

Supplementary Information

A Near-infrared Reversible Fluorescent Probe for Real-time Imaging of Redox Status Changes in vivo

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Supplementary Results

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Note: All animal experiments were carried out according to the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China.

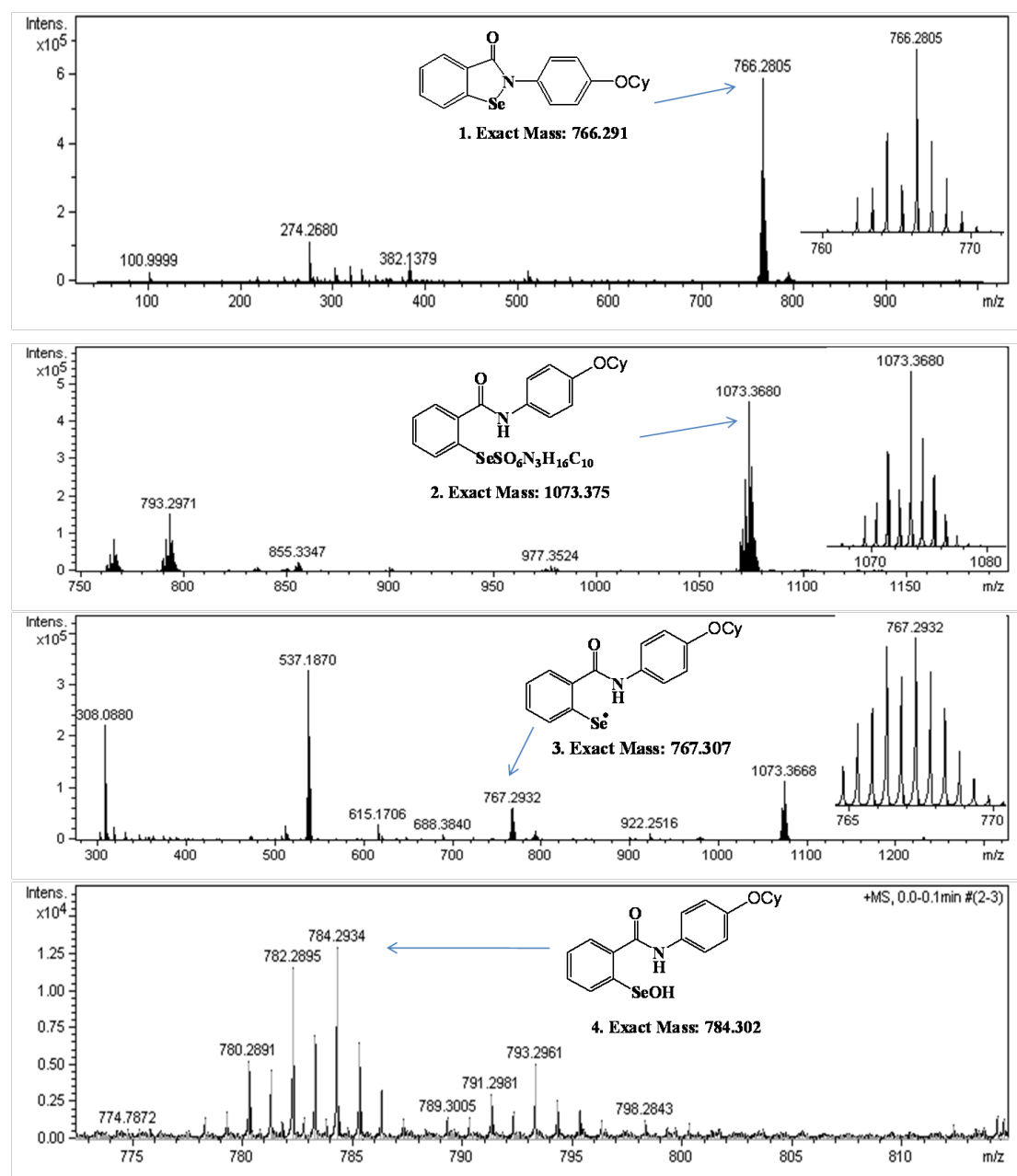
1. The synthesis of the compound 1^[1]

2,3,3-Trimethyl-1-ethyl-3H-indolium, inner salt (a): 2,3,3-trimethylindolenine (10 mL, 0.062 mol) and iodoethane (14.5 g, 0.093 mol) were mixed and heated under reflux for 24 h. Then the reaction mixture was cooled to room temperature and filtered. The precipitate was washed with ethyl acetate (3×10 mL). The pink crystals (15.2 g, yield 91.8%) were obtained.

2-Chloro-1-formyl-3-hydroxymethylenecyclohexene (b): Forty milliliters of dimethylformamide previously mixed with 40 mL of methylene chloride was chilled in an ice bath, and then 37 mL of phosphorus oxychloride dissolved in 35 mL of methylene chloride was added dropwise with stirring, followed by 10 g of cyclohexanone. The solution was refluxed for 3 h, cooled, poured onto 200 g of ice, and allowed to stand overnight. The yellow solid was crystallized from a small volume of acetone cooled with dry ice, to give 13 g (73.9%) with the melting point of 130–132 °C. Elemental analysis (%) calculated for C₈H₉ClO₂ (found): C 55.7 (55.3), H 5.3 (5.5), Cl 20.5 (20.5).

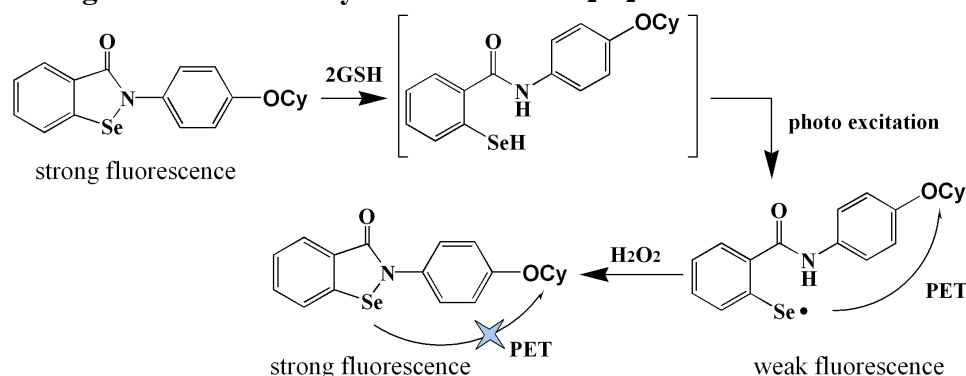
Compound 1: Compound (a) (3.66 g, 11.66 mmol), compound (b) (0.96 g, 5.44 mmol) and sodium acetate (0.47 g) were dissolved in acetic anhydride (30 mL). The mixture was stirred at room temperature for 2 h. Then the solvents were removed under vacuum. The residue was washed with ether to give 3.2 g of pure product with yield of 91%. ¹H NMR (CDCl₃, 300 MHz): δ1.47 (t, 6H), 1.72-1.78 (t, 12H), 1.99 (m, 2H), 2.79 (t, 4H), 4.26-4.28 (t, 4H), 6.23-6.30 (d, 2H), 7.17-7.41 (d, 8H), 8.21-8.38 (d, 2H).

2. MS data of Cy-O-Eb and reaction intermediates



Scheme S1. MS data of Cy-O-Eb and reaction intermediates

3. Detecting mechanism for Cy-O-Eb to GSH/H₂O₂



Scheme S2. A proposed detecting mechanism for Cy-O-Eb to GSH/H₂O₂^[3]

4. The pH dependence of the probe reaction

Because pH of the buffer solution plays generally an important role in the sensitivity of target molecular determination, we monitored the effect of pH on fluorescence intensity in the range of 6.5 ~ 8.4, as shown in Figure S1. The fluorescence intensities of both of the probe and the reaction product are relatively steady as the pH rised from 6.5 to 8.4. In order to apply in biological system, pH = 7.4 was selected for the following determination system.

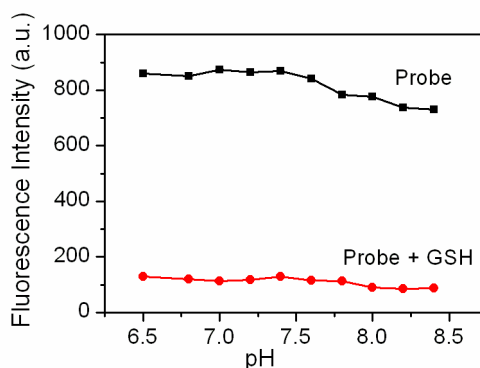


Figure S1. Effects of pH on fluorescence intensities of the probe and the reaction solution of Cy-O-Eb (10 μM) and GSH (20 μM) in HEPES buffer (10 mM)

5. Fluorescence quantum yields (Φ_F) of Cy-O-Eb

For measurement of the quantum yields of Cy-O-Eb, the solution of the probe was adjusted to an absorbance of ~ 0.05. The emission spectra were recorded using the maximal excitation wavelength^[2a], and the integrated areas of the fluorescence-corrected spectra were measured. Relative fluorescence quantum yield (Φ_F = 0.23) was obtained by comparing the area under the emission spectrum of the

test samples with that of a solution of heptamethine indocyanine IR-786 in methanol ($\Phi_F = 0.159$)^[2b].

6. Selectivity of Cy-O-Eb to GSH

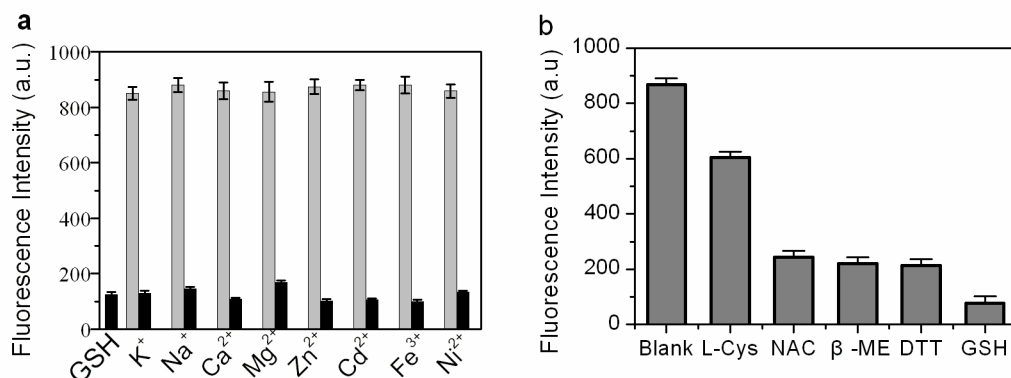


Figure S2. Selectivity of Cy-O-Eb to GSH (a) Fluorescence responses of 10 μM Cy-O-Eb to metal ions (grey bars) and metal ions plus 20 μM GSH (black bars): 0.4 mM for Ca²⁺ and Cd²⁺; 0.8 mM for Fe³⁺ and Ni²⁺; 0.2 mM for Zn²⁺; 1 mM for Mg²⁺ and 1.6 mM for K⁺ and Na⁺. (b) Fluorescence responses of 10 μM Cy-O-Eb to thiols: 5 mM for L-Cys, GSH, dithiothreitol (DTT) and N-acetylcysteine (NAC). All spectra were acquired in 10 mM HEPES buffer with pH 7.4 at 37 °C ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 768/794$ nm).

7. Selectivity of Cy-O-SeH to H₂O₂

The selectivity of Cy-O-SeH to H₂O₂ was accomplished by adding H₂O₂, ClO⁻ and *t*-BuOOH (each for 200 μM) to a mixture of 10 μM Cy-O-Eb and 20 μM GSH, respectively.

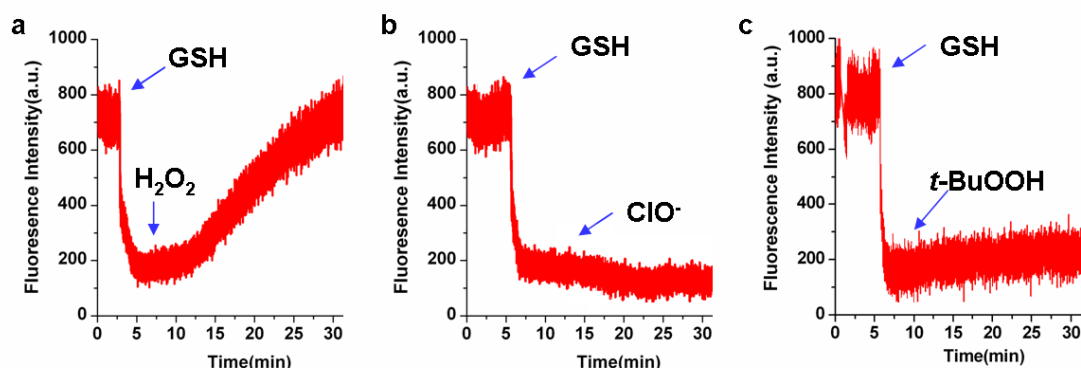
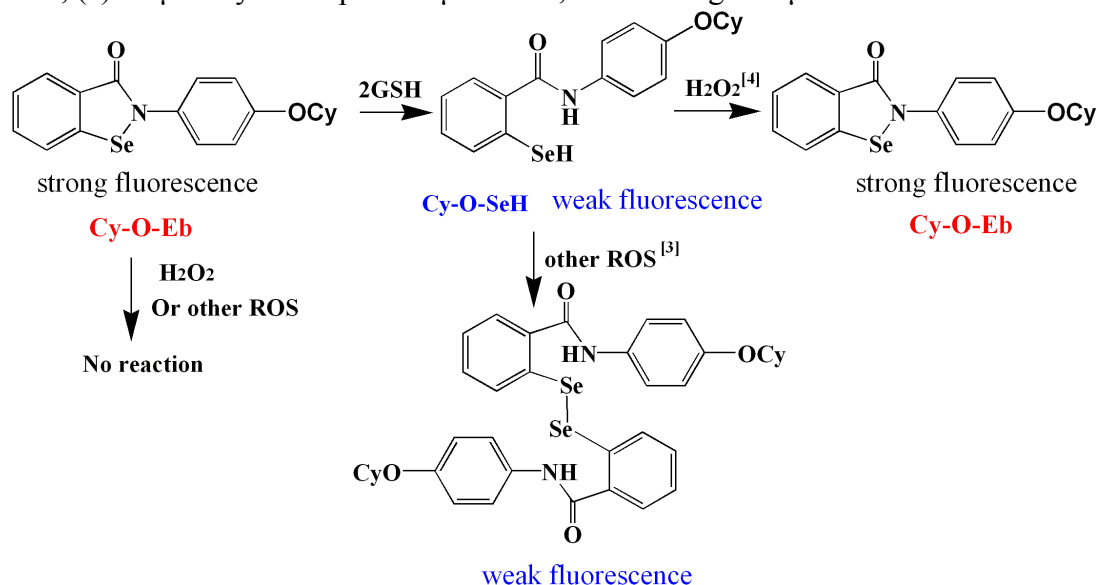


Fig. S3. Selectivity of Cy-O-SeH to H₂O₂ (a) 10 μM Cy-O-Eb plus 20 μM GSH, then adding 200 μM H₂O₂, (b) 10 μM Cy-O-Eb plus 20 μM GSH, then adding 200 μM

ClO^- , (c) 10 μM Cy-O-Eb plus 20 μM GSH, then adding 200 μM *t*-BuOOH



Scheme S3. The suggested mechanism for the reaction of Cy-O-SeH with ROS^[3,4]

8. MTT assay

To investigate Cy-O-Eb cytotoxicity, MTT assay were carried out when the probe existed HepG2 cells. Cells (10^6 cell mL^{-1}) were dispersed within replicate 96-well microtiter plates to a total volume of 200 μL well⁻¹. Plates were maintained at 37 °C in a 5% CO_2 /95% air incubator for 4 h. The probe was diluted to different concentrations of solution with medium and added to each well after the original medium has been removed. HepG2 cells were incubated with probe concentrations for 4 h. The concentrations of the probe were 1 μM to 500 μM . And then 200 μL MTT solution (5.0 mg mL^{-1} , HEPES) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a TRITURUS microplate reader (Figure S4). Calculation of IC_{50} values was done according to Huber and Koella.^[5] The probe IC_{50} value was calculated to be 288 μM , which demonstrated that Cy-O-Eb should be a low cytotoxic probe under experimental conditions at the concentration of 10 μM .

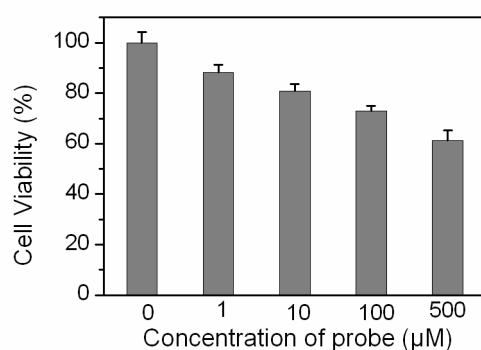


Figure S4. MTT assay of HepG2 cells in the presence of different concentrations of Cy-O-Eb

9. Co-staining Imaging in HepG2 cells with DAPI and PI

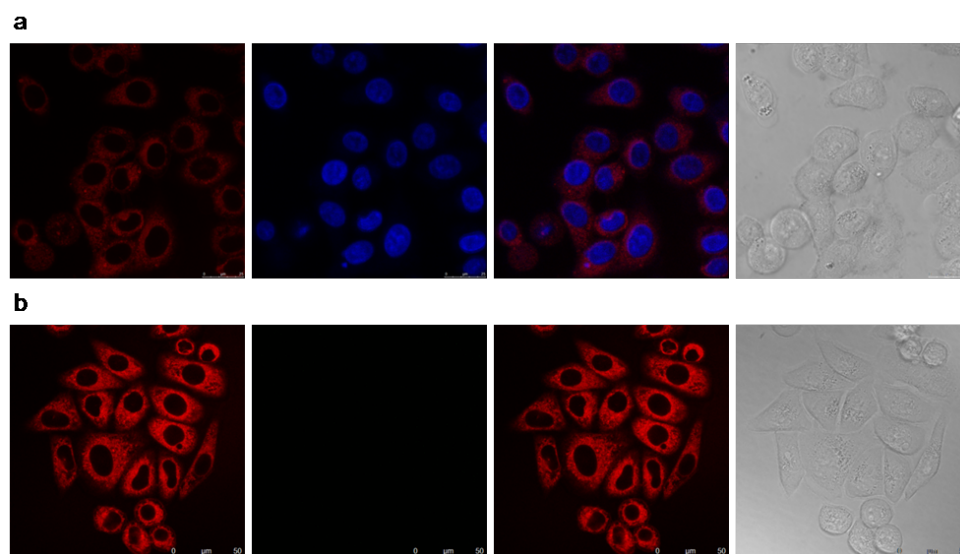


Figure S5. Co-staining Imaging in HepG2 cells **(a)** Cells were loaded with 10 μM Cy-O-Eb for 5 min at 37 °C, followed by DAPI for an additional 5 min at 37 °C, and were imaged using 633 nm He-Ne and 405 nm laser. **(b)** Using PI instead of DAPI. Images from left to right derived from probe, co-staining dye, overlay and brightfield, respectively. All cells were rinsed three times with 0.1 M HEPES buffer at room temperature before imaging. Scale bar = 50 μm.

10. Photo-bleaching

Photo-bleaching is an irreversible photochemical inversion of fluorescent molecule into a non-fluorescent state. The photo-stability of Cy-O-Eb was investigated by time-sequential scanning of the living cells. After 500 s of continuous irradiation with a 633-nm laser, no obvious changes were observed in fluorescence brightness of Cy-O-Eb (Figure S6). In order to quantitatively determine the photo-bleaching rate, we choose three regions, calculated the average intensity and obtained a curve with scanning time. The results in Figure S4b showed that the intensities of Cy-O-Eb after 0 ~ 500 s of time-sequential scanning were about 90% of the initial value. These data indicated that the probe is highly resistant to photo-bleaching.

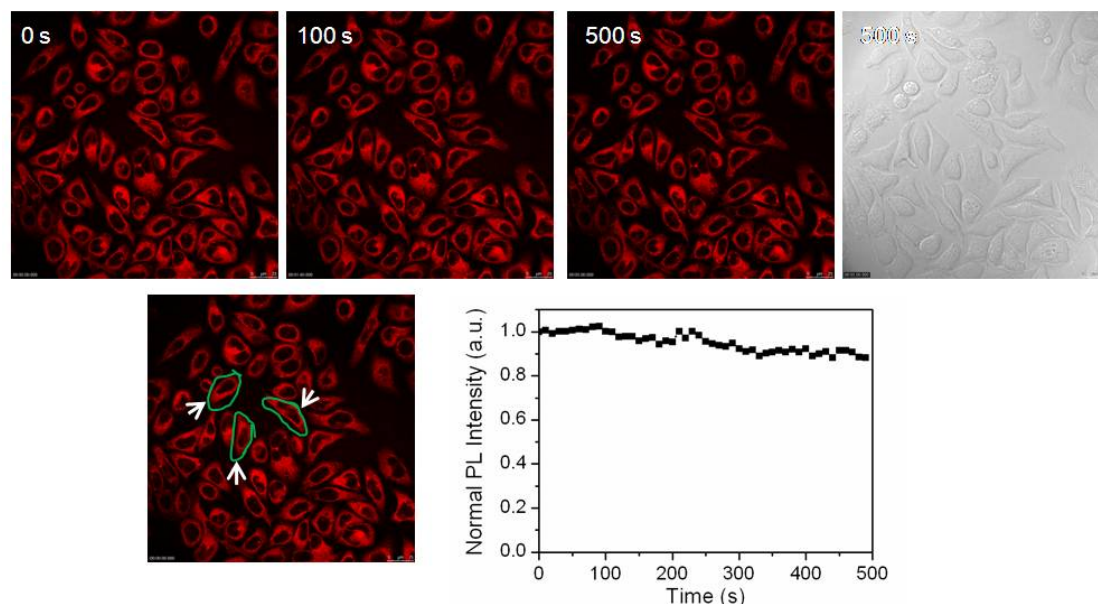


Figure S6. Test of photostability of Cy-O-Eb. Confocal fluorescence images (0 ~ 500s) were achieved by means of time-sequential scanning of the probe-loaded HepG2 cells incubated with 10 μ M Cy-O-Eb for 10 min. Normalized fluorescence intensity from three regions from 0 to 500s of time-sequential scanning

11. Leakage rate of the probe through the membranes of living cells

HepG2 cells incubated with 10 μ M Cy-O-Eb in 5 mL medium at 37°C for 5 min were washed quickly and further incubated in fresh medium. The 100 μ L sample taken from the above medium when incubated for 0, 3, 6 and 24 h was injected into a quartz cuvette (volume, 1.0 mL) where 10 μ M H₂O₂ in PBS was added in order to transform leaking Cy-O-SeH into Cy-O-Eb. And then the fluorescence was measured.

The leakage rate is calculated as follows^[6]: $f_F = (F - F_{\text{initial}})/(F_{\text{max}} - F_{\text{initial}}) \times 100\%$, where F is the measured fluorescence. F_{initial} is the initial fluorescence of medium at 0 h, and F_{max} is the fluorescence of 1 μM Cy-O-Eb solution, that is 100 μL /10 μM Cy-O-Eb in medium was diluted into 1 mL with PBS (corresponding to 100% leakage). Fig. S7 showed that the leakage rate is very low, approximately 0% ~ 1% under the concentrations used in the cell experiments.

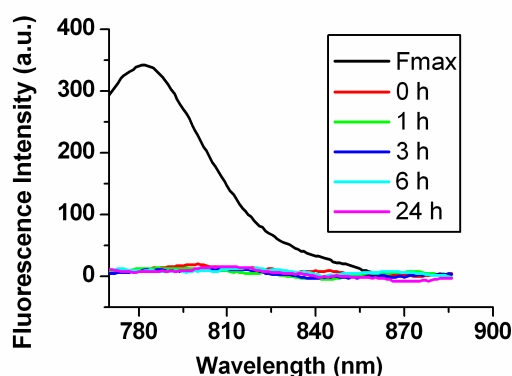


Fig. S7. Determination of the leakage rate of Cy-O-Eb when HepG2 cells were incubated with 10 μM Cy-O-Eb for 0, 3, 6 and 24 h

12. Normalized relationship of fluorescence intensity and GSH/H₂O₂ concentrations

The fluorescence intensity and GSH/H₂O₂ concentrations was normalized based on the experimental data of Fig.1.

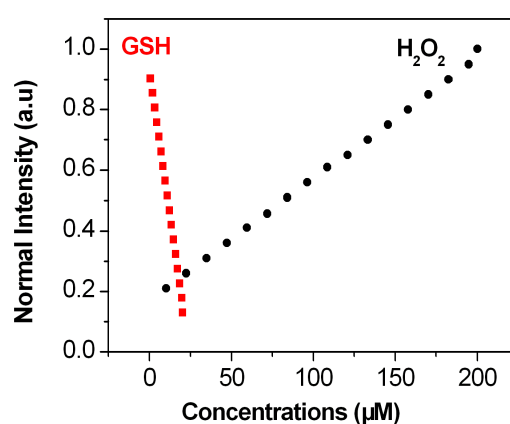
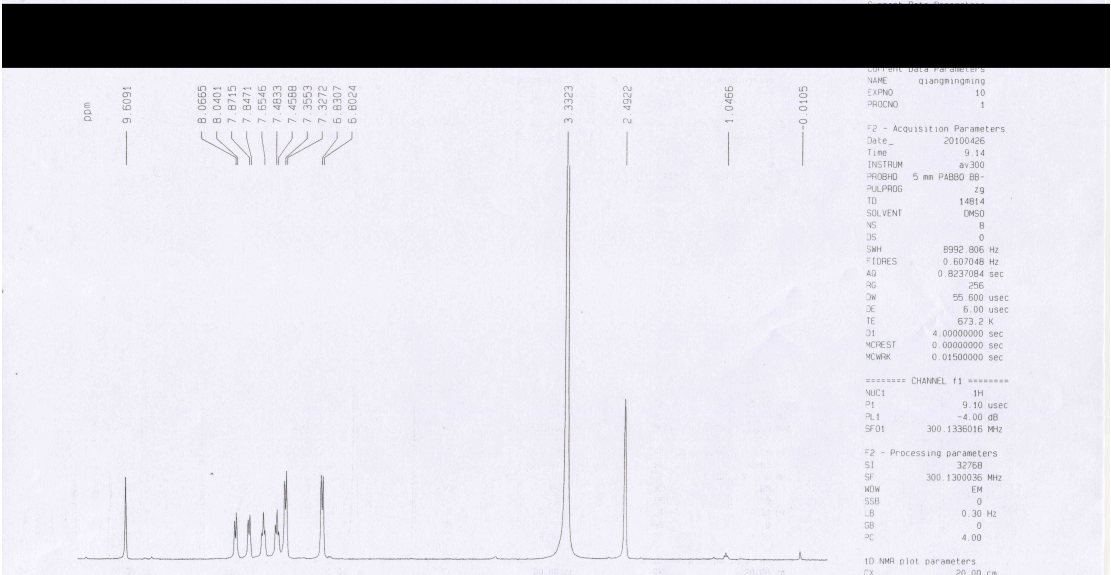


Fig. S8. Normalized fluorescence intensity from a series of solutions: 10 μM Cy-O-Eb to different concentrations of GSH from 0 to 20 μM , and Cy-O-SeH generated from 10 μM Cy-O-Eb with 20 μM GSH to different concentrations of H₂O₂ from 0 to 200 μM .

13. ¹H-NMR and IR of compound 2

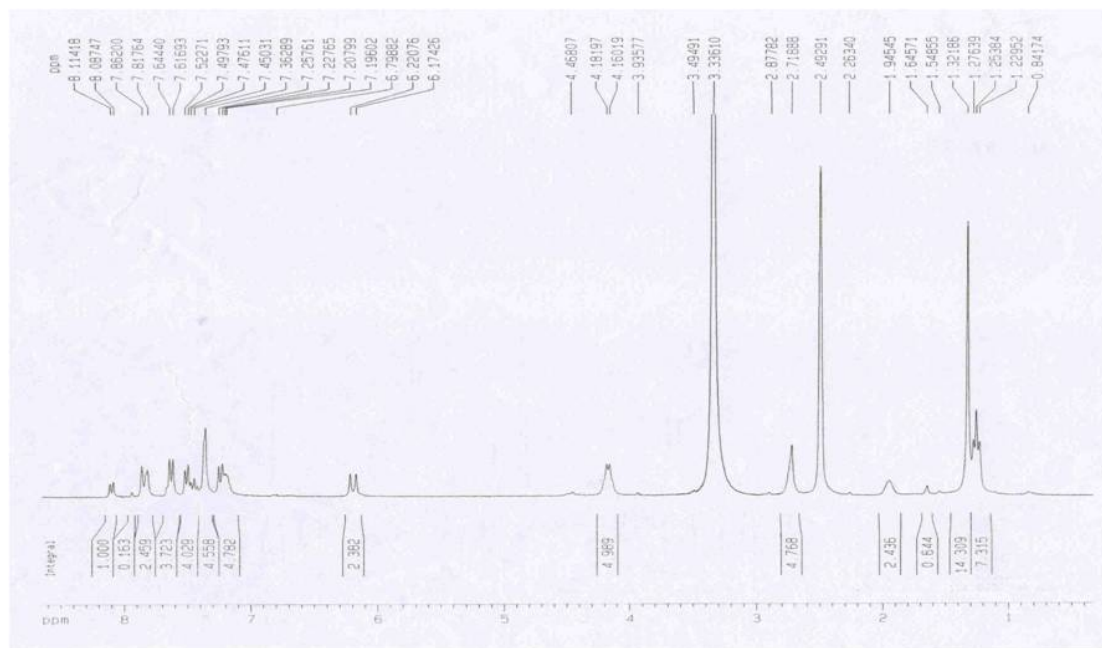


¹H-NMR of Ebselen-OH

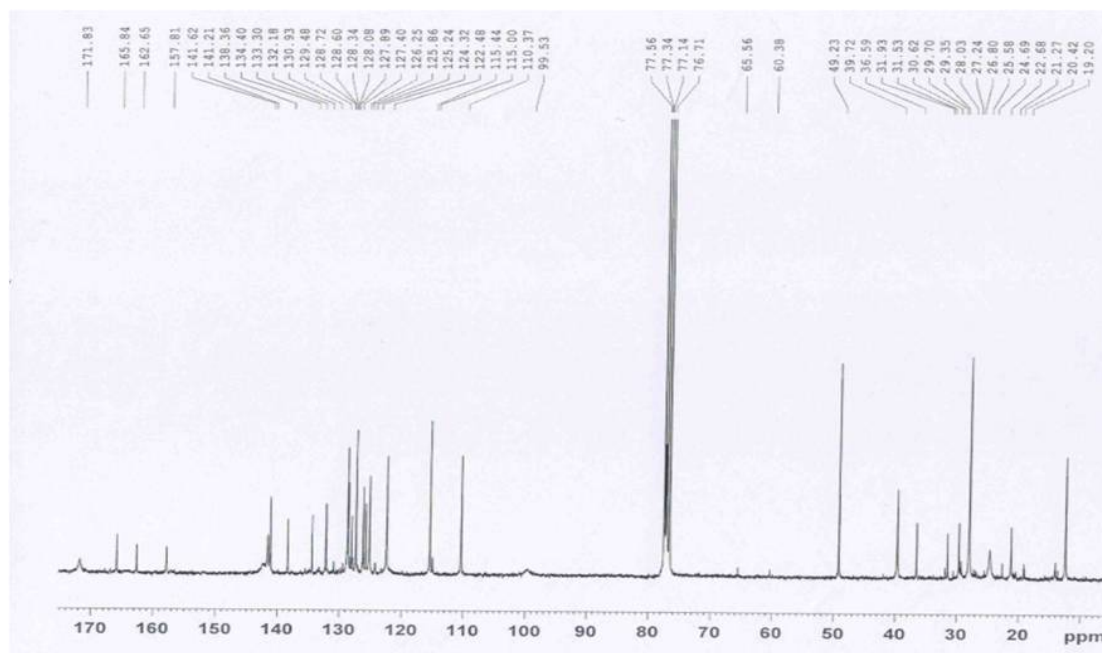


IR of Ebselen-OH

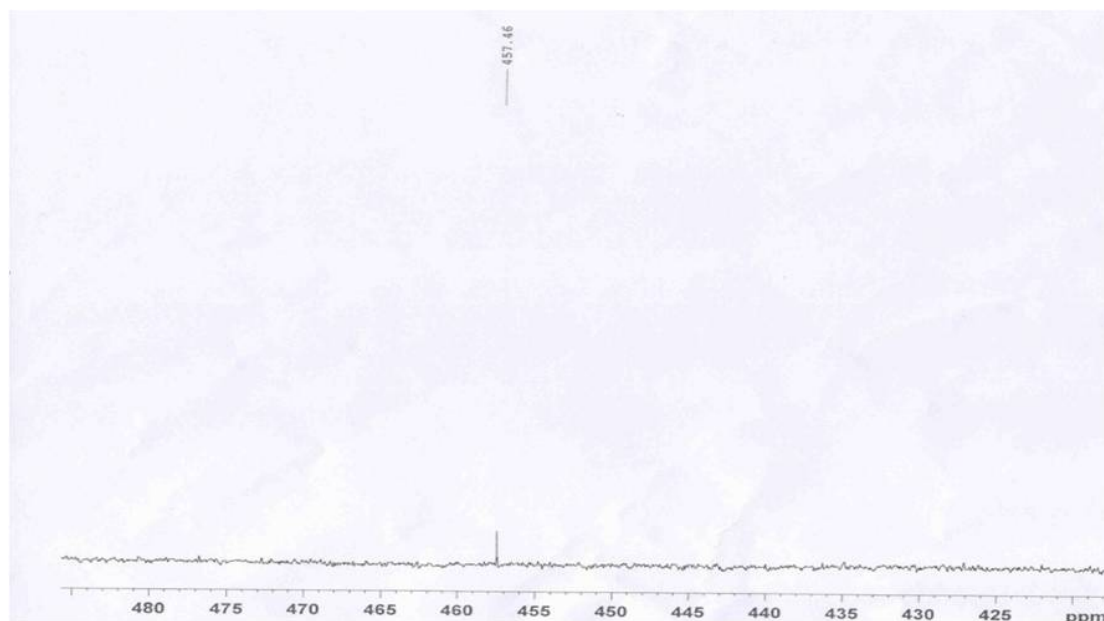
14. ^1H -NMR, ^{13}C -NMR and ^{77}Se -NMR of Cy-O-Eb



^1H NMR of Cy-O-Eb



^{13}C NMR of Cy-O-Eb



^{77}Se NMR of Cy-O-Eb

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