Electronic Supplementary Information

A near-infrared fluorescent probe for sensitive detection and imaging of sulfane sulfur in living cells and in vivo

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1. General Methods

1.1 Materials and instruments

BD-diSH (1 mM) was prepared in DMSO and stored at 4 °C in darkness. The purity of probe was tested on a Shimadzu LC-20AT HPLC system equipped with fluorescence and UV-vis absorption detectors. When it was used for imaging, the purity of our probe was greater than 99.89%. Stock solutions of cysteine (Cys), homocysteine (HCy), glutathione (GSH), cysteine (CysSSCys), oxidized glutathione (GSSG), $Na_2S_2O_3$, NaHS, $NaHSO_3$ and ascorbic acid were prepared to desired concentrations when needed. The stock solution of S_8 was prepared in CH_2Cl_2, and then used ethanol (EtOH) to dilute this solution, to get a 10 mM stock solution of S_8 in EtOH. Na_2S_4, Na_2S_2 and Cys-polysulfide were prepared using reported our reported paper[.1-4] Superoxide (O_2^{-}) was created by the enzymatic reaction of xanthine/xanthineoxidase (XA/XO; 6.0 μM/3 mU) at 25 °C for 5 min.[5,6] ·OH was generated by Fenton reaction between Fe^{II}(EDTA) and H_2O_2 quantitively, and Fe^{II}(EDTA) concentrations represented ·OH concentrations. *Tert*-butylhydroperoxide (*t*-BuOOH) and cumene hydroperoxide (CuOOH) could also be used to induce ROS in biological systems. The $ONOO^-$ source was the donor 3-Morpholinosydnonimine hydrochloride (SIN-1, 200 μmol/mL).[7] NO was generated in form of 3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5, 100 μM/mL).[8] NO_2^- was generated from $NaNO_2$. Methyl linoleate (MeLH) and 2,2'-azobis-(2,4-dimethyl)valeronitrile (AMVN) were used to produce MeLOOH.[9,10] H_2O_2 was determined at 240 nm ($\varepsilon_{240\ nm}$ = 43.6 $M^{-1}cm^{-1}$). $^1O_2$ was generated from 3,3'-(naphthalene-1,4-diyl)dipropionic acid.[11] Angeli's salt (a HNO donor) was prepared as reported by King and Nagasawa and stored dry at - 20°C in a refrigerator.[12] Stock solutions were diluted to desired concentrations when needed. All chemicals were purchased from Sigma-Aldrich or Aladdin and of analytical reagent grade. Double-distilled water purified from a Millipore Milli-Q system (Bedford, MA, USA) was used throughout all experiments. Mouse macrophage cell line (RAW 264.7), human lung carcinoma cell line (A549), human embryonic kidney cell line (HEK), human epitheloid cervix carcinoma cell line (HeLa), rat hepatoma cell line (RH-35), human hepatocellular carcinoma cell line (SMMC-7721), and human neuroblastoma cell line (SH-SY5Y) cells were purchased from the Committee on Type Culture Collection of the Chinese Academy of Sciences. Dulbecco's modified Eagle's medium (DMEM), RPMI (Roswell Park Memorial Institute) 1640 Medium, Fetal Bovine Serum (FBS) and trypsin were purchased from Gibco (Grand Island, USA). HEPES (4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) was obtained from Aladdin and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. All other chemicals were bought from commercial sources and of analytical reagent grade, unless indicated otherwise.

The absorption spectra were recorded on TU-1810DSPC UV-visible spectrophotometer (Beijing Persee).

Fluorescence spectra were measured by FluoroMax-4 Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. High-resolution mass spectral (HRMS) analyses were measured with Hybrid Ion Trap-Orbitrap Mass Spectrometer (LTQ Orbitrap XL,Thermo). ^{1}H and ^{13}C NMR were recorded on a 500 MHz Bruker Super Conducting NMR Spectrometer (AVANCE IIITM 500, Bruker). Cell imaging and hippocampus imaging were acquired with an Olympus Laser Scanning Confocal Microscope (FV1000, Olympus) at × 40 magnifications and × 10 magnifications, respectively. Flow cytometry data were collected by BD Biosciences FACSAria. Hippocampus slices were obtained from LeicaVT1200S. BALB/c mice fluorescence images were collected by Bruker In-vivo Imaging System.

1.2 Absorption and fluorescence analysis

Absorption spectra were obtained with 1.0-cm glass cells. Fluorescence emission spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. The fluorescence intensities were measured at $\lambda_{ex/em}$ = 710/736 nm. BD-diSH (0.10 mL, 1.0 mM) was added to a 10.0-mL color comparison tube. After dilution to 10 μM with 10 mM HEPES buffers, analytes were added. The mixtures were equilibrated 2 min before measurement.

1.3 Cell culture

RAW 264.7, A549, HEK, HeLa, RH-35, SMMC-7721 and SH-SY5Y cells were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All the cells were incubated in DMEM supplemented with 10 % FBS or RPMI 1640 Medium supplemented with 10 % FBS at 37 °C under a humidified atmosphere containing 5 % CO_2.

1.4 Flow cytometry analysis

The cells were cultured at 2.0×10^5 cells/well in 6-well plates, and then treated with probe as described in the paper. After harvest, cells were washed and suspended in fresh complete medium and analyzed by flow cytometry. The excitation wavelength was selected 633 nm and the collected wavelengths were selected 750 - 810 nm.

1.5 MTT assay

To access the potential toxicity of BD-diSH, MTT assays were carried out. RAW 264.7 cells were planted into 96-well microtiter plates in DMEM with 10% FBS. Then the cells were incubated for 24 h at 37 °C in a 5% CO_2/95% air upon different concentrations probe of 0 μM to 100 μM respectively. MTT solution (5.0 mg/mL, PBS) was then added to each well. After 4 h, the remaining MTT solution was removed, and 200 μL of DMSO was added to each well, shaking 10 min to dissolve the formazan crystals at room temperature. Absorbance was measured at 570 nm and 630 nm in a TECAN infinite M200pro microplate reader.

1.6 Statistical analysis

Statistical Product and Service Solutions (SPSS) software 19.0 was used for the statistical analysis. The error bars shown in the figures represented the mean ± s.d. Differences were determined with a one-way of variance (ANOVA) followed by LSD test. Statistical significance was assigned at $^{>>>}P < 0.001$. Sample size was chosen empirically based on our previous experiences and pre-test results. The numbers of animals or samples in every group were described in the corresponding Fig. legends. The distributions of the data were normal. All experiments were done with at least seven biological replicates. Experimental groups were balanced in terms of animal age, sex and weight. Animals were all caged together and treated in the same way. Appropriate tests were chosen according to the data distribution. Variance was comparable between groups in experiments described throughout the manuscript.

2. Selectivity of BD-diSH towards ROS and RNS

We further examed the selectivity of BD-diSH towards sulfane sulfur upon the addition of various bio-relevant reactive oxygen species (ROS) and reactive nitrogen species (RNS). The fluorescent responses were obtained at the time point of 5 min. As shown in Fig. S1, only sulfane sulfu could trigger strong fluorescence responses. Other ROS and RNS including $O_2{}^{\bullet-}$, $\bullet OH$, $^1O_2$, H_2O_2, HNO, $ONOO^-$, $NO_2{}^-$, NO, Tert-butylhydroperoxide (t-BuOOH) and cumene hydroperoxide (CuOOH) could not induce any fluorescence intensity changes.
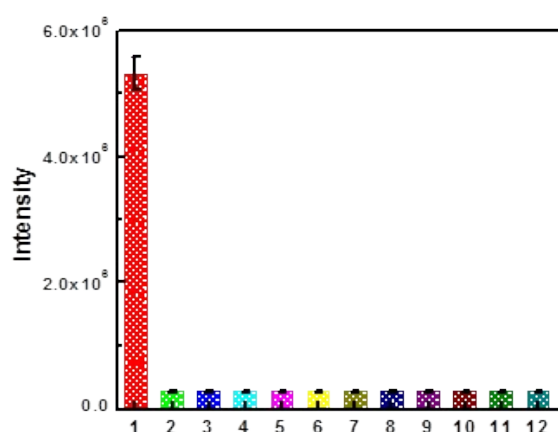

Fig. S1 Fluorescence responses of BD-diSH (10 μM) to biologically relevant ROS and RNS. In each group, the bars represent relative responses at 736 nm of BD-diSH to the analytes: 1, 20 μM Na_2S_4; 2, 10 μM $O_2{}^{\bullet-}$; 3, 10 μM $\bullet OH$; 4, 10 μM $^1O_2$; 5, 10 μM H_2O_2; 6, 10 μM HNO; 7, 10 μM $ONOO^-$; 8, 10 μM $NO_2{}^-$; 9, 10 μM NO; 10, 10 μM t-BuOOH; 11, 10 μM CuOOH; 12, black. The reaction was measured in 15 min at 37 °C. λ_{ex} = 710 nm, λ_{em} = 736 nm. The experiments were repeated seven times and the data were shown as mean (± s.d.).

3. Effects of pH values on probe

Standard fluorescence pH titrations were performed in HEPES buffer solution at a probe concentration of 10 μM. As shown in Fig. S1, the fluorophore BODIPY almost has no effect on the fluorescence by the pH of mediums within the range from 4.0 to 9.0. The probe itself could keep stable over pH range of 4.0 - 7.4. When the pH values were higher than 7.8, the fluorescence intensity decreased. These results indicate that the probe can work under physiological conditions (pH = 7.4, 0.5% Tw 80).
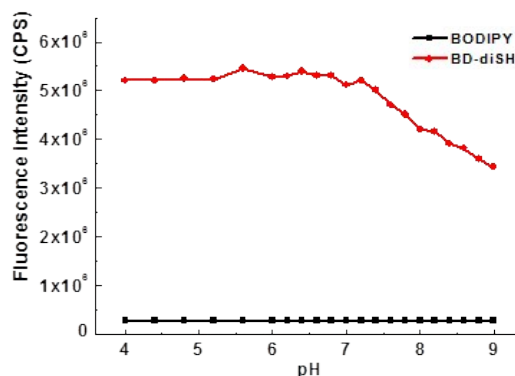

Fig. S2 Effects of pH value on the fluorescence intensity of probe BD-diSH (10 μM) and the fluorophore BODIPY (10 μM) in 10 mM HEPES (pH 7.4, 0.5% Tw 80) at 37 °C for 2 min, (λ_{ex} = 710 nm, λ_{em} = 736 nm). pH values: 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, 8.6, 8.8, 9.0.

4. Effect of Tween 80 to the fluorescence intensity

Tween 80 as a kind of common nonionic surfactant was used to form micelles to maximize the stimulation function of the cell. Then the effect of Tween 80 at different concentrations (0.1 %, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9% and 1.0%) on the fluorescent intensity of BD-diSH were evaluated in HEPES (10 mM, pH 7.4). As shown in Fig. S2, a visibly increasing of fluorescence intensity was obtained upon addition of Tween 80 (from 0% to 0.5%). After that, the fluorescence intensity reached saturation and threshold. Therefore, in this work, 0.5% Tween 80 was used for the study.
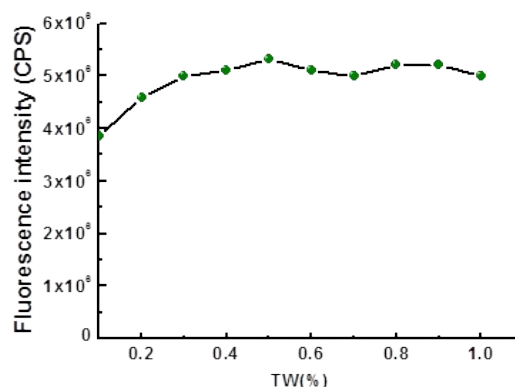

Fig. S3 Effect of Tween 80 on the fluorescence intensity (λ_{ex} = 710 nm, λ_{em} = 736 nm) of BD-diSH (10 μM). We evaluated the effect of Tween 80 (0.1 %, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, and 1.0%) on the

fluorescence intensity of BD-diSH in HEPES (10 mM, pH 7.4) at 37 °C for 2 min.

5. Fluorescence quantum yield of BD-diSH

The fluorescence quantum yield of BD-diSH was determined according to the following expression:

$$\varphi_u = \frac{(\varphi_s)(FA_u)(A_s)(\lambda_{exs})(\eta_u^2)}{(FA_s)(A_u)(\lambda_{exu})(\eta_s^2)}$$

Where φ is fluorescence quantum yield; the subscripts u and s refer to the unknown and the standard, respectively; F is integrated fluorescence intensity under the corrected emission spectra; A is the absorbance at the excitation wavelength; η is the refractive index of the solution.[13] We chose Mg-tetra-*tert*-butylphthalocyanine as standard, which has a fluorescence quantum yield of 0.84 according to the literature.[14]
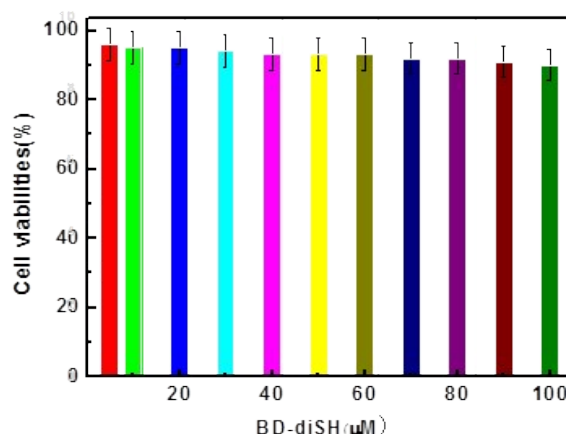
6. MTT Assay for BD-diSH

Fig. S4 Cell viabilities of BD-diSH using RAW264.7 cells as tested model. Cells were treated with different concentrations of BD-diSH for 24 h. Concentrations of BD-SH: 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μM.
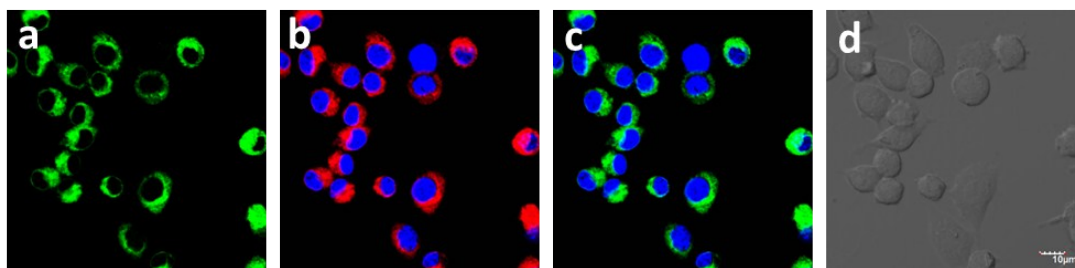
7. Images for Fig. 2

Fig.S5 a) Green channel for Fig. 2. RAW 264.7 cells stained with 1 μM Calcein-AM. Fluorescence collected window: λ_{ex} = 488 nm, λ_{em} = 500 - 550 nm. b) Overlay of red channel (Fig. 2i) and blue channel. c) Overlay of green and blue channel. d) Bright-field image. Scale bar: 10 μm.
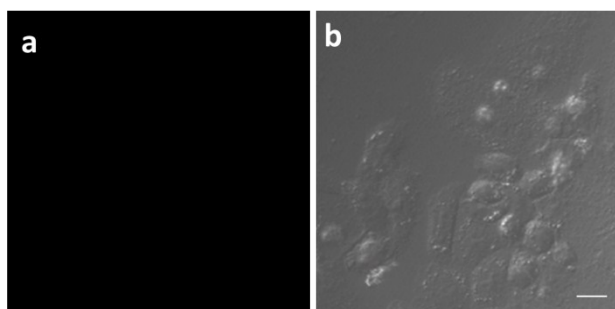
8. Bright-field images of Fig. 3

Fig. S6 Confocal fluorescence images of sulfane sulfur in RAW 264.7 cells stained with1 µM BD-diSH. a) Cells were incubated with 5 mM NEM for 30 min. b) Bright-field image. Scale bar: 10 µm.
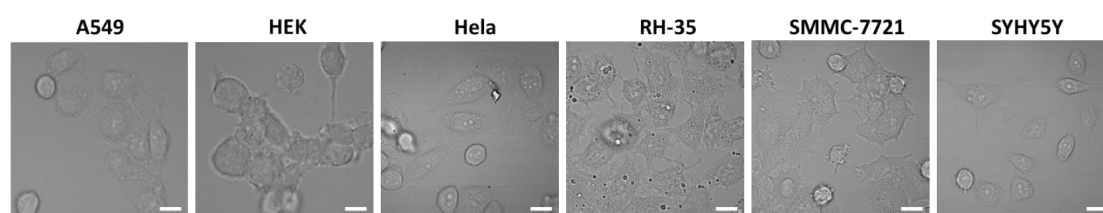
9. Bright-field images of Fig. 3

Fig.S7 Bright-field images of Fig. 3. Scale bar: 10 µm.
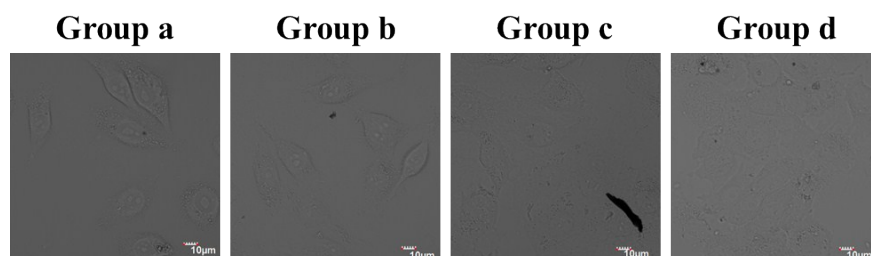
10. Bright-field images of Fig. 4

Fig. S8 Bright-field images of Fig. 4. Scale bar: 10 µm.

11. CSE overexpression

The siRNA sequence targeting human CSE gene was synthesized by Invitrogen. For transfection, cells were placed on six-well plates. Transfections were performed with Lipo2000 for 24 h as directed by the manufacturer (Invitrogen). The final siRNA concentration was 25 nM.

Western blot analysis the expression of CSE was performed to confirm the successful expression of CSE. The cell samples were washed for three times before lysis of cells. Protein extracts were prepared by suspending the cells in 200 µL RIPA lysis buffer containing 1% PMSF (Solarbio, China). Then the extracts were quantified with BCA protein assay kit (Biogot, China). After denatured, the equal amounts of protein were electrophoresed on 6–12% SDS-polyacrylamide gels (Bio-Rad, USA) and transferred to PVDF membranes. The membrane was incubated

with 5% BSA (Sigma-Aldrich, USA) and incubated with primary antibody (abcam) overnight at 4°C with gentle shake. A horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology, USA) was used to mirror the quantity of proteins and signals were detected with an enhanced chemiluminescence (ECL) detection system. The results were analyzed by Image J to acquire the grey value of every bond. As shown in Fig. S9, the expression of CSE in CSE-overexpress SMMC-7721cells was higher than control SMMC-7721 cells. The result indicated that the successful overexpression of CSE in SMMC-7721 cells.
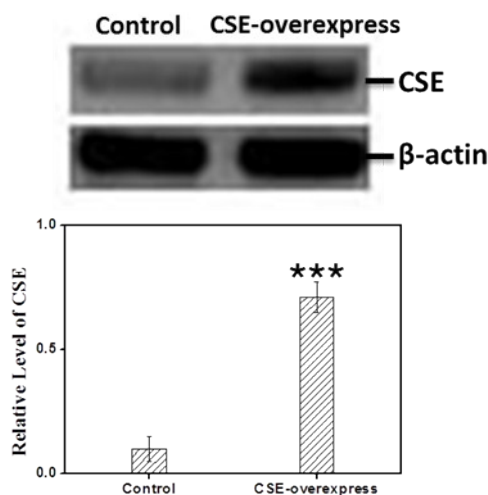

Fig. S9 Western blotting analysis of CSE. β-actin was used as a loading control. The data were shown as mean ($\pm$ s.d.) ($n = 3$).

12. Calculation of the sulfane sulfur levels in ex vivo-dissected organs

The actual sulfane sulfur concentrations are simply calculated via the detection values. The calculation details of actual sulfane sulfur concentrations in ex vivo-dissected organs have been listed as follows: The organs (brain, heart, liver, spleen, lung, and kidney) were treated as tissue homogenates (tissue 1 g in 10 mL of saline). And then BD-diSH (10 μM) is added for 15 min before testing. The fluorescence intensities of the ex-vivo organs are shown in Fig. 5.

[Na$_2$S$_4$] was calculated using the regression equation in Fig. 1c,

$$F_{736 \text{ nm}} = 2.95 \times 10^5 \text{ [Na}_2\text{S}_4] \text{ μM} + 3.89 \times 10^5 \text{ (r = 0.9985)},$$

$$[Na_2S_4] = \frac{F_{736nm} - 3.89 \times 10^5}{2.95 \times 10^5}$$

Then the sulfane sulfur concentrations in ex vivo-dissected organs were calculated as the following formula,

$$c = \frac{[Na_2S_4]v}{m}, (v = 10 \text{ mL}, m = 1 \text{ g})$$

According to the fluorescence intensities were 2.197×10^6, 2.395×10^6, 2.295×10^6, 2.27×10^6, 1.15×10^6, 4.49×10^6

in brain, heart, liver, spleen, lung, and kidney in Fig. 5, respectively. The [Na$_2$S$_4$] in tissue homogenates (tissue 1 g in 10 mL of saline) were 61.3 µM in brain, 68.0 µM in heart, 64.6 µM in liver, 62.3 µM in spleen, 25.7 µM in lung, and 139 µM in kidney. So the real sulfane sulfur level is 61.3 nmol/g in brain, 68.0 nmol/g in heart, 64.6 nmol/g in liver, 62.3 nmol/g in spleen, 25.7 nmol/g in lung, and 139 nmol/g in kidney.

13. H NMR, C NMR and HRMS for probe
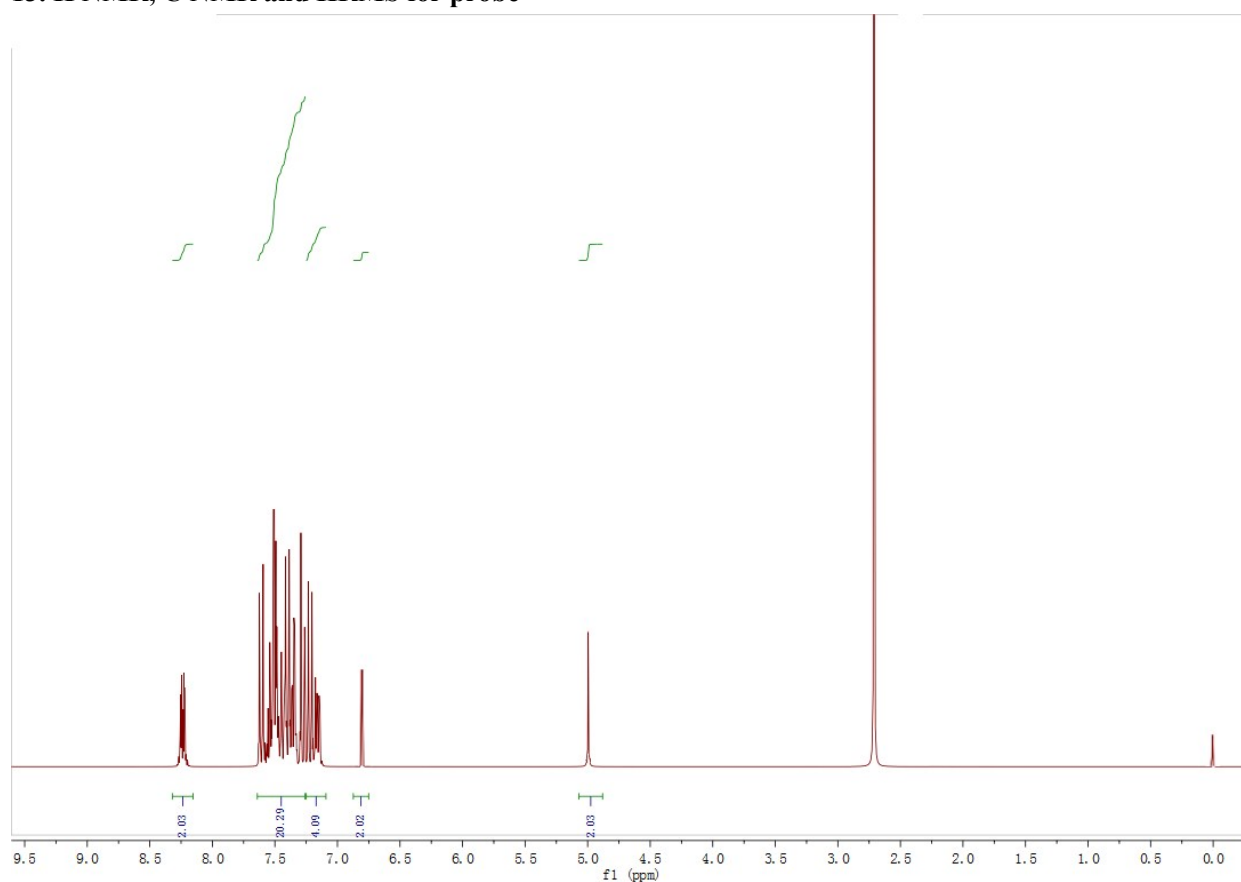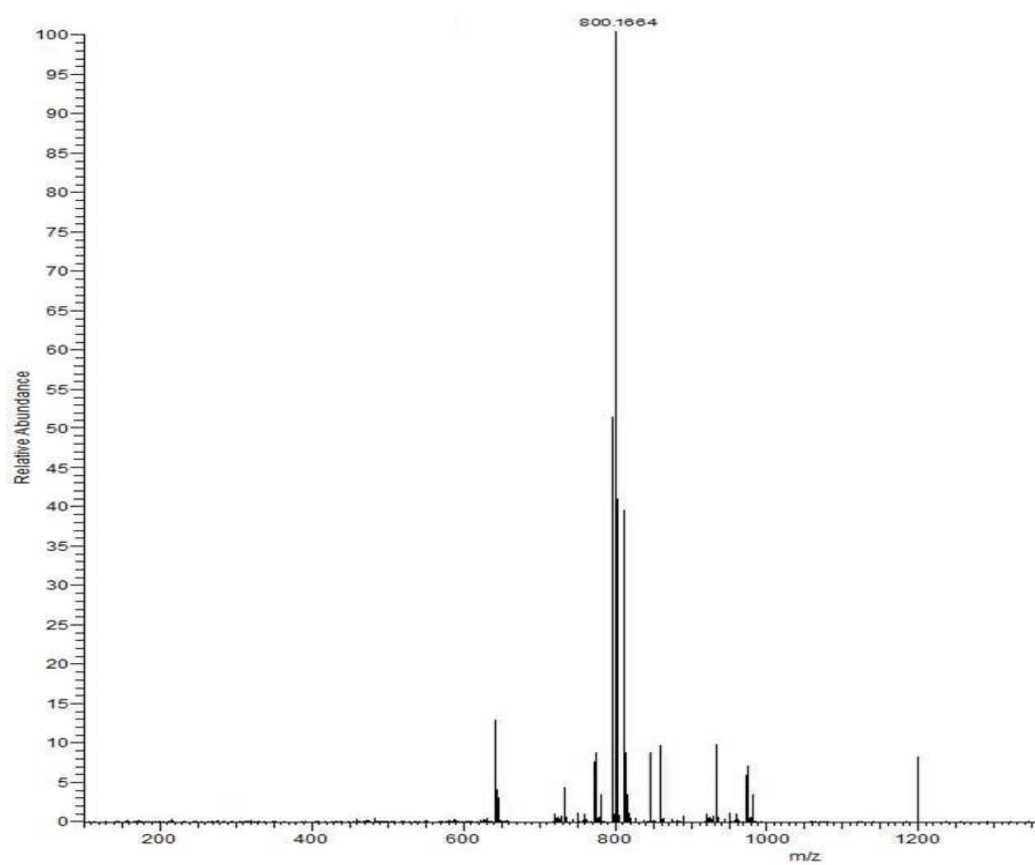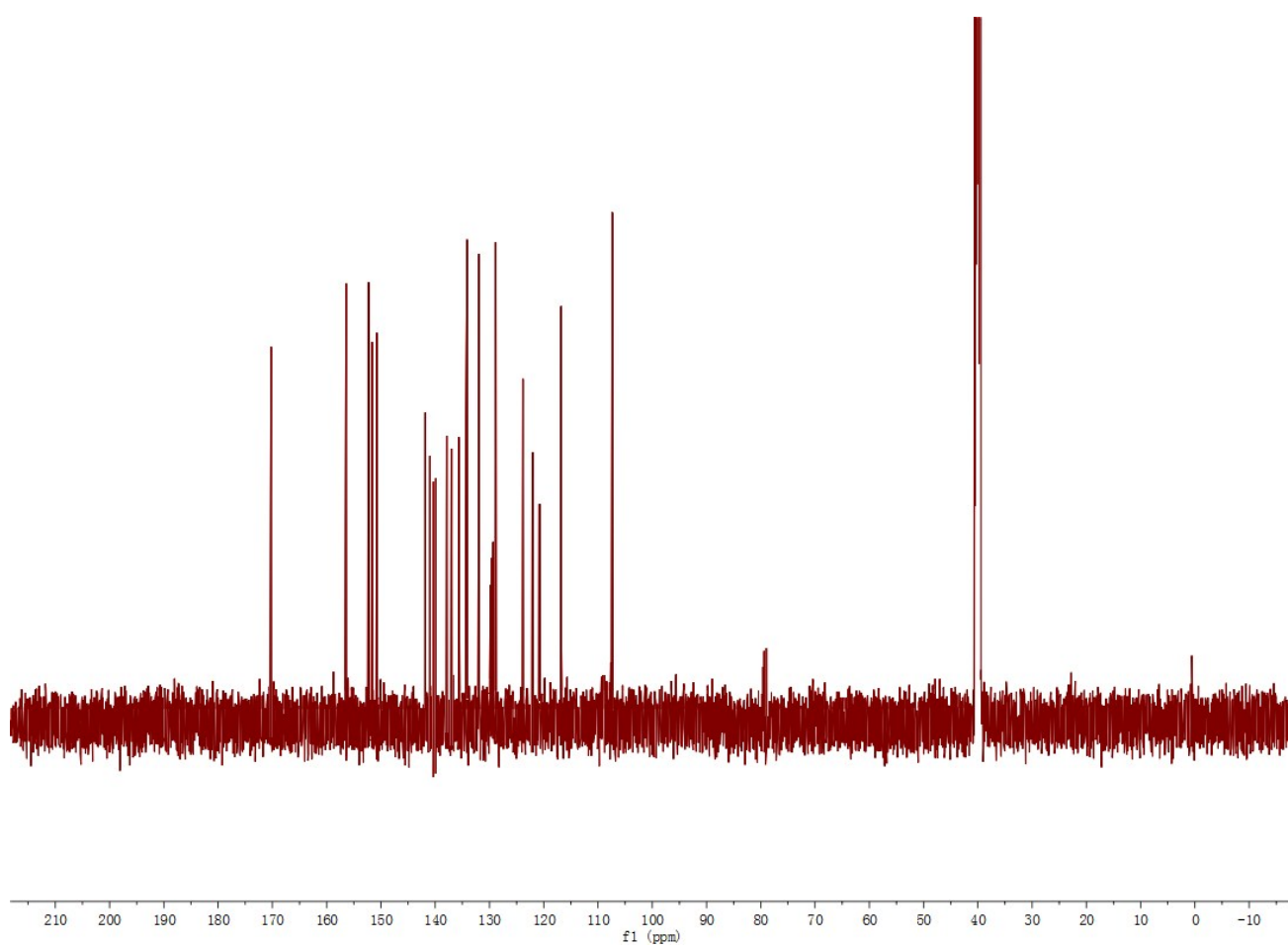
14. References

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