Electronic Supplementary Information

Fluorescence Imaging for Selenol in HepG2 Cell Apoptosis Induced by Na_2SeO_3

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Contents:

1. General Experimental Section

Materials. Solvents were dried by distillation before use. All other reagents were of commercial quality and used without further purification. L-Selenocystine, N-bromosuccinimide (NBS) and benzoyl peroxide (BPO) was purchased from BaiLing Wei Chemical Company. Cysteine (Cys), glutathione (GSH), TrxR (thioredoxin reductase from rat liver), S-nitroso-N-acetyl-dl- penicillamine (SNAP), xanthine (1 mm) in 10 mm NaOH solution, xanthine oxidase (5 UmL^{-1}), H_2O_2, 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT), DAPI, PI working solution, JC-1, 2, 1, 3-Benzoselenadiazole and sodium selenite were purchased from Sigma Chemical Company. Ascorbic acid, *beta*-mercaptoethanol (β-ME), N-acetyl-L-cystein (NAC), Cystine (CySSCy), ammonium dihydrogen phosphate, tetrabutylammonium bromide and dithiothreitol (DTT) were obtained from Sinopharm Chemical Regent Co., Ltd. HepG2 (Human hepatocellular liver carcinoma cell line) cells and HL-7702 (Human hepatocellular liver cell line) cells were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Sartorius ultrapure water (18.2 MΩ cm) water was used throughout the analytical experiments.

$O_2^{-\cdot}$ were created by the enzymaticreaction of xanthine/xanthine oxidase (XA/XO) at 25 °C for 5 min. NaClO was used as the source of HClO. NO was generated from SNAP.

Instruments. Cary Eclipse Fluorescence Spectrometer (Varian, Inc. USA) with a 1.0 cm quartz cells at the slits of 10/10 nm. High-resolution mass spectral analyses were carried out on Bruker maxis UHR-TOF Ultra High Resolution Quadrupole-time of flight mass spectrometer (Bruker Co., Ltd., Germany). Melting points were measured using X-6 micro-melting point apparatus and are uncorrected. IR spectra were measured using a Bruker Tensor-27 FTIR spectrometer using the KBr pellet. ^{1}H-NMR and ^{13}C-NMR spectra were taken on a Varian Advance 600-MHz or Bruker Advance 300-MHz spectrometer, δ values are in ppm relative to TMS. The fluorescence images of cells were taken using a TCS SP5 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with an objective lens (×40). All pH measurements were performed

with a pH-3c digital pH-meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass-calomel electrode. Absorbance was measured in a TRITURUS microplate reader in the MTT assay.

Fluorescence analysis. Fluorescence spectra were obtained with Cary Eclipse Fluorescence Spectrometer (Varian, Inc. USA) with a 1.0 cm quartz cells at the slits of 10/10 nm. After dilution to 10 μM with 10 mM PBS buffer solution, various amounts of Sec were added. In addition, the Sec was prepared by the reaction of Cys with L-Selenocystine for 30 min at 37°C before use every time. The fluorescence intensity was measured at $\lambda_{ex}/_{em}$ = 460 /580 nm.

HPLC analysis

The separation was performed on an LC-20AT liquid chromatographic system (Shimadzu, Japan) equipped with a degasser, a pump, a manual injector provided with a 20 μL loop, a column oven and a diode array detector (DAD). The chromatographic separation was performed on a guard and analytical cartridge system (WondaSil C18 column, 5 μm average particle size, 200 mm × 4.6 mm i.d., GL Sciences Inc., Japan) with column temperature set at 40 ◦C. 220 nm was employed as the detection wavelength. Samples were separated using the mobile phase consisting of solvent A [ammonium dihydrogen phosphate with 1.5% (m/v) tetrabutylammonium bromide, pH = 5.0 ± 0.05] and 5% solvent B (methanol). The injection volume was 20 μL.

Cell culture. HepG2 and HL-7702 cells were maintained following the protocols provided by the American Type Tissue Culture Collection. Cells were first grown in a circular petri dish (60 mm) using high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), $NaHCO_3$ (2 g/L) and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cultures were maintained in a humidified incubator at 37°C, in 5% CO_2 /95% air. One day before imaging, cells were passed and plated on 18 mm glass coverslips in culture dish. The culture medium was refreshed every 24 h. All cells used were in the exponential growth phase.

Confocal imaging. Fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscope (Germany Leica Co., Ltd) with an objective lens

(×40). Excitation of probe-loaded cells at 488 nm was carried out with an Argon laser, and emission was collected using a META detector between 500 and 600 nm. Prior to imaging, the medium was removed. Cell imaging was carried out after washing cells with PBS (pH 7.4, 0.10 M) three times.

Mass spectrometry analysis: MS spectra were obtained with LCQ Deca XP MAX (Thermo Finnigan). A MS solution was prepared as follows: 100 μL 10 mM of CySe-SeCy in H_2O, 0.01% $NH_3 \cdot H_2O$ as cosolvent, was mixed with 20 μL 54 mM Cys in H_2O or DTT in CH_3CN under 37°C for 30 min. 50 μL 1 mM of BS was added to 22 μL of mixture above mentioned, and diluted to the total volume of 200 μL by using MeOH : H_2O : FA = 50 : 50 : 1%. Additional 5 μL of formic acid was added and then detected by MS.

The quantum yields of the probe before and after reacting with CysSe were determined according to the literature[1]:

$$\Phi_x=\Phi_s(F_x/F_s)(A_s/A_x)(\lambda_{exs}/\lambda_{exx})(n_x/n_s)^1$$

Where Φ is quantum yield; F is the integrated area under the corrected emission spectrum; A is the absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; n is the refractive index of the solution; the subscripts x and s refer to the unknown and the standard, respectively. Fluorescein (Φ_F = 0.90) in 0.1mol/L NaOH was used as the standard[1].

2. Quantum chemical calculation: All calculations were carried out using DFT as implemented in Gaussian 09.[2] The structures of BS and Ebselen were optimized at M06-2X [3]/ 6-31+G(d) level. Natural bond orbital (NBO) analyses [4] were performed at the M06-2X/6-31++G (d,p) level to measure the Wiberg bond indices (WBI). According to the results, the WBI of Se-N in BS is larger than that of Ebselen (1.145 *vs* 0.832), indicating Se-N bond in BS is stronger than Se-N bond in Ebselen.

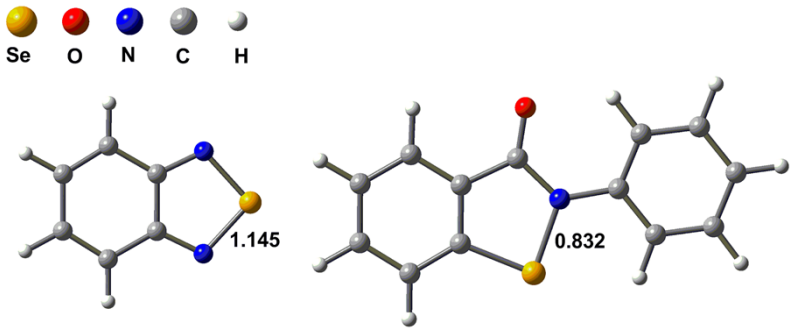


Figure S1. Wiberg bond indices of Se-N bonds in BS and Ebselen

3. Quantitative analysis of Sec generated from selenocystine with cysteine by HPLC method

Reduction of selenocystine by cysteine takes place in the following series of reactions:

$$\text{Cys} + \text{CySeSeCy} \longrightarrow \text{CySeSCy} + \text{Sec}$$

$$\text{CySeSCy} + \text{Cys} \longrightarrow \text{CySSCy} + \text{Sec}$$

Selenocysteine (Sec), as a highly reactive compound, is difficult to be detected. Based on above reaction equations, the content of Sec is equivalent to the consumption of Cys. Thus, in our experiment, the consumption of Cys, the production of CySSCy were detected by HPLC assay. 100 mM stock solution of CySeSeCy was prepared by weighing exactly 167.0 mg CySeSeCy and transferring it into 5 mL ultrapure water, 0.01% $NH_3 \cdot H_2O$ as cosolvent. The stock solutions of Cys (2 mM) and CySSCy (2 mM) were prepared by weighing exactly 2.4 mg Cys, 4.8 mg CySSCy, and transferring them into 10 mL ultrapure water, respectively. The ultrapure water before used was deoxygenated by vigorous sparging with nitrogen for at least 30 min. The Sec (2 mM) solutions were prepared by adding 50 μL of CySeSeCy (100 mM) to 5 ml nitrogen-saturated solution of Cys (2 mM), and incubated at 37 ℃ for 30 min. The preparations of other Sec samples with different concentrations were similar to what has been described above.

Sec was generated by the reaction of CySeSeCy with Cys at equivalence ratio of 1:2 and the content of Sec generated from the reduction reaction was detected by HPLC

assay. Four series of the Sec and reference samples with different concentrations (0.33 mM, 0.5 mM, 1 mM, 2 mM) were determined, and the similar results were obtained. As shown in Figure S2, in the generated Sec sample (0.16 mM of CySeSeCy plus 0.33 mM of Cys, final concentrations), the Cys fully converts to CySSCy, which indicates that the reduction of CySeSeCy with Cys yields Sec almost quantitatively at 37 ℃ for 30 min.

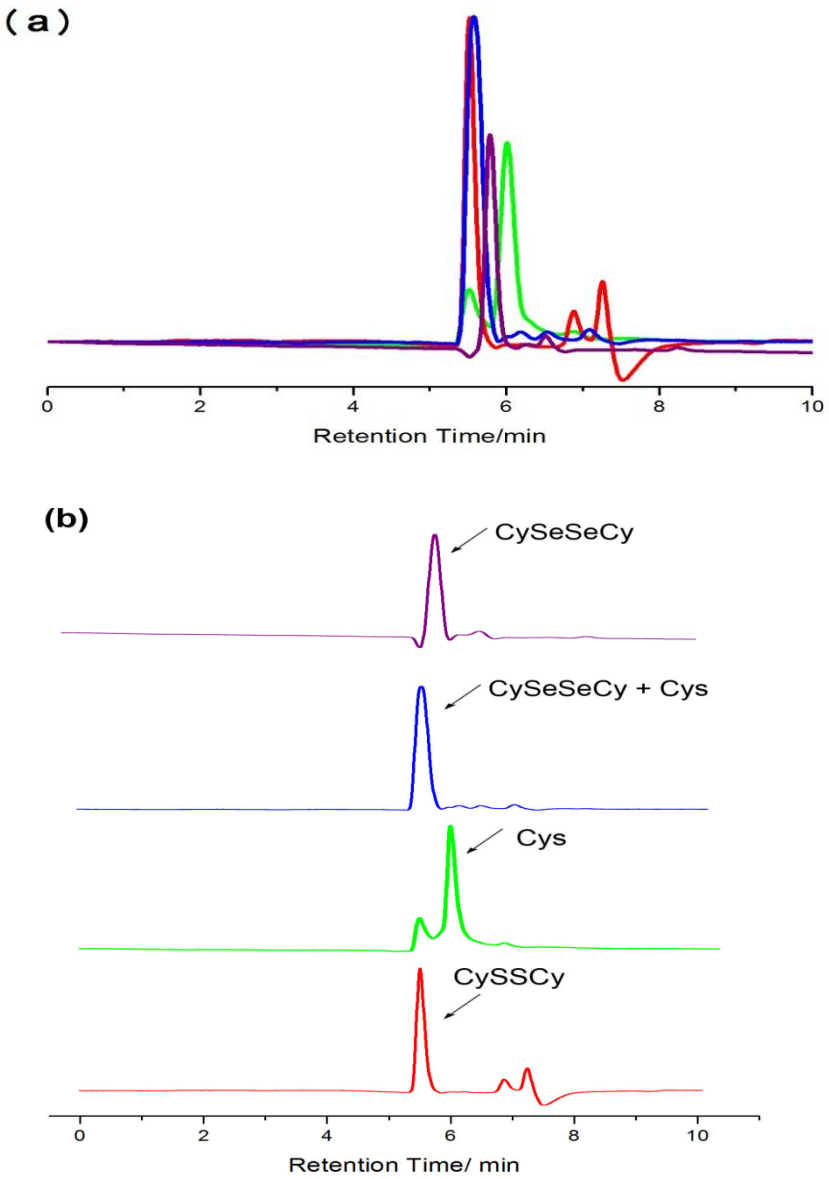


Figure S2. The HPLC spectrogram of Cys (green line), CySSCy (red line), CySeSeCy (purple line), and the reaction mixture of Cys and CySeSeCy (blue line). (a) Overlay; (b) Each respectively

4. Product Analysis of the reaction of BS with Sec:

The reactive products from the reaction of BS and Sec were examined by mass spectrometry, as shown in Figure S3 (inset: theoretical peak distribution in top right), which does show the formation of the reduced BS product, *o*-phenylenediamine, and CysSeSeSeCys. Sec was generated by the reaction of $(CysSe)_2$ with Cys. A mixture of BS and Cys was also tested by MS under the same condition, and no product peaks appeared except *m/z* 122 of Cys (data no shown).

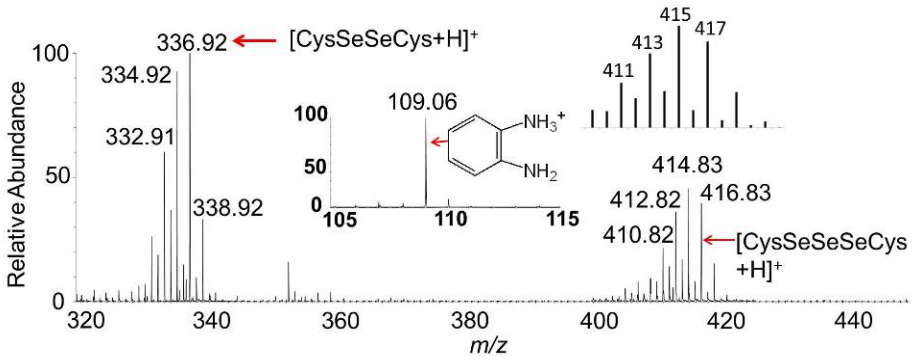


Figure S3. ESI-MS spectrum showing the reactive products of BS with Sec. A MS solution was prepared as follows: 100 μL of 10 mM $(CysSe)_2$ in H_2O, 0.01% $NH_3 \bullet H_2O$ as cosolvent, was mixed with 10 μL of 55 mM Cys in H_2O under 37°C for 30 min. 50 μL of 1 mM BS was added to 20 μL of mixture above mentioned, and diluted to the total volume of 200 μL by using MeOH : H_2O : FA = 50 : 50 : 1%. Additional 5 μL of formic acid was added and then detected by MS.

5. Discuss on the reaction of 2,1,3-benzoselenadiazole with Sec selectively

The reactions of 2,1,3-benzoselenadiazole (BS) with Sec and Cys were discussed by fluorescence method, mass spectrometry and quantum chemical calculation, as shown in Figure S1, Figure S3 and Figure S4. The details were given below.

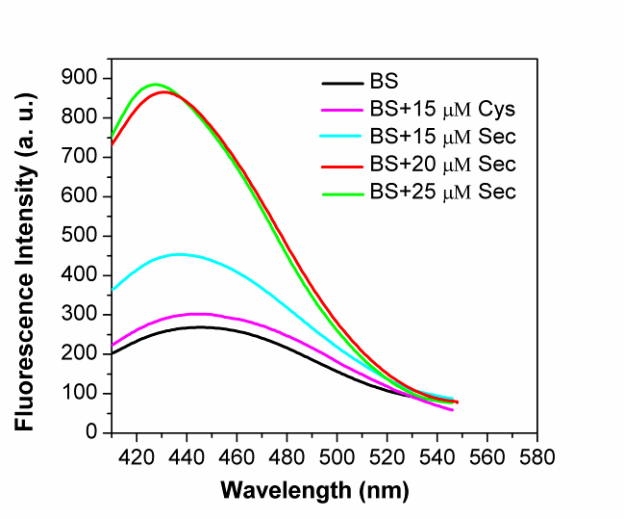


Figure S4. Fluorescence responses of 10 μM BS to diferent concentrations of Sec (15 μM, 20 μM and 25 μM) and 15 μM of Cys at pH 7.4 (PBS buffer, 10 mM) at 37 °C. Fluorescence intensity was measured at $\lambda_{ex/em}$ = 260/430 nm.

6. Product Analysis of the reaction of HB with Sec:

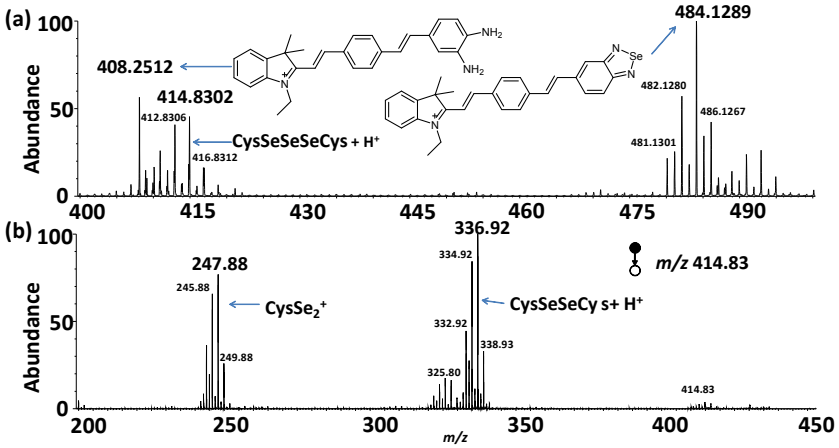
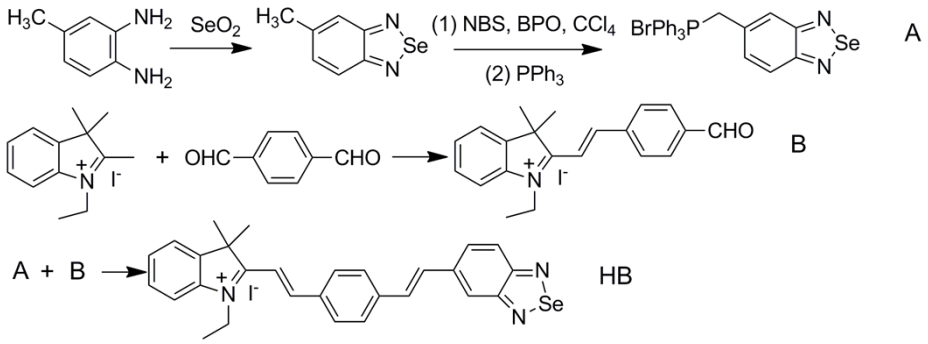


Fig. S5 (a) ESI-MS spectrum showing the reaction of HB with Sec. (b) CID MS2 spectrum of the CysSeSeSeCys product ion (m/z 414).

The reaction products of HB and Sec were fully characterized by mass spectrometry and the collision-induced dissociation (CID) MS^2 spectrum. As shown in Figure S5a. The probe showed a characteristic peak of m/z at 484.1289. For the mixture of HB and Sec, a new peak of m/z at 408.2512 was observed, which corresponded to the diamine product. Besides, the other product CySeSeSeCy (m/z at 414.83) was further confirmated by CID MS2 spectrum (Figure S5b).

7. Synthesis of Fluorescent Probe (HB):

Scheme S1. Synthesis of HB



7.1 Synthesis and Characterization of the probe (HB)

7.1.1. 5-methylbenzoselenadiazole

To a porcelain mortar was added 3, 4-diaminotoluene (1.22 g, 1 mmol), SeO_2 (1.10 g, 1 mmol). The mixture was fully grinded for 30 min, and TLC showed full conversion of 3, 4-diaminotoluene to 5-methylbenzoselenadiazole. The resulting mixture was extracted by cyclohexane (50 ml), then the solution was concentrated on a rotary evaporator to afford the crude product as a light pink solid 1.64 g, yield: 83.2%. Mp 68-70 ˚C; ^{1}H NMR (300Mz, $CDCl_3$): δ 7.72(d, J = 9 Hz, 1H), 7.58(s, 1H), 7.33(d, J = 12 Hz, 1H), 2.47(s, 3H). ^{13}C NMR (75Mz, DMSO-d6): 160.6, 159.2, 140.0, 133.0, 122.8, 121.4, 21.5. HRMS (ESI-TOF): m/z $[M+H]^+$ ($C_7H_7N_2Se$): Calculated 198.9769; found 198.9770.

7.1.1. 5-((bromotriphenylphosphoranyl)methyl)benzo[c][1,2,5]selenadiazole (Compound A)

NBS (1.33 g, 7.5 mmol) and BPO (cat. 0.17 g) were added to a solution of the 5-methylbenzoselenadiazole(1.02 g, 5 mmol) in CCl_4, and the resulting mixture was stirred at 78 °C for 4 h under argon. The solution was allowed to reach ambient temperature and filtered to remove insoluble materials. Then, the insoluble substance was washed by 20 ml CCl_4. The combined organic solution was concentrated under reduced pressure to provide the pale yellow product.

1.0 g (3.6 mmol) of the crude product was then added to 1.14 g (4.3 mmol)triphenylphosphine, refluxed in chloroform for 1.5 h under argon until TLC analysis indicated the complete consumption of the starting material. Evaporation *in*

vacuo gave the crude product as a pale yellow solid. Crystallization from cold diethyl ether afforded 1.01 g the pure product, yield: 52%. Mp 147-149 ˚C; ^{1}H NMR(600Mz, DMSO-d_6):δ 7.92-7.94 (m, 3H), 7.75-7.82 (m, 12H), 7.70(d, *J* = 9.0 Hz, 1H), 7.54(d, *J* = 3.6 Hz, 1H), 7.08(d, *J* = 9.6 Hz, 1H), 5.45(d, *J* = 16.2Hz, 2H).^{13}C NMR(150Mz, DMSO-d6):159.4, 159.1, 135.6, 134.5, 131.6, 130.7, 130.6, 130.2, 125.9, 123.6, 118.2, 117.7, 56.4. HRMS (ESI-TOF): m/z $[M-Br]^+$ ($C_{25}H_{20}N_2PSe$):. Calculated 459.0528; found 459.0519.

7.1.3.(E)-1-ethyl-2-(4-formylstyryl)-3,3-dimethyl-3H-indolium iodide (Compound B)

To a 25 ml round-bottomed flask charged with a magnetic stir bar was added 2, 3, 3-trimethylindolenine (2.0 g, 6 mmol), 1, 4-phthalaldehyde (4.25 g, 3 mmol) and anhydrous sodium acetate (0.3 g). The mixture was stirred at 60 °C for 3 h. The crude product was purified by flash chromatography (silica; 10:1 dichloromethane/methanol) to afford of 1.82 g (70%) of target product as a red solid. Mp 189-192 ˚C; IR 3160, 1684, 1638, 1532 cm^{-1}.^{1}H NMR(600Mz, DMSO-d_6): δ 10.14(s, 1H), 8.55(d, *J* = 16.8 Hz, 1H), 8.49(d, *J* = 8.4 Hz, 2H), 8.11(d, *J* = 8.4 Hz, 2H), 8.03-8.04(m, 1H), 7.95-7.96(m, 1H), 7.89(d, *J* = 16.8 Hz, 1H), 7.68-7.69(m, 2H), 4.80-4.84(m, 2H), 1.91(s, 6H), 1.51(t, *J* = 7.2, 3H). ^{13}C NMR(150Mz, DMSO-d_6):193.2, 181.9, 151.9, 144.8, 140.8, 139.9, 138.7, 131.3, 130.4, 130.2, 129.7, 123.6, 116.1, 115.7, 53.1, 43.2, 25.7, 14.4. HRMS (ESI-TOF): m/z $[M-I]^+$ ($C_{21}H_{22}NO$): calculated 304.1696; found 304.1701.

7.1.4.2-((E)-4-((E)-2-(benzo[c][1,2,5]selenadiazol-5-yl)vinyl)styryl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium iodide (HB)

A mixture of compound A (0.8 g, 1.48 mmol), compound B (0.64 g, 1.48 mmol), and potassium tert-butoxide (0.4 g, 3.6 mmol) in dichloromethane (30 mL) and was heated at reflux under nitrogen for 1.5 h. The reaction mixture was then filtered through Celite, the solvent was removed in vacuo to yield a red solid, which was purified by column chromatography on silica gel (15:1 dichloromethane/methanol).

Recrystallization from methanol and diethyl ether gave the pure product 0.41g, yield: 45%. Mp 179-181 ˚C; IR 3166, 1618, 1527cm^{-1}. ^{1}H NMR(600Mz, DMSO-d_6): δ 8.51(d, J = 16.2 Hz, 1H), 8.33(d, J = 7.8 Hz, 2H), 8.07(d, J = 9.0 Hz, 1H), 8.01(s, 1H), 7.91-7.98(m, 4H), 7.65-7.79(m, 6H), 4.71(m, 2H), 1.84(s, 6H), 1.49(t, J = 7.2, 3H). ^{13}C NMR(150Mz, DMSO-d_6):181.6, 160.7, 160.1, 153.6, 144.4, 142.2, 140.8, 138.2, 134.6, 131.8, 131.5, 131.2, 130.8, 129.9, 129.6, 127.9, 127.8, 123.8, 123.6, 123.2, 122.2, 115.6, 112.7, 52.7, 25.8, 14.3. HRMS (ESI-TOF): m/z [M-I]$^+$ ($C_{28}H_{26}N_3Se$): calculated 484.1288; found 484.1289.

8. Excitation and emission spectra of HB with Sec

Excitation and emission spectra were recorded before (black) and after (green) the reaction of HB with Sec in phosphate buffer at pH 7.4.

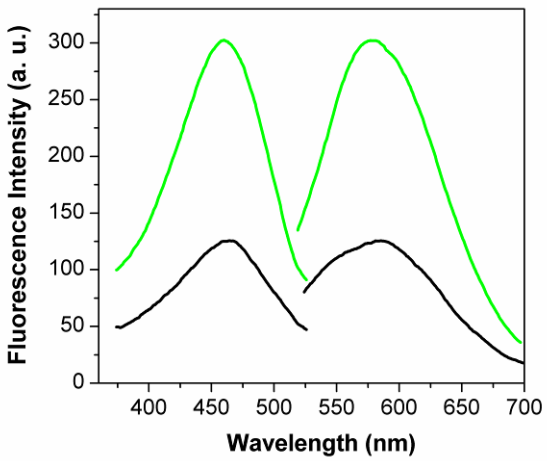


Figure S6. Fluorescence responses of 10 μM HB to 15 μM Sec at pH 7.4 (PBS buffer, 10 mM, 0.5% DMSO) at 37°C, $\lambda_{ex}/\lambda_{em}$ = 460 /580 nm.

9. Optimization of pH and the probe concentration

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 ~ 8.6, as shown in Figure S7a. The fluorescence intensities of the probe and the reaction product were relatively steady as the pH raised from 6.5 to 8.6. In order to apply in biological system, pH = 7.4 was

selected for the following determination system. In addition, the probe concentrations used were also optimized, as shown in Figure S7b.

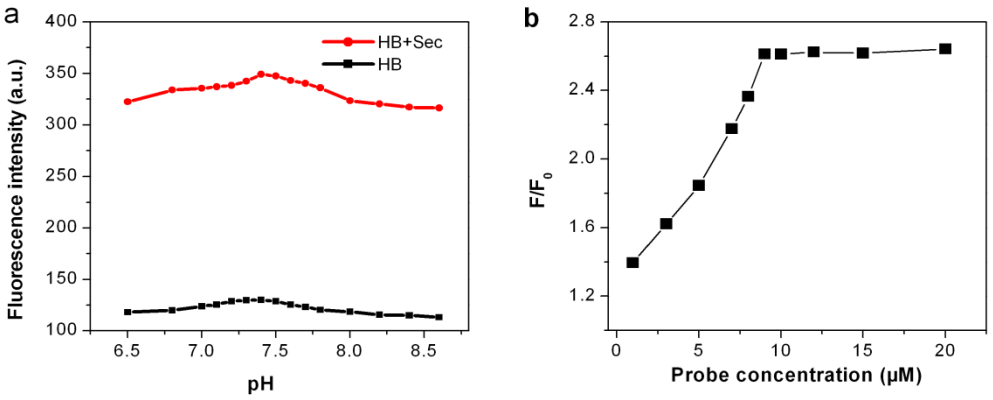


Figure S7. (a) Effects of pH on fluorescence intensities of the probe and the reaction solution of HB (10 μM) and Sec (20 μM) in PBS buffer (10 mM). (b) The effect of probe concentrations on fluorescence intensities.

10. Measurement of Sec residues in TrxR

TrxR from Sigma Chemical Company (221μL, 0.25 mg protein/mL was received as a solution in 50 mM Tris buffer (pH 7.5) containing 300 mM NaCl, 1 mM EDTA, and 10% glycerol. A TrxR solution 2.5 μg/ mL and an 8 M guanidine solution (final concentration) were incubated at 37 °C for 30 min. The probe solution (10^{-3} M,10 μL), PBS (pH 7.4, 100 μL, 100 μM), Cys (100 μM, 10 μL) and H_2O (770 μL) were added in turn, final volume was 1000 μL. Parallel to the above procedure, TrxR solution 2.5 μg/ mL (0.25 mg protein/ mL, 10 μL), probe solution(10^{-3} M, 10 μL), PBS (pH 7.4, 100 μL, 100 μM), H_2O (880 μL). Then the fluorescence intensity was measured in Figure S8.

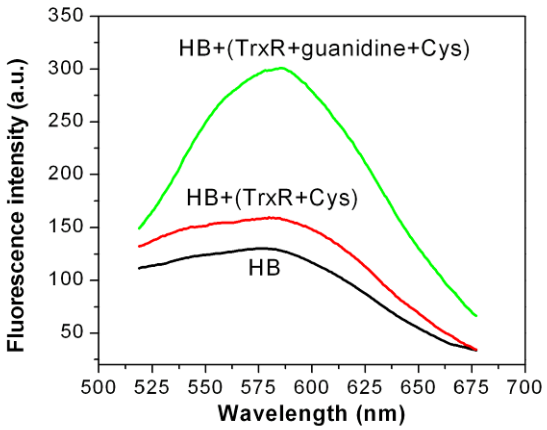


Figure S8**.** Fluorescence responses of 10 μM HB to the denatured TrxR

In order to confirm that the Sec residues could react with HB, the SePs were pretreated with a denaturing reagent as follows: TrxR were incubated in the presence of guanidine at 37 ºC for 30 min, and then the denatured SePs were mixed with Cys (keeping reductive conditions) and the probe solution prepared in phosphate buffer at pH 7.4 containing 0.4 M guanidine. The fluorescence of the mixture was measured immediately (Figure S8). The result shows that HB is available to detect the Sec residues in denatured SePs.

11. Selectivity of HB toward Sec

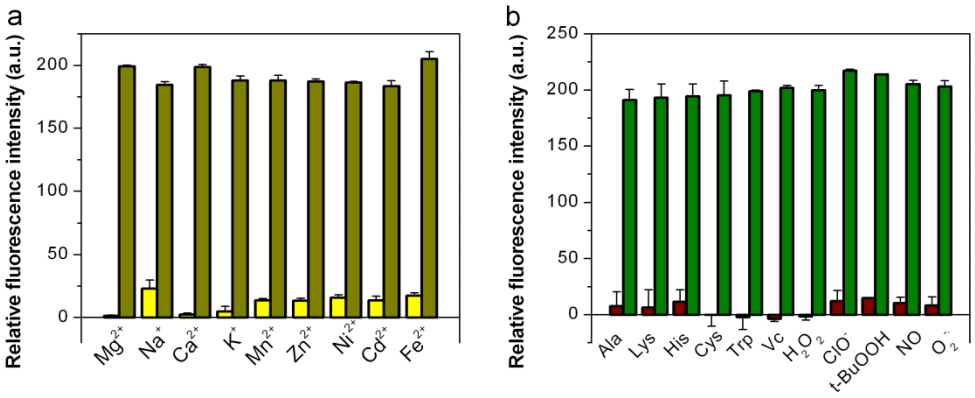


Figure S9**.** Selectivity of HB to Sec. (a) Fluorescence responses of 10 μM HB to metal ions (yellow bars) and metal ions plus 20 μM Sec (dark yellow bars). 10 mM for Na^+ and K^+; 5 mM for Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Ni^+, Cd^{2+} and Fe^{2+}. (b) Fluorescence responses of 10 μM HB to amino acids, Vc and reactive oxygen species (ROS) (purple bars), and the above solution plus 20 μM Sec (olive bars): 100 μM for Ala, Lys, His, Cys and Trp; 2.5 mM for Vc; 100 μM for H_2O_2, *t*-BuOOH

and 5 mM NO; 50 μM for ClO^- and $O_2^{\cdot-}$. All spectra were acquired in 10 mM PBS buffer with pH 7.4 at 37 °C ($\lambda_{ex/em}$ = 460 / 580 nm).

12. MTT assay

To investigate HB cytotoxicity, MTT assay were carried out when the probe existed HepG2 cells in Figure S10. 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, respectively. MTT solution (5.0 mg mL^{-1} in PBS) was then added to each well. After 4 h, the remaining MTT solution was removed and DMSO (150 μL) was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a TRITURUS microplate reader. Calculation of IC_{50} values was done according to Huber and Koella.[5] The probe IC_{50} value was calculated to be 321 μM, which demonstrated that HB should be a low cytotoxic probe under experimental conditions at the concentration of 10 μM.

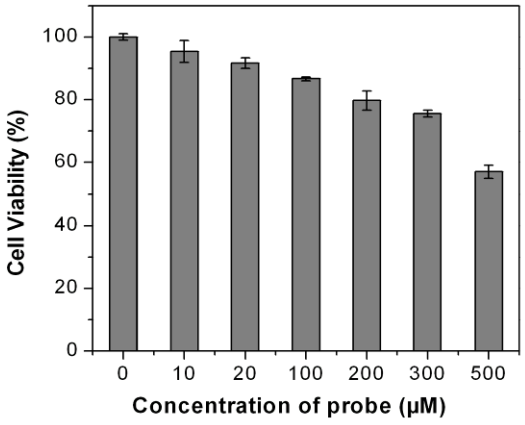


Figure S10. MTT assay of HepG2 cells in the presence of different concentrations of HB

13. Photo-bleaching test of the reaction product of HB with Sec

Photo-bleaching is an irreversible photochemical inversion of fluorescent molecule into a non-fluorescent state. The photo-stability of HB was investigated by time-sequential scanning of the living cells. After 500 s of continuous irradiation with a

488-nm laser, no obvious changes were observed in fluorescence brightness of HB. In order to quantitatively determine the photo-bleaching rate, we choose three regions, calculated the average intensity and obtained a curve with scanning time in Figure S11. The results showed that the intensities of HB 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.

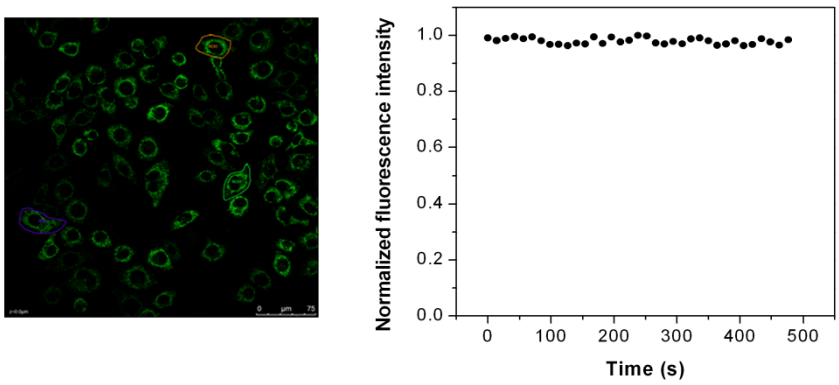


Figure S11. Test of photo-stability. Fluorescence images were achieved by means of time-sequential scanning of the probe-loaded HepG2 cells incubated with Sec for 10 min. Normalized fluorescence intensity from three regions from 0 to 500s of time-sequential scanning.

14. **Fluorescence imaging of Sec in living cells**

HepG2 and/or HL-7702 cells were seeded on glass coverslips in 24 well culture plates at 2×10^4 cells per well in 1 mL growth medium and incubated at 37°C for 24 h, and then cells were exposed to different treatment conditions. Cells were divided into four groups. One group was chosen as control, and the three other groups were treated with Selenocystine $(CysSe)_2$ (20 μM), Cys (20 μM), and Sec obtained by $(CysSe)_2$ (10 μM) plus Cys (20 μM) for 10 min, respectively. Then the fluorescence in situ changes were observed within 0-1.5h by confocal microscopy.

The other cells were exposed to different concentrations sodium selenite (2 to 20 μM) or different time (0-48h). After treatment, cells were incubated with 10 μM of HB probe for 15 min and nuclei were stained with 1 μmol/L DAPI for 5 min. Finally, the fluorescence was detected under confocal microscopy (Leica, Germany).

15. Fluorescence imaging of Sec *in vivo*

Kunmin male mice (15-20 g) were anesthetized with 4% chloral hydrate [6](3 mL/ kg) by intraperitoneal injection. Ten minutes later, their abdominal fur was removed and HB (100 μM) was injected into their peritoneal cavity. Then the left mice was given 500 μL of physiological saline as control and the right mice was given 500 μL of a mixture of Cys (200 μM) plus $(CysSe)_2$ (100 μM) in the abdomen. The mice were imaged by using IVIS Lumina III imaging system, as shown in Figure S12. The strong fluorescence appeared in right mouse, which displays that selenocysteine level *in vivo* can be successfully assessed by the probe.

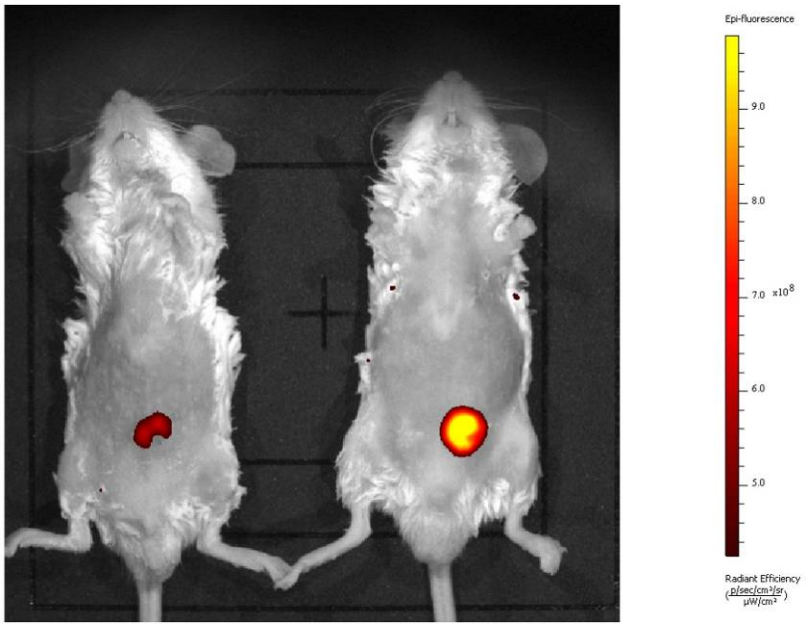


Figure S12. Imaging of selenocysteine in peritoneal cavity of the mice. Left: 500 μL of physiological saline as control; Right: 500 μL of a mixture of Cys plus $(CysSe)_2$ (λ_{ex}/ λ_{em} = 460/ 570 nm)

16. Cell Cycle Analysis

The cell cycle distribution was analyzed by flow cytometry. HepG2 cells were seeded in 60 mm cell culture dish at 2×10^4 cells /mL growth medium with 5 mL, and incubated at 37°C for 24 h, and then cells were exposed to 5 μM of sodium selenite up to different time (6-24h). After treatment, the cells were harvested and divided into two equal groups. One group was stained with 10 μM of HB for 15 min and the fluorescence intensity of cells was detected using flow cytometer with CellQuest

analysis software (Becton Dickinson). The other group was fixed with 70% ethanol overnight at 4°C, and then cells were washed with PBS and stained with PI working solution (Beyotime, China) for 30min in darkness at 37°C. The DNA contents were analyzed with flow cytometer. Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). The proportions of cells in G1, S, and G2 phases were represented as DNA histograms.

17. Mitochondrial Membrane Potential (MMP) Analysis

The values of mitochondrial membrane potential were determined by flow cytometry using JC-1 staining. HepG2 cells were seeded in 60 mm cell culture dish at 2×10^4 cells /mL growth medium with 5 mL, and incubated at 37 °C for 24h, then cells were exposed to different concentrations of sodium selenite (2-10 μM) up to 12h. After treatment, the cells were harvested and divided into two equal groups. One group was stained with 10 μM of HB probe for 15 min and the fluorescence intensity of cells was detected using flow cytometer. The other group was incubated with 10 μM JC-1 for 30 min at 37°C in the dark according to the manufacturer's instructions (Beyotime, China). The positive cells were then detected by flow cytometer with CellQuest analysis software.

18. Apoptosis assays of HepG2 cells induced by Na_2SeO_3

The apoptosis detection was performed by the AnnexinV-FITC/PI Apoptosis Detection Kit (BD Biosciences, USA). Briefly, HepG2 cells were seeded in 6-well plates and incubated for 24 h, then exposed to 2-10μM Na_2SeO_3 for 12h. After treatment, approximately 1×10^6 cells were harvested, washed twice with PBS, and then stained with Annexin V-FITC and PI according to the manufacturer's instructions. The resulting fluorescence was detected by flow cytometry with CellQuest analysis software. As shown in Figure S13, the number of apoptotic cells significantly increased in 10μM treatment group.

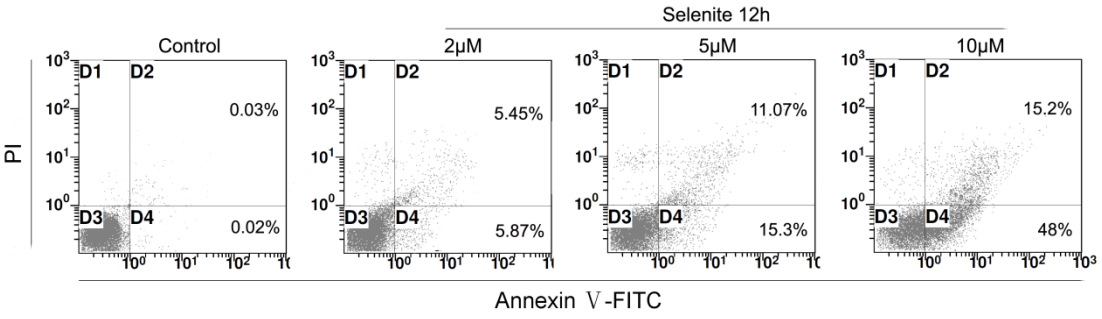


Figure S13. Cell apoptosis rate was analyzed by the Annexin V-FITC/PI assay by flow cytometry

19. References

[1] R. A. Velapoldi and H. H. Tonnesen, *J. Fluoresc.*, 2004, ***14***, 465.

[2] M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr, J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. S. J. J. Dannenberg, S. Dapprich, A. D. Daniels, O. Farkas, J. B. Foresman, J. V. Ortiz, J.Cioslowski and D. J. Fox, *Gaussian, Inc., Wallingford, CT.*, 2010.

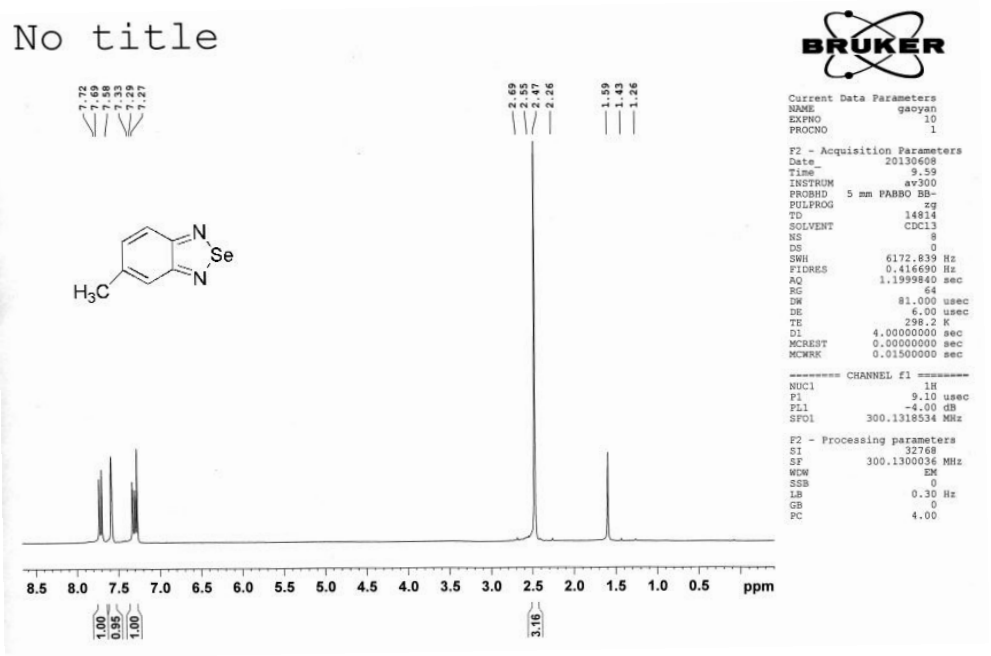
[3] Y. Zhao and D. G. Truhlar, *Theor. Chem. Acc.*, 2008, ***120***, 215.

[4] a) J. E. Carpenter and F. Weinhold, *J. Mol. Struc. Theochem.*, 1988, ***69***, 41; b) A. E.Reed and F. Weinhold, *J. Chem. Phys.*, 1983, ***78***, 4066; c) J. P. Foster and F. Weinhold, *J. Am. Chem. Soc.*, 1980, ***102***, 7211; d) E. D. Glendening, A. E. Reed, J. E. Carpenter and F. Weinhold, *NBO Version 3.1*.

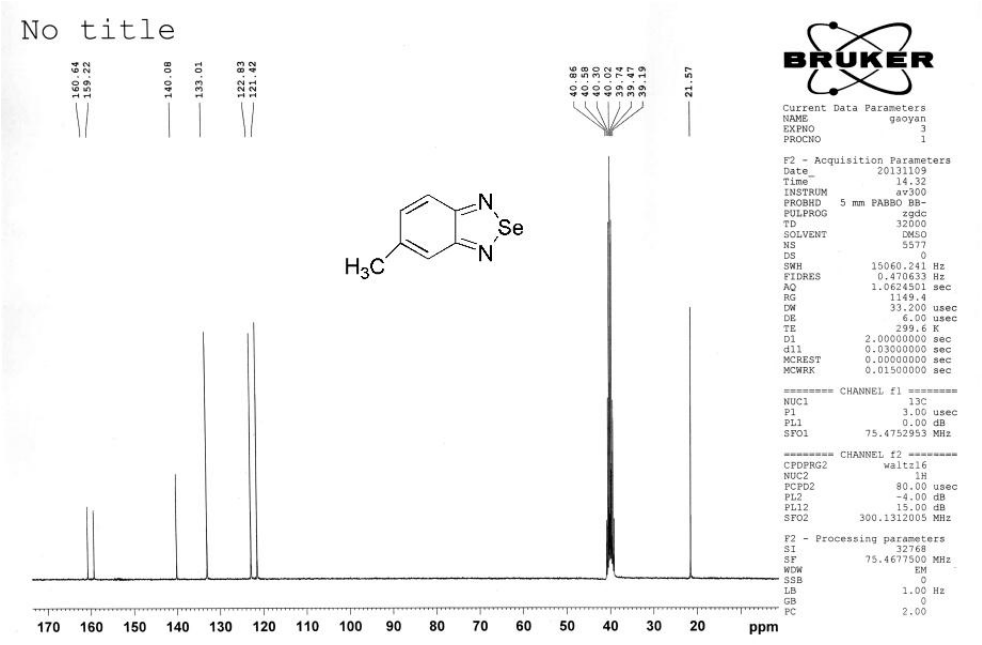
[5] G. L. Gessa, M. Melis, A. L. Muntoni and M. Diana, *Eur J Pharmacol*, 1998, ***341***, 39.

[6] W. D. Huber and J. C. Koella, *Acta Trop.*, 1993, ***55***, 257.

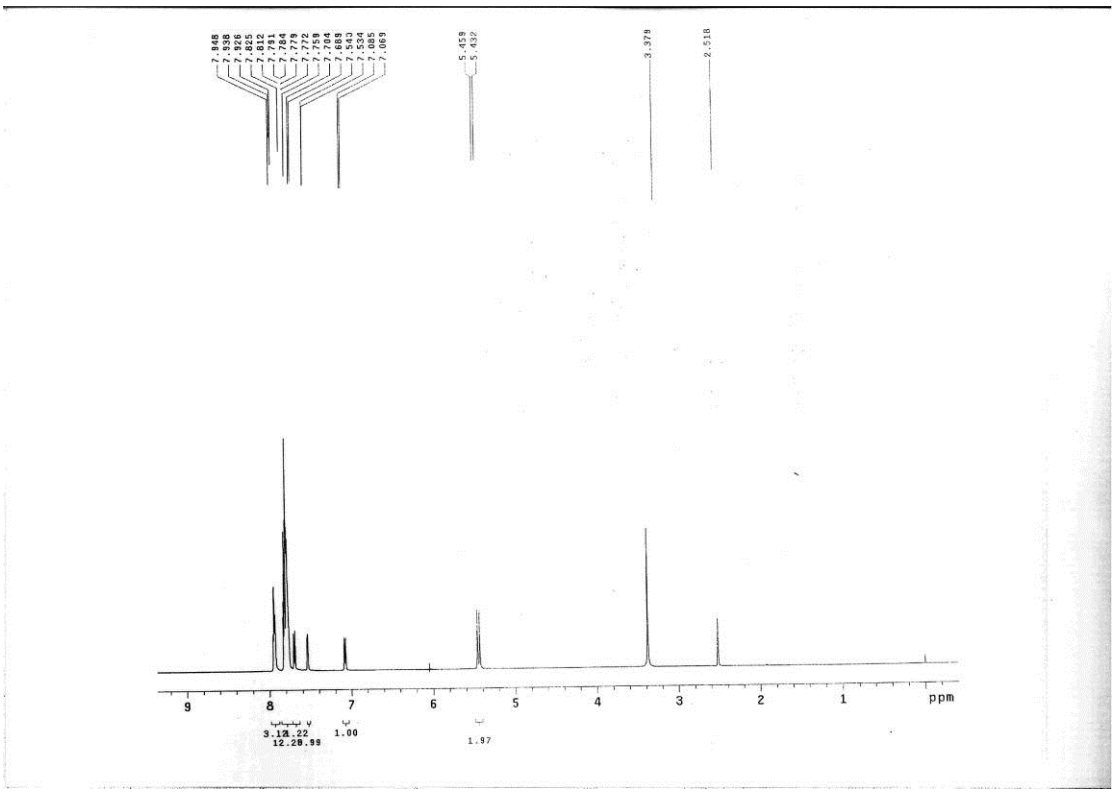
20. Spectrogram of compound 5-methylbenzoselenadiazole, A, B and HB



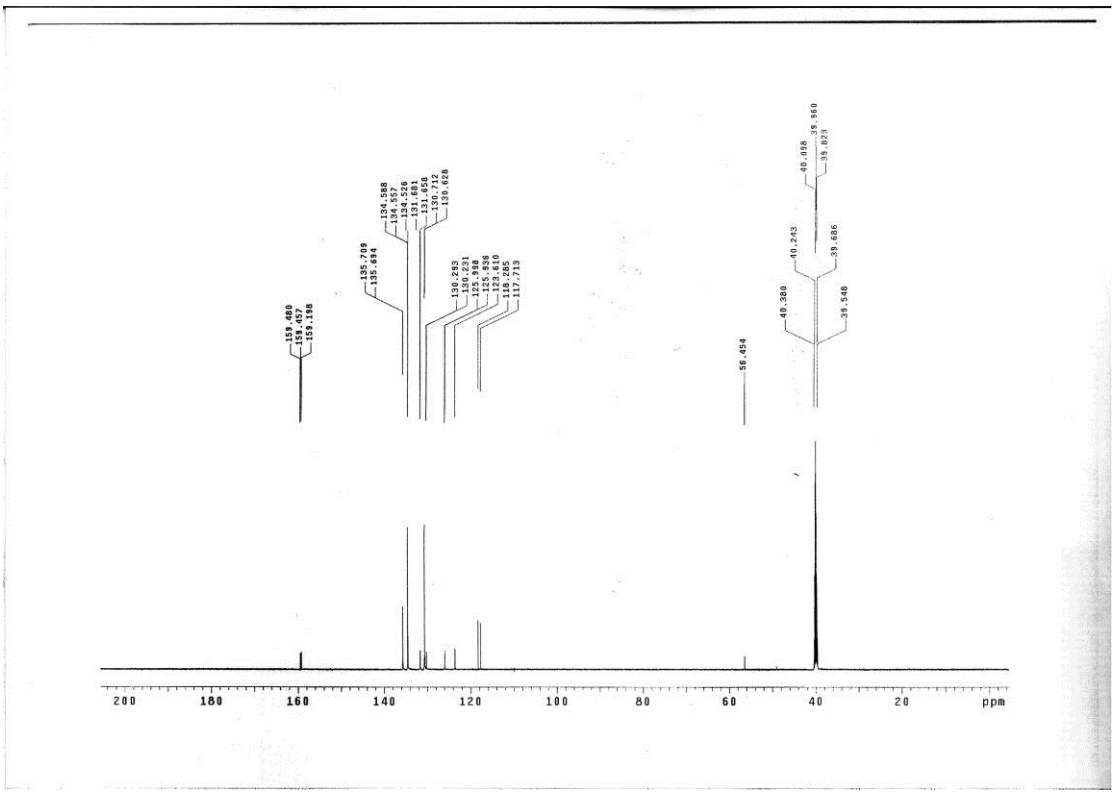
^{1}H NMR of 5-methylbenzoselenadiazole



^{13}C NMR of 5-methylbenzoselenadiazole



^{1}H NMR of compound A



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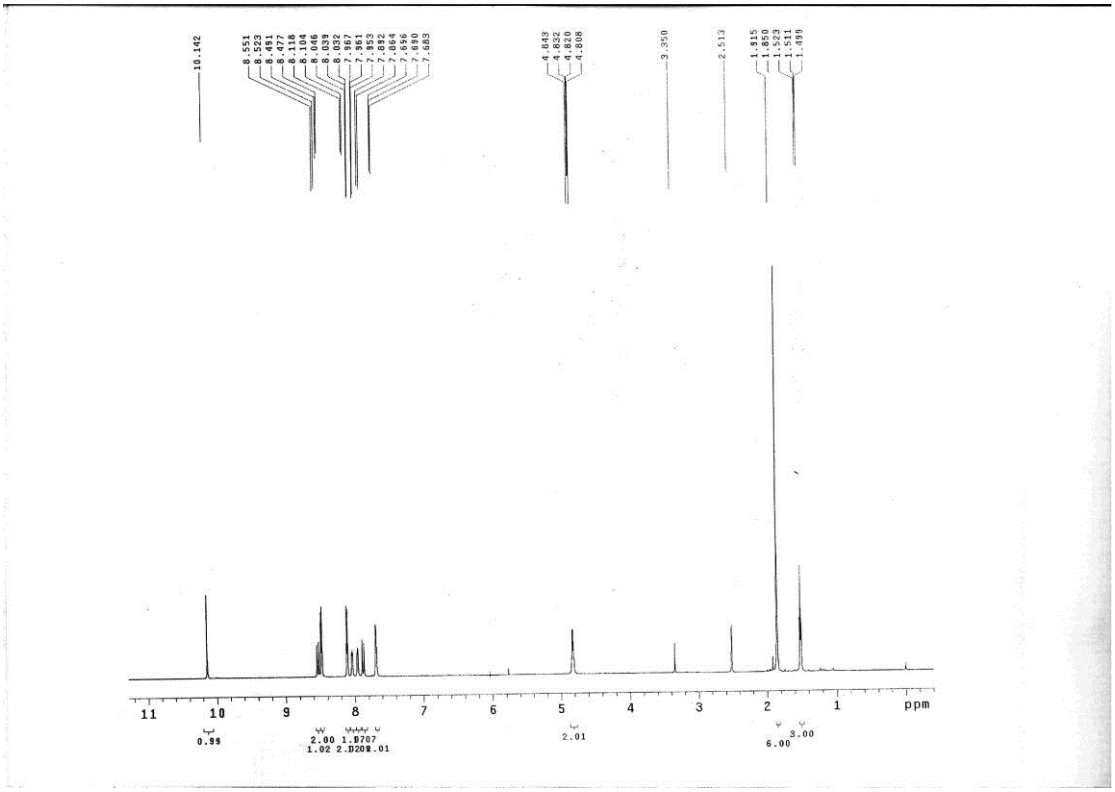

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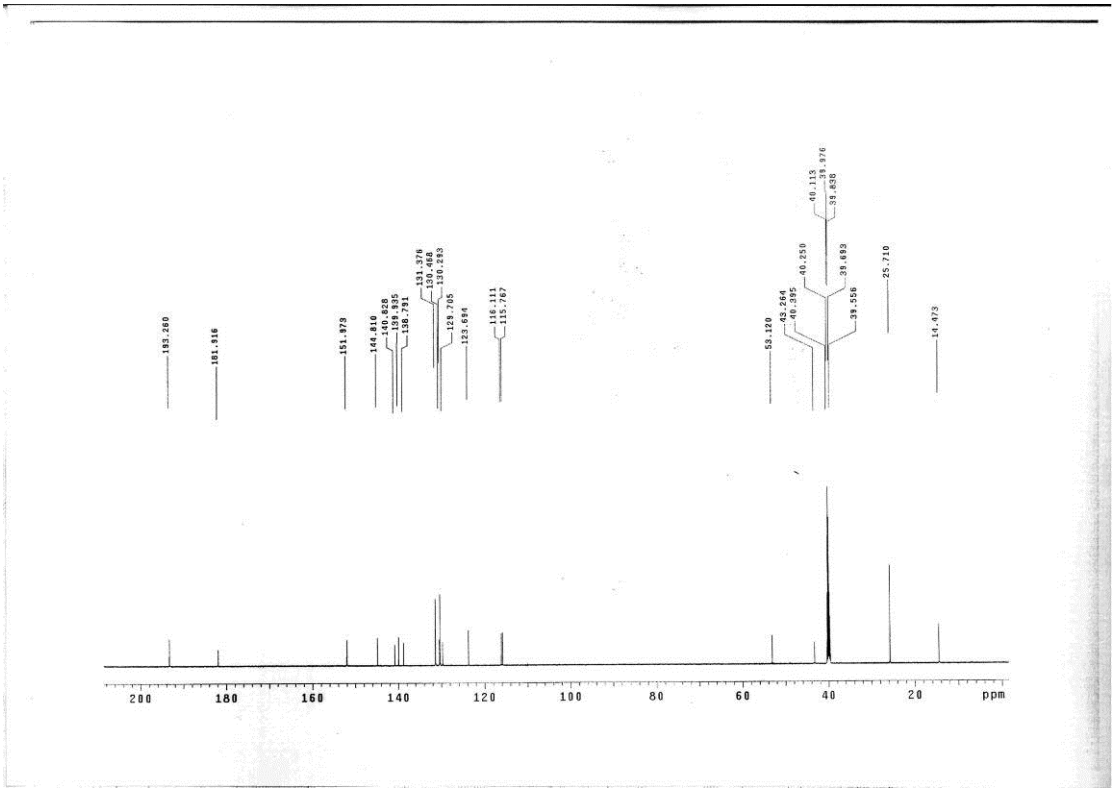

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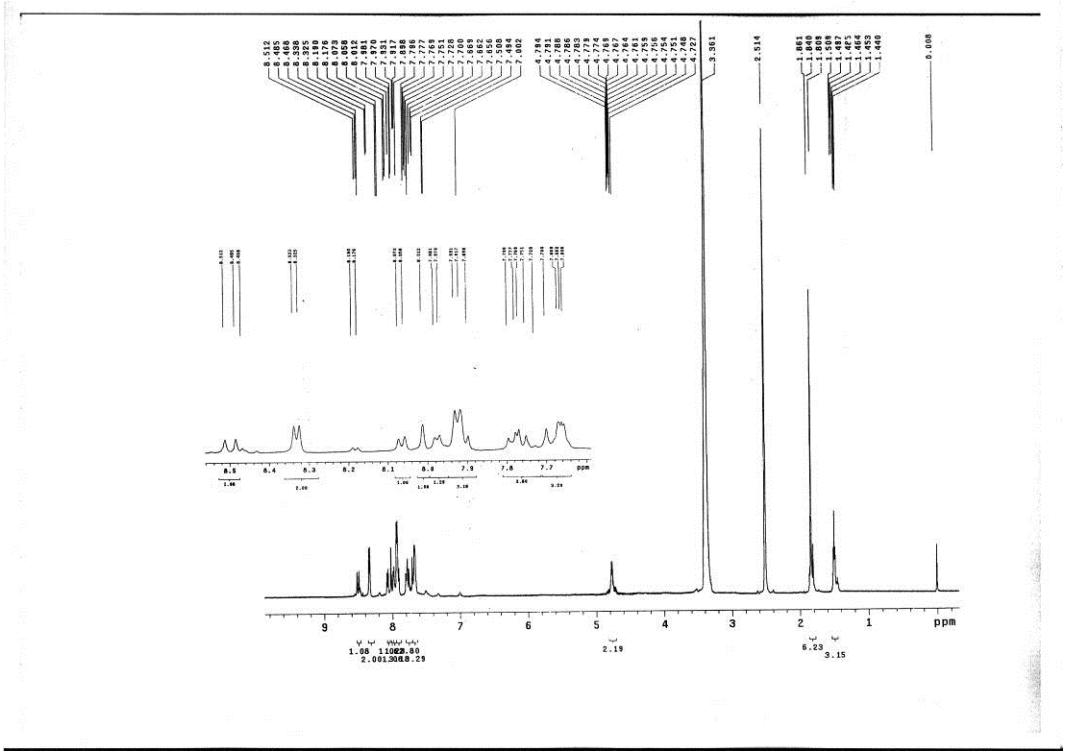
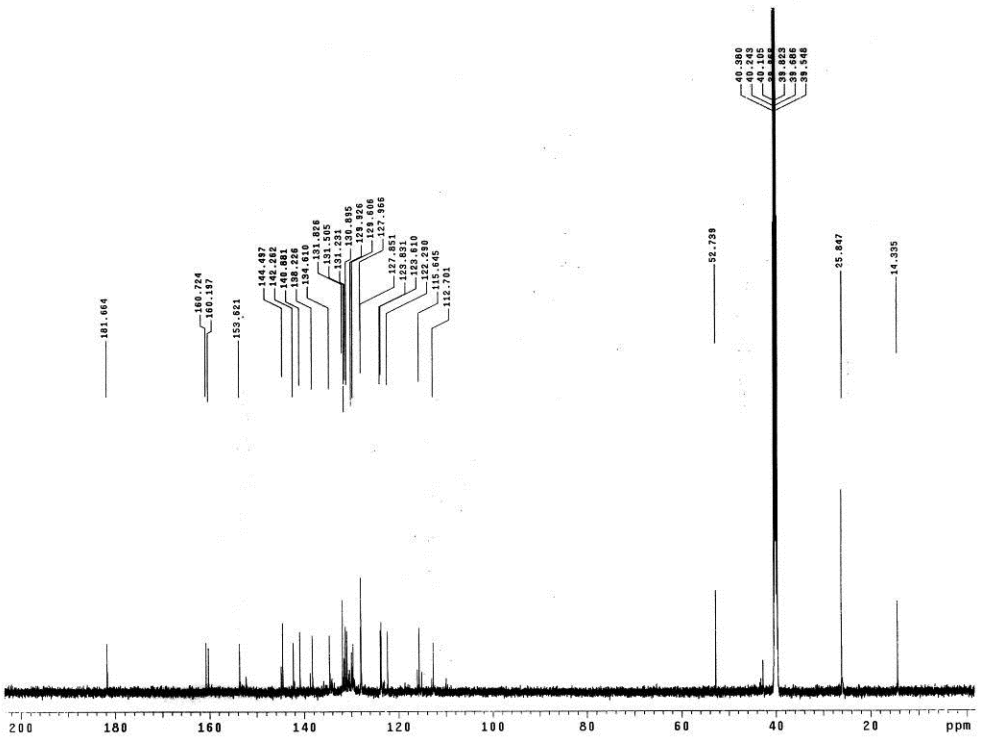


^{1}H NMR of compound HB



^{13}C NMR of compound HB

Mass Spectrum List Report

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Comment			

Acquisition Parameter

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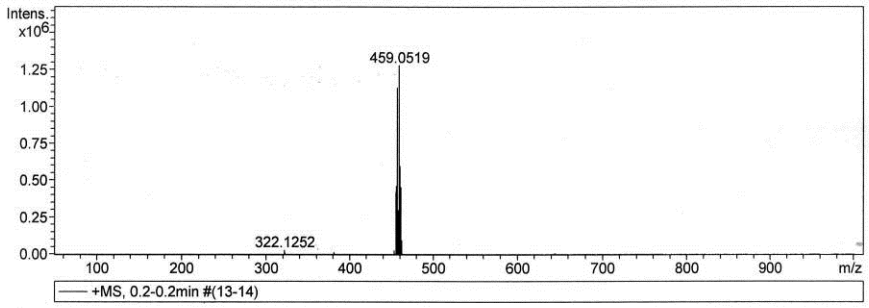

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4	123.9923	17035	601.0	601	0.0073
5	144.0638	22497	988.5	989	0.0064
6	144.9883	21262	744.5	745	0.0068
7	150.9519	22392	734.0	734	0.0067
8	192.9624	23013	576.5	577	0.0084
9	243.1170	19384	405.0	405	0.0125
10	274.2737	23400	918.0	918	0.0117
11	291.1297	23438	1024.0	1024	0.0124
12	318.2999	21337	1015.0	1015	0.0149
13	322.1252	22193	28665.5	28666	0.0145
14	322.6268	21852	10718.0	10718	0.0148
15	323.1278	22182	2159.5	2160	0.0146
16	381.1513	24425	11124.0	11124	0.0156
17	382.1543	25189	1699.0	1699	0.0152
18	453.0578	25518	23403.5	23404	0.0178
19	454.0605	24630	2894.5	2895	0.0184
20	455.0546	19550	420171.0	420171	0.0233
21	456.0558	19991	458269.0	458269	0.0228
22	457.0530	18752	1123428.5	1123429	0.0244
23	458.0559	20302	296041.0	296041	0.0226
24	459.0519	13544	1274991.5	1274992	0.0339
25	460.0547	19668	592488.5	592489	0.0234
26	461.0529	19344	450960.5	450961	0.0238
27	462.0554	23830	91372.0	91372	0.0194
28	463.0582	26385	3066.5	3067	0.0176
29	643.2403	28880	5652.0	5652	0.0223
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Bruker Compass DataAnalysis 4.0 printed: 11/6/2013 9:27:17 AM Page 1 of 1

HRMS of compound A

Mass Spectrum List Report

Analysis Info

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Sample Name		Instrument / Ser#	maXis 49
Comment			

Acquisition Parameter

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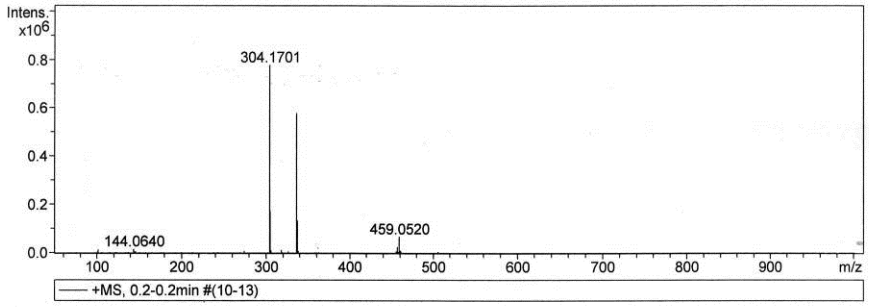

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7	146.0073	19634	9830.5	4915	0.0074
8	150.9517	22011	5436.0	2718	0.0069
9	243.1171	20687	4527.0	2264	0.0118
10	274.2745	22745	14934.5	7467	0.0121
11	304.1701	18343	1549889.5	774945	0.0166
12	304.2606	29886	10421.5	5211	0.0102
13	305.1734	20006	341984.5	170992	0.0153
14	306.1762	22055	17321.0	8661	0.0139
15	318.3004	23495	21591.0	10796	0.0135
16	326.1514	23970	9591.0	4796	0.0136
17	336.1961	18597	1152639.0	576320	0.0181
18	337.1994	20845	264224.5	132112	0.0162
19	338.2021	24464	14754.0	7377	0.0138
20	358.1776	23712	3976.5	1988	0.0151
21	360.3231	25624	4469.5	2235	0.0141
22	362.3260	23098	4537.0	2269	0.0157
23	455.0542	25312	9473.0	4737	0.0180
24	456.0556	26443	11307.5	5654	0.0172
25	457.0529	25090	49132.0	24566	0.0182
26	458.0554	26234	5667.5	2834	0.0175
27	459.0520	24507	134151.5	67076	0.0187
28	460.0549	27589	17983.0	8992	0.0167
29	461.0527	23857	10536.0	5268	0.0193
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HRMS of compound B

Mass Spectrum List Report

Analysis Info

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Comment			

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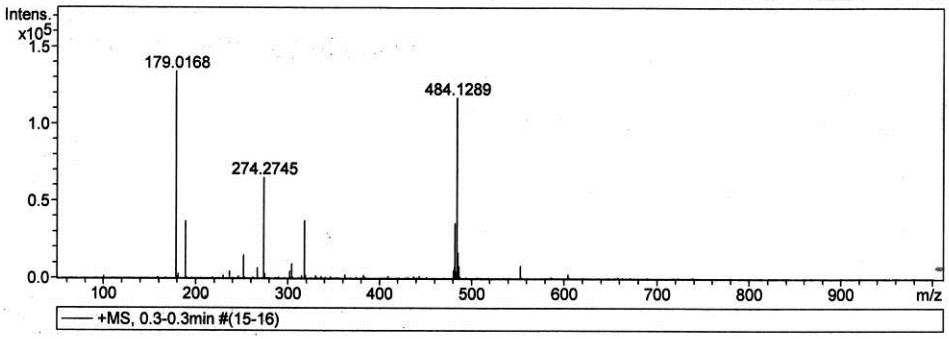

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4	230.2492	27511	284.3	1564	0.0084
5	236.9787	24312	812.4	4468	0.0097
6	252.1707	28436	2709.9	14905	0.0089
7	267.0801	26176	1202.5	6614	0.0102
8	274.1536	30962	2307.9	12694	0.0089
9	274.2745	29348	11821.0	65016	0.0093
10	275.2786	22982	530.7	2919	0.0120
11	302.2471	25948	416.5	2291	0.0116
12	302.3069	35101	795.9	4378	0.0086
13	304.2620	35238	1650.5	9078	0.0086
14	314.9936	25987	285.8	1572	0.0121
15	318.3008	39751	6746.0	37103	0.0080
16	319.3050	25577	320.4	1762	0.0125
17	330.3385	29109	268.4	1476	0.0113
18	336.1974	23609	216.3	1190	0.0142
19	362.3281	29040	400.4	2202	0.0125
20	382.2772	28596	358.2	1970	0.0134
21	409.1643	28136	252.6	1390	0.0145
22	480.1322	31175	922.8	5076	0.0154
23	481.1341	34749	1326.5	7296	0.0138
24	482.1302	33848	6459.4	35527	0.0142
25	483.1342	31733	729.5	4013	0.0152
26	484.1289	51371	21178.0	116479	0.0094
27	485.1323	38261	3014.9	16582	0.0127
28	486.1310	34454	1385.4	7620	0.0141
29	553.1608	35786	1417.5	7796	0.0155
30	604.2685	32024	480.5	2643	0.0189

HRMS of compound HB