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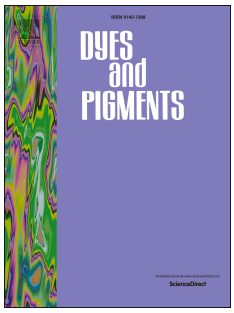

A high signal-to-background ratio H_2S-specific fluorescent probe based on nucleophilic substitution and its bioimaging for generation H_2S induced by Ca^{2+} in *vivo*

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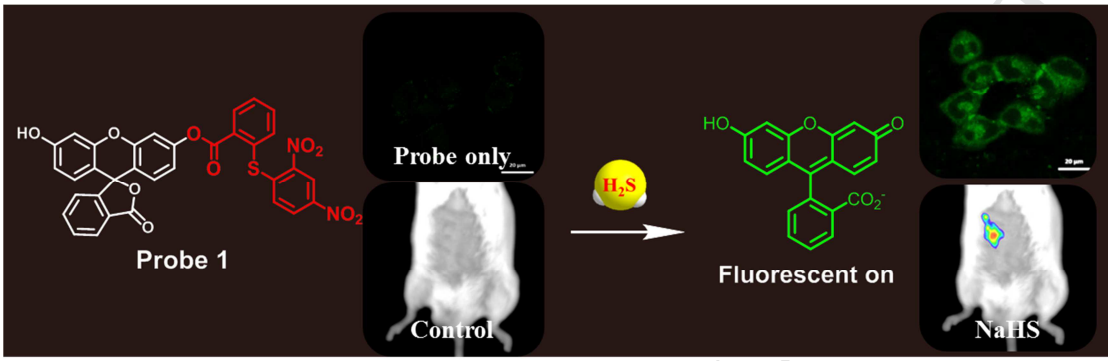
Highlights

1. This probe exhibit high signal-to-background ratio in response to H_2S.
2. This probe was used to detect endogenous generation H_2S induced by Ca^{2+} mediated cystathionine γ-lyase (CSE).
3. This probe was successfully used to detect exogenous hydrogen sulfide in mice.

Abstract Graphic

The statement:



A novel fluorescence H_2S probe based on nucleophilic substitution reactions, which with high signal-to-background ratios. This probe was successfully used to imaging ex-/endogenous H_2S in living cells, and exogenous H_2S in mice.

A high signal-to-background ratio H_2S-specific fluorescent probe based on nucleophilic substitution and its bioimaging for generation H_2S induced by Ca^{2+} in *vivo*

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Abstract Hydrogen sulfide (H_2S), a new endothelium-derived relaxing factor (*EDRF*), which plays vital roles in regulating intracellular redoxstatus and other fundamental signaling processes involved in human health and disease. In this work, we designed a fluorescent probe for H_2S successive nucleophilic reaction with high signal-to-background (S/B) ratio. In the probe, we utilized 2-mercaptobenzoic acid as the distinguish reaction site and introduced 2,4-dinitrophenyl to provide appropriate steric hindrance to realize specific response on well-structured H_2S. Other substances sulfur-containing do not disturb the detection of H_2S. The max emission with 197-fold enhancement exhibits high signal-to-background ratio. The detection limit was calculated to be 64 nM. Imaging assays in living cells showed that the probe could penetrate cells membrane easily and allowed to detect endogenous generation H_2S induced by Ca^{2+} mediated cystathionine γ-lyase (CSE). Moreover, the probe was successfully used to detect exogenous H_2S in mice.

Keywords: H_2S-specific; Two nucleophilic; Ca^{2+} mediated; Vivo

1. Intruduction

H_2S is extensively exist in a wide variety of industries such as oil exploitation, natural gas well, tunnel and sewage disposal [1-4]. Exposure to high level H_2S atmosphere may cause respiratory symptoms, cardiovascular abnormalities and neurological disorders [5-7]. H_2S was found as the third multifunctional gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO) since last decades [8-10]. H_2S influences a wide range of physiological and pathophysiological processes, including its ability to act as a neurotransmitter [11], regulator of blood pressure [12], immunomodulator [13] or anti-apoptotic agent [14], together with its great pharmacological potential, such as cardioprotection [15], endogenous stimulation of angiogenesis [16] and mitochondrial bioenergetics [17]. However, abnormally H_2S concentrations have been significant associated with alzheimer's disease, down's syndrome, diabetes and liver cirrhosis [18-21]. Physiological effects of H_2S are concentration-dependent, thus, in order to benefit from H_2S, the concentration of H_2S must be maintained at the appropriate level. In mammals, endogenous H_2S is synthesized naturally by several enzymes, including cystathionine γ-lyase (CSE), cystathionine β-synthetase (CBS) and 3-mercaptopyruvate sulfurtransferase (MST)/cysteine aminotransferase (CAT) [22-25]. Yang et al. reported that the activity of cystathionine γ-lyase (CSE), an important enzyme responsible for H_2S physiological generation in animals, could be activated by calcium-calmodulin pathway [26]. However, the mechanism of Ca^{2+} induced intracellular H_2S production in living cells has not been elucidated. Therefore, developing selective and sensitive

detection tools for H_2S in complex biological systems is important.

Traditional methods of H_2S detection, including methylene blue method [27], lead acetate [28], electrochemical sensors [29], gas chromatography [30] and monobromobimane derivatization are often limited by poor compatibility with biosystem, limited temporal resolution or rigorous preparation requirements [31-32]. In contrast, fluorescent probes with highly sensitive, selective, nondestructive are suitable for detection of this volatile, gaseous molecule with readily available instruments [33-36]. In the past several years, a number of fluorescent probes for H_2S have been reported [37-41]. However, probes with high signal-to-background (S/B) ratio are rare. In bio-imaging, the probe with high S/B ratio is benefit for obtaining more accurate and reliable signals. There are two ways to improve the probe's S/B ratio: (1) Increase extent of fluorescence enhancement; (2) Improve selectivity. Moreover, the application of fluorescent probes to the H_2S signaling pathway is still rare.

The 2,4-dinitrophenyl (DNP) ether moiety has been employed extensively as a protecting group for tyrosines in peptide synthesis [42]. Direct assemble the DNP group onto various kinds of fluorophores have yielded a series H_2S probes [43-47]. Indeed, biothiols can also remove DNP group, the selectivity of these probes are unsatisfactory [48-49]. In this work, we employed the DNP derivative 2-((2,4-dinitrophenyl)thio)benzoic acid as the masking group link to the fluorescein and obtained the probe (**Probe 1**) (Scheme 1). The probe exhibits high selectivity and significant fluorescence enhancement up to 197-fold upon the detection of H_2S.

Furthermore, Ca^{2+} induced H_2S production in Hela cells was directly visual demonstration use the probe.

2. Preparation and characterization of compounds

*2.1. Preparation of **Probe 1**.*

The **Probe 1** was synthesized from the compound 1 via an ester moiety link to fluorescein (Scheme 1). The compound 1 was synthesized use the same methods as previous literature [50].

<Inserted Scheme 1>

Compound 1 (1.0 mmol, 0.32 g) and fluorescein (1.0 mmol, 0.33 g) were dissolved into 25 mL dichloromethane, added N-(3-dimethylaminopropyl)-N-ethyl carbodi imide hydrochloride (EDC) (1.0 mmol, 0.19 g) and 4-dimethylaminopyridine (DMAP) (0.10 mmol, 0.01 g). Surrounded by Ar, the reactant stired overnight (r.t.). Then solvent was evaporated and resulted residue was subjected to column chromatography. Probe was obtained as a yellow powder (0.19 g, yield: 30 %).

*2.2. Characterization of **Probe 1***

^{1}H NMR (600 MHz, DMSO-d_6) δ 8.88 (d, J = 2.5 Hz, 1H), 8.34 (t, J = 8.0 Hz, 3H), 8.05 (d, J = 7.6 Hz, 1H), 7.85 (d, J = 5.7 Hz, 4H), 7.80 (t, J = 7.5 Hz, 1H), 7.75 (t, J = 7.3 Hz, 1H), 7.37-7.34 (m, 2H), 7.15 (d, J = 9.0 Hz, 2H), 6.96 (d, J = 8.7 Hz, 2H), 6.91 (d, J = 8.7 Hz, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 163.4, 151.5, 150.6, 144.9, 144.4, 144.3, 137.5, 134.4, 133.5, 132.1, 131.3, 130.2, 129.9, 129.3, 127.6, 124.9, 123.9, 121.0, 118.3, 116.5, 110.3. ESI-MS m/z: [probe + H]$^+$ Calcd for 635.07604, Found 635.07525; [probe + Na]$^+$ Calcd for 657.05804, Found 657.05683.

3. Results and Discussion

*3.1 Specta properties of **Probe 1** response to H_2S.*

Firstly, we evaluated the UV-vis spectra properties of **Probe 1**. As shown in Fig. 1, in 2 mL DMSO : PBS (v/v, 7 : 3, pH = 7.4) solution of **Probe 1** (25 μM), gradually added HS^- (0-300 μM), the absorbance at 338 nm increased with concomitant appearance of absorbance at 475 nm in a short time frame (10 min). Similarly, we also studied the reaction beween **Probe 1** and biothiols (Cys, Hcy and GSH) in UV-vis spectrometer (Fig. S6 a, b, c). Under the same conditions, the addition of Cys (500 μM) caused a neglectable absorbance enhancement at 338 nm, and Hcy (500 μM)/GSH (500 μM) couldn't cause obvious changes in UV-vis spectra.

<Inserted Figure 1>

The **Probe 1** is considered to be a high S/B ratio probe. On the one hand, high S/B ratio probe depend on the extent of fluorescence enhancement. As predicted, the incremental addition of HS^- (0-70 μM) to the solution DMSO : PBS (v/v, 7 : 3, pH = 7.4) of **Probe 1** (5 μM) resulted in a dramatic enhancement of the emitting band centered at 538 nm in 10 min (Fig. 2a). Finally, the max emission has 197-fold enhancement. On the other hand, the high selectivity is very important condition for high S/B ratio probe. The **Probe 1** was treated with HS^- and various relevant analyte (100 eq.) in DMSO : PBS (v/v, 7 : 3, pH = 7.4). As shown in Fig. 2b, the **Probe 1** was highly selective for HS^- versus biological relevant thiols or HSO_3^-. These results demonstrate the excellent selectivity of the probe to H_2S. These above experiment results, the emission of **Probe 1** has a large enhancement (197-fold) and the high

selective of the **Probe 1** fulfilled the requirements of high S/B ratio probe. Hence, the probe is a H_2S-specific fluorescent probe with high S/B ratio.

<Inserted Figure 2>

3.2 *Working curve and time dependents*

Furthermore, a plot of fluorescent intensities at 538 nm versus the concentrations of H_2S showed a good linearity (R^2 = 0.991): $F-F_0 = 94.24c - 293.60$ (Fig. 3a). With the definition: Detection limit = $3\sigma/k$, the detection limit evaluated as 64 nM. In order to evaluate the response speed of **Probe 1** towards H_2S, we examined the reactivity of probe (5 μM) towards the HS^- (10 eq.) through time-dependent fluorescence spectroscopy in DMSO : PBS (v/v, 7 : 3, pH = 7.4). As the Fig. 3b depicted, the reaction could be completed within 10 min.

<Inserted Figure 3>

3.3. Proposed mechanism

The proposed mechanism of **Probe 1** was depicted in the Scheme 2. The H_2S nucleophilic substitution the carbonyl group of **Probe 1**, result in the protect group cleaved and released the fluorescein and compound 2 [51]. Furthermore, the mechanism was proved by the ESI-MS spectra analysis (Fig. S9). ESI-MS of a solution mixture of **Probe 1** and NaHS exhibited m/z peaks at 331.0611, 198.9816 in accordance with the fluorescein and compound 2 respectively.

<Inserted Scheme 2>

3.4 Cytotoxicity experiments

HepG2 cells were cultured in 96-well plates. The cell number was determined and a

serial dilution on **Probe 1** was performed in culture medium (without serum), incubated for 5 or 10 h. Subsequently, CCK-8 (10 % in serum free culture medium) was added to each well, which was washed with PBS two times and the plate was incubated for another 1 h. Then measure optical densities at 450 nm. Cytotoxicity assay demonstrating the **Probe 1** was benign to cells and has the potential to be used in biological applications (Fig. S8).

3.5. Imaging of living cells

The HepG2 cells incubated with **Probe 1** (5 μM) only for 20 min showed no fluorescence in the green channel (Fig. 4 a1). In contrast, treatment of **Probe 1**-loaded cells with 20 μM NaHS for 20 min triggered an obvious increased in green fluorescence signal (Fig. 4 b1).

<Inserted Figure 4>

Encouraged by its good sensing performances for H_2S in vitro, we applied **Probe 1** to image the endogenously generated H_2S by exogenous compound stimulation. S-Nitroso-N-acetyl-DL-penicillamine (SNP) was used to stimulate the generation of endogenous H_2S in HepG2 cells. As we expected, after incubation with 10 μM SNP for 1 h, the fluorescence intensities from the green channel clearly increased, which are similar to that of the addition of exogenous NaHS (Fig. 5 d1),. These results indicated that **Probe 1** capable of visualization of endogenous H_2S generation in HepG2 cells. These cell experiments showed the **Probe 1** can thus be used to imaging ex/endogenous H_2S in living cells.

<Inserted Figure 5>

3.6. Imaging the Ca^{2+}-dependent H_2S

Ca^{2+} is the second messenger and involved in a variety of intracellular signaling pathways. According to reported, that calcium-calmodulin regulating the activity of cystathionine γ-lyase (CSE) responsible for H_2S physiological generation in animals. To demonstrate the mechanism of Ca^{2+} induced intracellular H_2S production in cells, we conducted imaging experiments with **Probe 1** in Hela cells under conditions of both negative and positive controls. No fluorescent signal was observed in green channel when the cells were pre-incubated with DL-propargylglycine (PAG, which suppresses CSE) along with **Probe 1** and Ca^{2+}. In contrast, obvious fluorescent was observed when the cells were pre-incubated with Ca^{2+} and **Probe 1**, or cells pre-incubated with aminooxyacetic acid (AOAA, a potent inhibitor of CBS) along with **Probe 1** and Ca^{2+}. This indicated that CSE contributes to the observed H_2S generation upon Ca^{2+} stimulation.

<Inserted Figure 6>

3.7. Imaging of living mice

Furthermore, we applied **Probe 1** imaging in mice. The Kunming mice were fed commercial mouse chow in individual cages and left to freely wander in their housing for two weeks with 12 h dark/light cycles for acclimatization before the experiment. A living animal imaging system is used in imaging the mice. After all the preparatory work was completed, the mice were under anesthesia with 100 mL pentobarbital intraperitoneal injection. As Fig. 7 shows, the mice were injected with 50 mM of

Probe 1 in the abdomen, HS^- was carefully injected into the same location. Then, the fluorescence images were recorded at different periods of time (0, 5, 10, 15 min). These results displayed that **Probe 1** could visualize the endogenous H_2S in vivo.

<Inserted Figure 7>

4. Conclusions

In conclusion, we prepared a fluorescein-based H_2S-specific probe **Probe 1** with high S/B ratio. The results of UV-vis spectrum and fluorescence emission spectrum studies showed that **Probe 1** had good selectivity for H_2S and having a 64 nM detection limit for H_2S. Kinetics studies showed that the response can be completed in 10 min. Furthermore, this probe was used to directly image Ca^{2+}-dependent H_2S production, and in vivo imaging.

5. Acknowledgments

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Figure captions

Scheme 1 Synthesis of **Probe 1**.

Figure 1 UV-vis responses of **Probe 1** (25 μM) in DMSO : PBS (v/v, 7 : 3, pH = 7.4) solution, added HS^- (0-300 μM).

Figure 2 (a) fluorescent responses of **Probe 1** (5 μM) toward HS^-; Inset: Visual fluorescence change photograph for **Probe 1** only and upon addition of NaHS under illumination with a 365 nm UV lamp; (b) the selective of **Probe 1** (5 μM) towards HS^- and various relevant analyte (100 eq.), λ_{ex} = 512 nm, slit: 5 nm/5 nm.

Figure 3 (a) The working curve of **Probe 1** (5 μM) in the presence of various concentrations of HS^-(0-70 μM); (b) Time dependent of **Probe 1** (5 μM) to HS^- (10 eq.). λ_{ex} = 512 nm, slit: 5 nm/5 nm.

Scheme 2 The mechanism of the **Probe 1** responsing to HS^-.

Figure 4 Confocal fluorescence imaging the H_2S in HepG2 cells of **Probe 1** 20 min: (a1-a3) Incubated with the **Probe 1** only: (a1) green channel; (a2) brightfield image; (a3) Overlay. (b1-b3) Incubated with the **Probe 1** 20 min, then treat with HS^- 20 min: (b1) green channel; (b2) brighfield image; (b3) Overlay. Excitation at 488 nm, the green channel was set at 545 ± 15 nm scale bar = 20 μm.

Figure 5. Confocal fluorescence imaging the endogenously generated H_2S in HepG2 cells of **Probe 1**: (c1-c3) Incubated with the **Probe 1** only: (c1) green channel; (c2) brightfield image; (c3) Overlay. (d1-d3) Incubated with the SNP for 1 h, and then incubated with **Probe 1** for 20 min: (d1) green channel; (d2) brighfield image; (d3) Overlay. Excitation at 488 nm, the green channel was set at 545 ± 15 nm, scale bar =

20 μm.

Figure 6. CLSM images of Ca^{2+} regulated H_2S production pathways. (e1-e3) Incubated with the **Probe 1** only: (e1) green channel; (e2) brightfield image; (e3) Overlay. (f1-f3) Incubated with $CaCl_2$ (200 μM, 1 h), then incubated with **Probe 1** 20 min: (f1) green channel; (f2) brighfield image; (f3) Overlay. (g1-g3) pre-incubated with AOAA (100 μM, 3 h), incubated with $CaCl_2$ (200 μM, 1 h), then incubated with **Probe 1** 20 min: (g1) green channel; (g2) brighfield image; (g3) Overlay. (h1-h3) pre-incubated with PAG (100 μM, 3 h), incubated with $CaCl_2$ (200 μM, 1 h), then incubated with **Probe 1** 20 min: (h1) green channel; (h2) brighfield image; (h3) Overlay. Excitation at 488 nm, the green channel was set at 545 ± 15 nm scale bar = 20 μm.

Figure 7. The *in vivo* imaging of H_2S in a nude mice model of **Probe 1** (20 μM). (a) Control group; (b, c, d) First injected 40 μM **Probe 1** solution, then injected 50 μM HS^- solution for 5, 10, 15 min. Excitation at 475 nm, the green channel was collected at 520±5 nm.

Scheme 1

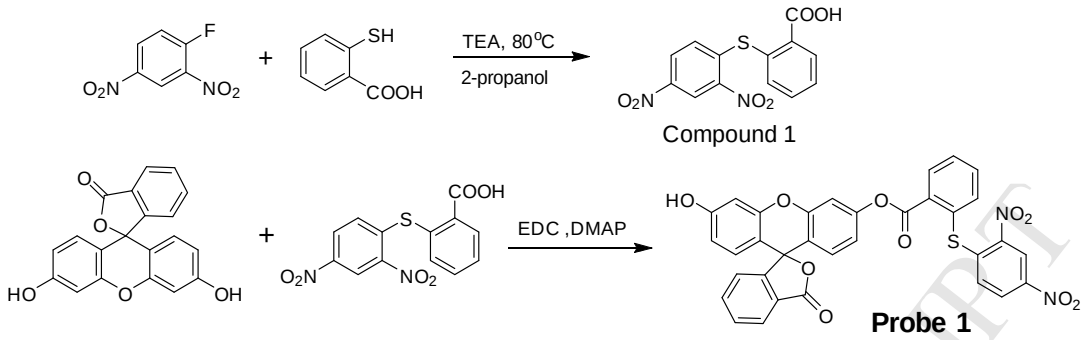


Figure 1

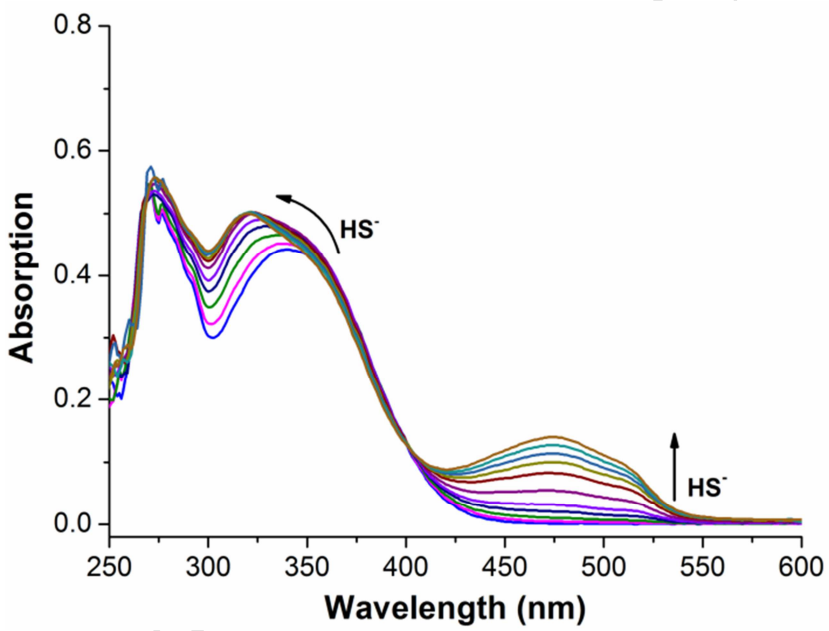


Figure 2

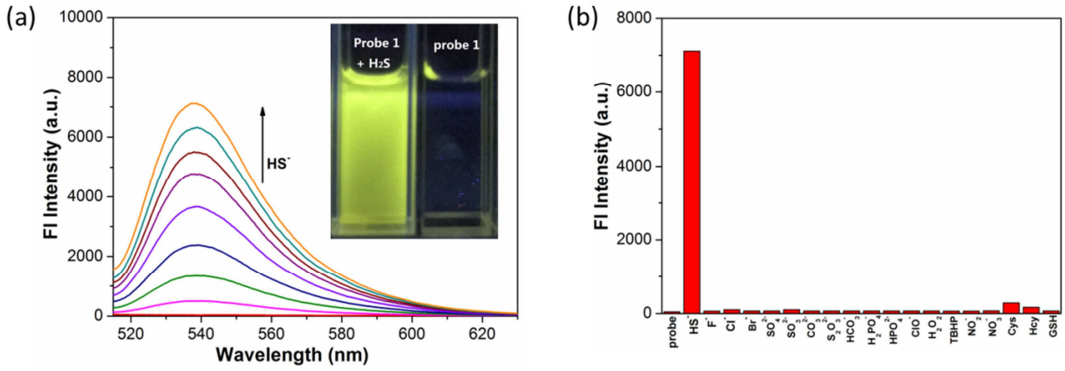
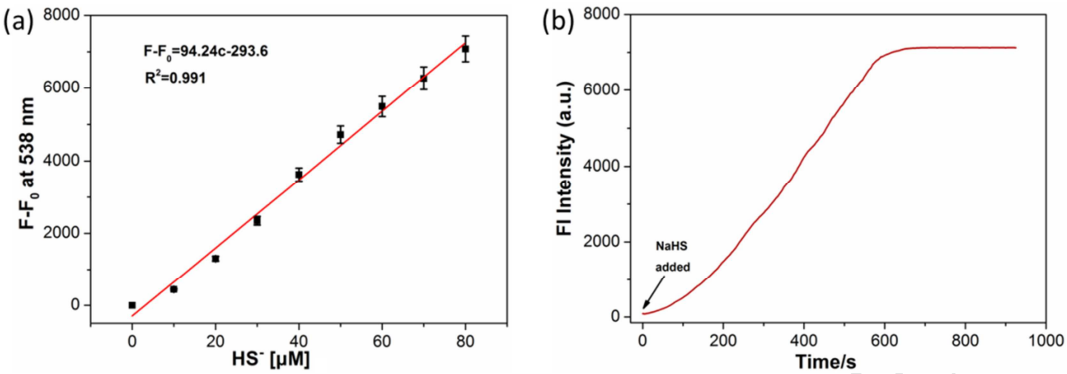


Figure 3



Scheme 2

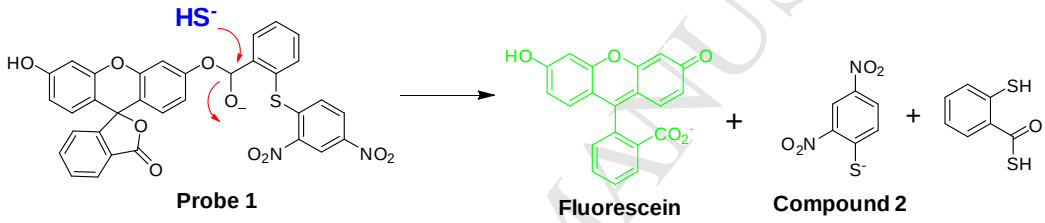


Figure 4

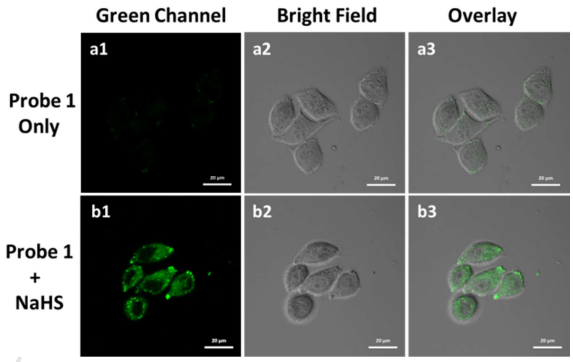


Figure 5

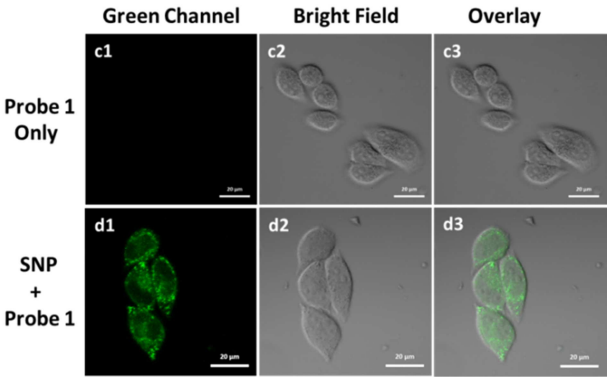


Figure 6

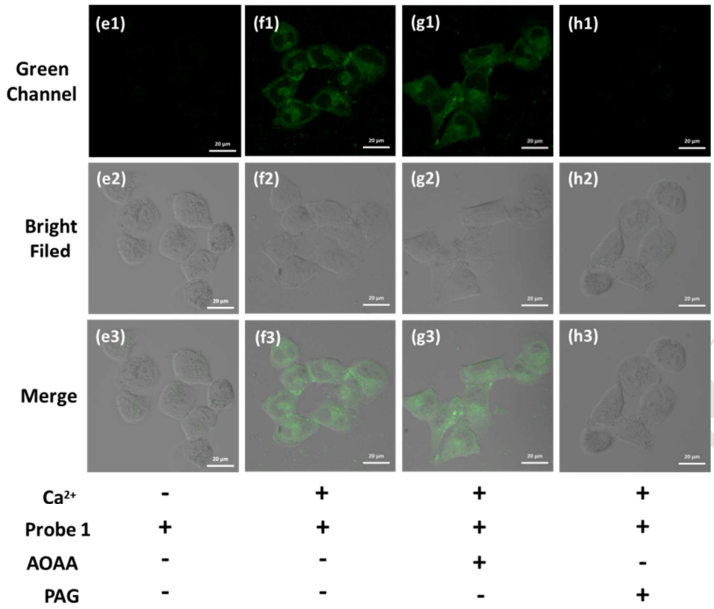
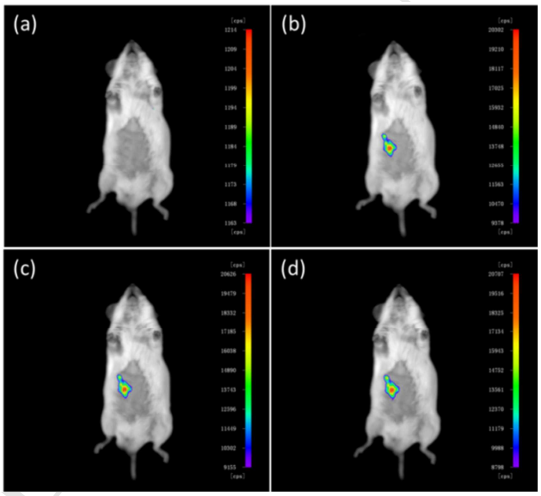


Figure 7



Supporting Information

Table S1: Compare of reported fluorescent hydrogen sulfide probes in recent years.

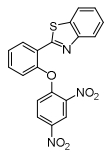
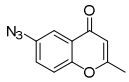
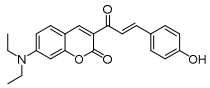
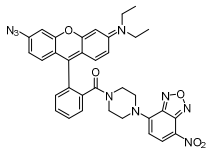
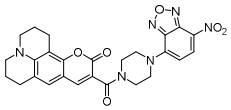
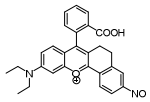
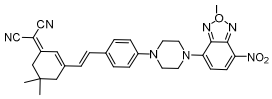
I Material and Methods

II (Fig.S1-S5): Copies of NMR and ESI-MS of related compounds.

III(Fig. S6-S9): Spectroscopic studies.

Table S1:

Properties of the reported fluorescent hydrogen sulfide probes in recent years.

probes	S/B ratio	detection limit	response time	Ref.
	60	10.5 nM	2.5 min	[1]
	--	18 nM	30 min	[2]
	--	50 nM	<1 min	[3]
	40	120 nM	15 min	[4]
	200	90 nM	15 min	[5]
	--	29 nM	5 min	[6]
	--	30 nM	60 min	[7]
This work (probe 1)	197	64 nM	10 min	

[1] Lin L, Zeng XQ, Shen YN, Zhu HL, Qian Y. An ultrasensitive fluorescent probe for rapid determination of thiophenols. Talanta 2017;165:321-325.

[2] Qiao Z, Zhang HY, Wang KW, Zhang YR. A highly sensitive and responsive fluorescent probe based on 6-azidechroman dye for detection and imaging of hydrogen sulfde in cells. Talanta 2019;195:850-856.

[3] Yang Y, Feng Y, Jiang Y, Qiu FZ, Wang YZ, Song XR, Tang XL, Zhang GL, Liu WS. A coumarin-based colorimetric fluorescent probe for rapid response and highly sensitive detection of

hydrogen sulfde in living cells. Talanta 2019;197:122-129.

[4] Ma C, Wei C, Li XY, Zheng XY, Chen B, Wang M, Zhang PZ, Li XL. A mitochondria-targeted dual-reactable fluorescent probe for fast detection of H2S in living cells. Dyes and Pigments 2019; 162:624-631.

[5] Ismail I, Wang DW, Wang ZH, Wang D, Zhang CY, Yi L, Xi Z. A julolidine-fused coumarin-NBD dyad for highly selective and sensitive detection of H_2S in biological samples. Dyes and Pigments 2019;163:700-706.

[6] Chen HH, Gong XY, Liu XW, Li Z, Zhang JJ, Yang XF. A nitroso-based fluorogenic probe for rapid detection of hydrogen sulfide in living cells. Sensors Actuators: B. Chemical 2019;281:542-548.

[7] Huang XT, Liu HY, Zhang JW, Xiao BR, Wu FX, Zhang YY, Tan Y, Jiang YY. A novel near-infrared fluorescent hydrogen sulfide probe for live cell and tissue imaging. New J. Chem., 2019;43:6848-6855.

I. Materials and apparatus.

Reagents with analytical grades were purchased from commercial and used without further purification.

UV-vis spectra were taken on a HITACHI U-3900 spectrophotometer and fluorescence spectra were recorded using a HITACHI F-7000 spectrophotometer. ^{1}H NMR and ^{13}C NMR experiments were measured by a Bruker AVANCE-600 MHz spectrometer (Bruker, Billerica, MA). Electrospray ionization (ESI) mass spectra were

acquired using an AB Triple TOF 5600plus System (AB SCIEX, Framingham, USA). The cell imaging experiments used Zeiss LSM880 CLSM.

Imaging Experiments

HepG2 cells and HeLa cells were cultured in dulbecco's modified eagle's medium (DMEM, Gibco) in an atmosphere of 5% CO_2 and 95% air at 37 °C. Before the CLSM imaging, the cells were plated on 14 mm glass coverslips and were incubated overnight. The cells were washed with PBS and then incubated with **Probe 1** in DMSO/PBS (0.5 %, v/v) for 3 h at 37 °C. After washing three times, the cells were subjected to CLSM imaging.

Imaging procedures were conducted with adult nude mice under general anesthesia by injection of sodium pentobarbital (0.5 mL/0.03%). Images were taken using an excitation of 475 nm and emission was collected 520±5 nm.

Ⅱ: Copies of NMR and ESI-MS of related compounds

Fig. S1

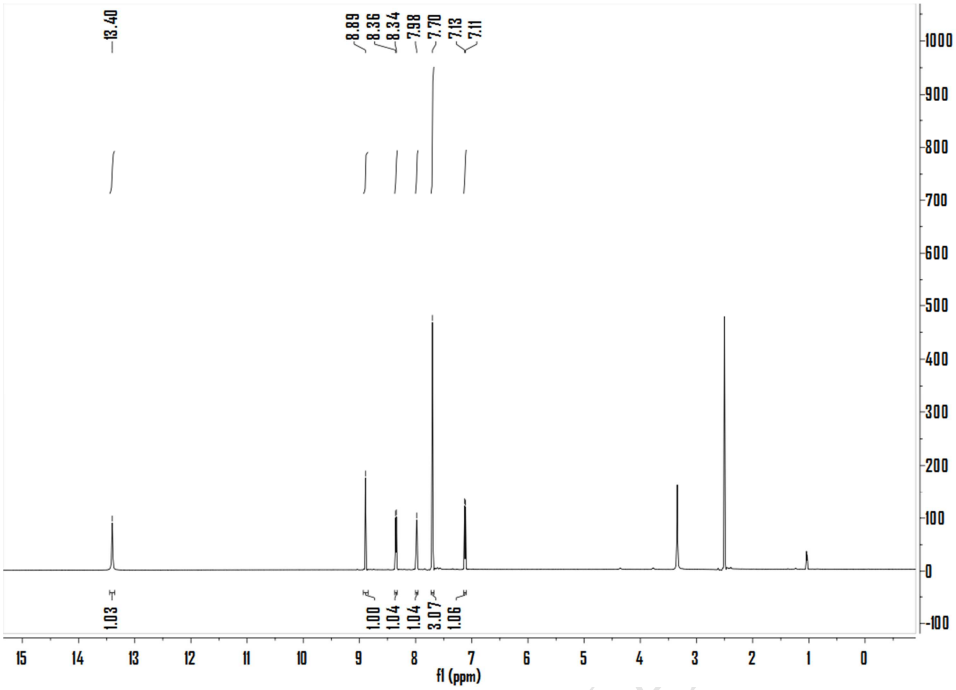


Figure S1. The ^{1}H NMR (600 MHz) spectra of the compound 1 in DMSO-d_6.

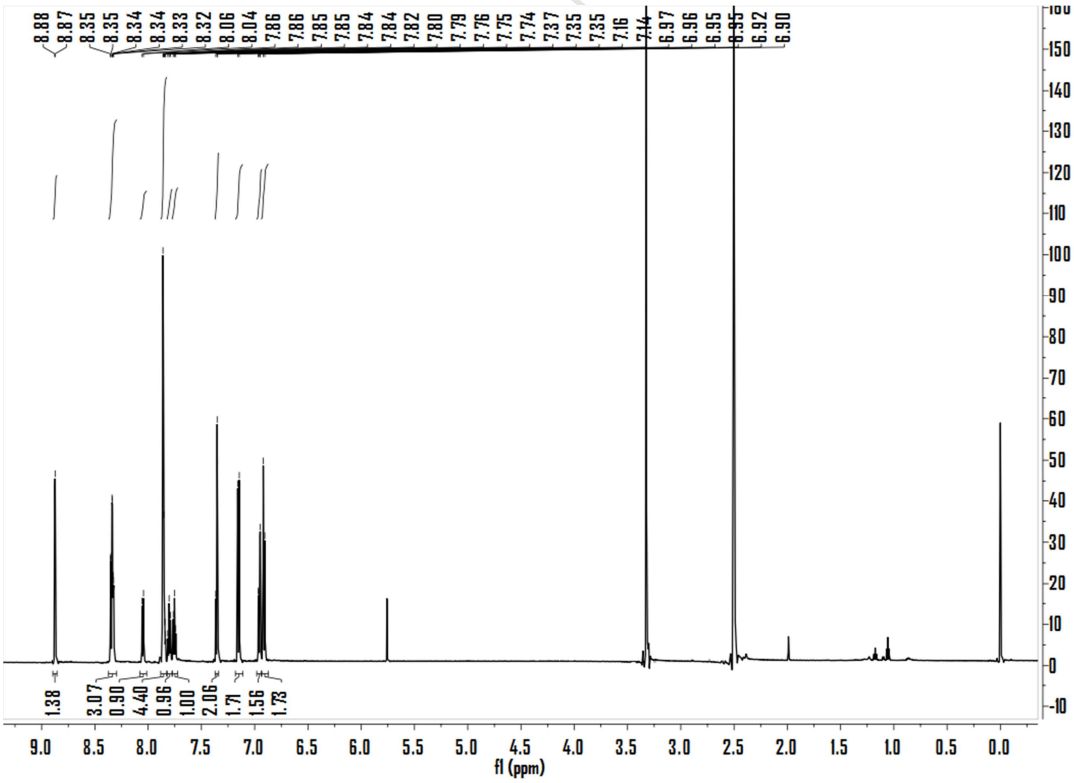


Figure S2. The ^{1}H NMR (600 MHz) spectra of the **Probe 1** in DMSO-d_6.

Fig. S3

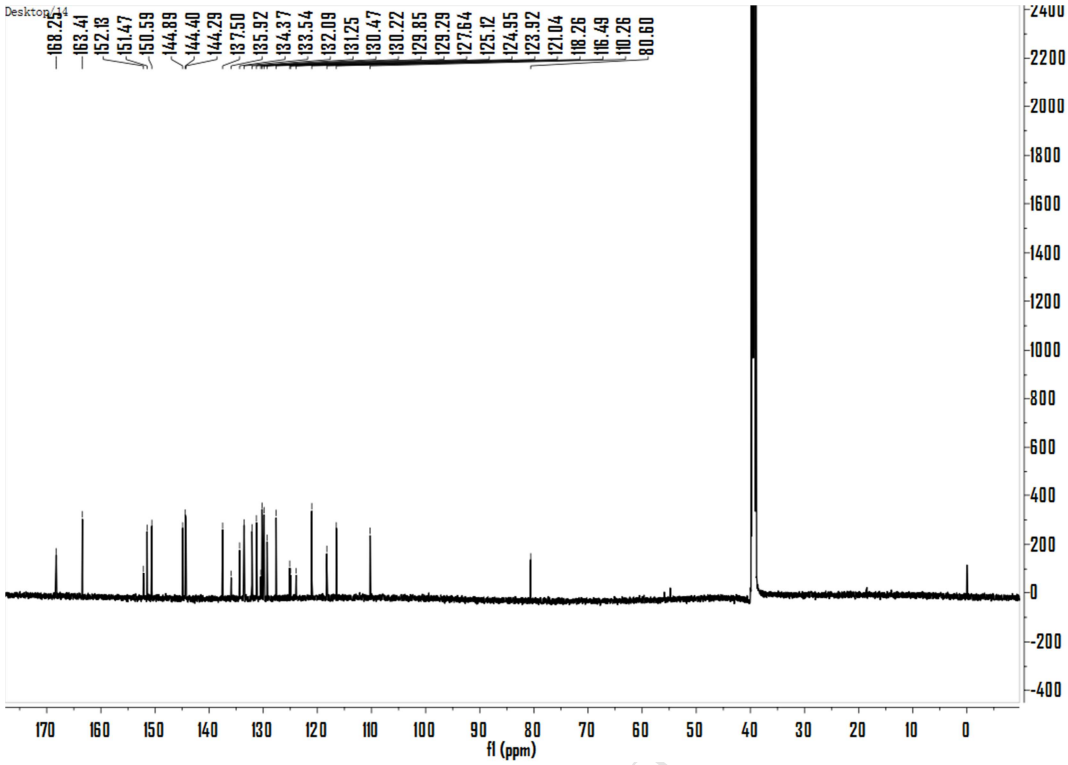


Figure S3. The ^{13}C NMR (150 MHz) spectra of the **Probe 1** in DMSO-d_6.

Fig. S4

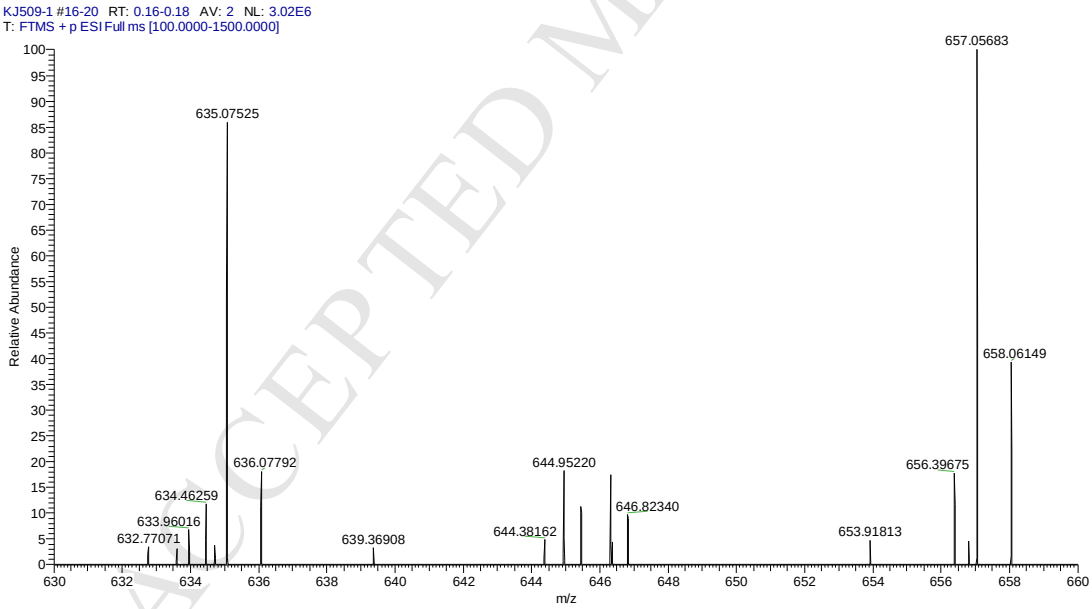


Figure S4: ESI-MS of the **Probe 1**: [**Probe 1** + H]+ Calcd for 635.076, Found 635.076; [probe 1 + Na]+ Calcd for 657.058, Found 657.057.

Fig. S5

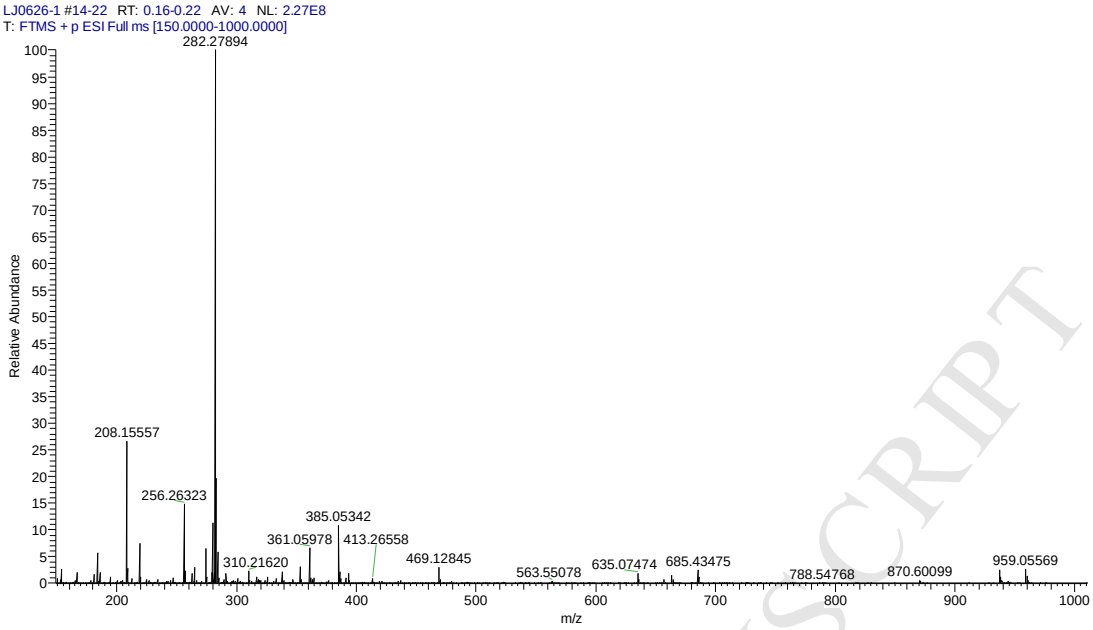


Figure S5: ESI-MS of the complete whole m/z range of the **Probe 1**: [**Probe 1** + H]+ Calcd for 635.076, Found 635.076.

Ⅲ. Spectroscopic Studies

Fig. S6

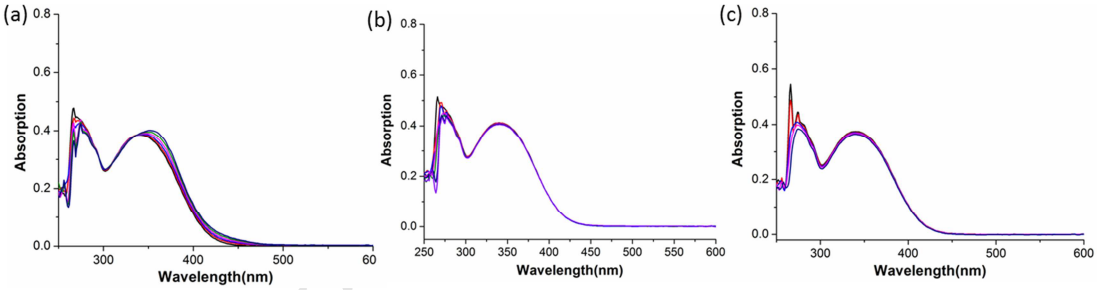


Figure S7. In 2 mL DMSO : PBS (v/v, 7 : 3, pH = 7.4) solution of **Probe 1** (25 μM), added of 500 μM (a) Cys; (b) Hcy; (c) GSH.

Fig. S8

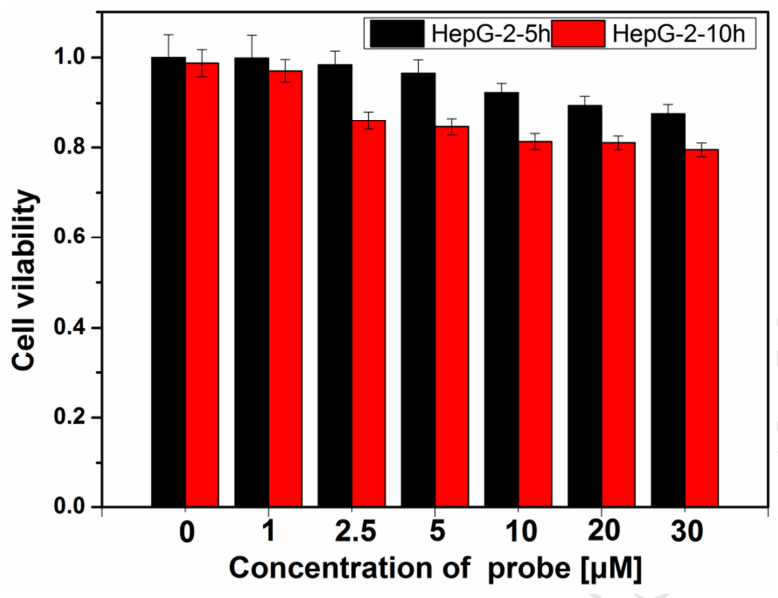
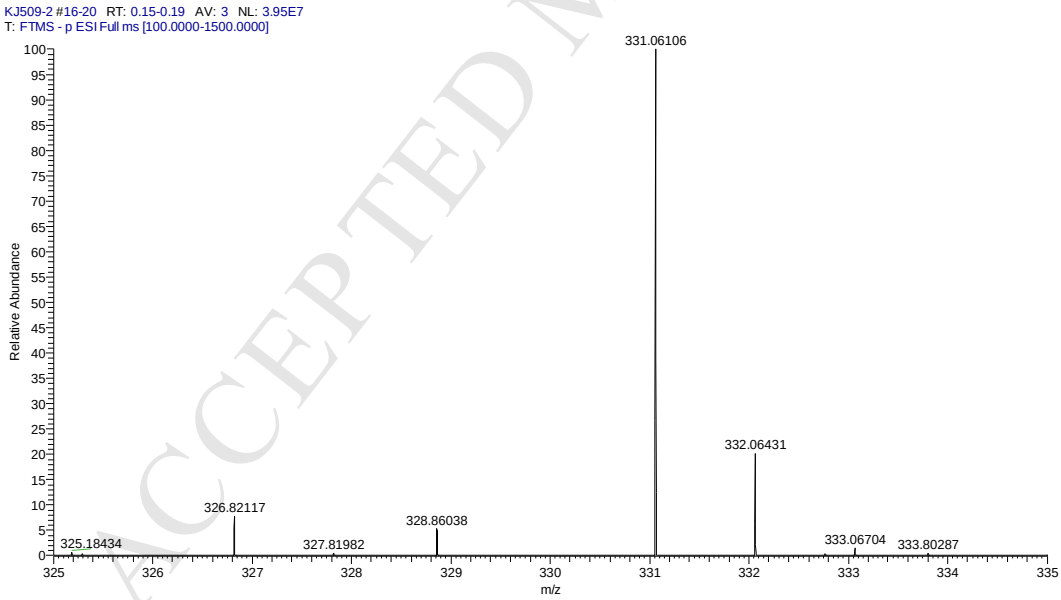


Figure S8. Cytotoxicity of **Probe 1** toward HepG2 cells.

Fig. S9



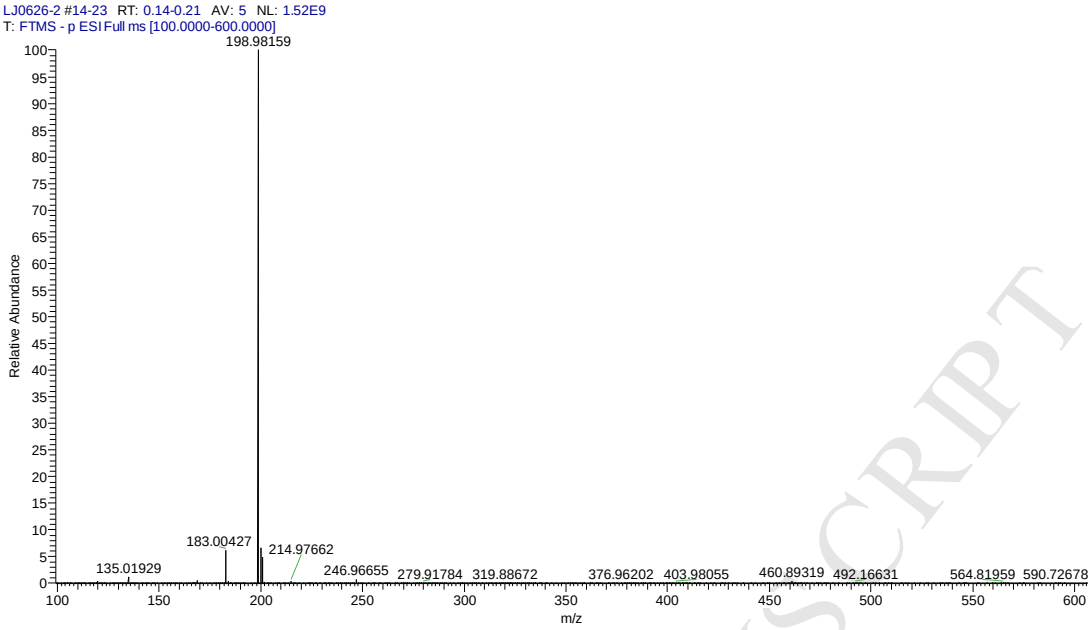


Figure S9. ESI-MS of a solution mixture of **Probe 1** and NaHS exhibited dominant m/z peaks at 331.06, 198.98, 168.95 in accordance with the fluorescein: 331.06, compound 2: 198.98.