Electronic Supplementary Information

A near-infrared fluorescent probe for the detection of hydrogen polysulfides biosynthetic pathways in living cells and in vivo

Min Gao,[ab†] Rui Wang,[b†] Fabiao Yu,[b*] Jinmao You[a], and Lingxin Chen[ab*]

[a]The Key Laboratory of Life-Organic Analysis, College of Chemistry and Chemical Engineering, Qufu Normal University, Qufu 273165, China

[b]Key Laboratory of Coastal Environmental Processes and Ecological Remediation, The Research Center for Coastal Environmental Engineering and Technology, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

E-mail: fbyu@yic.ac.cn, lxchen@yic.ac.cn

[†]These authors contributed equally.

Contents:

1. General experimental section

Instruments: Fluorescence spectra were obtained by FluoroMax-4 Spectrofluorometer with a Xenon lamp and 1.0-cm quartz cells. Absorption spectra were measured on NANO Drop 2000c UV-visible spectrophotometer (Thermo Fisher Scientific). 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. Mass spectra were taken on LCQ Fleet LC-MS System (Thermo Fisher Scientific). ^{1}H NMR, ^{13}C NMR spectra were taken on a Bruker spectrometer. The fluorescence images of cells were taken using a confocal laser scanning microscope (Japan Olympus Co., Ltd) with an objective lens (×40).

Materials: 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and HEPES were purchased from Sigma-Aldrich. Mouse leukaemic monocyte macrophage cell line (RAW264.7) was obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). BD-ss (1.0 mM, 10 mL) was prepared in DMSO and stored at 4 ^{o}C in darkness. All other reagents and chemicals were from commercial sources and of analytical reagent grade, and used without further purification. Solvents used for spectroscopic studies were purified and dried by standard procedures before use. Ultrapure water (Millipore, Bedford, MA, USA) was used throughout.

Absorption analysis: Absorption spectra were obtained with 1.0-cm glass cells. We firstly added 0.4 mL Tween 80 to color comparison tube, and then 1 mL various concentrations of Na_2S_2 was added and diluted to 10.0 mL with 10 mM HEPES buffer. Finally, probe BD-ss was added. The mixture was equilibrated for 5 min before measurement.

Fluorescence analysis: Fluorescence spectra were obtained with a Xenon lamp and 1.0-cm quartz cells. We firstly added 0.4 mL Tween 80 to color comparison tube, and then 1 mL various concentrations of Na_2S_2 was added and diluted to 10.0 mL with 10 mM HEPES buffer. Finally, probe BD-ss was added. The mixture was equilibrated for 5 min before measurement.

Confocal imaging: Fluorescent images were acquired on an Olympus Fluo View FV1000 confocal laser-scanning microscope (Japan) with an objective lens (×40). The excitation wavelength was 635 nm. Cell imaging was carried out after being washed with RPMI-1640 for three times.

Cell culture: Mouse leukaemic monocyte macrophage cell line (RAW264.7) was obtained from the cell bank of the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS) at 37 ^{o}C in a humidified atmosphere containing 5% CO_2.

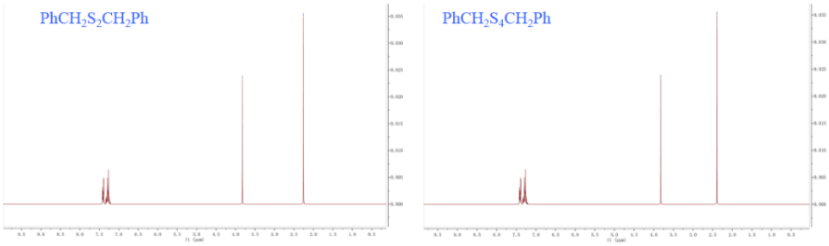
Serum treatment: BALB/c mice were anesthetized with xylazine and blood was drawn with a syringe from the inferior vena cava. Then the blood was prepared by centrifugation at 3000 rpm for 10 min, the supernatant is fresh serum. We prepared a solution containing 20% mouse fresh serum. Then BD-ss (10 μM) was added to the samples to evaluate the concentration of H_2S_n. The resultant fluorescence intensity multiplied by a factor 5 to obtain the final result (The concentration of H_2S_n in 100% mouse fresh serum).

Visualization of H_2S_n in Mice. BALB/c mice, 20-25 g, were obtained from Binzhou Medical University. Mice were group-housed on a 12:12 light–dark cycle at 22 °C with free access to food and water. Images were taken by Bruker In-vivo Imaging System.

2. Preparation of analytes

Stock solutions of cysteine (Cys), homocysteine (Hcy), glutathione (GSH), cystine (CysSSCys), GSSG, NaHS, $Na_2S_2O_3$, $NaHSO_3$, ascorbic acid, tocopherol were prepared in distilled water (2.5 mM, respectively). The stock solution of S_8 (200 mM) was prepared in CH_2Cl_2, and then used EtOH to dilute this solution, to get a 10 mM stock solution of S_8 in EtOH. The stock solutions were diluted to desired concentrations when needed. H_2O_2 was determined at 240 nm (ε= 43.6 $M^{-1}cm^{-1}$). Na_2S_2 and Cys-polysulfide was prepared using reported procedures.[1] A heterogeneous mixture of Na (25 mg-atom) and S_8 (25 mg-atom) in 10 mL anhydrous 1,2-dimethoxyethane under Ar atmosphere. The heterogeneous mixture could immediately turn dark brown at room temperature. However, the reaction need to be continued for 3h at 70 °C to give anhydrous Na_2S_2. Filtrated then washed by ether to yield a yellow solid 30 mg, 60%. Na_2S_4 was also synthesized with Na (25 mg-atom) and S_8 (50 mg-atom) via the same method (yield 15%). Then the products were derived by benzyl chloride to convert sodium oligosulfide into dibenzyl oligosulfane. The Na_2S_2 (Na_2S_4) derived by benzyl chloride to convert Na_2S_2 into $PhCH_2S_2CH_2Ph$ ($PhCH_2S_4CH_2Ph$). In order to confirm the purities of Na_2S_2 and Na_2S_4, we also purchased the Na_2S_2 and Na_2S_4 from Angene International Limited as standards. After derived the samples by benzyl chloride, the purities of our products were up to 99.91% by GC. Retention time of $PhCH_2S_2CH_2Ph$ and $PhCH_2S_4CH_2Ph$ were at 12.1 min and 12.8 min, respectively. $PhCH_2S_2CH_2Ph$ ^1H NMR (500 MHz, DMSO-D_6) δ(ppm): 7.43-7.37 (m, 4H), 7.33-7.20 (m, 6H), 3.81 (S, 4H). GC-MS (ESI^+): m/z $C_{14}H_{14}S_2$ calcd. 246.0537, found [M^+] 246.0535. $PhCH_2S_4CH_2Ph$ ^1H NMR (500 MHz, DMSO-D_6) δ(ppm): 7.42-7.35 (m, 4H), 7.33-7.21 (m, 6H), 3.82 (S, 4H). GC-MS (ESI^+): m/z $C_{14}H_{14}S_4$ calcd. 309.9978, found [M^+] 309.9978.

1 (a) T. Takata, K. Saeki, Y. Makita, N. Yamada and N. Kihara, *Inorg. Chem*. 2003, **42**, 3712; (b) N. Yamada, M. Furukawa, M. Nishi and T. Takata, *Chem. Lett*. 2002, 454; (c) K. Stensaas, A. Brownell, S. Ahuja, J. Harriss and S. Herman, *J. Sulfur Chem.* 2008, **29**, 433; (d) W. Chen, C. Liu, B. Peng, Y. Zhao, A. Pacheco and M. Xian. *Chem. Sci.* 2013, **4**, 2892.



3. The dynamic response of BD-ss to Na_2S_2

To confirm whether BD-ss (10 μM) could rapidly react with H_2S_n, time-dependent test had been carried out in the presence of Na_2S_2 (20 μM). As shown in Fig. S1, BD-ss exhibited almost no fluorescence in absence of Na_2S_2. However, a rapid increase of fluorescence intensity was obtained upon addition of Na_2S_2. After the reaction of Na_2S_2 for 10 s, fluorescence intensity reached saturation with a 34-fold turn-on emission. Therefore, BD-ss could provide a rapid response time to Na_2S_2. Taking the rapid metabolism of endogenous H_2S_n into account, the rapid response time to H_2S_n indicated that our probe had potential for real-time intracellular imaging.

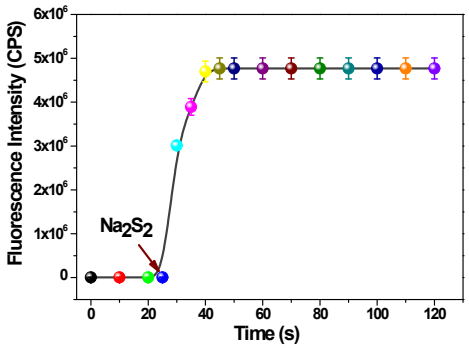


Fig. S1 Time-dependent fluorescence intensity changes of BD-ss (10 μM) towards Na_2S_2 (20 μM). Na_2S_2 was added at the reaction time of 25s in 10 mM HEPES buffer (pH 7.4, 0.4% Tween 80). The reactions were measured during 120 s at 37 °C. λ_{ex} = 707 nm, λ_{em} = 737 nm.

4. Effect of pH values

Standard fluorescence pH titrations were performed in HEPES buffer solution at a probe concentration of 10 μM. As shown in Fig. S2, the probe itself almost has no effect on the fluorescence by the pH of mediums within the range from 4.0 to 10.0. The fluorophore BODIPY could keep stable over pH range of 4.0-7.4. When the pH values were higher than 7.4, the fluorescence intensity decreased. These results indicate that the probe can work under physiological conditions.

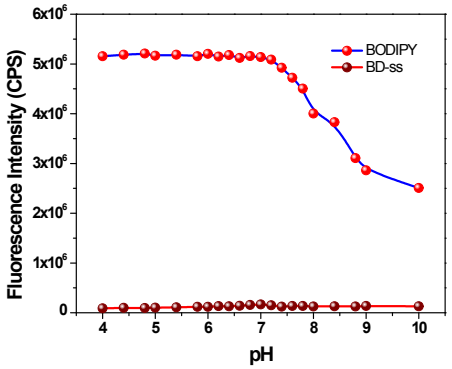


Fig. S2 The effects of pH value on the fluorescence intensity of probe (10 μM) in 10 mM HEPES. pH values: 4.0, 4.4, 4.8, 5.0, 5.4, 5.8, 6.0, 6.2, 6.4, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.4, 8.8, 9.0, 10.0.

5. Effect of Tween 80 to the fluorescence intensity

As is well known, the cell membrane is composed by phospholipids, glycolipids with other lipids, and protein. Micelles commonly play an important role in mimetic bio-membrane research. Therefore, in order to maximize the stimulation function of the cell, Tween 80 was used to form micelles, which was a common nonionic surfactant. Thus, we applied BD-ss to assess the effect of Tween 80 at different concentrations (0%, 0.1 %, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.8% and 1.0%) in HEPES (10 mM, pH 7.4). As shown in Fig. S3, a visibly increasing of fluorescence intensity was obtained upon addition of Tween 80 (from 0% to 0.4%). After that, the fluorescence intensity reached saturation and threshold. Therefore, in this work, 0.4% Tween 80 was used for the studies.

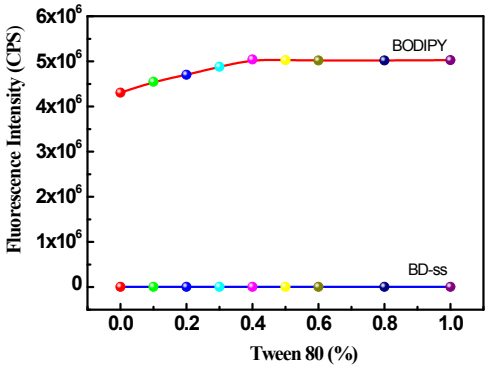


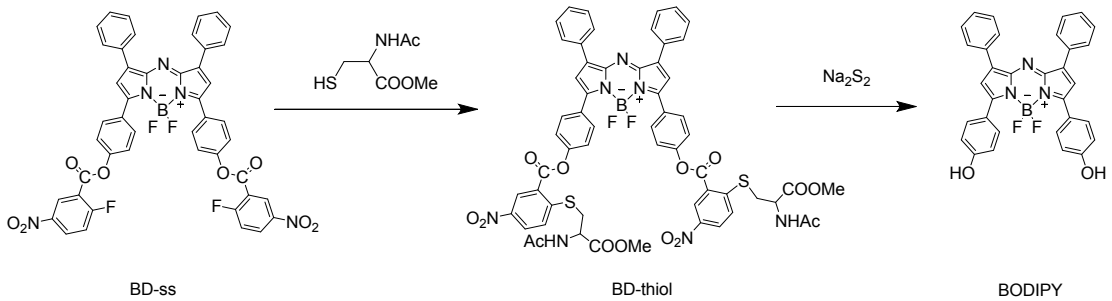
Fig. S3 The effect of Tween 80 concentrations on the fluorescence intensity of BD-ss (10 μM) in presence of Na_2S_2 (20 μM). The data was obtained in 10 mM HEPES (pH 7.4) at 37 °C for 35 min. λ_{ex} = 707 nm, λ_{em} = 737 nm.

6. Reaction of BD-ss with H_2S_n

A solution of BD-ss (0.1 mmol) in DMSO was treated with Na_2S_2 (0.2 mmol) in HEPES buffer (10 mM, pH = 7.4). The mixture was stirred for 10 min at room temperature. Then solvent was extracted by CH_2Cl_2 and washed the organic phase with H_2O, the organic layer evaporated to dryness. The resulted residue was purified by column chromatography. Compound BODIPY was obtained as a green solid. The formation of BODIPY was confirmed by HRMS (ESI^+). LC-MS (ESI^+): m/z $C_{32}H_{22}BF_2N_3O_2$ calcd. 529.1773, found $[M-H]^-$ 528.1695.

7. Reaction of BD-ss with N-Acetyl-L-cysteine methyl ester

Scheme S1. Model reaction of BD-ss with N-Acetyl-L-cysteine methyl ester



A solution of BD-ss (0.1 mmol) in acetonitrile was treated with N-Acetyl-L-cysteine methyl ester in HEPES buffer (10 mM, pH = 7.4). The mixture was stirred for 1 hour at room temperature. Then solvent was evaporated and partitioned between CH_2Cl_2 and H_2O, the organic layer evaporated to dryness. The resulted residue was purified by column chromatography. We obtained BD-thoil as a blue-green solid. The formation of BD-thoil was confirmed by HRMS (ESI^+). LC-MS (ESI^+): m/z $C_{58}H_{46}BF_2N_7O_{14}S_2$ calcd. 1177.2605, found $[M^+]$ 1177.2604.

Then the solution of BD-thoil in acetonitrile was treated with Na_2S_2 (0.2 mmol) in HEPES buffer (10 mM, pH = 7.4). The mixture was stirred for 10 min at room temperature. Then solvent was extracted by CH_2Cl_2 and washed the organic phase with H_2O, the organic layer evaporated to dryness. The resulted residue was purified by column chromatography. We obtained BODIPY as a green solid. The formation of BODIPY was confirmed by HRMS (ESI^+). LC-MS (ESI^+): m/z $C_{32}H_{22}BF_2N_3O_2$ calcd. 529.1773, found $[M-H]^-$ 528.1695.

8. Fluorescence response to H_2S_n at low concentration

We have performed an experiment for the fluorescence response of BD-ss to H_2S_n at low concentration. We have fitted the lines in the narrow range again. The fluorescence intensities at 737 nm were linearly related to the concentrations of Na_2S_2 under the given range (Fig. S4). The regression equation in solution was $F_{737\ nm} = 8.14\times10^4\ [Na_2S_2]\ \mu M + 1787$ with r = 0.9910. The regression equation in 20% fetal bovine serum was $F_{737\ nm} = 6.04 \times 10^4\ [Na_2S_2]\ \mu M + 2399$, with r = 0.9988. As shown in Fig. S4, our probe can respond to H_2S_n at low concentration (nM level).

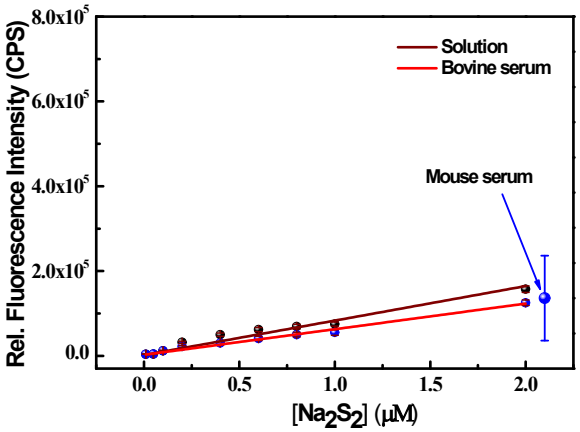


Fig. S4 Fluorescence response of BD-ss (10 μM) upon addition of Na_2S_2: 10 nM, 50 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM. Fluorescence intensities were acquired in 10 mM HEPES buffer (pH 7.4, 0.4% Tween 80) after incubation with various concentrations of Na_2S_2 for 5 min at 37 °C.

9. Effect of common physiological metal ions and anions

To verify whether there is fluorescence response to common metal ions and anions or not, BD-ss (10 μM) was treated with various analytes in HEPES buffer (10 mM, pH 7.4, 0.4% Tween 80). As shown in Fig. S5, the K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Cl^-, Br^-, SO_4^{2-}, CO_3^{2-}, and $H_2PO_4^-$ did not trigger any fluorescence enhancement.

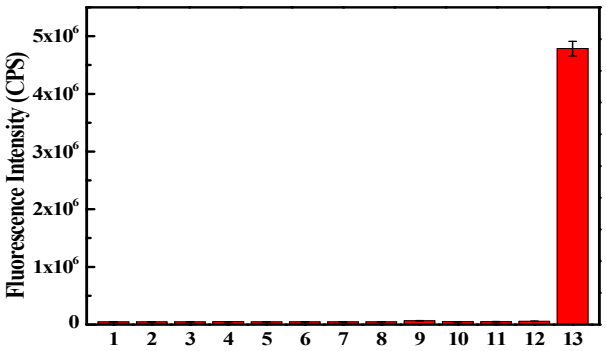


Fig. S5 Fluorescence responses of BD-ss to various metal ions and anions. Legend: (1) K^+ (1 mM), (2) Na^+ (1 mM), (3) Ca^{2+} (1 mM), (4) Mg^{2+} (1 mM), (5) Zn^{2+} (1 mM), (6) Cu^{2+} (1 mM), (7) Fe^{2+} (1 mM), (8) Cl^- (1 mM), (9) Br^- (1 mM), (10) SO_4^{2-} (1 mM), (11) CO_3^{2-} (1 mM), (12) $H_2PO_4^-$ (1 mM), (13) Na_2S_2 (20 μM). Data were recorded in 10 mM HEPES buffer (pH 7.4, 0.4% Tween 80) at 37 ℃ for 35 min. λ_{ex} = 707 nm, λ_{em} = 737 nm.

10. More images of Fig. 5

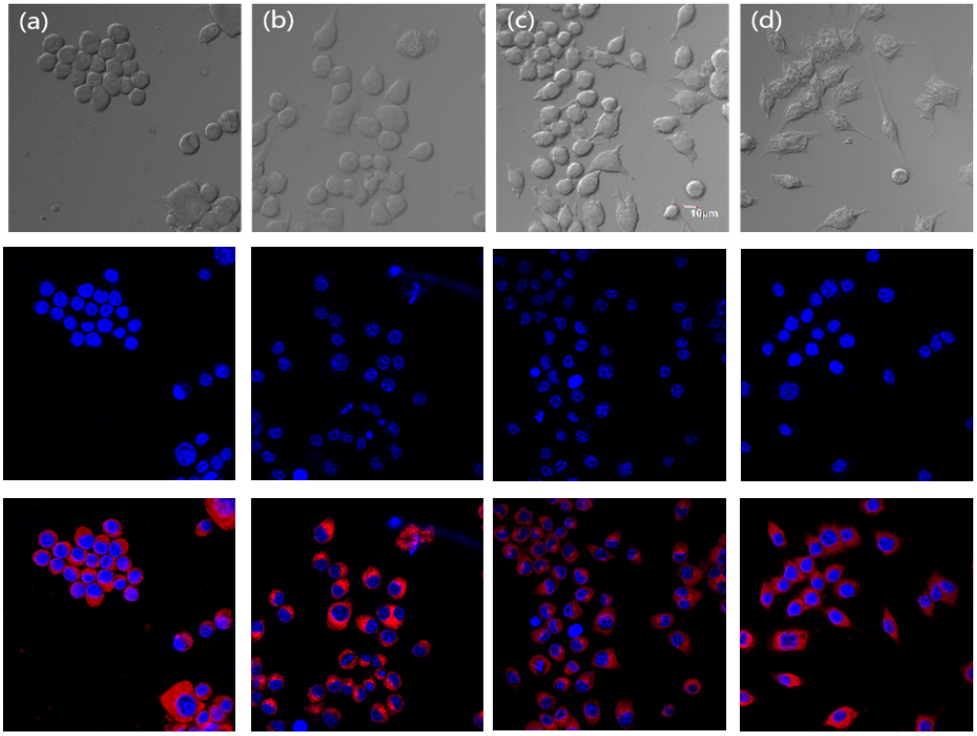


Fig. S6 Bright-field images of Fig. 5 (a) Brightfield image of Fig. 5a; (b) Brightfield image of Fig. 5f; (c) Brightfield image of Fig. 5k; (d) Brightfield image of Fig. 5p. The cells were incubated with Hoechst 33342 (a commercial nuclear dye) for 30 min.

11. MTT Assay

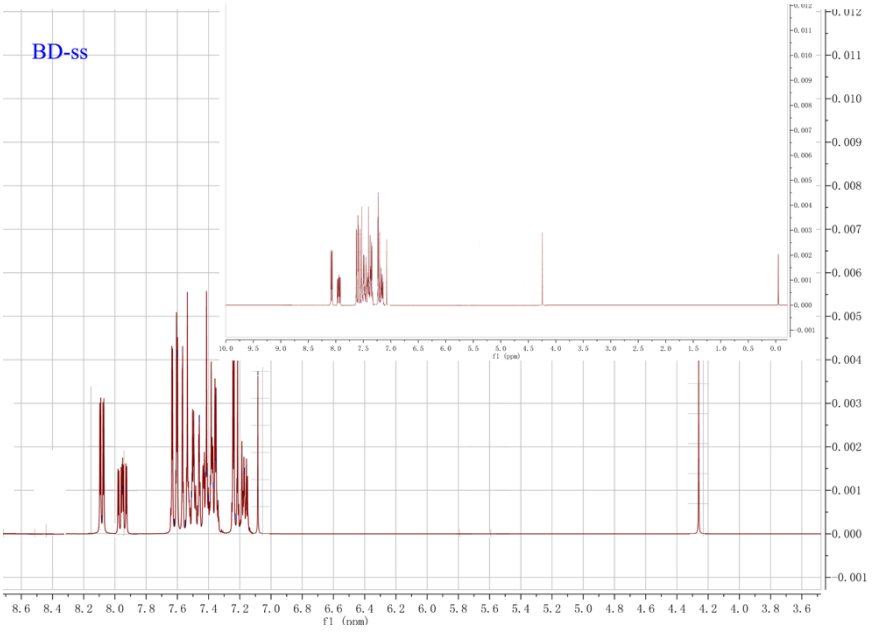
To evaluate the cytotoxicity of BD-ss, we performed an MTT assay on RAW 264.7 cells with

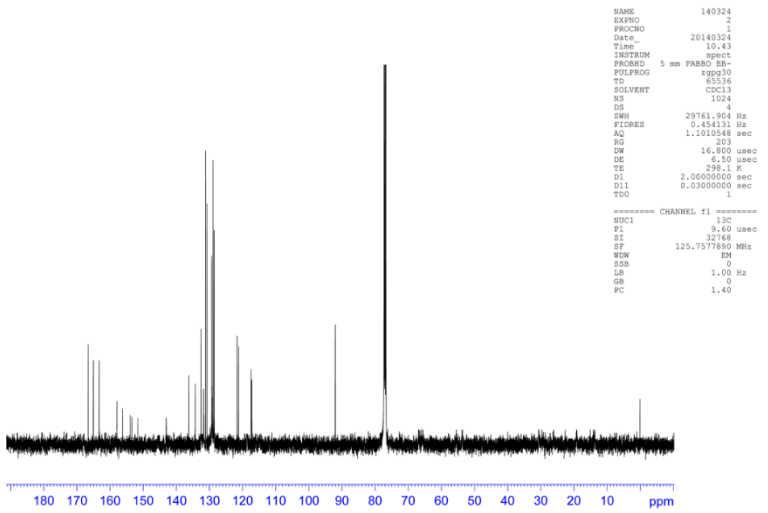
probe concentrations from 10^{-7} to 10^{-3} M. The result showed IC_{50} values of 233 μM for BD-ss, that is, BD-ss is of low toxicity towards cell cultures under experimental conditions.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay: Cytotoxicity in vitro was measured by using the methyl thiazolyl tetrazolium (MTT) assay in RAW264.7 cells. Cells were seeded into 96-well cell culture plate at 6000 /well, cultured at 37 °C and 5% CO_2 for 48 h, and then different concentrations of probe BD-ss (0, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M) were added to the wells. For MTT experiments, solutions of BD-ss were prepared in CH_3CN (10 mM and 1 mM). The final volume of cell culture medium was 600 μL. Accordingly, the amounts of solvent added in the absence of probe were correspondingly 0, 60 μL, 6 μL (the above used 10 mM solution), 6 μL, 0.6 μL, 0.06 μL (the last three used 1 mM solution). To verify whether 10% methanol might cause significant cell loss or not, 60 μL methanol was added into the medium. The cells were then incubated for 48 h at 37 °C under 5% CO_2. Subsequently, 20 μL MTT (5 mg/mL) was added to each well and incubated for an additional 4 h at 37 °C under 5% CO_2. Cells were lysed in triple liquid (10% SDS, 0.012 M HCl, 5% isopropanol), and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (Tecan, Austria). Calculation of IC_{50} values was done according to Huber and Koella.[4] The following formula was used to calculate the inhibition of cell growth: Cell viability (%) = (mean of Abs. value of treatment group / mean Abs. value of control) • 100%.

4. W. Huber and J. Koella, *Acta Trop.* 1993, **55**, 257.

12. ^{1}H NMR, ^{13}C NMR and MS of BD-ss

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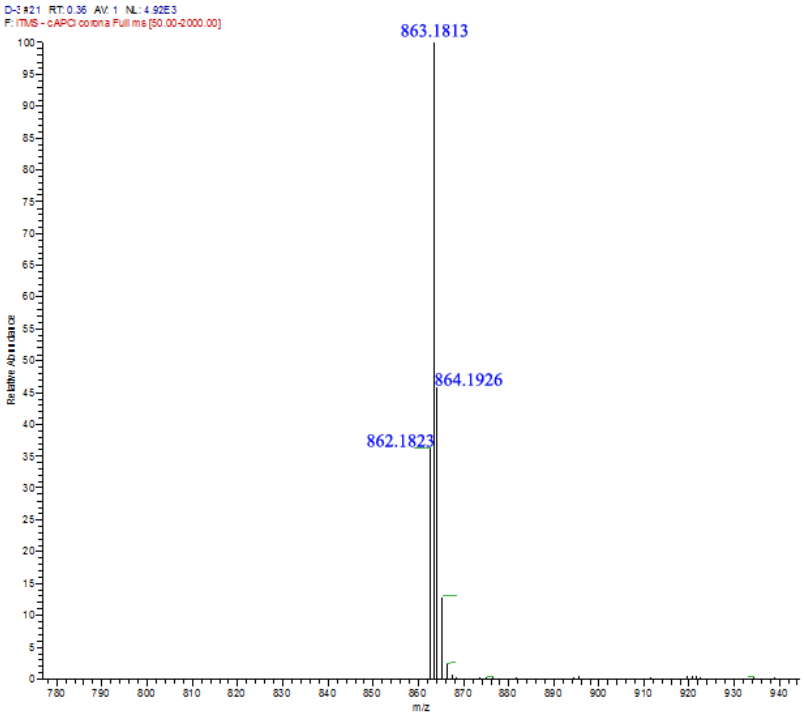
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