Supporting Information

Elucidating Cuproptosis-Linked Redox Dynamics in Wilson Disease via a Reversible Ratiometric Fluorescence Strategy

Shunping Zang,‡ Qingguo He,‡ Yuyang Zeng, Yuyang Huang, Benhua Wang,* Qing Liu* and Xiangzhi Song*

College of Chemistry & Chemical Engineering, Central South University, Changsha 410083, PR China.

‡These authors equally contribute to this work.

*Corresponding authors.

Emails: benhuawang@csu.edu.cn; CSU-LQ@csu.edu.cn; xzsong@csu.edu.cn.

1. Materials and Instruments

All commercial chemicals from suppliers were used without further purification. HeLa cells were obtained from Xiangya Hospital, Central South University, China. Male C57BL/6 mice (9~10 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker 400 spectrometer, and mass spectra were acquired using a Bruker Daltonics micr-OTOF-Q II spectrometer. High performance liquid chromatography (HPLC) experiments were performed on an Essentia LC-20AR system (Shimadzu Corporation, Japan). Emission spectra were recorded on a Hitachi F-7000 fluorometer, and UV-vis absorption spectra were obtained using a Shimadzu UV-2450 spectrophotometer. A Leici PHS-3C pH meter was used for pH measurements. Fluorescence imaging experiments were conducted with Olympus FV3000 confocal microscope.

2. Preparation of reactive oxygen species

Hydrogen peroxide (H_2O_2)

The commercial 30% H_2O_2 aqueous solution was used as the source, then diluted by ultrapure water and its concentration was determined by measuring the absorbance at 240 nm (ε = 43.6 $mol^{-1}\cdot cm^{-1}$).

Hypochlorous acid (HOCl)

A commercial 35% NaOCl aqueous solution was used as the source, then diluted by ultrapure water and its concentration was determined by measuring the absorbance at 292 nm with ε = 391 $mol^{-1}\cdot cm^{-1}$.

Superoxide solution ($O_2^{\bullet-}$)

71.1 mg of KO_2 was dissolved in 10 mL dry DMSO and the resulting solution was stirred vigorously for 20 min to obtain a 0.1 mol/L $O_2^{\bullet-}$ (0.1 mol/L) stock solution.

Hydroxyl radical (·OH)

19.9 mg of $FeCl_2\cdot 4H_2O$ (1.0 mmol) was dissolved in 10 mL PBS containing 37.2 mg of $Na_2EDTA\cdot 2H_2O$ (10.0 mmol). To the above solution was added 10 mL H_2O_2 (1 mol/L) to obtain a 1.0 mol/L ·OH stock solution.

Nitric oxide (NO)

15.5 mg of diethylamine NONOate sodium salt hydrate was dissolved into 10 mL dry DMSO to obtain a 0.01 mol/L NO stock solution.

Peroxynitrite ($ONOO^-$)

At 0 °C, 5 mL hydrogen peroxide (0.7 mol/L) was acidified with 1 mL concentrated hydrochloric acid, and then mixed with 5 mL sodium nitrite solution (0.6 mol/L). To the resulting solution was quickly added into 5 mL sodium hydroxide aqueous solution (1.5 mol/L) within 2 s to prepare the $ONOO^-$ stock solution. Its concentration was determined by measuring the absorption absorbance at 302 nm with ε = 1670 $mol^{-1}\cdot cm^{-1}$.

3. Optical Measurements

A stock solution of probe **WD-Redox** in CH_3CN was prepared at a concentration of 1.0 mM. All optical tests were carried out in PBS buffer (10.0 mM, pH = 7.4) with a probe concentration of 10.0 μM. UV-vis absorption spectra were recorded over the range of 350-700 nm. Fluorescence spectra were acquired with λ_{ex} = 500 nm, using slit widths of 5 nm for both excitation and emission.

4. Cell Imaging

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin, in a humidified atmosphere containing 5% CO_2 and 95% air.

For exogenous HOCl/GSH cell imaging experiments, HeLa cells were pre-treated with HOCl (50.0 μM) for 10 min and then stained with probe **WD-Redox** (10.0 μM) for 30 min. Another set of cells was pre-treated with HOCl (50.0 μM) for 10 min, then incubated with GSH (1.0 mM) for 10 min, and finally stained with probe **WD-Redox** (10.0 μM) for 30 min.

For cuproptosis cell imaging experiments, HeLa cells were pre-treated with elesclomol-Cu (20.0 μM) for different durations (1, 2, 3 and 4 h), which each group subsequently stained with probe **WD-Redox** (10.0 μM) for 30 min.

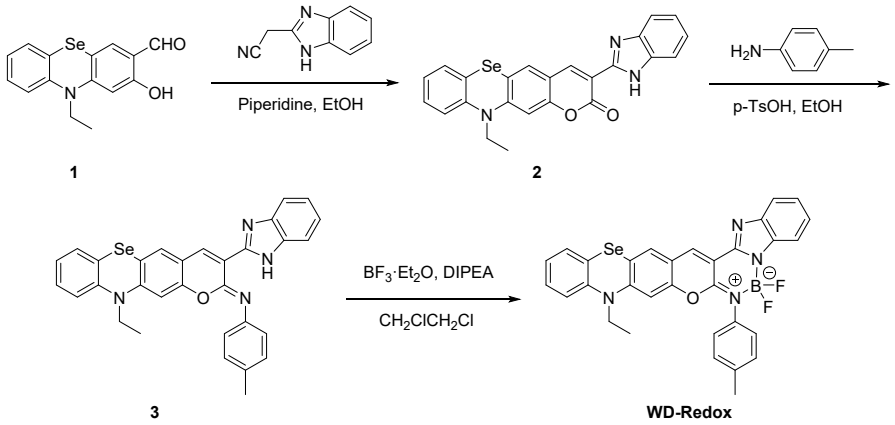
After washing all cell groups three times with PBS (1.0 mL each), confocal imaging experiments were performed using an Olympus FV3000 confocal microscope.

5. Liver tissue imaging

Male C57BL/6 mice (9 ~10 weeks) were purchased from Hunan SJA Laboratory Animal Co., Ltd (No. SYXK (Xiang) 2020-0012). All animal experiments were carried out in accordance with guidelines and all procedures were performed in accordance with national guidelines of the ethical committee of Central South University (No. 430727211101478756). Male C57BL/6 mice (9~10 weeks) were randomly divided into three groups (n = 3 per group). Control group: mice were fed a standard diet for 4 weeks. WD model group: mice were fed with a diet supplemented with $CuSO_4$ (1.0 g/kg) and provided 0.185% $CuSO_4$ in drinking water for 4 weeks to establish a copper overload model of Wilson's disease. Treatment group: WD model mice were treated with D-penicillamine (DPA, 500 mg/kg) for additional 4 weeks. After euthanasia, liver tissues slices (approximately 20 μm thick) were collected, sectioned, stained with probe **WD-Redox** (10.0 μM) for 30 min, and imaged using an Olympus FV3000 confocal microscope.

6. Synthesis

The synthesis of probe **WD-Redox** was illustrated in Scheme S1.



Scheme S1 Synthesis route of probe **WD-Redox**.

Synthesis of compound 1. Compound **1** was synthesized according to the literature method. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.25 (s, 1H), 9.58 (s, 1H), 7.32 – 7.26 (m, 2H), 7.22 – 7.17 (m, 1H), 6.98 – 6.93 (m, 2H), 6.44 (s, 1H), 3.92 (q, J = 7.0 Hz, 2H), 1.35 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.70, 163.02, 153.60, 143.00, 134.00, 130.14, 127.82, 124.17, 120.88, 117.72, 116.79, 110.60, 104.46, 43.76, 13.45. HRMS (ESI) m/z calcd for $C_{15}H_{14}NO_2Se$ $[M+H]^+$, 320.0190; found, 320.0233.

Synthesis of compound 2. Under an argon atmosphere, compound **1** (50.0 mg, 0.18 mmol) and 1H-benzo[*d*]imidazole-2-carbonitrile (163.0 mg, 0.55 mmol) were dissolved in anhydrous ethanol (10.0 mL), followed by the addition of a catalytic amount of piperidine (80.0 μL). The solution was refluxed at 100°C for 8 h. Next, the reaction mixture was poured into ice water (100.0 mL). Then, the obtained mixture was extracted with dichloromethane three times (40.0 mL × 3). The combined organic layers were washed with saturated saline (50.0 mL), dried over anhydrous Na_2SO_4. After removal of the solvent, the obtained residue was purified by column chromatography (silica gel, using petroleum ether/dichloromethane = 1:1-1:4 (v/v) as eluent) to give compound **2** as a dark red solid (65.0 mg, 90.0%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.00 (s, 1H), 7.93 (s, 1H), 7.69 (dd, *J* = 6.0, 3.2 Hz, 2H), 7.42 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.40 – 7.23 (m, 4H), 7.21 (d, *J* = 7.0 Hz, 2H), 7.06 (td, *J* = 7.5, 1.2 Hz, 1H), 4.09 (q, *J* = 6.9 Hz, 2H), 1.33 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.17, 154.87, 150.58, 146.48, 143.04, 141.72, 130.23, 128.88, 128.11, 124.46, 123.52, 120.34, 118.87, 117.68, 114.67, 103.69, 44.01, 29.74, 13.35. HRMS (ESI) m/z calcd for $C_{24}H_{18}N_3O_2Se$ $[M+H]^+$, 460.0564; found, 460.0576.

Synthesis of compound 3. Under an argon atmosphere, a catalytic amount of *p*-toluenesulfonic acid (8.6 mg) was added to a solution of compound **2** (50.0 mg, 0.11 mmol) and *p*-toluidine (23.6 mg, 0.22 mmol) in anhydrous ethanol (15.0 mL). The mixture was stirred at 90°C for 10 h. After cooling to room temperature, the reaction mixture was poured into brine (80.0 mL). Next, the obtained mixture was extracted with dichloromethane three times (50.0 mL × 3). The combined organic layers were dried over anhydrous Na_2SO_4 and then concentrated to yield the crude compound **3** (52.0 mg), which was used directly in the next step without further purification.

Synthesis of WD-Redox. To a solution of crude compound **3** (45 mg, 0.08 mmol) and *N*, *N*-diisopropylethylamine (72.0 μL, 0.41 mmol) in 1, 2-dichloroethane (5.0 mL) was added $BF_3{\cdot}Et_2O$ (98%, 105.0 μL, 0.80 mmol). The mixture was refluxed for 30 min. Next, the solvent was removed under vacuum. The obtained residue was purified by column chromatography (silica gel, using

dichloromethane/ methanol = 50:1-30:1 (v/v) as eluent) to give **WD-Redox** as a dark red solid (26.6 mg, 54.4%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.13 (s, 1H), 7.72 (d, *J* = 8.2 Hz, 1H), 7.62 (d, *J* = 8.1 Hz, 1H), 7.57 (s, 1H), 7.53 – 7.38 (m, 5H), 7.35 (dd, *J* = 7.6, 1.5 Hz, 1H), 7.29 (d, *J* = 1.5 Hz, 1H), 7.20 – 7.16 (m, 1H), 7.06 (td, *J* = 7.5, 1.2 Hz, 2H), 6.98 (dd, *J* = 8.3, 1.2 Hz, 1H), 3.95 (q, *J* = 7.0 Hz, 2H), 2.52 (s, 3H), 1.36 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 152.38, 151.69, 142.11, 138.58, 136.05, 133.61, 130.36, 130.18, 129.33, 128.13, 126.34, 124.93, 124.48, 123.42, 121.42, 120.52, 118.00, 117.90, 114.70, 114.27, 103.16, 44.10, 29.69, 21.34, 13.37. HRMS (ESI) m/z calcd for $C_{31}H_{24}BF_2N_4OSe$ $[M+H]^+$, 597.1176; found, 597.1110.

7. Experiments for mechanism study

WD-Redox (10.0 μM) was treated with HOCl (50.0 μM) in 3 mL CH_3CN: H_2O = 2: 8 solution. In addition, we successively treated **WD-Redox** (10.0 μM) with 50.0 μM HOCl and 1.0 mM of GSH in 3 mL CH_3CN: H_2O = 2: 8 solution. Each of these two samples was extracted with ethyl acetate twice (1 mL × 2) and concentrated. The extracted products were designated as product **1** (**WD-Redox** with HOCl) and product **2** (**WD-Redox** with HOCl and GSH). Then, we performed TLC analysis on products **1** and **2**.

Moreover, we scraped the main spots from TLC plates for products **1** and **2**, and measured their absorption and fluorescence spectra in CH_3CN/PBS buffer (10.0 mM, pH = 7.4, v/v = 2: 8).

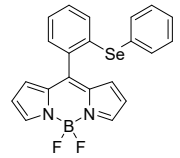
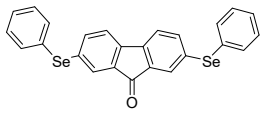
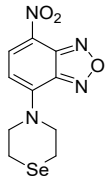
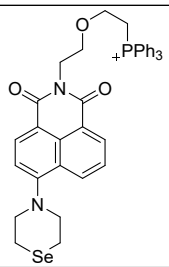
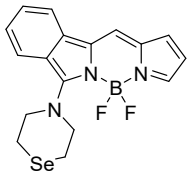
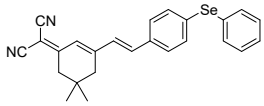
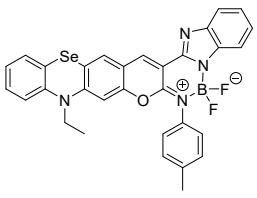
WD-Redox (10.0 μM) was treated with different concentrations of HOCl (0.0, 10.0, 20.0 and 50.0 μM) in 3 mL CH_3CN: H_2O = 2: 8 solutions. In addition, we successively treated **WD-Redox** (10.0 μM) with 50.0 μM HOCl and different concentrations of GSH (0.2, 0.5 and 1.0 mM) in 3 mL CH_3CN: H_2O = 2: 8 solutions. Each of these seven samples was extracted with ethyl acetate twice (1 mL × 2), concentrated, and re-dissolved in 1 mL CH_3CN for high performance liquid chromatography (HPLC).

Furthermore, the eluates from HPLC fraction of sample (a), (d) and (g) were subjected to HRMS analysis.

On TLC plate, **WD-Redox** exhibited an orange-red fluorescence while HOCl-oxidized product showed a yellow fluorescence, and subsequent GSH treatment restored the original fluorescence of **WD-Redox** (Fig. S4). The HPLC chromatogram of **WD-Redox** showed a single peak at 11.95 min (Fig. S5). After the addition of excessive HOCl, in contrast, only a single peak at 5.52 min was seen. Further treatment with a high concentration of GSH restored the single peak at 11.95 min. HRMS analysis on the reaction mixtures showed that HOCl-oxidized sample exhibited a peak at m/z = 613.1360, corresponding to the molecular weight of the expected product, **WD-Redox-O** (Fig. S6). Subsequent treatment with GSH resulted in the reappearance of the peak for **WD-Redox** at m/z = 597.1220.

8. Supplemental data

Table S1. Comparison of selenium-based fluorescence probe for HOCl monitoring.

Structure of probe	Detection species	Detection manner, fluorescence wavelength(s)	Application	Ref.
	HOCl	Off-on, Reversible, 526 nm	Exogenous and endogenous cell imaging	Org. Lett. 2013, 15, 878–881
	HOCl/GSH	Off-on, Reversible, 520 nm	Mice, zebrafish and cell imaging	Chem. Commun., 2015, 51, 10150-10153
	HOCl/GSH	Off-on, Reversible, 544 nm	Cell imaging	RSC Adv., 2015, 5, 79519-79524
	HOCl/GSH	Off-on, Reversible, 540 nm	Cell imaging	New J. Chem., 2018, 42, 15105-15110
	HOCl	Off-on, 552 nm	Zebrafish and cell imaging	J. Photochem. Photobiol. A Chem. 2024, 447, 115253
	HOCl/H_2S	Ratiometric, Reversible, 575/ 460 nm	Food detection	Spectrochim. Acta A Mol. Biomol. Spectrosc. 2025, 329, 125570
	HOCl/GSH	Ratiometric, Reversible, 555/712 nm	Cuproptosis and Wilson Disease tissue imaging	This work

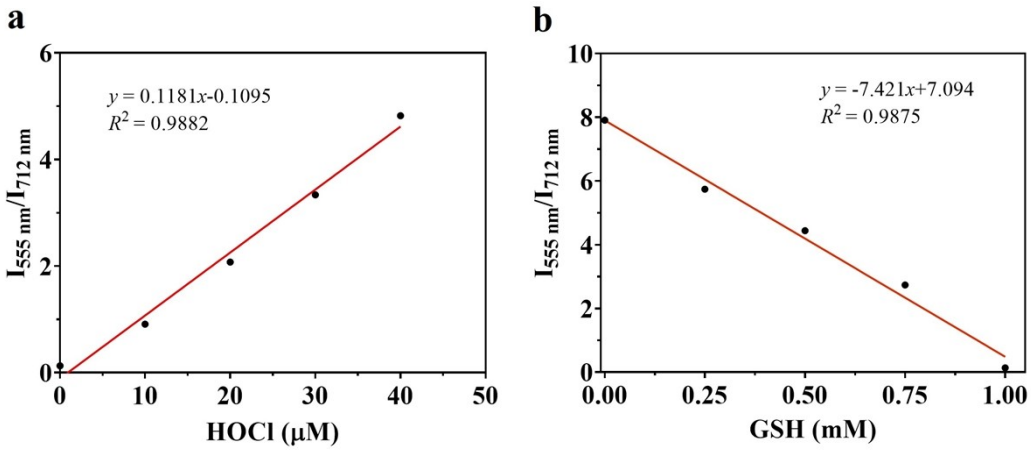


Fig. S1 (a) Linear correlation between the fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of probe **WD-Redox** and HOCl concentrations in CH_3CN/PBS buffer (10.0 mM, pH = 7.4, v/v = 2: 8). (b) Linear correlation between the fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of probe **WD-Redox** with HOCl (50.0 μM) and GSH concentrations in CH_3CN/PBS buffer (10.0 mM, pH = 7.4, v/v = 2: 8).

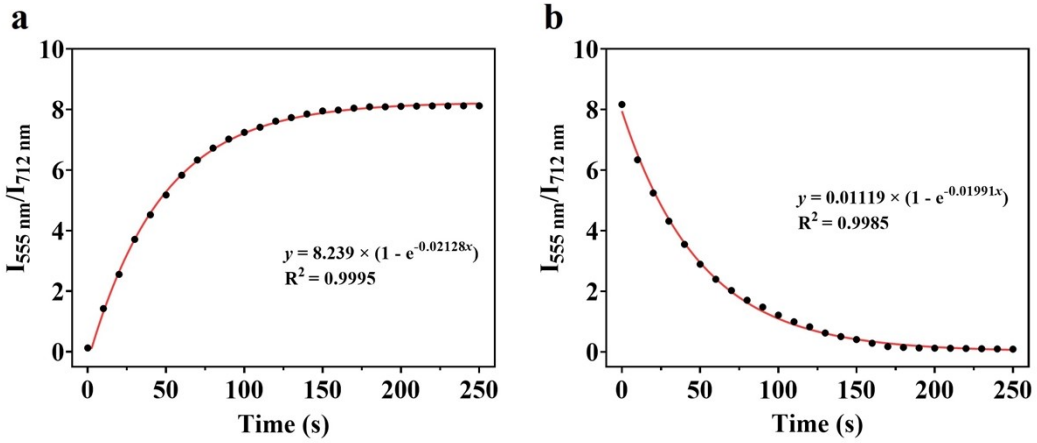


Fig. S2 *Pseudo-first-order* kinetic curves of fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of **WD-Redox** (10.0 μM) with the addition of different species in CH_3CN/PBS buffer (10.0 mM, pH = 7.4, v/v = 2: 8). (a) HOCl (50.0 μM) only. (b) HOCl (50.0 μM) first, followed by addition of GSH (1.0 mM) after 250 s.

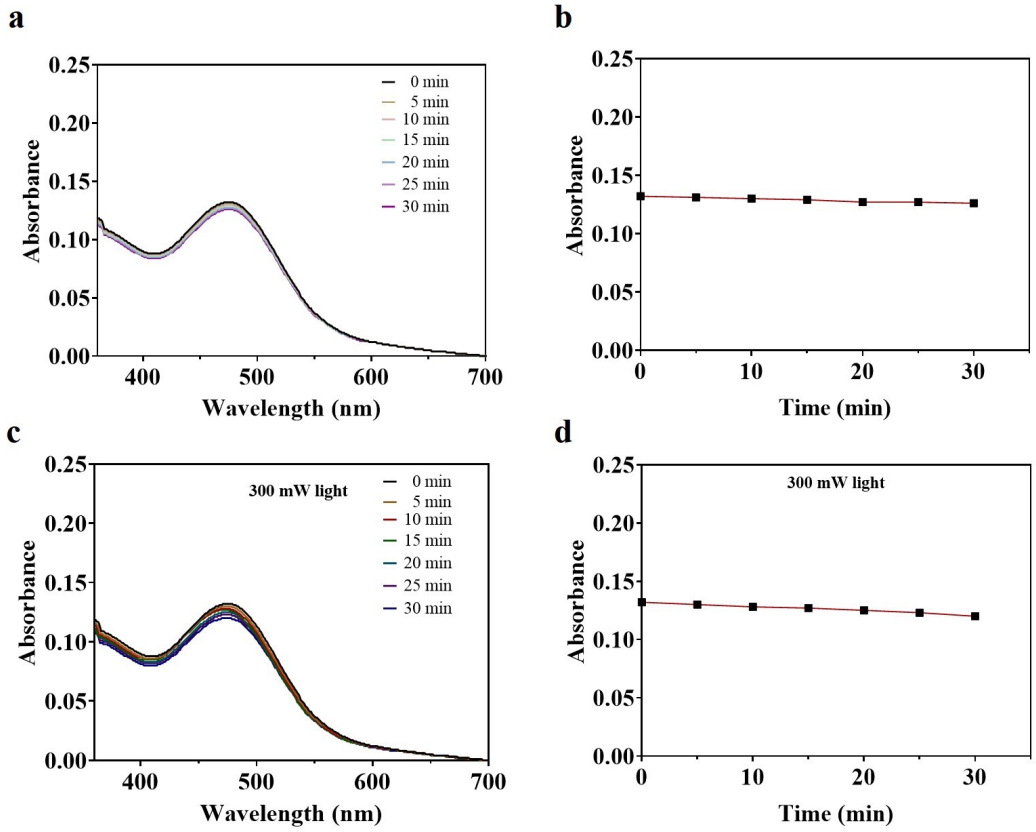


Fig. S3 (a, c) Absorption spectra of **WD-Redox** solutions in PBS buffer (10.0 mM, pH = 7.4) in the absence and presence of light exposure. (b, d) Relative absorbance changes corresponding to the data in (a) and (c).

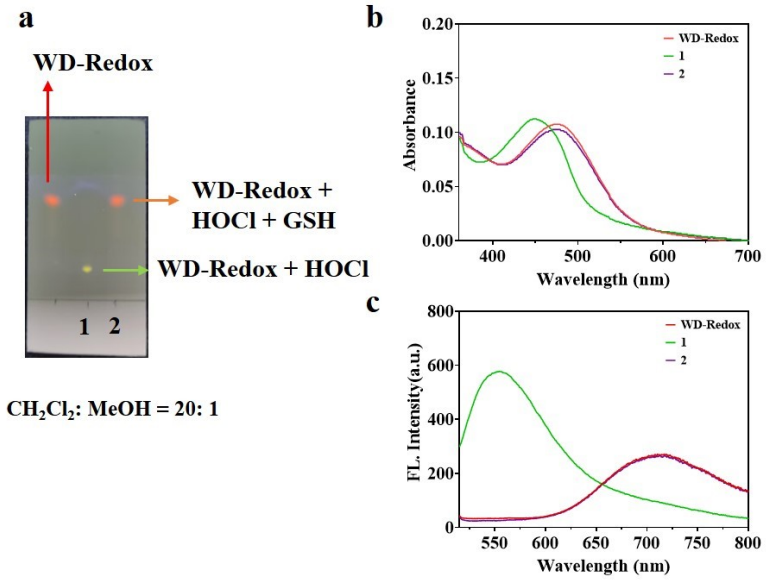


Fig. S4 (a) TLC analysis **WD-Redox** and the reaction products of **WD-Redox** with HOCl and GSH. (**1**: **WD-Redox** + HOCl; **2**: **WD-Redox** + HOCl + GSH; mobile phase used: CH_2Cl_2: MeOH = 20: 1). (b) UV-vis spectra of **WD-Redox**, the TLC spots from products **1** and **2**. (c) Fluorescence spectra of **WD-Redox**, the TLC spots from products **1** and **2**.

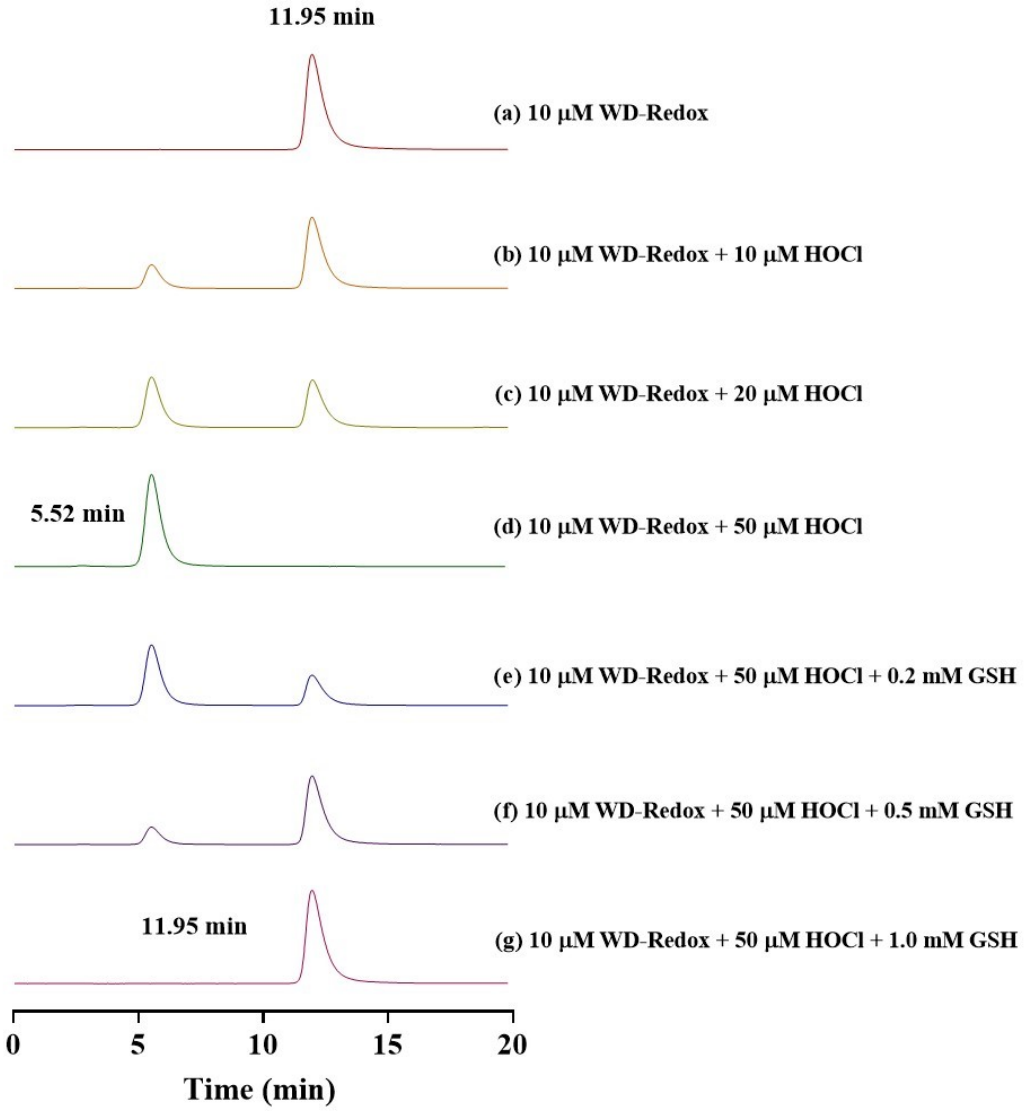


Fig. S5 HPLC chromatograms of **WD-Redox** (10.0 μM) with HOCl (0.0, 10.0, 20.0, 50.0 μM, 5 min incubation) and subsequent treatment with GSH (0.2, 0.5, 1.0 mM, 5 min incubation). Conditions: eluent, CH_3CN: MeOH = 95: 5; flow rate, 5.00 mL/min; column, 20 mm ID × 250 mm (Buckyprep, Nacalai Tesque Inc.); detection wavelength = 470 nm; inject volume, 100 μL.

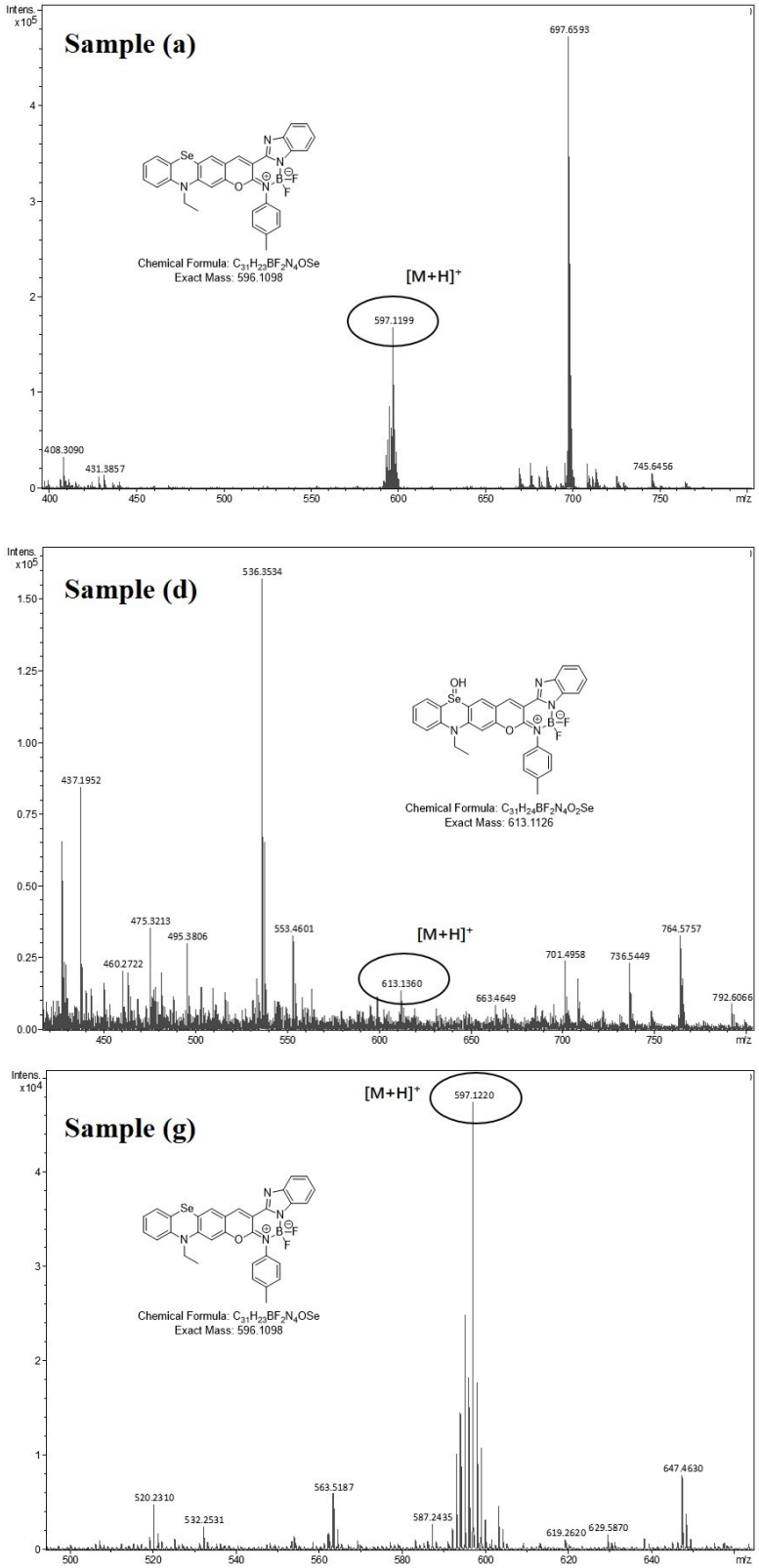


Fig. S6 HRMS spectra of sample (a), (d) and (g) in Fig. S5, respectively.

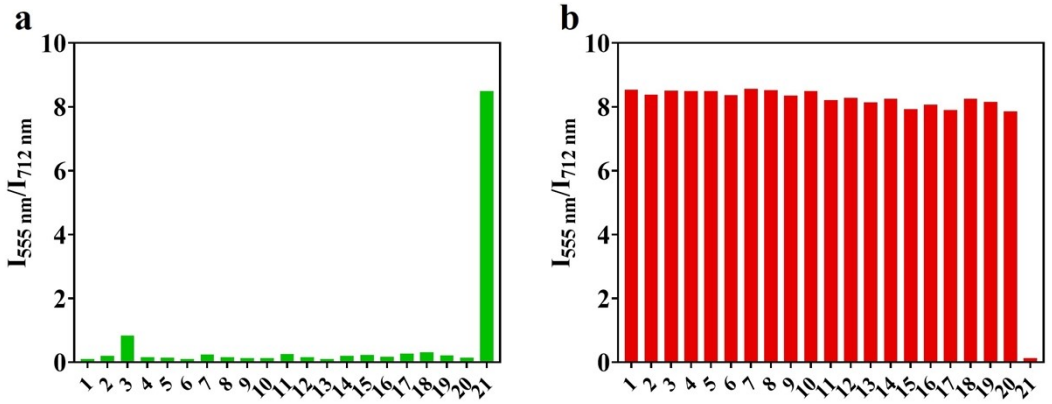


Fig. S7 (a) Fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of **WD-Redox** (10.0 μM) in the presence of various species (50.0 μM) after 5 min incubation: 1, GSH; 2, Cys; 3, Hcy; 4, Cu^{2+}; 5, Ca^{2+}; 6, Mg^{2+}; 7, CO_3^{2-}; 8, NO_3^-; 9, NO_2^-; 10, S^{2-}; 11, HS^-; 12, HSO_3^-; 13, SO_3^{2-}; 14, NO; 15, $ONOO^-$; 16, TBHP; 17, $O_2^{\cdot-}$; 18, $HO\cdot$; 19, H_2O_2, 20, elesclomol-Cu complex; 21, HOCl. (b) Fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of **WD-Redox** (10.0 μM) pretreated with HOCl (50.0 μM) and then treated with other analytes (1.0 mM) for 5 min: 1, blank; 2, Cys; 3, Hcy; 4, Cu^{2+}; 5, Ca^{2+}; 6, Mg^{2+}; 7, CO_3^{2-}; 8, NO_3^-; 9, NO_2^-; 10, S^{2-}; 11, HS^-; 12, HSO_3^-; 13, SO_3^{2-}; 14, NO; 15, $ONOO^-$; 16, TBHP; 17, $O_2^{\cdot-}$; 18, $HO\cdot$; 19, H_2O_2; 20, elesclomol-Cu complex; 21, GSH. Testing media: CH_3CN/PBS buffer (10.0 mM, pH = 7.4, v/v = 2: 8). λ_{ex} = 500 nm.

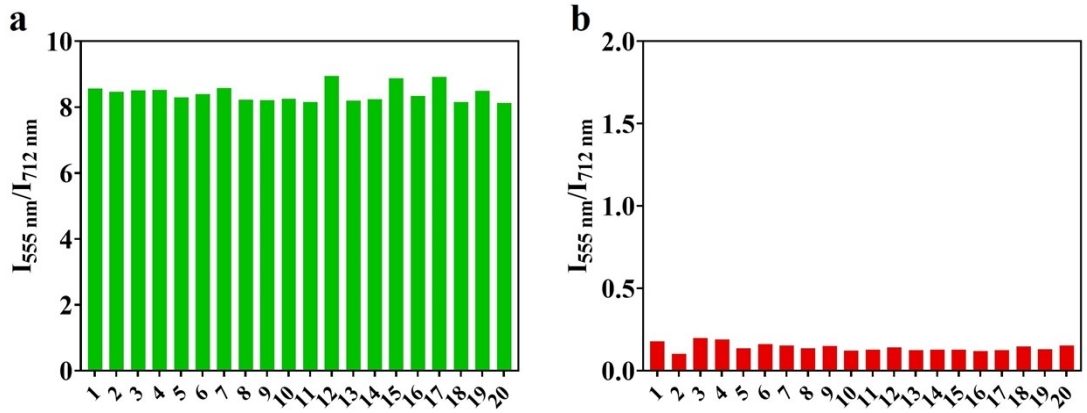


Fig. S8 (a) Fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of **WD-Redox** (10.0 μM) in the presence of various substances (50.0 μM) following the addition of HOCl (50.0 μM). (b) Fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of **WD-Redox** (10.0 μM) after initial reaction with various substances and HOCl (both 50.0 μM), followed by treatment with GSH (1.0 mM). Substances: 1, Blank; 2, Cys; 3, Hcy; 4, Cu^{2+}; 5, Ca^{2+}; 6, Mg^{2+}; 7, CO_3^{2-}; 8, NO_3^-; 9, NO_2^-; 10, S^{2-}; 11, HS^-; 12, HSO_3^-; 13, SO_3^{2-}; 14, NO; 15, $ONOO^-$; 16, TBHP; 17, $O_2^{\cdot-}$; 18, $HO\cdot$; 19, H_2O_2; 20, elesclomol-Cu complex. Testing media: CH_3CN/PBS buffer (10.0 mM, pH = 7.4, v/v = 2: 8). Incubation time: 5 min each for HOCl and GSH. λ_{ex} = 500 nm.

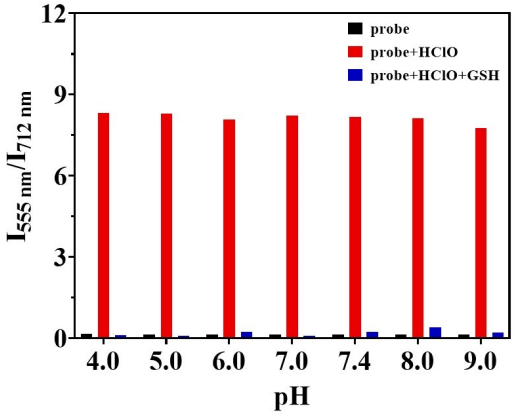


Fig. S9 Fluorescence intensity ratio ($I_{555\ nm}/I_{712\ nm}$) of **WD-Redox** (10.0 μM) upon addition of HOCl (50.0 μM) for 5 min (red lines), followed by addition of GSH (1.0 mM) for 5 min (blue lines) in PBS buffers at various pH values.

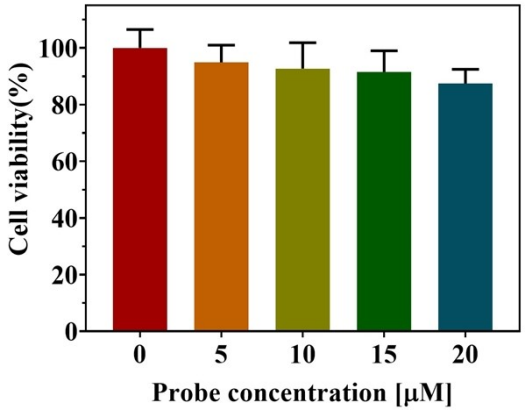


Fig. S10 Survival rates of HeLa cells treated with various concentrations of **WD-Redox** for 24 h.

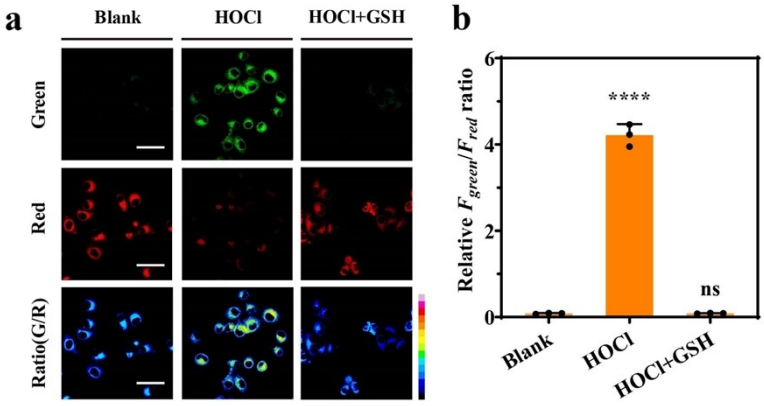


Fig. S11 (a) Fluorescence (green and red) and ratio (G/R) images of cells subjected to different treatments and followed by staining with **WD-Redox**. Red channel: λ_{em} = 650-750 nm, λ_{ex} = 488 nm; green channel: λ_{em} = 500-600 nm, λ_{ex} = 488 nm; G/R: green to red. Scale bar: 50 μm. (b) Relative fluorescence intensity ratio (F_{green}/F_{red}, green to red) corresponding to images in (a). Error bars: SD, n = 3. ****p<0.0001.

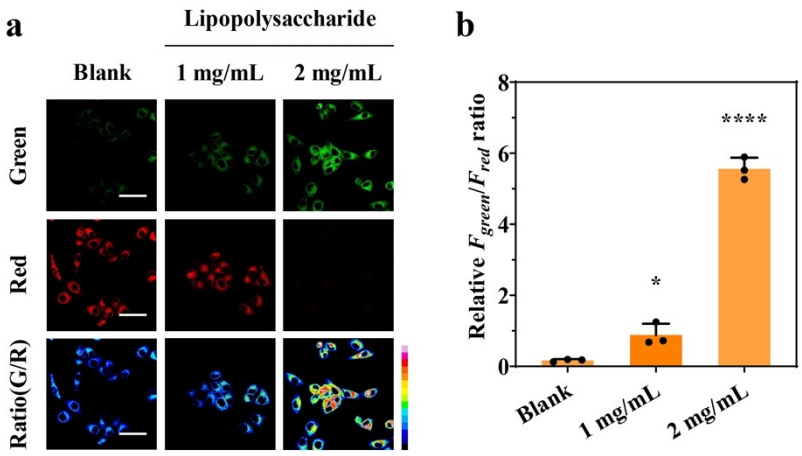


Fig. S12 (a) Fluorescence (green and red) and ratio (G/R) images of cells subjected to different concentrations of lipopolysaccharide (1.0 and 2.0 mg/mL) and followed by staining with **WD-Redox**. Red channel: λ_{em} = 650-750 nm, λ_{ex} = 488 nm; green channel: λ_{em} = 500-600 nm, λ_{ex} = 488 nm; G/R: green to red. Scale bar: 50 μm. (b) Relative fluorescence intensity ratio (F_{green}/F_{red}, green to red) corresponding to images in (a). Error bars: SD, n = 3. **** p<0.0001, * p<0.05.

4. ^{1}H NMR, ^{13}C NMR and HRMS Spectra

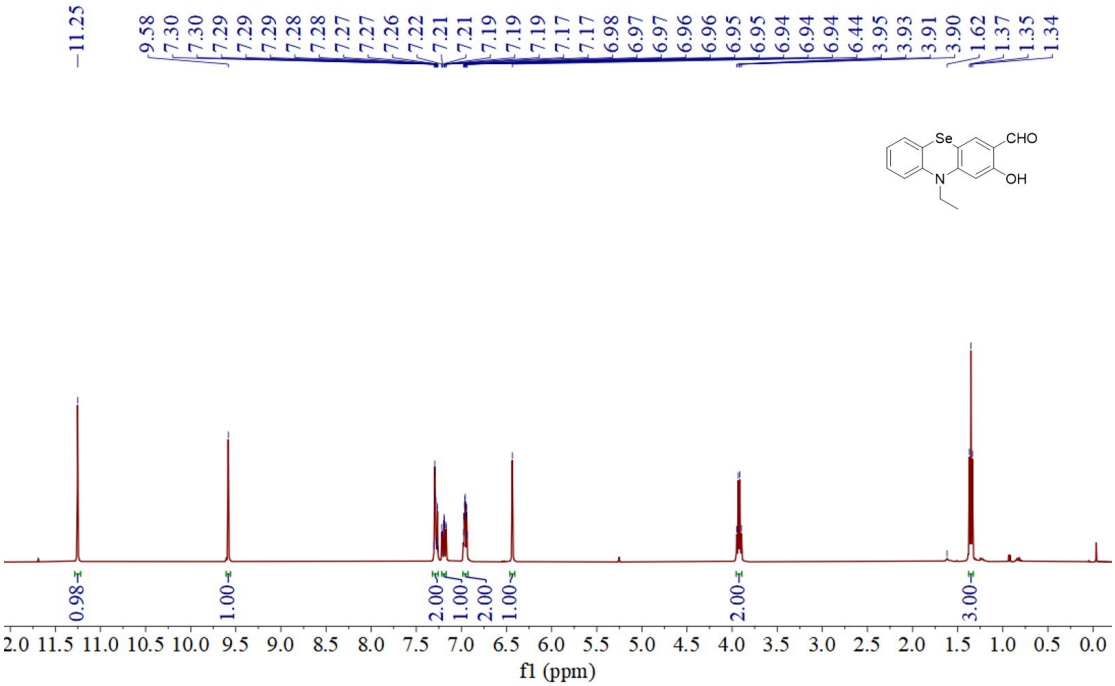


Fig. S13 ^{1}H NMR spectrum of compound **1** in $CDCl_3$.

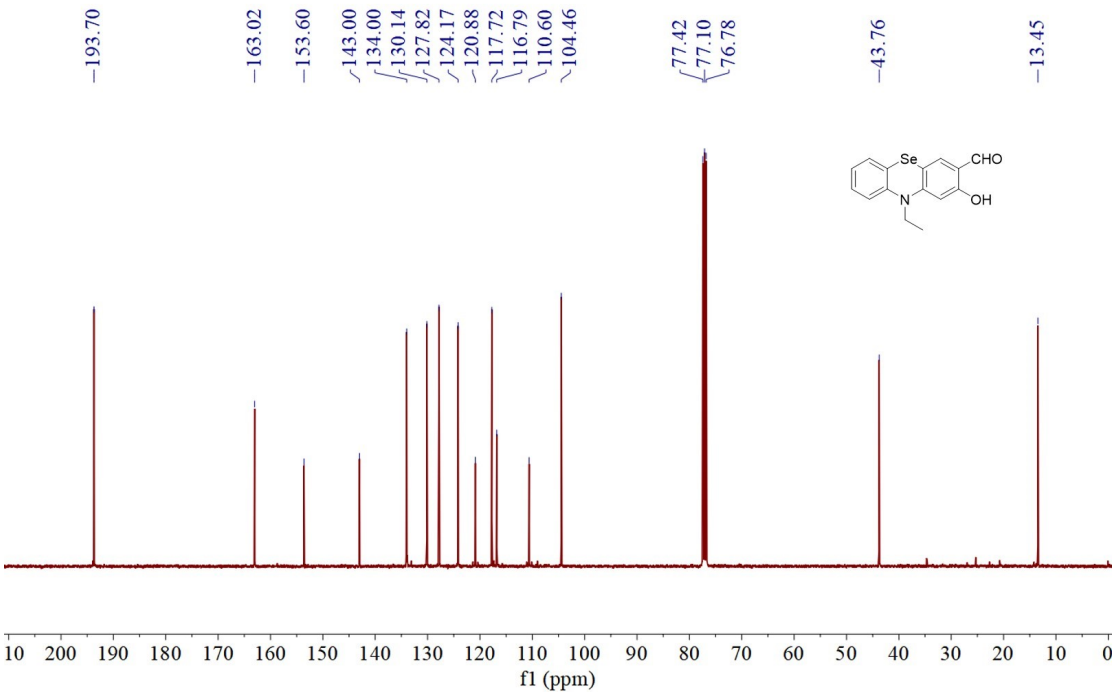


Fig. S14 ^{13}C NMR spectrum of compound **1** in $CDCl_3$.

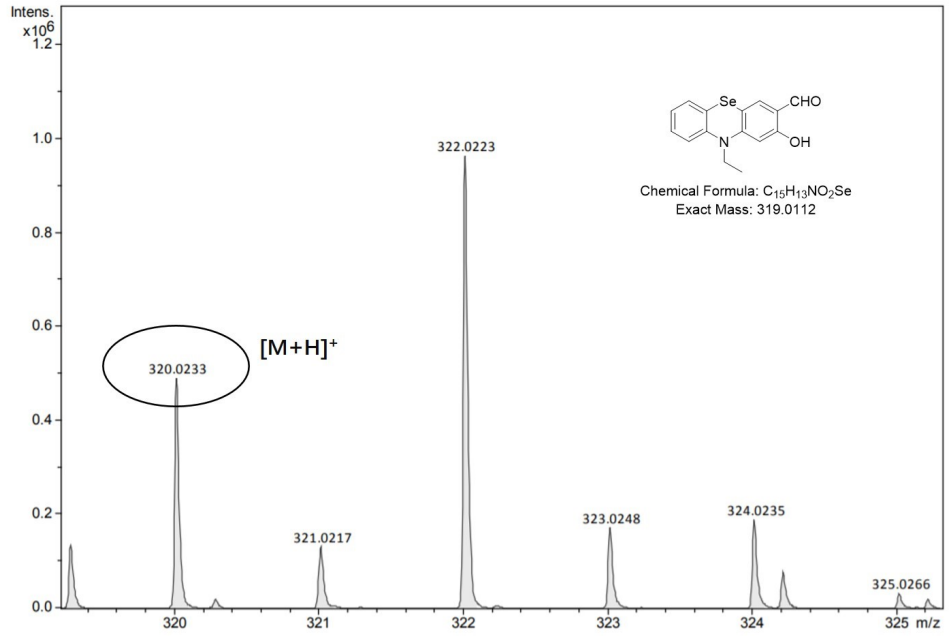


Fig. S15 HRMS spectrum of compound **1**.

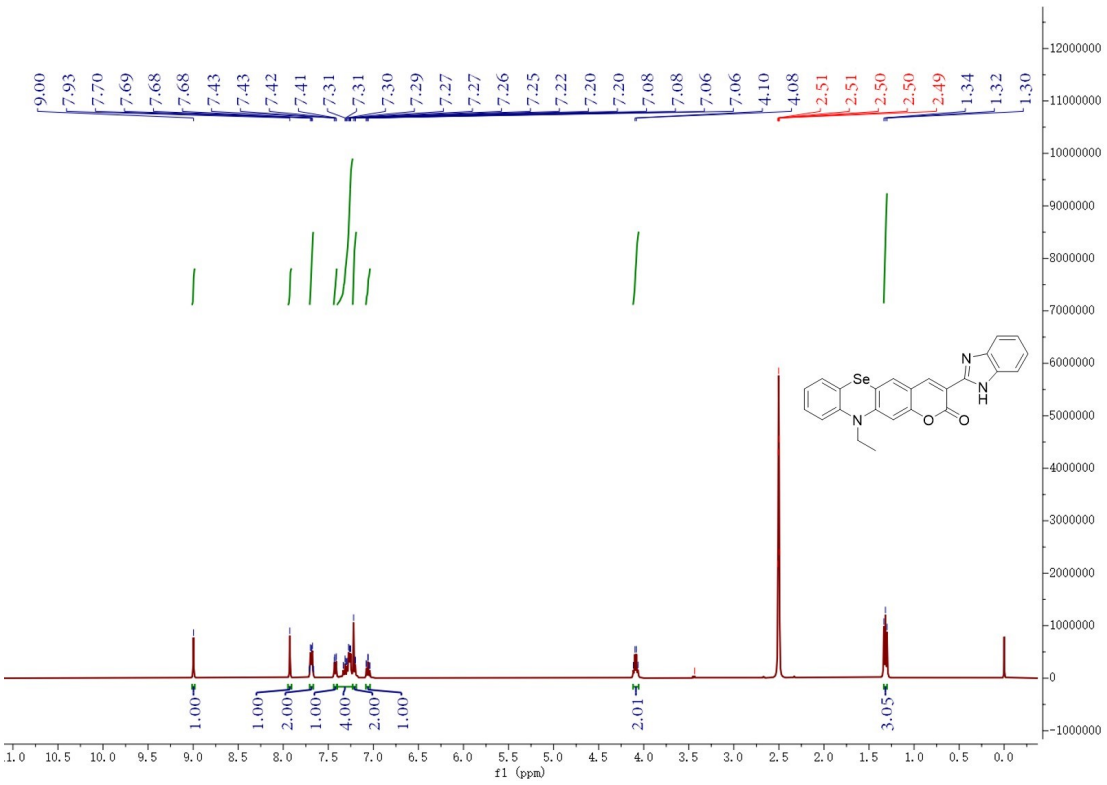


Fig. S16 ^{1}H NMR spectrum of compound **2** in DMSO-d_6.

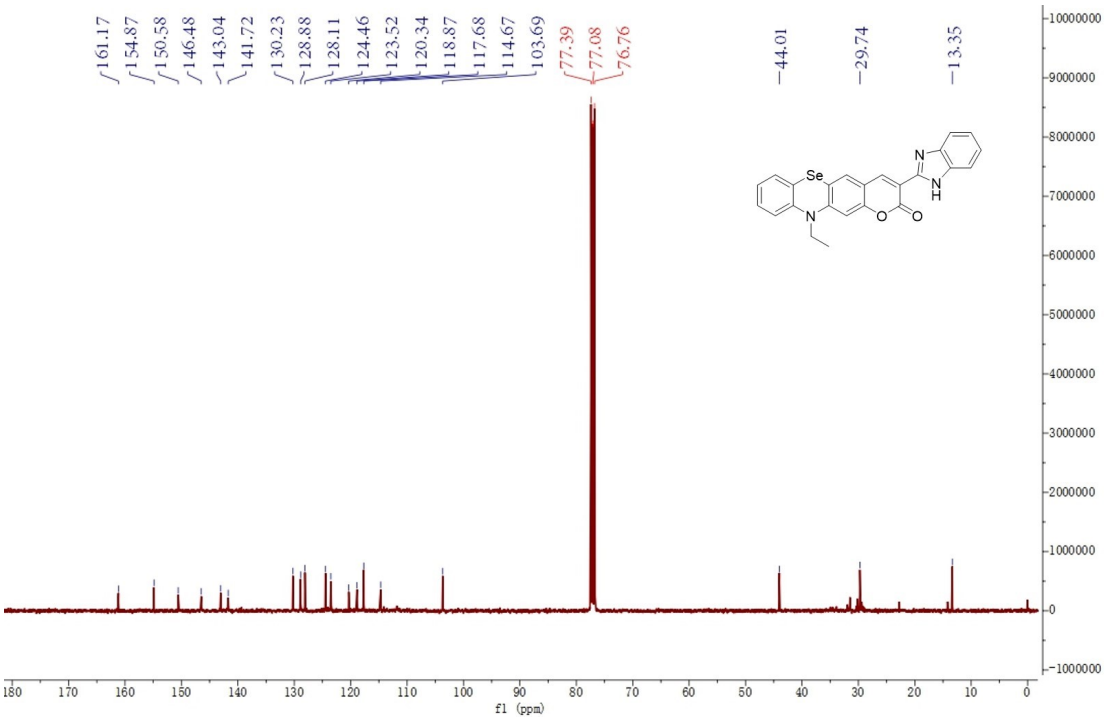


Fig. S17 ^{13}C NMR spectrum of compound **2** in $CDCl_3$.

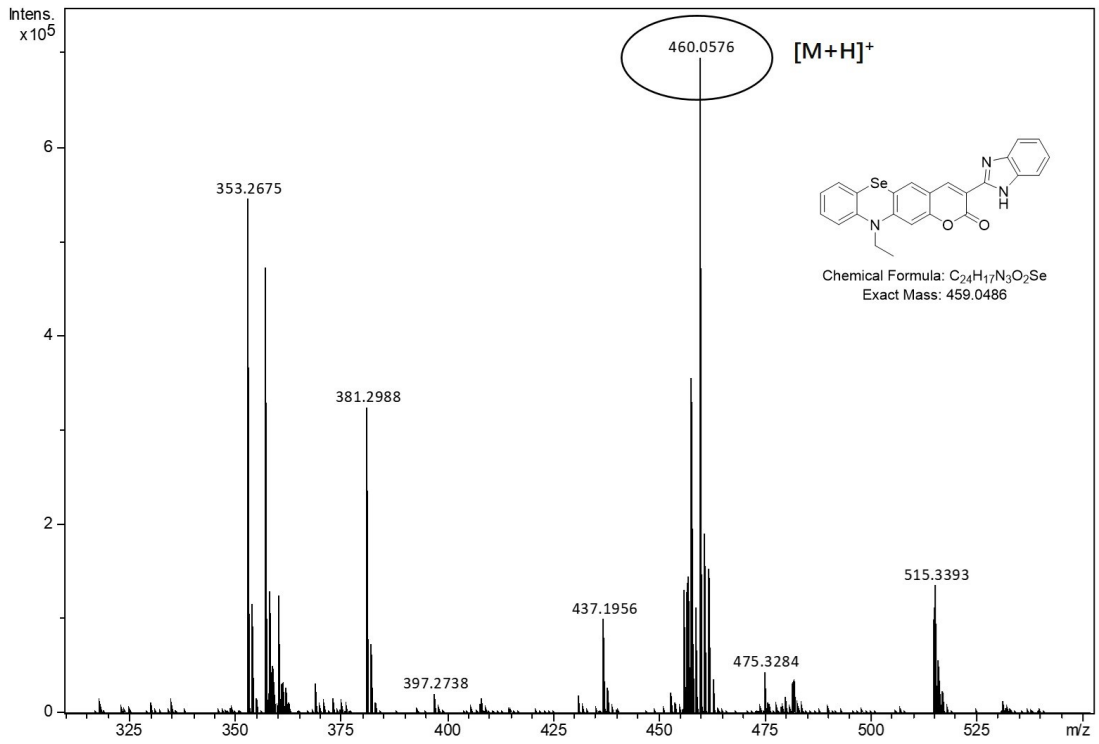


Fig. S18 HRMS spectrum of compound **2**.

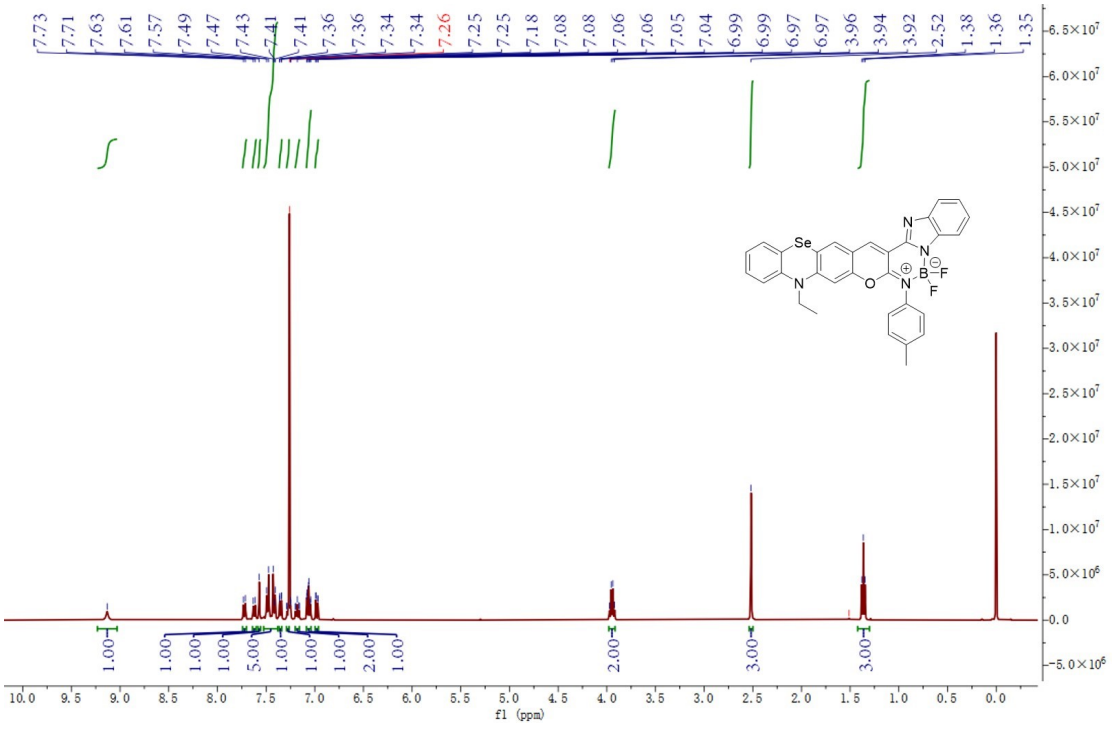


Fig. S19 ^{1}H NMR spectrum of **WD-Redox** in $CDCl_3$.

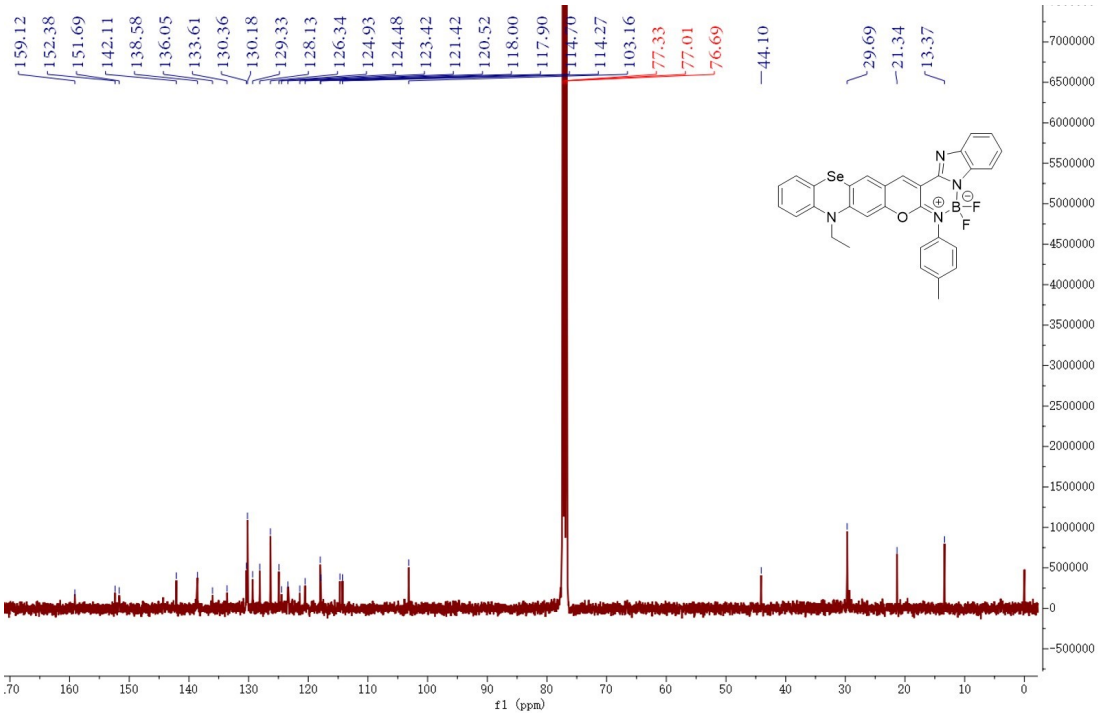


Fig. S20 ^{13}C NMR spectrum of **WD-Redox** in $CDCl_3$.

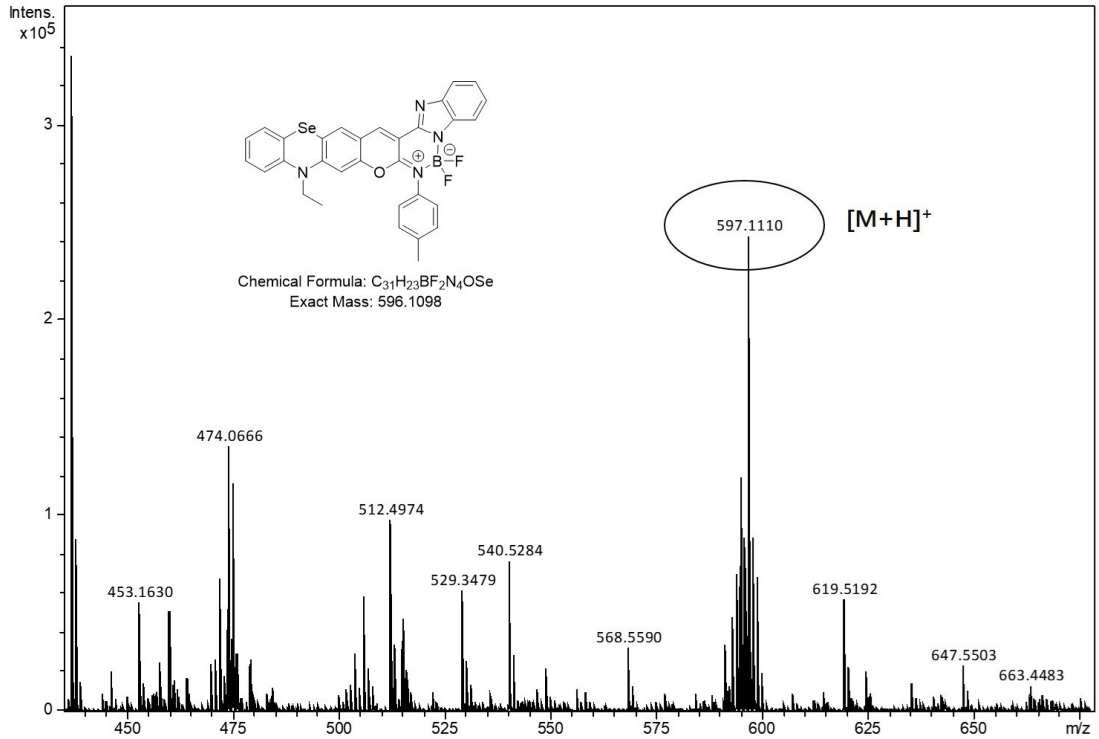


Fig. S21 HRMS spectrum of **WD-Redox**.