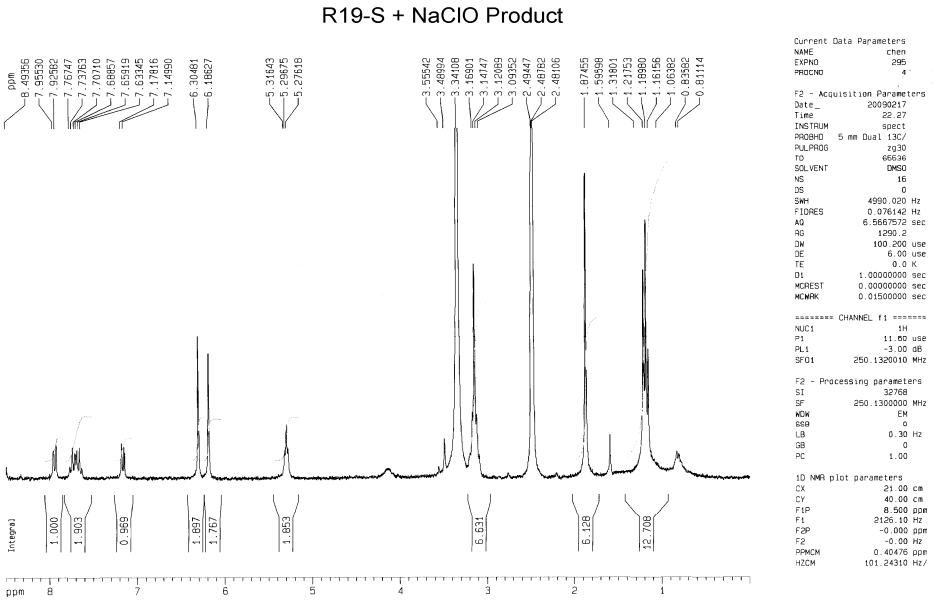


Figure S4. The [1]H NMR spectrum of product **R19** from the reaction of **R19-S** and HOCl.

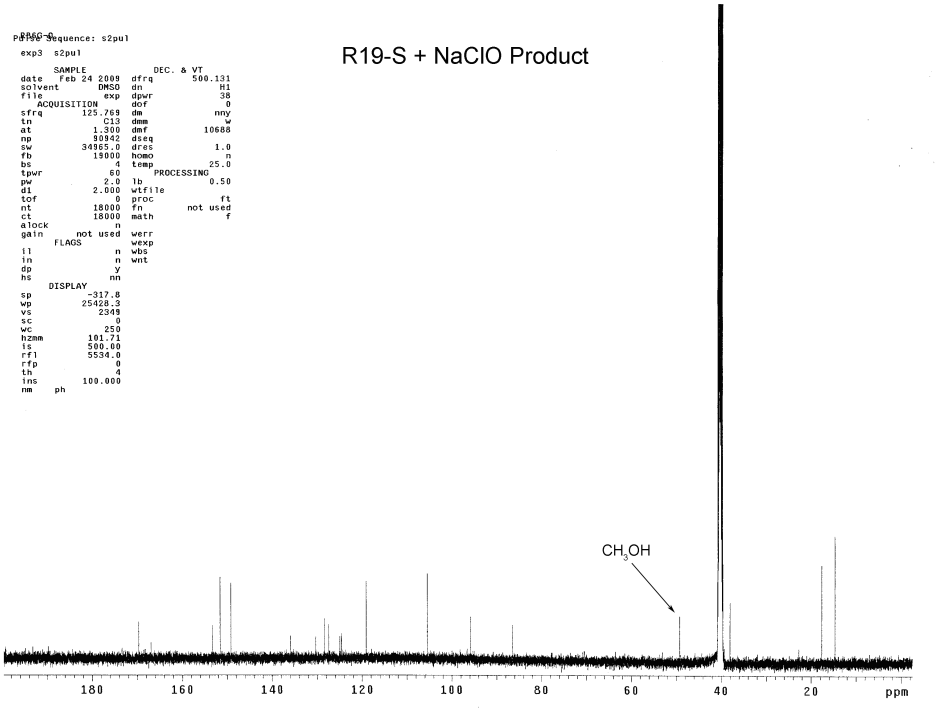


Figure S5. The ^{13}C NMR spectrum of product **R19** from the reaction of **R19-S** and HOCl.

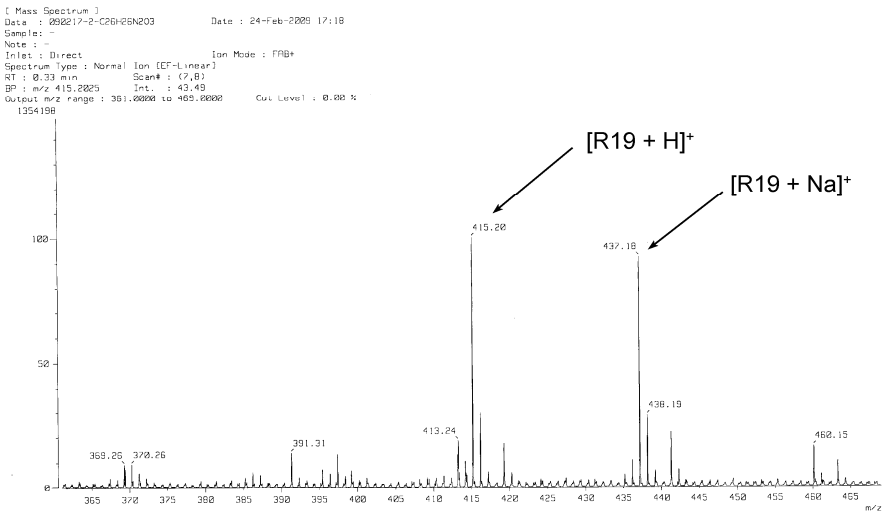


Figure S6. The mass spectrum of product **R19** from the reaction of **R19-S** and HOCl.

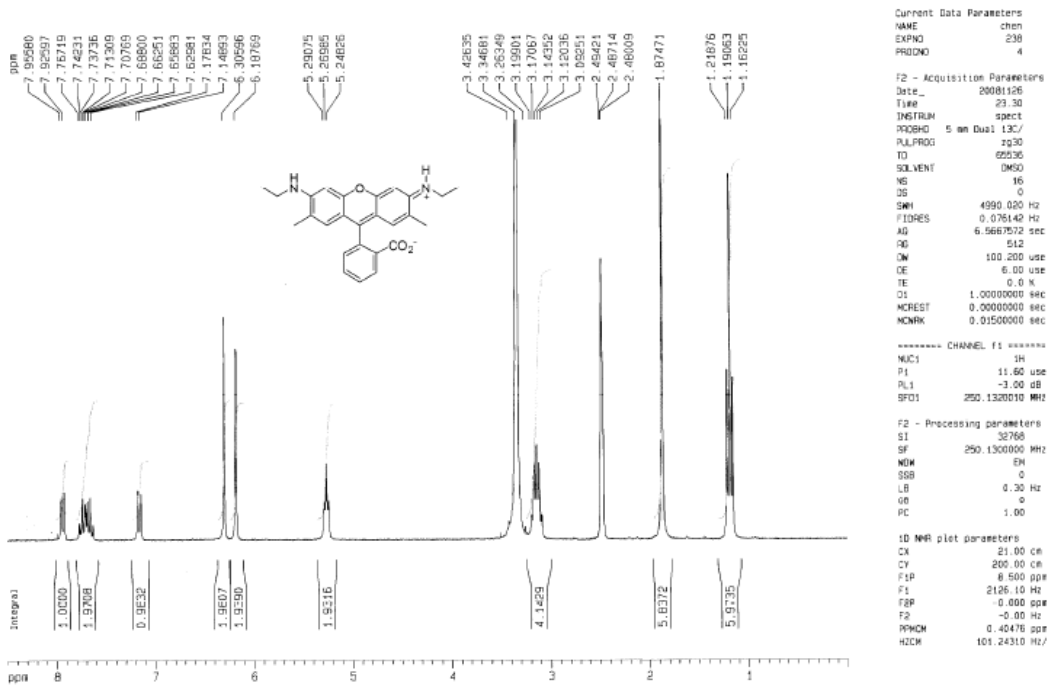


Figure S7 The ^{1}H NMR spectrum of **R19**.

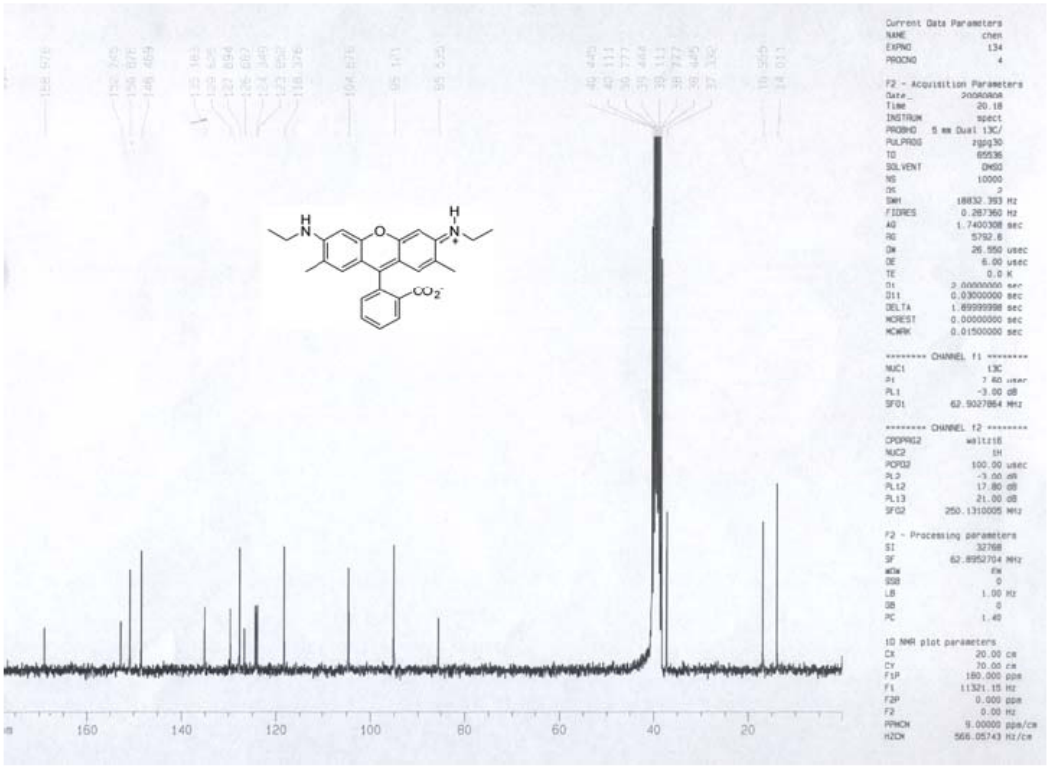


Figure S8 The ^{13}C NMR spectrum of **R19**.

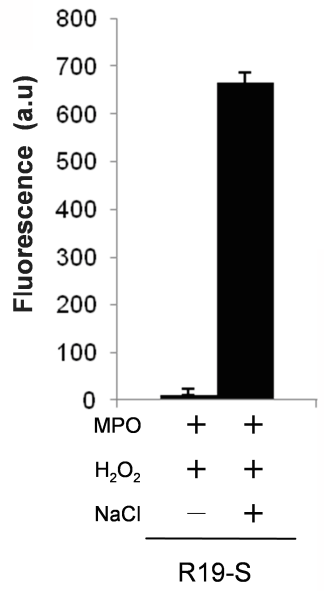


Figure S9. The fluorescence response of **R19-S** to HOCl generated by MPO. The fluorescence intensity of **R19-S** was measured in 0.2 M Na_2HPO_4/0.1 M citric acid (pH 4.6) containing 0.01 Unit of purified MPO (Sigma) and H_2O_2 (100 μM) in the presence or absence of NaCl (100 mM). The fluorescence change was measured using a fluorescence spectrophotometer (excitation at 515 nm, slit: 3 nm/1.5 nm). Data represent from three independent experiments (mean and s.d.).

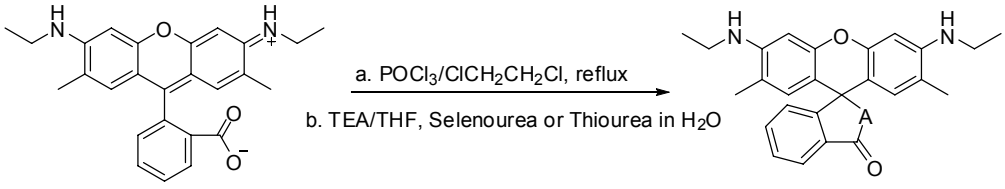
Synthesis(Scheme S1).

R19-S was prepared as describe previously.[7] The NMR spectra were shown as Figure S10 and Figure S11.

R101-S: To a stirred solution of rhodamine 101 (2 g, 3.5 mmol) in 1,2-dichloroethane (15 mL), phosphorus oxychloride (3 mL) was added dropwise. After refluxed for 4 h, the reaction mixture was cooled and evaporated in vacuo. The crude acid chloride was dissolved in THF (6 mL), and the resulting solution was then added dropwise to a mixed solution of thiourea (1.5 g, 20 mmol) and triethylamine (12 mL) in THF (50 mL)/water (10 mL) at room temperature. After stirring over night, the solvent was removed under reduced pressure. Then, 50 mL of water was added, and the formed precipitate was filtered. The precipitate was washed several times with water and dried. The crude product was purified by silica-gel column chromatography with CH_2Cl_2 as eluent, affording 860 mg of **R101-S** (yield 42%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 7.96 (d, 1H, J = 7.4 Hz), 7.48-7.35 (m, 2H), 7.18 (d, 1H, J = 7.4 Hz), 6.18 (s, 2H), 3.07-2.99 (m, 8H), 2.80 (t, 4H, J = 6.3 Hz), 2.52-2.35 (m, 4H), 1.95 (t, 4H, J = 4.3 Hz), 1.79 (t, 4H, J = 5.3 Hz). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ (ppm): 198.211, 157.908, 147.480, 143.329,

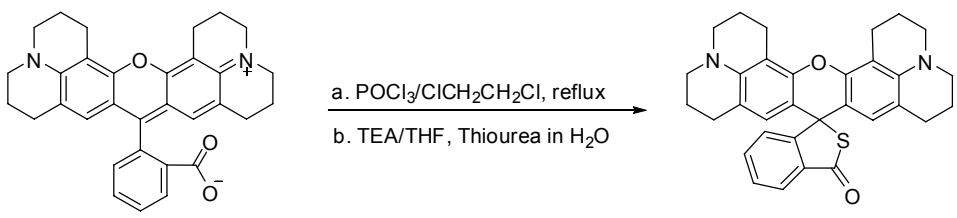
135.834, 134.154, 128.055, 127.502, 125.612, 122.504, 117.444, 108.751, 107.518, 64.008, 49.886, 49.427, 27.189, 21.988, 21.481, 21.186. FAB MS m/z = 507.2108 $[M + H]^+$, calc. for $C_{23}H_{31}N_2O_2Se$ = 507.2106. The NMR spectra were shown as Figure S12 and Figure S13.

R19-Se: To a stirred solution of rhodamine 19 (1 g, 2.4 mmol) in 1,2-dichloroethane (15 mL), phosphorus oxychloride (2 mL) was added dropwise. After refluxed for 4 h, the reaction mixture was cooled and evaporated in vacuo. THF (6 mL) was added to the crude acid chloride, and the suspension was then added dropwise to a mixed solution of selenourea (500 mg, 4.1 mmol) and triethylamine (6 mL) in THF (25 mL)/water (5 mL) at room temperature. After stirring over night, the solvent was removed under reduced pressure. Then, 30 mL of water was added, and the formed precipitate was filtered. The precipitate was washed several times with water and dried. The crude product was purified by silica-gel column chromatography with CH_2Cl_2 as eluent, affording 240 mg of **R19-Se** (yield 21%). ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 7.81 (d, 1H, *J* = 7.5 Hz), 7.49-7.32 (m, 2H), 7.14 (d, 1H, *J* = 7.5 Hz), 6.44 (s, 2H), 6.20 (s, 2H), 3.43 (s, 2H), 3.14 (q, 4H, *J* = 7.0 Hz), 1.84 (s, 6H), 1.24 (t, 6H, *J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$, 62.5 MHz) δ (ppm): 201.875, 159.023, 150.583, 146.893, 140.698, 134.217, 130.010, 128.405, 128.131, 122.520, 118.010, 111.235, 96.305, 62.953, 38.359, 16.826, 14.760. FAB MS m/z = 479.1241 $[M + H]^+$, calc. for $C_{26}H_{27}N_2O_2Se$ = 479.1239. The NMR spectra were shown as Figure S14 and Figure S15.



R19

R19-S: **A = S**
R19-Se: **S= Se**



Rhodamine 101 inner salt

R101-S

Scheme S1. Synthesis of rhodamine derivates.

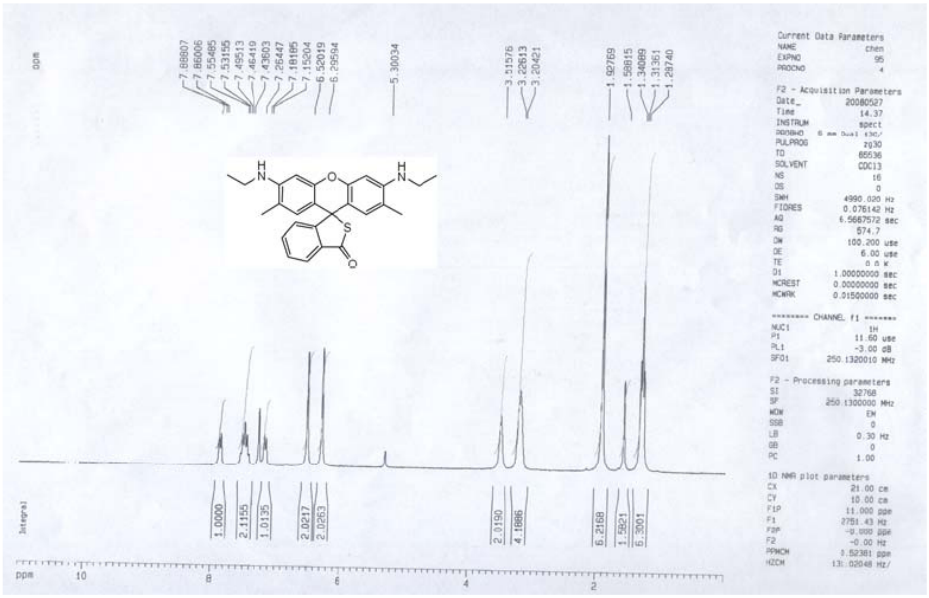


Figure S10 The ^{1}H NMR (250 MHz) spectrum of **R19-S**.

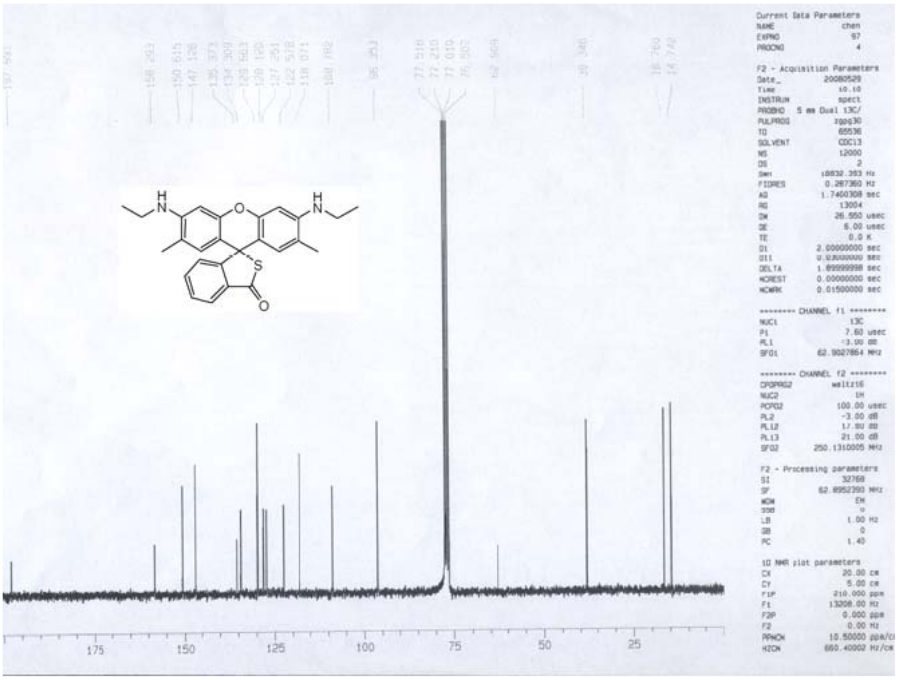


Figure S11 The ^{13}C NMR (62.5 MHz) spectrum of **R19-S**.

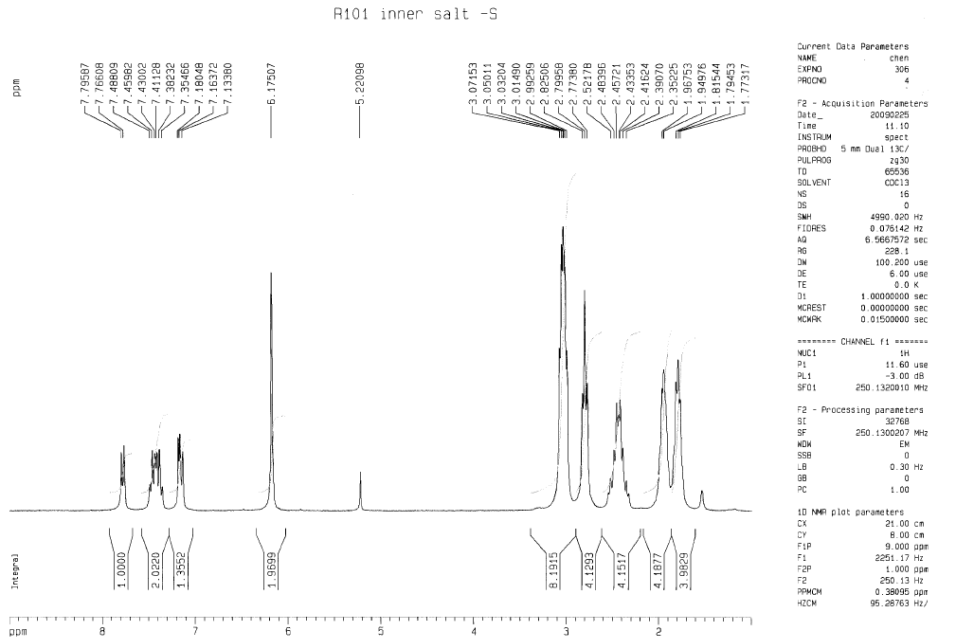


Figure S12. ^{1}H NMR (250 MHz) of **R101-S**.

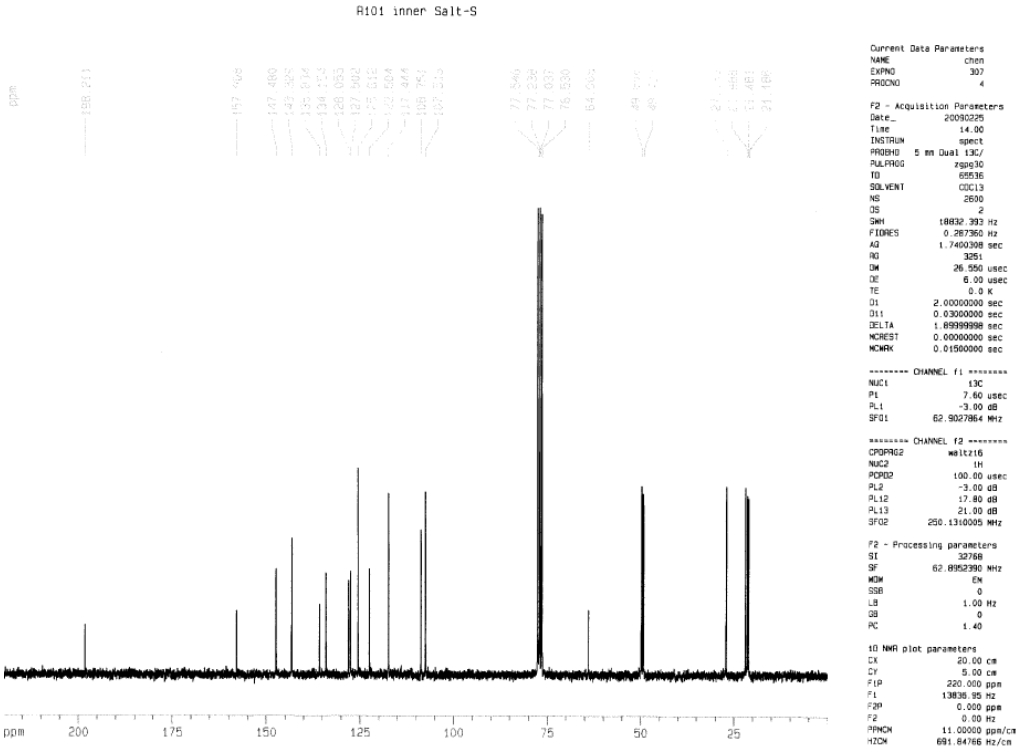


Figure S13. ^{13}C NMR (62.5 MHz) of **R101-S**.

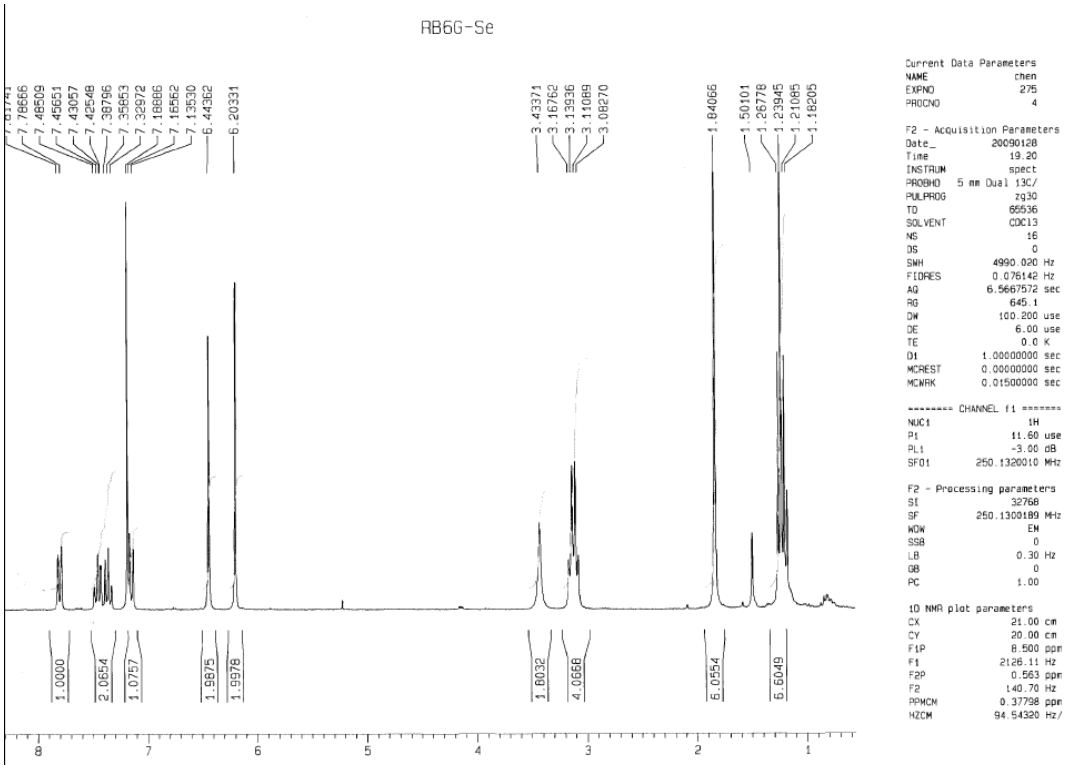


Figure S14. [1]H NMR (250 MHz) of **R19-Se**.

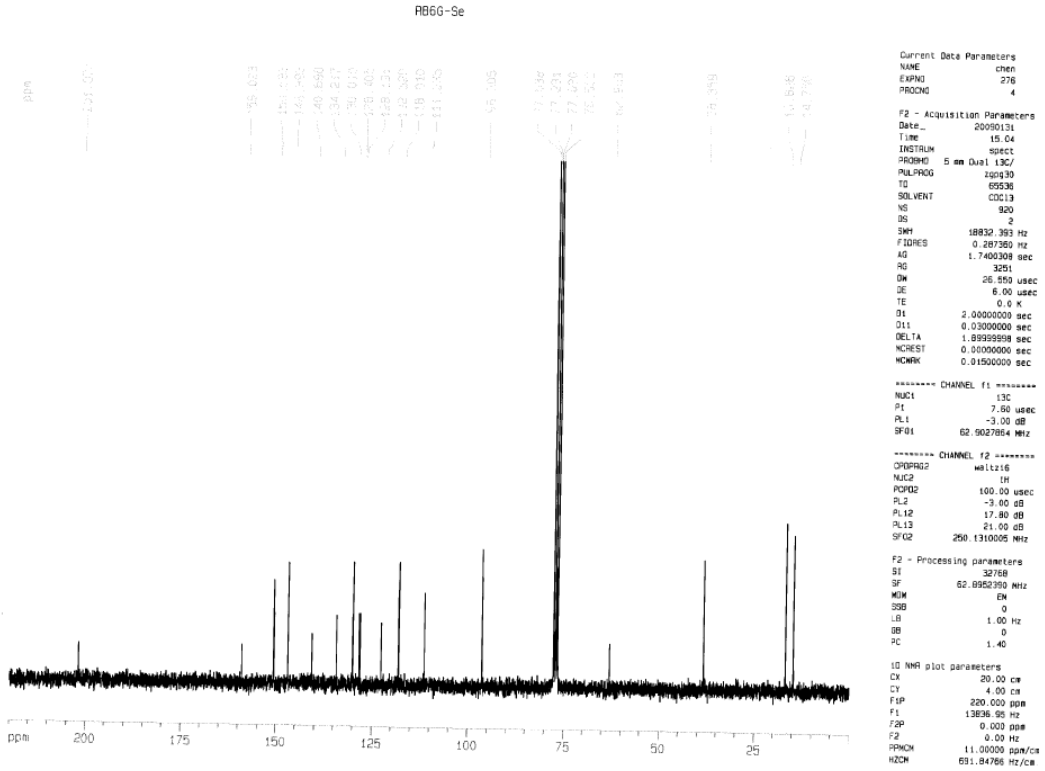


Figure S15. [13]C NMR (62.5 MHz) of **R19-Se**.

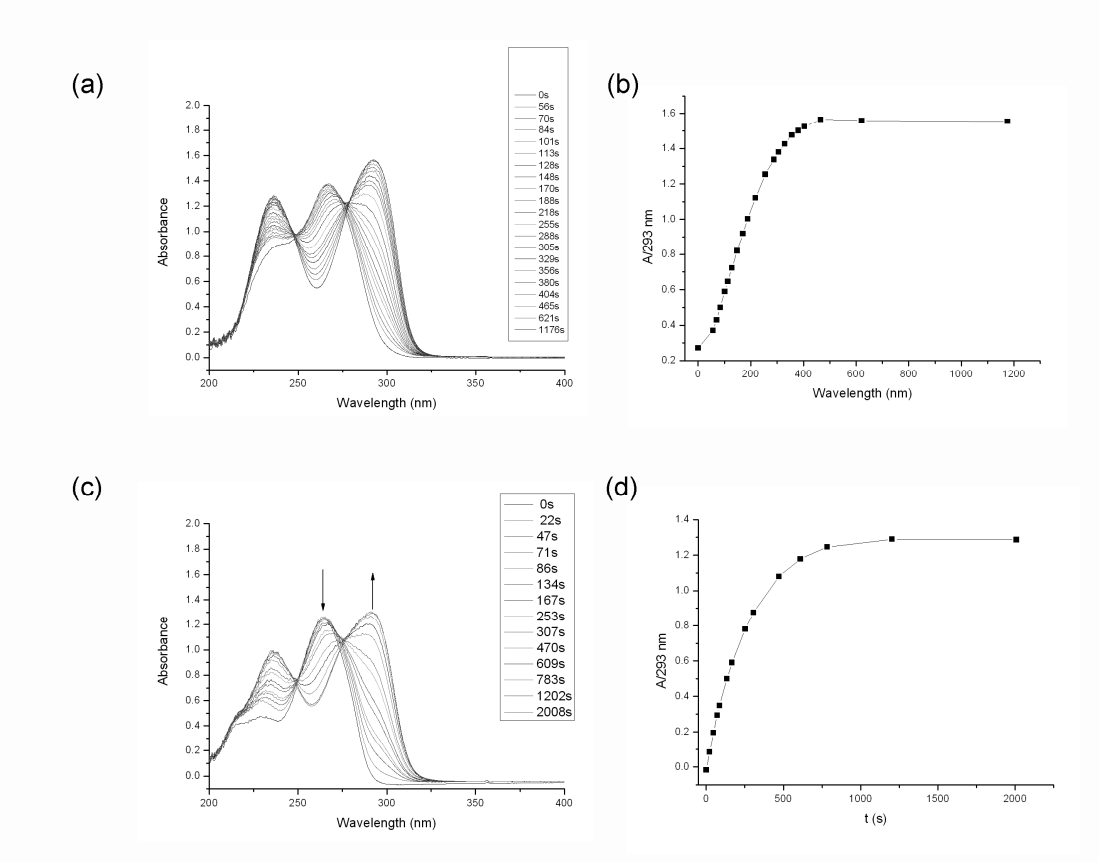


Figure S16. The activity assays of xanthine oxidase: (a) and (b), 200 μM xanthine was incubated with 0.1 unit xanthine oxidase in 0.01 HEPES buffer (pH 7.4); (c) and (d), 200 μM xanthine was incubated with 0.1 unit xanthine oxidase in 0.1 KH_2PO_4 buffer (pH 5.5).

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