Supplementary information
for

Cell-specific activation of gemcitabine by endogenous H_2S and tracking through simultaneous fluorescence turn-on

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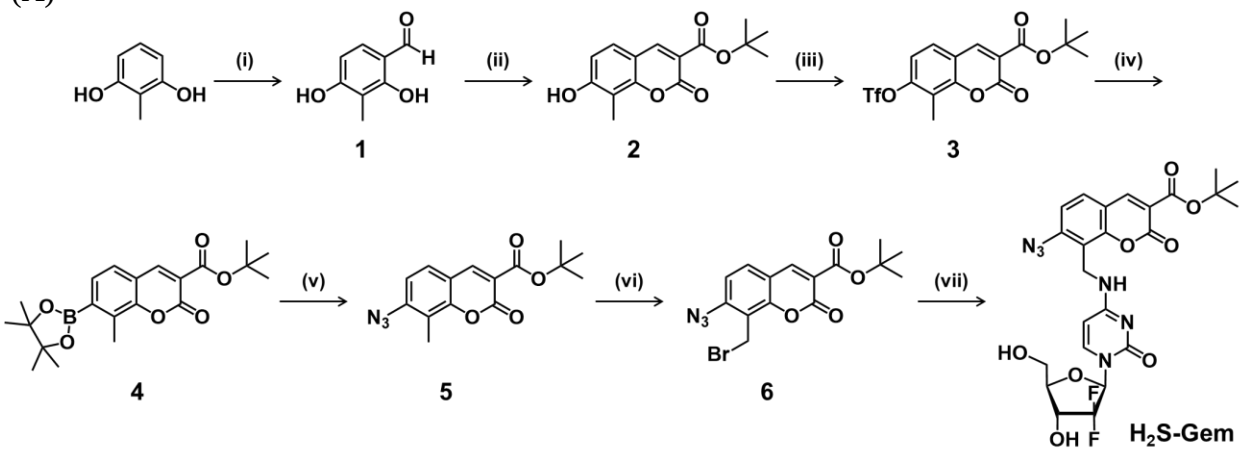
Contents

1. General information on synthetic materials and instrumentation

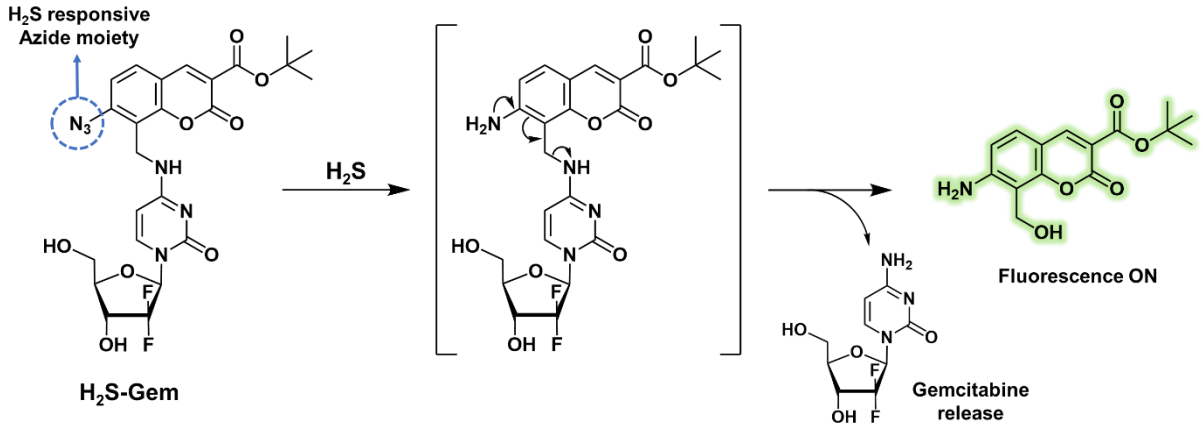
2-Methyl resorcinol, tert-butyl ethyl maloate, piperidine, phosphorous oxychloride, *N*-phenyl-bis(trifluoromethanesulfonimide), potassium carbonate, bis(pinacolato) diboron, 1,1'-bis(diphenylphosphino)ferrocene-palladium(II) dichloride dichloro methane complex, potassium acetate, sodium azide, copper acetate, *N*-bromo succinimide, AIBN, sodium carbonate, gemcitabine, acetonitrile, diethyl ether, hexane, ethyl acetate, methanol and DCM were purchased from Aldrich (Aldrich, St. Louis, MO, USA), Alfa (Alfa, Heysham, LA3 2XY, United Kingdom) and TCI (TCI, Tokyo, Japan) and used without further purification. All the materials for UV/Vis and fluorescence spectroscopy, DMSO (J.T. Baker), were purchased from commercial suppliers and were used without further purification. NMR spectra were recorded on Bruker (500 MHz) instrument. HR-ESI-MS data were obtained using liquid chromatography mass spectrometer (LC/MS) at the Korea Basic Science Institute (Seoul).

2. Synthetic scheme and procedures

(A)



(B)



Scheme S1. (A) Synthetic scheme for **H_2S-Gem**. (B) Activation mode in presence of H_2S followed by release of gemcitabine and fluorophore. Reagents and reaction conditions; Scheme (A) : (i) DMF, $POCl_3$, RT, 2 h; (ii) tert-butyl ethyl malonate, 2,4-dihydroxy-3-methylbezaldehyde, piperidine, EtOH, RT, 6 h; (iii) *N*-phenyl-bis(trifluoromethane sulfonimide), Na_2CO_3, DMF, RT, 2 h; (iv) Bis(pinacolato)diboron, $PdCl_2$(dppf)-DCM complex, CH_3COOK, toluene, reflux, 4 h; (v) NaN_3, $Cu(OAc)_3$, MeOH, 55 °C, 30 min; (vi) NBS, AIBN, CCl_4, reflux, 5 h; (vii) Gemcitabine, K_2CO_3, ACN, 12 h.

Synthesis of 1: Phosphorous oxychloride (5.63 mL, 0.06 mol) was added dropwise with stirring to DMF (20 mL) in ice-bath for 30 min under nitrogen atmosphere. Then, 2,5-dimethylbenzene-1,3-diol (5 g, 0.04 mol) was added slowly to the reaction mixture and warm to room temperature. After 2 h, the reaction completion was checked using TLC and quenched with ice. Next, 10% aqueous NaOH was added to the reaction mixture until pH 13 and the mixture was refluxed for 10 min. The reaction mixture was cooled to room temperature and diluted with aqueous HCl to pH 3. Then, crystalline solid was observed, filtered and dried to give compound **1** (3.15 g, 51 % yield) as brown solid. HRMS $[M+H]^+$ calc. 153.0546 m/z, $[M+H]^+$ obs. 153.0557 m/z. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 2.14 (s, 3H); 5.59 (s, 1H); 6.47 (d, J = 8.5, 1H); 7.28 (t, J = 9.3 Hz, 1H); 9.69 (s, 1H); 11.67 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ 194.7, 162.3, 161.2, 133.0, 115.1, 111.1, 108.0, 7.1 ppm.

Synthesis of 2: tert-butyl ethyl malonate (2.23 mL, 13.10 mmol) was added dropwise to a stirred solution of compound **1** (0.9 g, 5.92 mmol) in ethanol (10 mL) followed by addition of piperidine (2.16 mL, 21.87 mmol) under nitrogen atmosphere. The reaction mixture was further stirred for 6 h at ambient temperature. The progress of the reaction was monitored by checking TLC. After reaction completion, the reaction mixture was concentrated under reduced pressure, diluted with water and extracted with ethyl acetate. The organic phase was washed with brine solution, dried over anhydrous Na_2SO_4 and concentrated to get crude compound, purified by recrystallization using DCM and hexanes to give compound **2** (0.76 g, 47 % yield) as yellow solid. HRMS $[M+Na]^+$ calc. 299.0890 m/z, $[M+Na]^+$ obs. 299.0898 m/z. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.52 (s, 9H); 2.14 (s, 3H); 6.89 (d, J = 9.0 Hz, 1H); 7.57 (d, J = 9.0 Hz, 1H); 8.53 (s, 1H); 10.93 (s, 1H) ppm.

^{13}C NMR (DMSO-d_6, 125 MHz): δ 162.7, 162.1, 157.2, 155.2, 149.3, 129.2, 113.6, 113.6, 110.9, 110.9, 81.6, 28.3, 8.3 ppm.

Synthesis of 3: To a DMF (15 mL) solution of compound **2** (1.65 g, 5.98 mmol) in a round bottom flask, *N*-phenyl bis-(trifluoromethanesulphonimide) (4.28 g, 11.98 mmol) and sodium carbonate (0.32g, 3.02 mmol) were added and stirred under nitrogen atmosphere for 2 h at room temperature. After reaction completion, the reaction mixture was poured into ice water. Precipitation was formed, filtered, washed with water, and dried in vacuo to give compound **3** (3.8 g, 85 %) as white solid. HRMS $[M+Na]^+$ calc. 431.0383 m/z, $[M+Na]^+$ obs. 431.0405 m/z. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.61 (s, 9H); 2.47 (s, 3H); 7.25 (d, J = 8.5 Hz, 1H); 7.51 (d, J = 8.5 Hz, 1H); 8.35 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ 161.5, 155.7, 154.2, 151.0, 146.3, 127.6, 120.6, 120.4, 117.8, 117.5, 83.3, 28.1, 9.5 ppm.

Synthesis of 4: To a dry toluene purged with nitrogen for 20 min, compound **3** (1.43 g, 3.49 mmol), potassium acetate (1.03 g, 10.46 mmol), bis(pinacolato)diboron (1.16 g, 4.57 mmol) were added. The reaction mixture was further purged with nitrogen for 10 min. Pd(dppf)Cl_2-DCM complex (0.86 g, 1.05 mmol) was added and degassed again for 5 min. The reaction mixture was stirred at 110 °C for 4 h. After reaction completion, the reaction mixture was cooled to room temperature, concentrated under reduced pressure. The crude compound was dissolved in ethyl acetate, filtered with celite, and filtrate was concentrated. Then, the crude compound was subjected to a silica gel column chromatography using ethyl acetate/hexanes (1:1, v/v) as an eluent and recrystalized with DCM/hexanes to afford compound **4** (0.47 g, 35 %) as brown solid. HRMS $[M+Na]^+$ calc. 409.1793 m/z, $[M+Na]^+$ obs. 409.1814 m/z. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 1.37 (s, 12H); 1.60 (s, 9H); 2.65 (s, 3H); 7.38 (d, J = 7.5 Hz, 1H); 7.65 (d, J = 7.5 Hz, 1H); 8.35 (s, 1H) ppm. ^{13}C NMR

($CDCl_3$, 125 MHz): δ 162.0, 157.2, 153.0, 147.6, 132.8, 130.9, 125.6, 120.0, 119.2, 84.3, 82.7, 28.1, 24.9, 14.2 ppm.

Synthesis of 5: Compound **4** (0.51 g, 1.32 mmol) in methanol (10 mL) was placed in a round bottom flask, and sodium azide (0.21 g, 3.23 mmol), copper acetate (0.05 g, 0.28 mmol) were added. The reaction mixture was heated at 55 °C for 30 min. After reaction completion, the reaction was cooled to room temperature, concentrated in vacuo to give crude compound. The crude compound was subjected to a silica column chromatography using ethyl acetate/hexanes (1:7, v/v) as an eluent to afford compound **5** (0.34 g, 87 %) as yellow solid. HRMS $[M+Na]^+$ calc. 324.0955 m/z, $[M+Na]^+$ obs. 324.0961 m/z. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.60 (s, 9H); 2.29 (s, 3H); 7.10 (d, J = 8.5 Hz, 1H); 7.45 (d, J = 8.0 Hz, 1H); 8.35 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ 162.0, 156.8, 154.3, 147.5, 144.4, 127.6, 117.7, 117.6, 114.6, 114.4, 82.7, 28.1, 9.7 ppm.

Synthesis of 6: To a solution of compound **5** (0.34 g, 1.14 mmol) in carbon tetrachloride (10 mL) was added *N*-bromo succinimide (0.80 g, 4.49 mmol) and 12 wt% AIBN in acetone (1.60 mL, 0.91 mmol). The reaction mixture was refluxed at 78 °C for 5 h. After reaction completion, the reaction mixture was cooled to room temperature, quenched with water, and extracted with DCM three times. The collected organic solvents were rinsed with a brine solution and dried with anhydrous Na_2SO_4. The organic solvents were concentrated and the crude product was purified by a silica gel column chromatography using ethyl acetate/hexanes (1:7, v/v) as an eluent to afford compound **6** (0.24 g, 55 %) as light-yellow solid. HRMS $[M+Na]^+$ calc. 402.0060, 404.0039 m/z, $[M+Na]^+$ obs. 402.0050, 404.0033 m/z. ^1H NMR ($CDCl_3$, 500 MHz): δ 1.60 (s, 9H); 4.87 (s, 2H); 7.13 (d, J =

8.5 Hz, 1H); 7.57 (d, *J* = 8.5 Hz, 1H); 8.35 (s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): 161.7, 155.8, 153.9, 147.0, 144.5, 130.3, 118.4, 117.1, 114.9, 114.7, 83.0, 28.1, 19.7 ppm.

Synthesis of H_2S-Gem: To an acetonitrile (10 mL) solution of compound **5** (0.15 g, 0.39 mmol) and gemcitabine (0.24 g, 0.8 mmol) was added K_2CO_3 (0.27 g, 1.95 mmol) at 0 °C under nitrogen atmosphere. The reaction mixture was further stirred for 12 h at ambient temperature. After reaction completion, the reaction mixture was concentrated under reduced pressure, and diluted with water and ethyl acetate, extracted with ethyl acetate three times. The organic layer was washed with a brine solution, dried with anhydrous Na_2SO_4. The organic solvents were concentrated in vacuo and crude compound was purified through a silica gel column chromatography using ethyl acetate to give **H_2S-Gem** (43 mg, 20 %) as light orange solid. HRMS $[M+H]^+$ calc. 563.1696 m/z, [M+Na]+ calc. 585.1516 m/z, $[M+H]^+$ obs. 563.1708 m/z, $[M+Na]^+$ obs. 585.1516 m/z. ^{1}H NMR (DMSO-d_6, 500 MHz,): δ 1.53 (s, 9H); 3.63-3.60 (m, 1H); 3.81-3.76 (m, 1H); 4.19-4.11 (m, 1H); 5.29-5.20 (m, 3H); 5.81 (d, *J* = 8.0 Hz, 1H); 6.05 (t, *J* = 8.0 Hz, 1H); 6.26 (d, *J* = 6.5 Hz, 1H); 7.20 (d, *J* = 8.0 Hz, 1H); 7.32 (d, *J* = 8.5 Hz, 1H); 7.80 (s, 1H); 7.82 (d, *J* = 8.5 Hz, 1H); 8.61 (s, 1H) ppm. ^{13}C NMR (DMSO-d_6, 125 MHz): δ 162.2, 155.9, 154.0, 150.8, 148.2, 143.4, 130.7, 130.1, 125.6, 124.9, 123.5, 121.5, 117.5, 116.3, 115.7, 114.9, 102.2, 84.1, 82.3, 21.0, 69.0, 59.3, 36.4, 28.2 ppm.

3. UV/Vis absorption and fluorescence spectroscopic methods, reagents, and instrumentation

All reagents such as metals (chloride salts of Na^+, K^+, Mg^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Cu^+, Zn^{2+}, Fe^{2+}, and Fe^{3+} ions), reactive oxygen species ($^{\bullet}O_2^-$, $^{\bullet}OH$, t-BuO$^{\bullet}$, H_2O_2, t-BuOOH, and ClO^-), thiols (cysteine (Cys), homocysteine (Hcy), glutathione (GSH), and NaHS (H_2S precursor)), $NaNO_2$,

Na_2CO_3, and PBS (phosphate buffered saline) pellet were purchased from Aldrich (Aldrich, St. Louis, MO, USA), Alfa (Alfa, Heysham, LA3 2XY, United Kingdom) and TCI (TCI, Tokyo, Japan), and used without further purification. Stock solutions of metal chloride salts, thiols and reactive oxygen species (ROS) were prepared in deionized water. ROS stock solutions were prepared by using literature procedures.[1] Stock solutions of **H_2S-Gem** and NaHS were prepared in DMSO (HPLC grade) and PBS buffer (10 mM, pH 7.4), respectively.

All absorption and fluorescence spectra were obtained on UV-2600 (Shimadzu Corporation, Kyoto, Kyoto Prefecture, Japan) and RF-6000 (Shimadzu Corporation, Kyoto, Kyoto Prefecture, Japan) spectrophotometer, respectively. All data were recorded in a PBS solution (10 mM, pH 7.4) containing 1% (v/v) of DMSO. Excitation wavelength was 410 nm with slit width set at 5 nm.

4. Cell culture and confocal microscopic methods

A human cervical cancer cells (HeLa) and normal human fibroblast cell line (WI38) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Gibco® fetal bovine serum (FBS), and 100 U/mL penicillin-streptomycin. Adenocarcinoma human alveolar basal epithelial cells (A549) were cultured Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% FBS, and 100 U/mL penicillin-streptomycin. At 2 days before the microscopic experiments, the cells were transferred on cover glass-bottom dish. The cells were seeded at 10^5 per dish and maintained at 37 °C in a humidified atmosphere consisting of 5% (v/v) CO_2 containing air. All cells were purchased from Korean Cell Line Bank (Seoul, South Korea). For cell culture media, DMEM, RPMI, FBS, trypsin 0.25%-EDTA and penicillin-streptomycin

1. Oushiki, D.; Kojima, H.; Terai, T.; Arita, M.; Hanaoka, K.; Urano, Y.; Nagano, T. Development and application of a near-infrared fluorescence probe for oxidative stress based on differential reactivity of linked cyanine dyes. *J. Am. Chem. Soc.* **2010**, *132*, 2795-2801.

were purchased from BIOWEST (Nuaillé-France). The clear and adhesion-typed confocal dishes (diameter = 35 mm) were purchased from SPL (Phocheon-si, Korea).

For confocal microscopy images, the culture medium was removed, and the cells were washed with phosphate buffered saline (PBS 1X, pH 7.4). Cells were incubated with **H_2S-Gem** (15 μM) for 1 h and washed with PBS to remove the excess probes from the medium. For dose-dependent imaging, the cells were incubated with various concentration of **H_2S-Gem** (1-20 μM) for 1 h. To perform exogenous H_2S monitoring, the cells were pretreated with **H_2S-Gem** (15 μM) for 1h and then incubated with NaHS (600 μM) for 30 min and washed with PBS prior to imaging. To monitoring the endogenous levels of H_2S, the cells were preincubated with cysteine (200 μM) for 1 h and then with **H_2S-Gem** for 1 h. To reduce the endogenous concentration of H_2S, cells were pretreated with AOAA/PAG (2 mM, respectively) for 1 h and then incubated with **H_2S-Gem** for 1 h. Confocal microscopy imaging were performed under Zeiss LSM-700 (Carl Zeiss, Oberkochen, Germany). All images were collected by using an excitation laser of 405 nm and band-path filters for 505-600 nm.

5. MTT assay

Cell viability was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells at 1.5×10^4/mL were treated with different concentrations of **H_2S-Gem** and gemcitabine in 96-well plates for 24, 48, and 72 h at 37 °C, respectively. For H_2S boosting and inhibitory study, cells were pretreated with NaHS (1 mM) and PAG (1 mM) for 12 h, which then incubated with **H_2S-Gem** for 48 h. Then, a solution of MTT in serum free media (5 mg/mL) was added to each well, which was then further incubated for 3 h. The water-insoluble formazan was formed during the incubation, and then DMSO was added to each well. The amount of formazan

was then measured by checking the absorbance at 540 nm using a Spectra Max i3x microplate reader (Molecular devices, San Jose, CA). MTT was purchased from Sigma-Aldrich (St. Louis, MO) and used as received without further purification.

6. ^{1}H and ^{13}C NMR and ESI-MS analysis

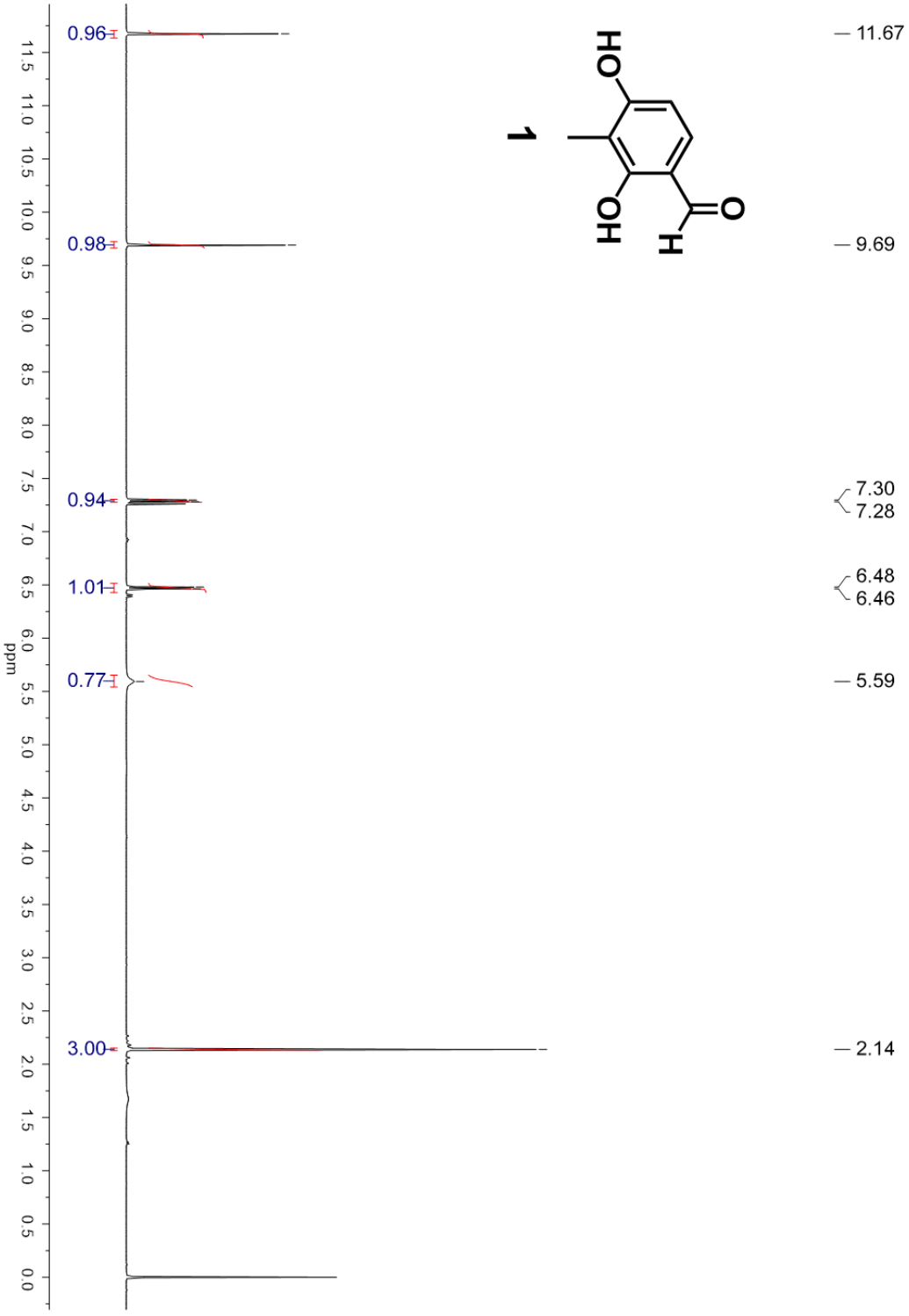


Fig. S1 ^{1}H NMR of compound **1** in $CDCl_3$.

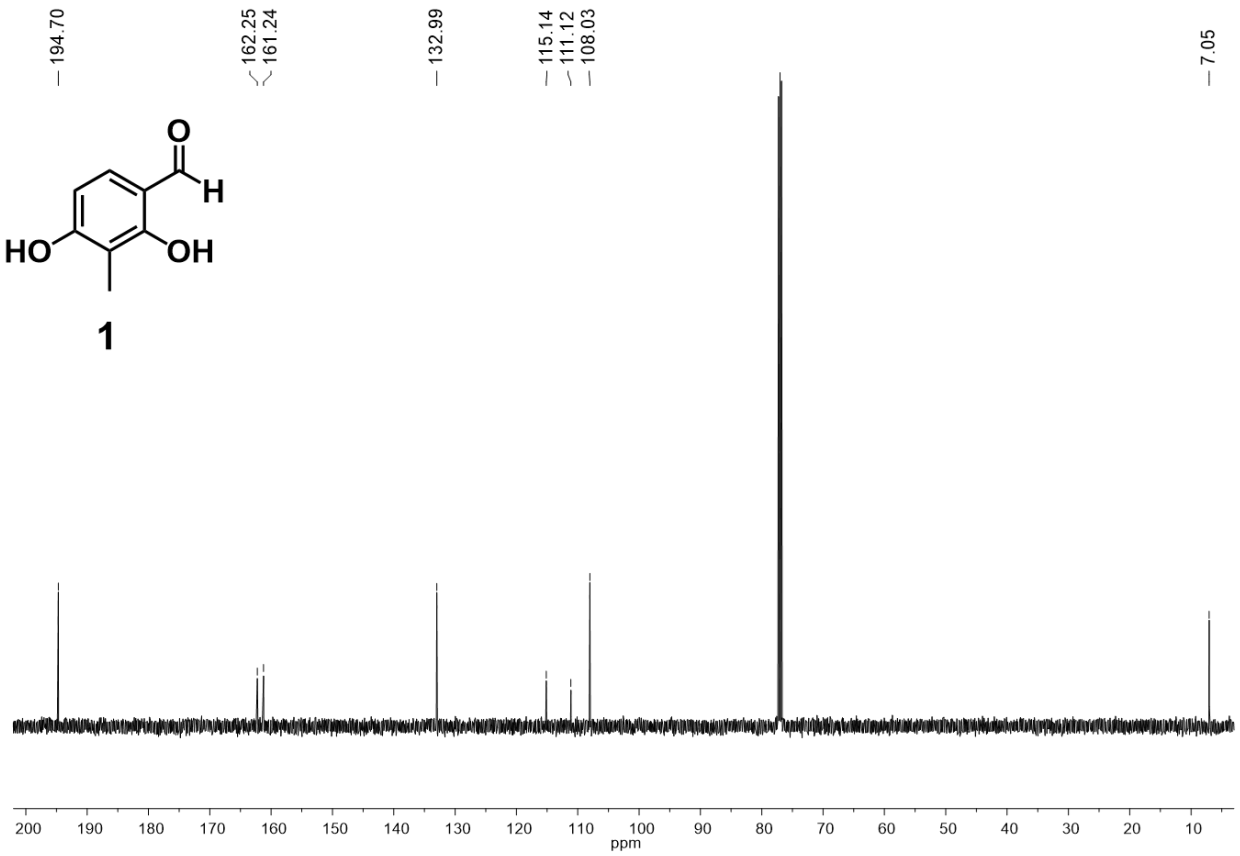


Fig. S2 ^{13}C NMR of compound **1** in $CDCl_3$.

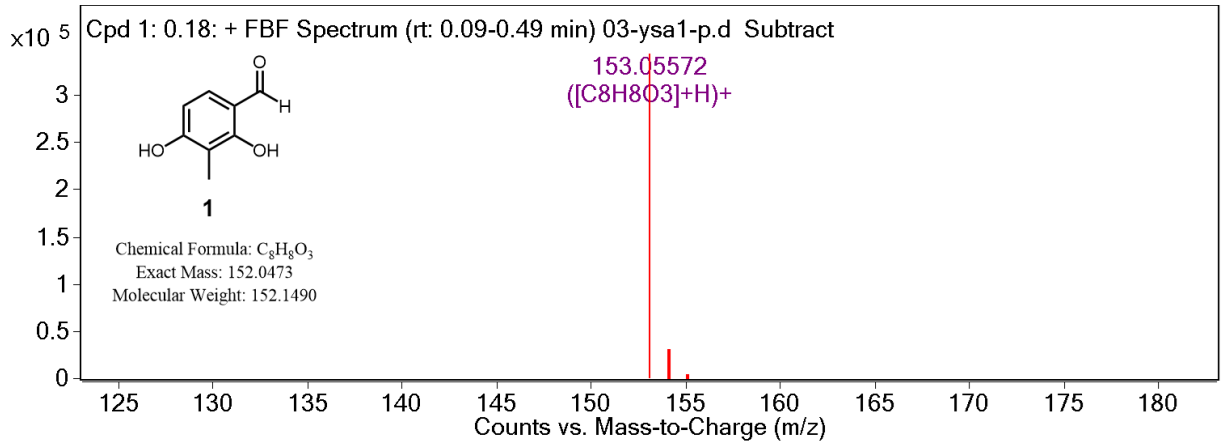


Fig. S3 ESI-MS of compound **1**.

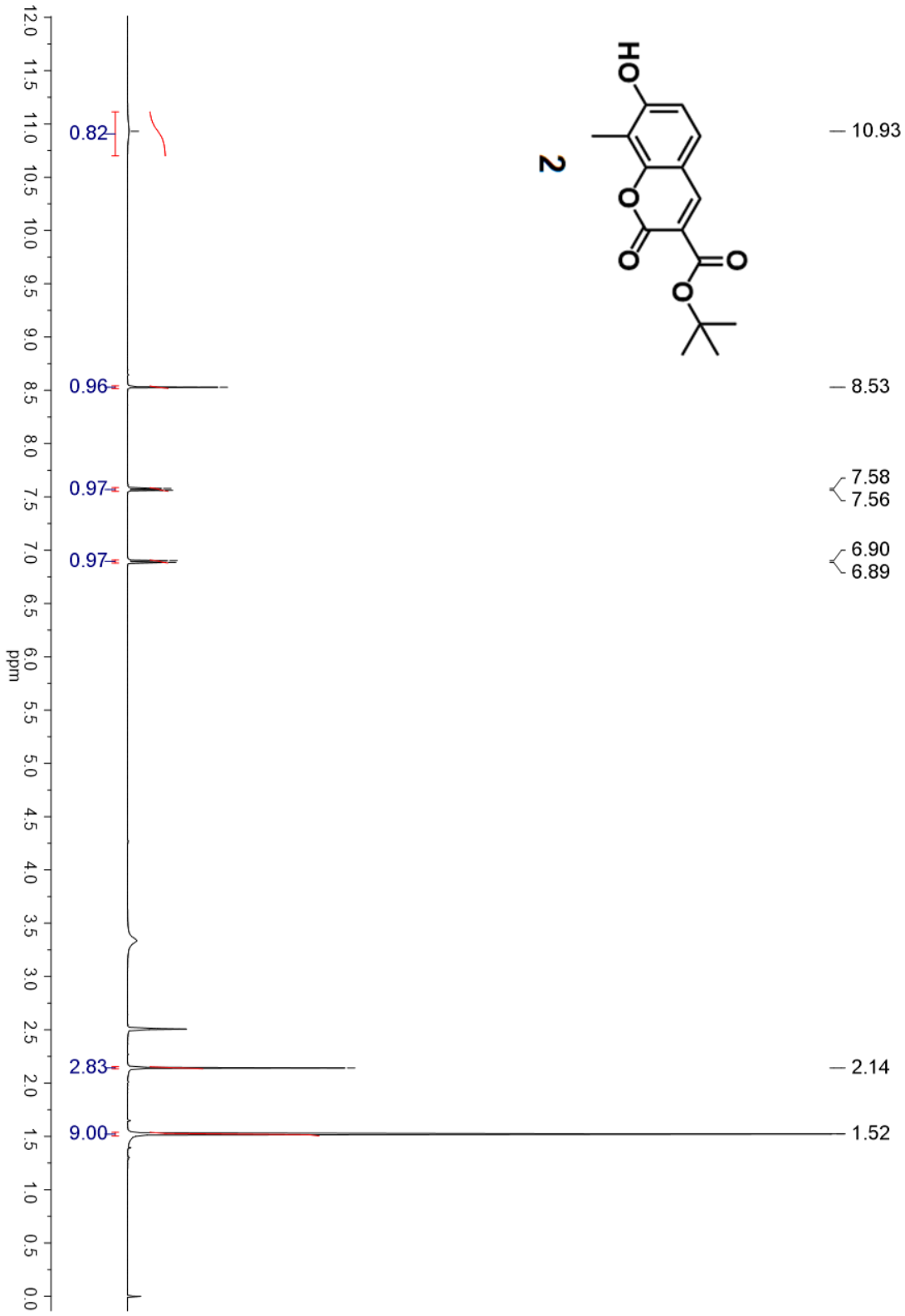


Fig. S4 ^{1}H NMR of compound **2** in DMSO-d_6.

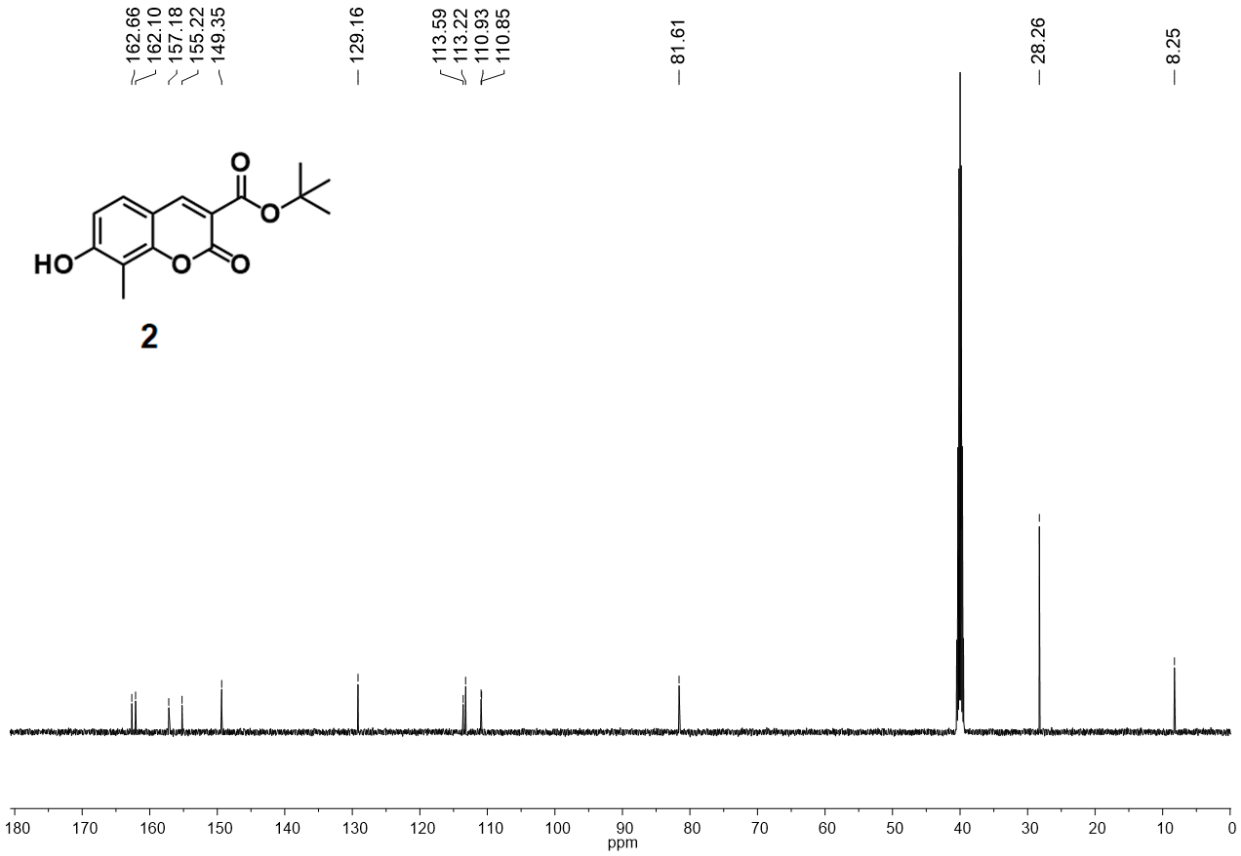


Fig. S5 ^{13}C NMR of compound **2** in DMSO-*d*$_6$.

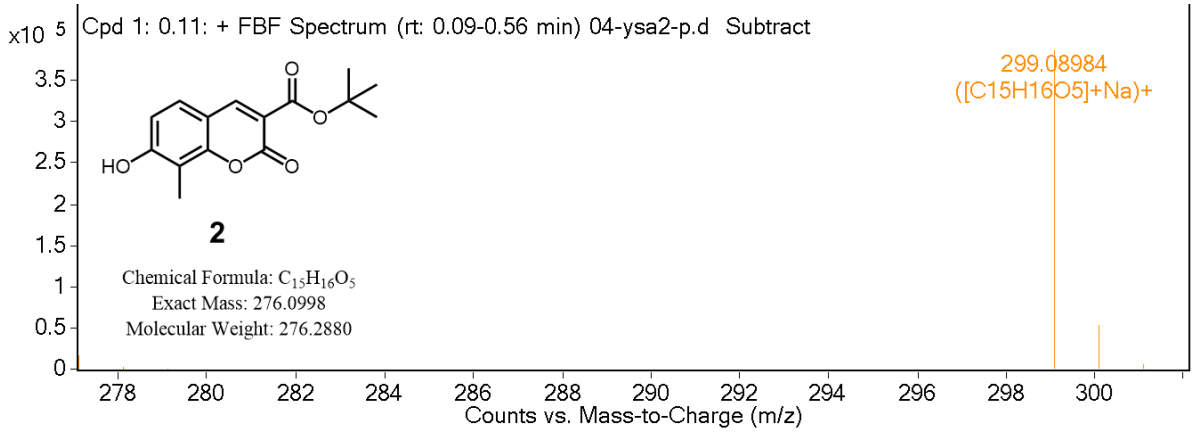


Fig. S6 ESI-MS of compound **2**.

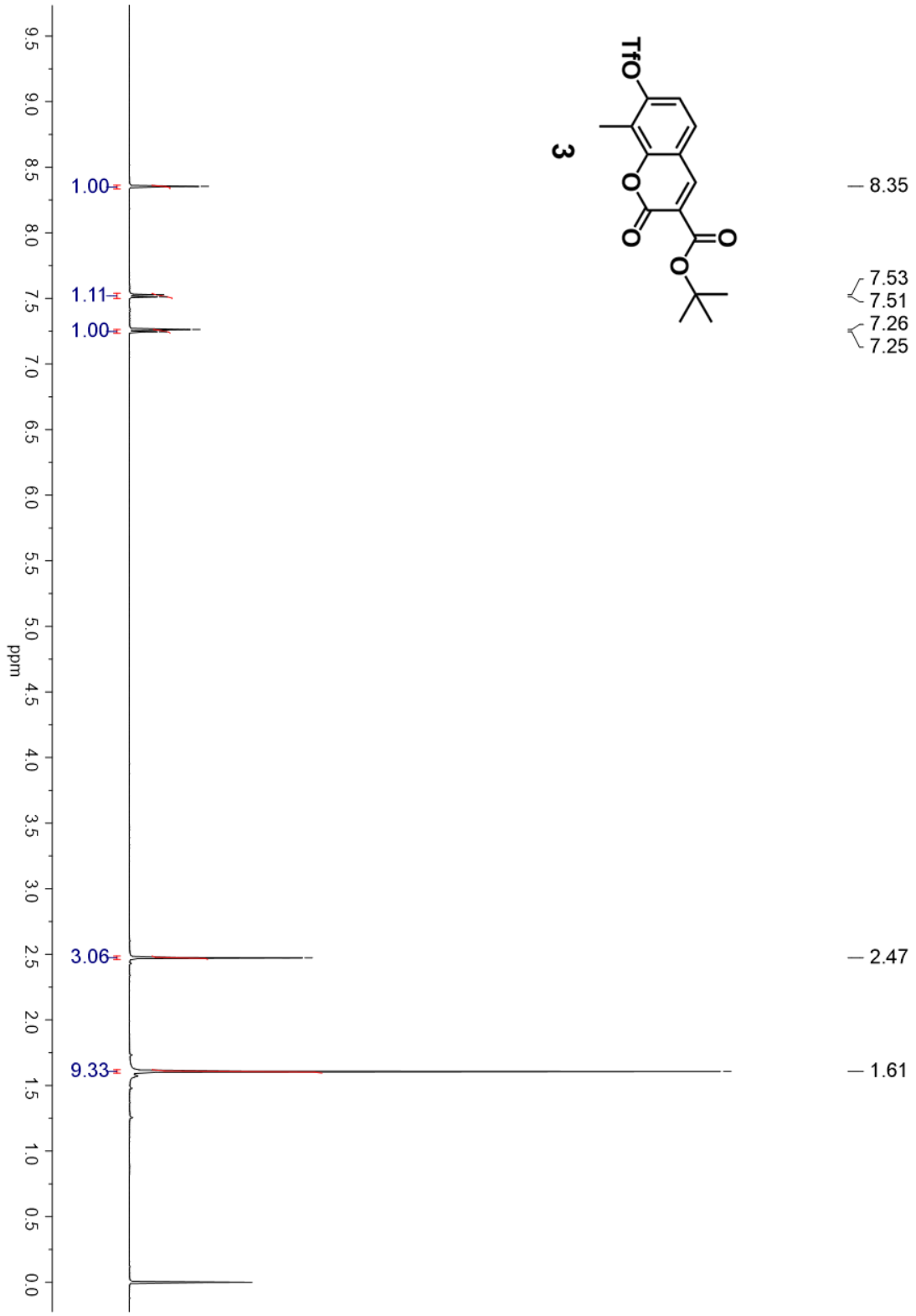


Fig. S7 ^{1}H NMR of compound **3** in $CDCl_3$.

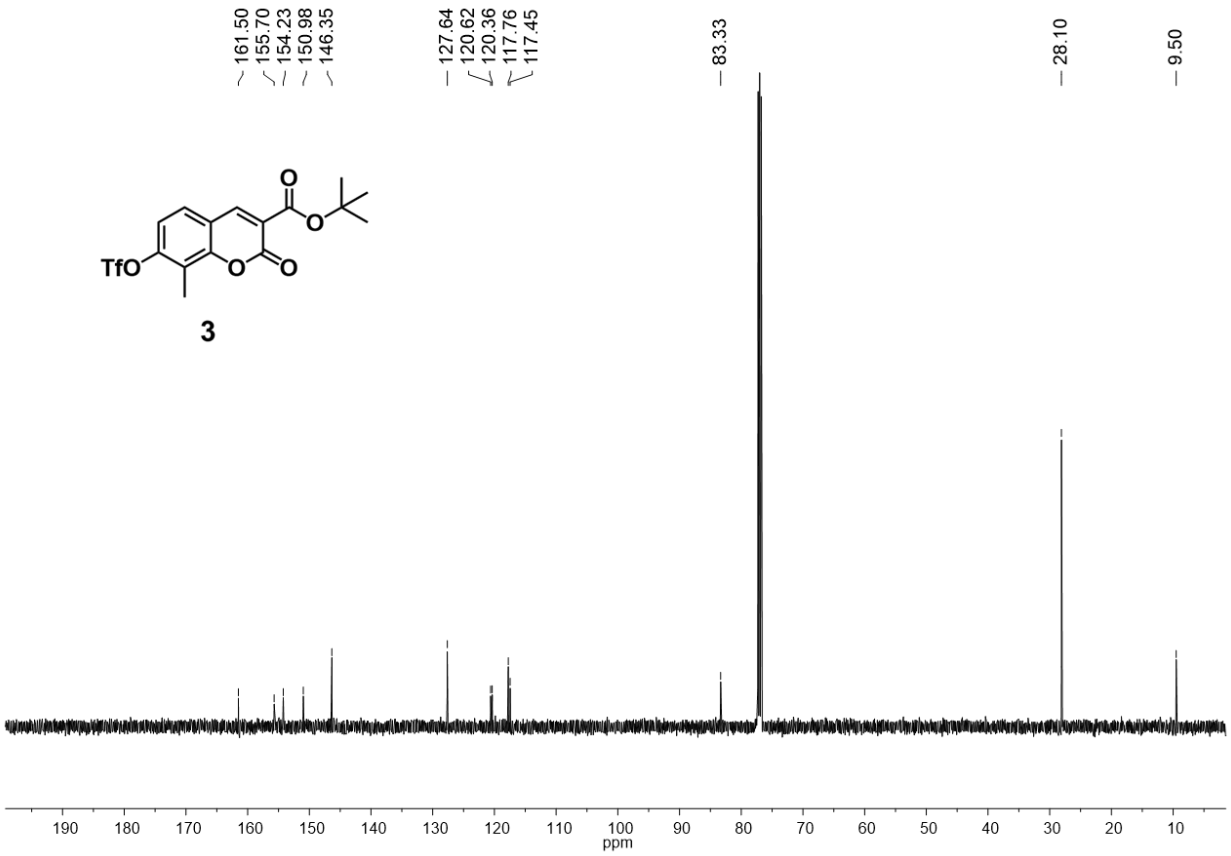


Fig. S8 ^{13}C NMR of compound **3** in $CDCl_3$.

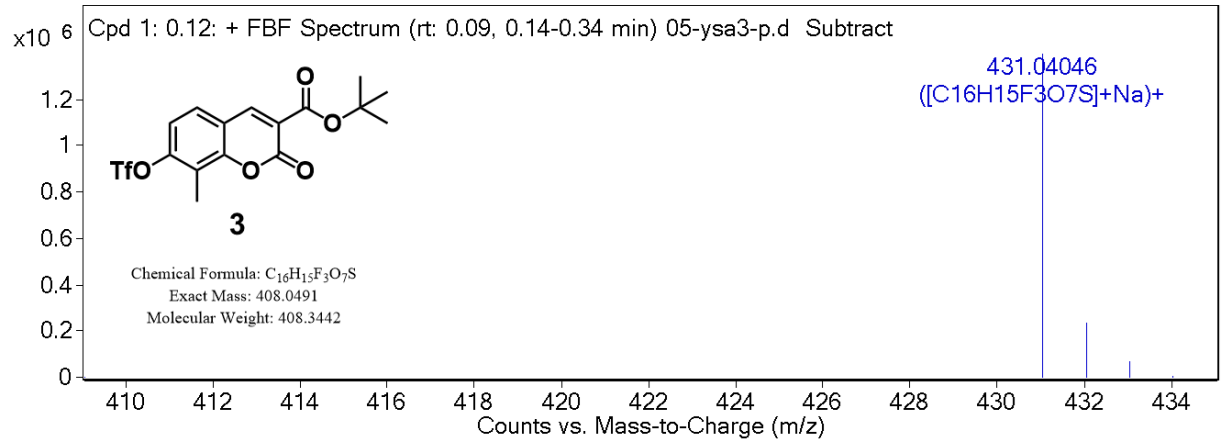


Fig. S9 ESI-MS of compound **3**.

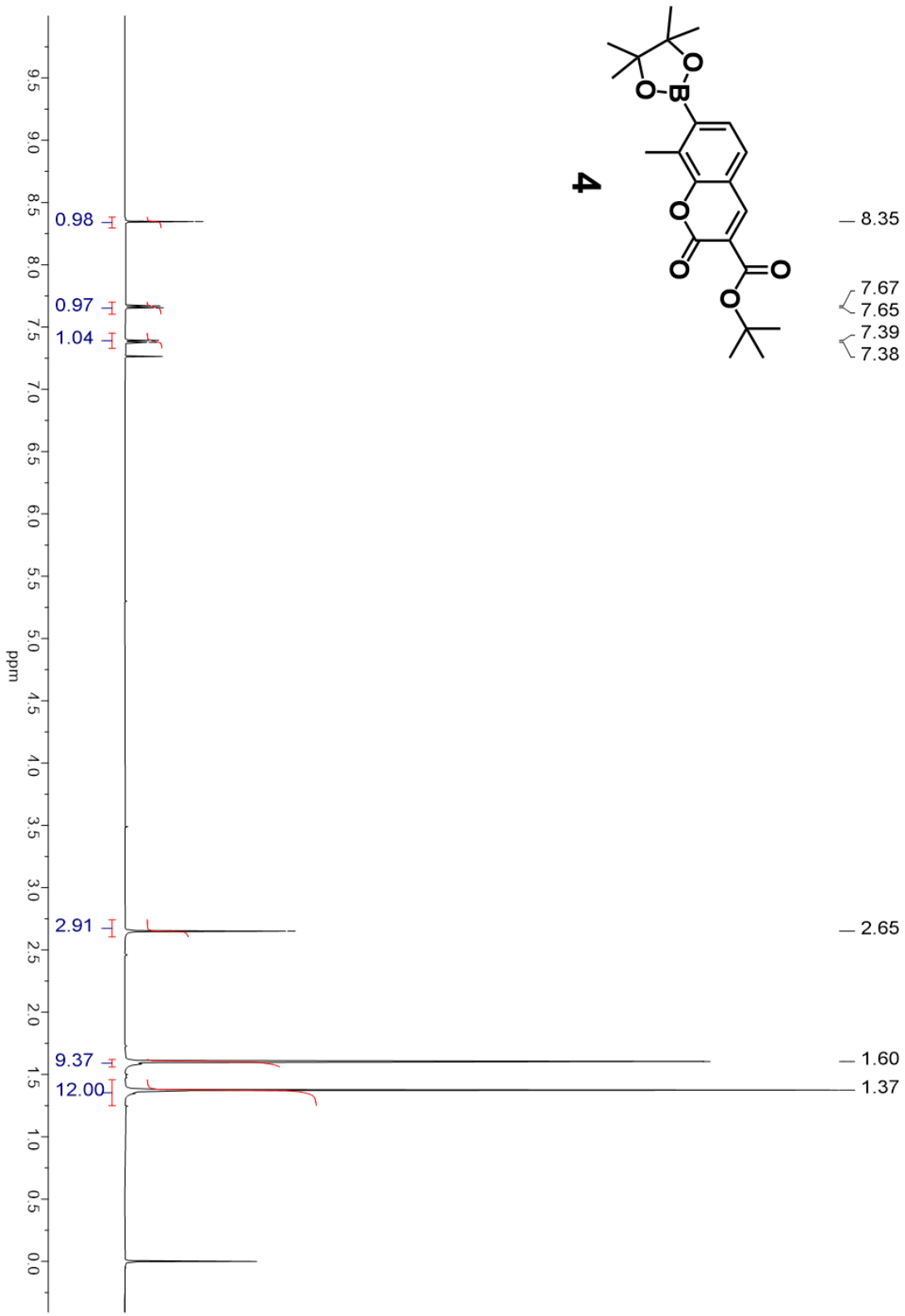


Fig. S10 ^{1}H NMR of compound **4** in $CDCl_3$.

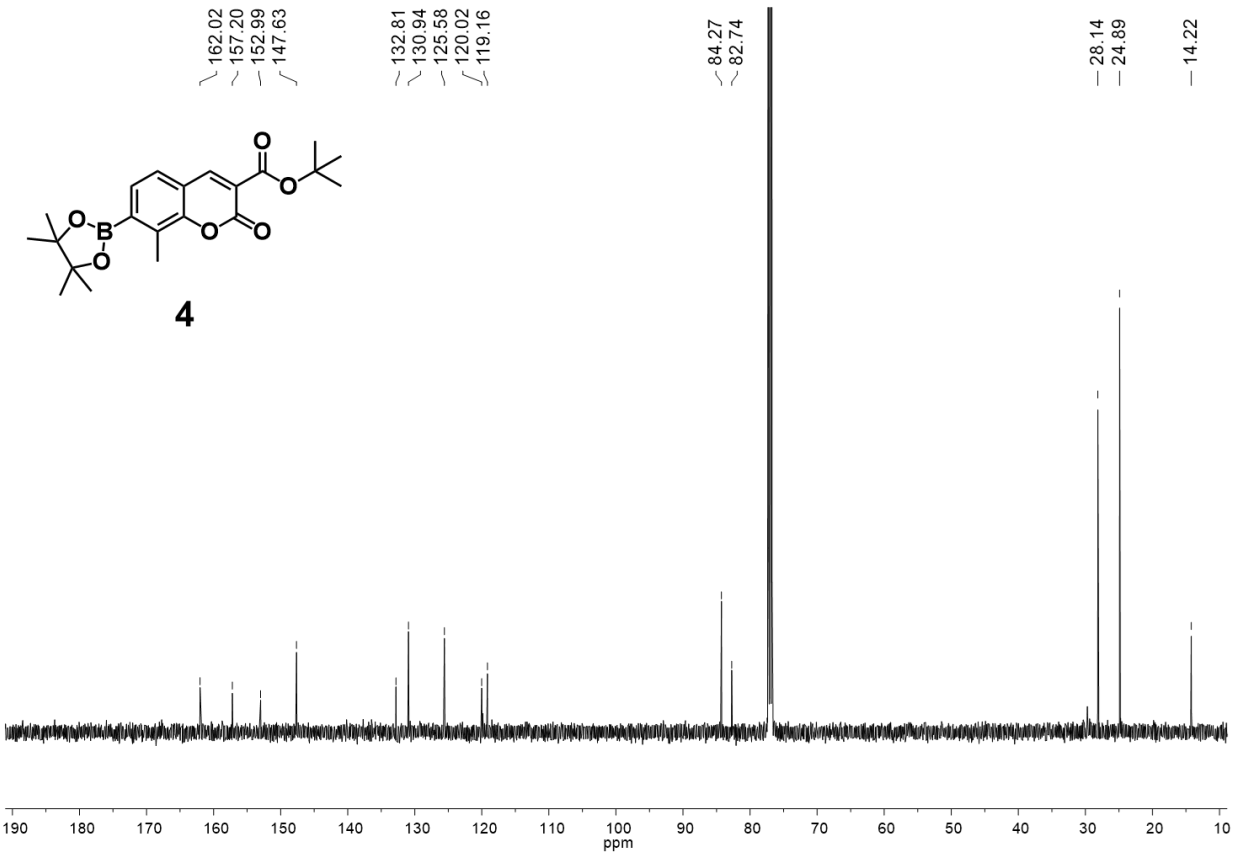


Fig. S11 ^{13}C NMR of compound **4** in $CDCl_3$.

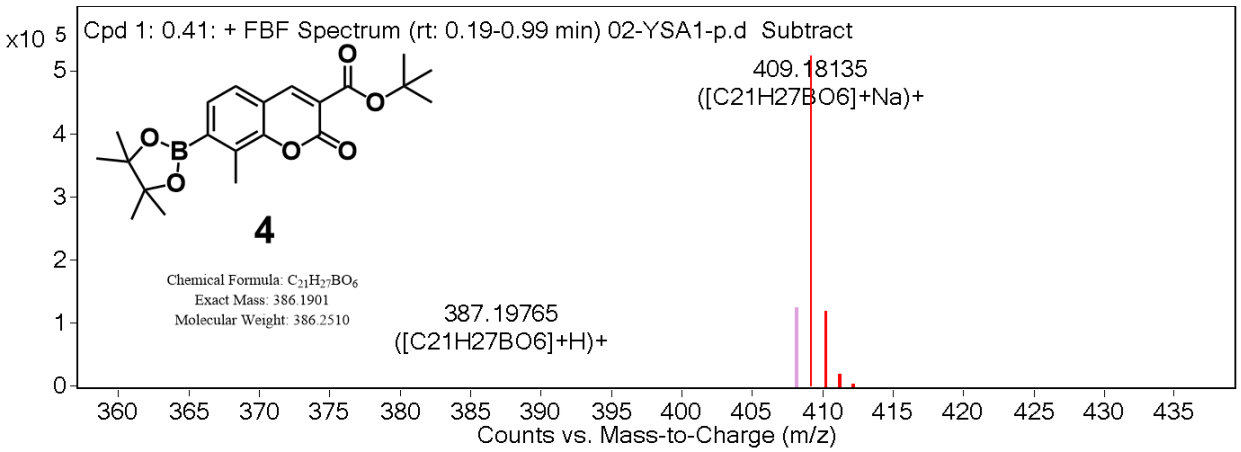


Fig. S12 ESI-MS of compound **4**.

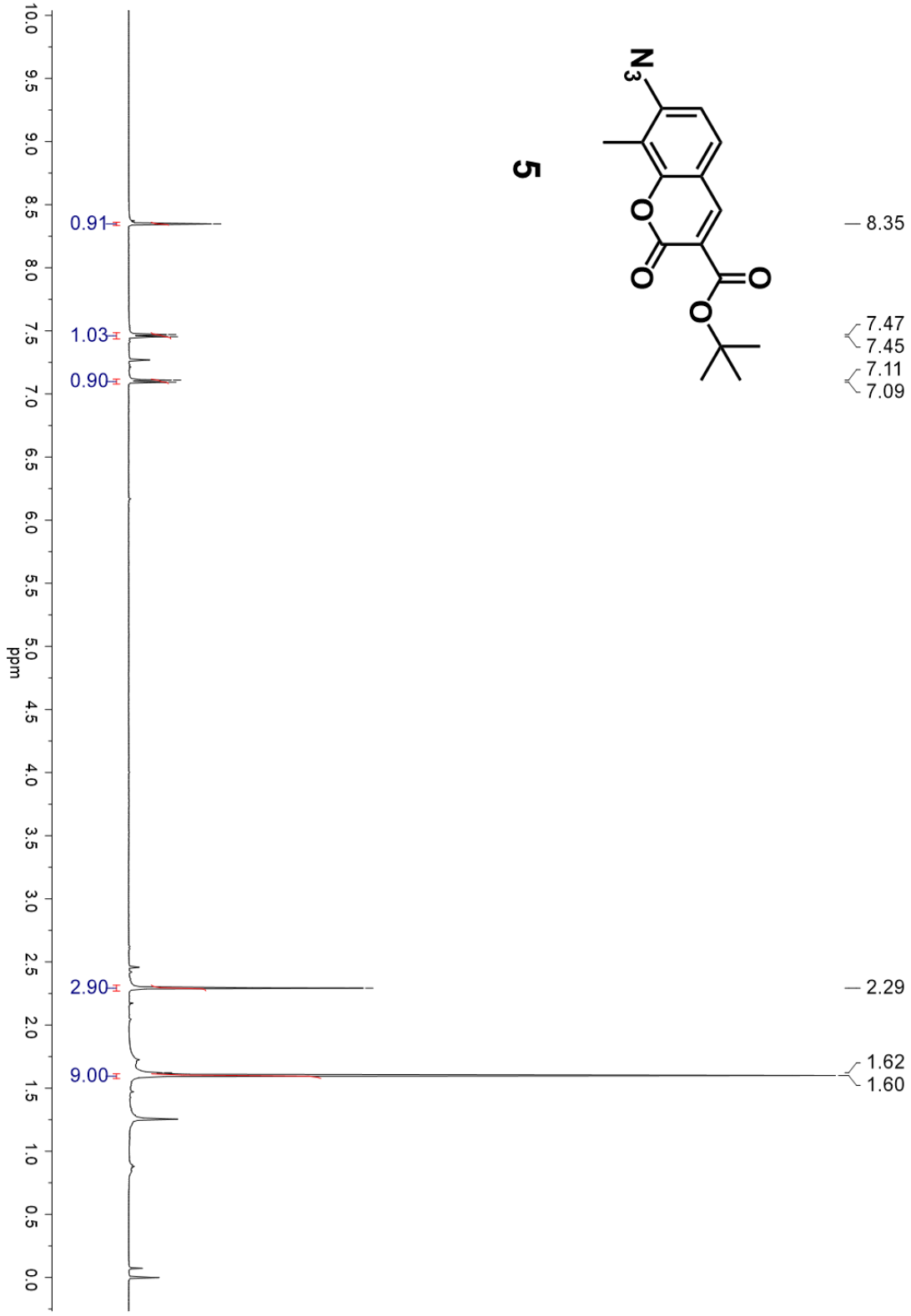


Fig. S13 ^{1}H NMR of compound **5** in $CDCl_3$.

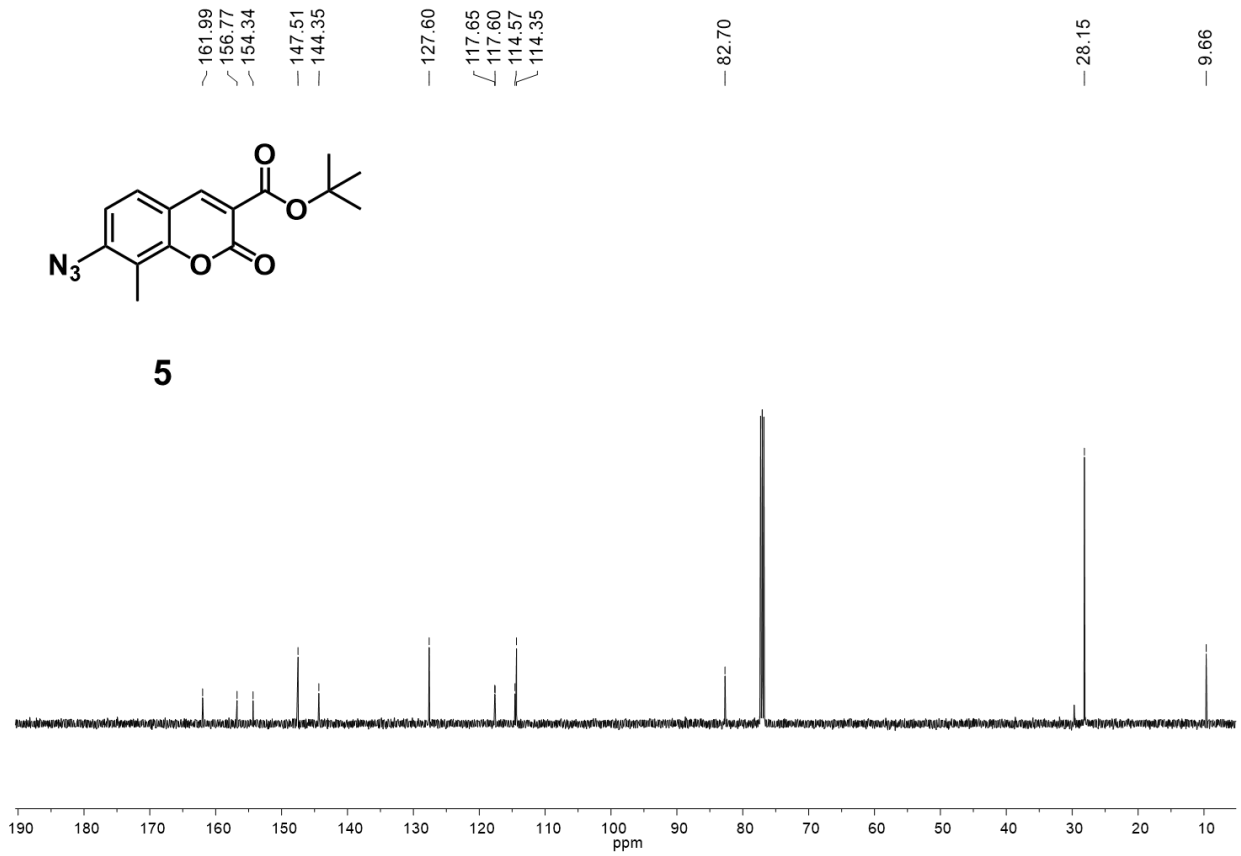


Fig. S14 ^{13}C NMR of compound **5** in $CDCl_3$.

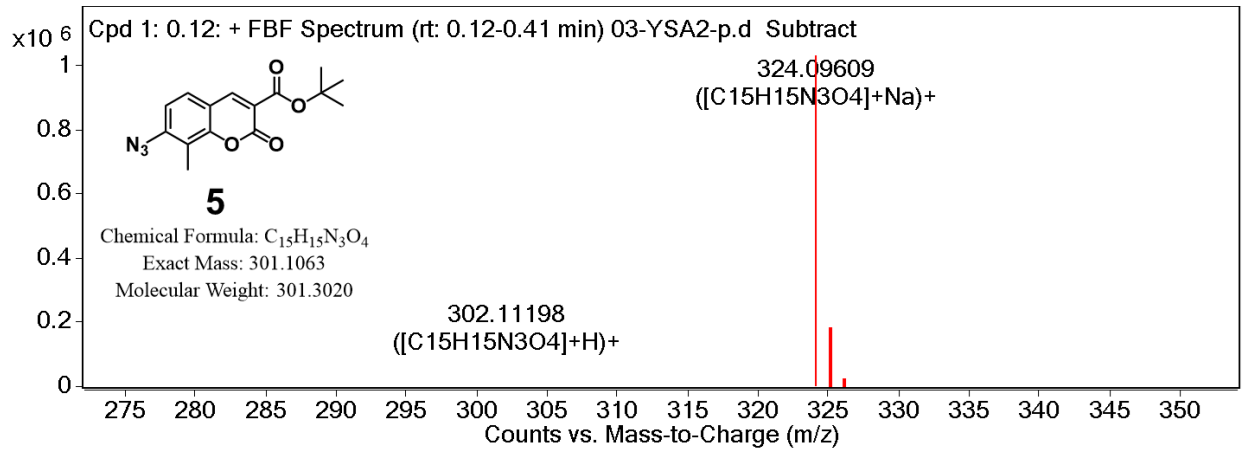


Fig. S15 ESI-MS of compound **5**.

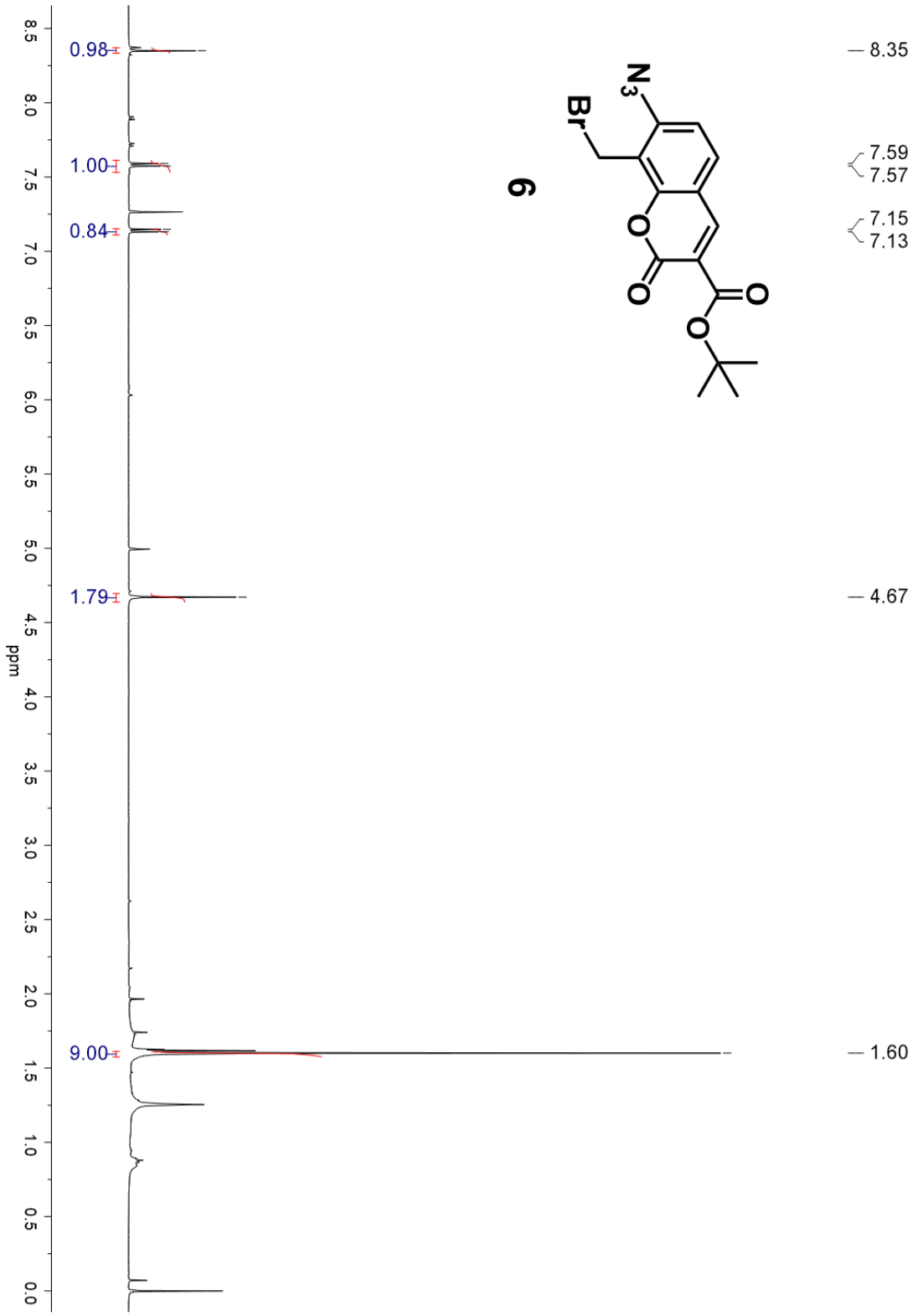


Fig. S16 ^{1}H NMR of compound **6** in $CDCl_3$.

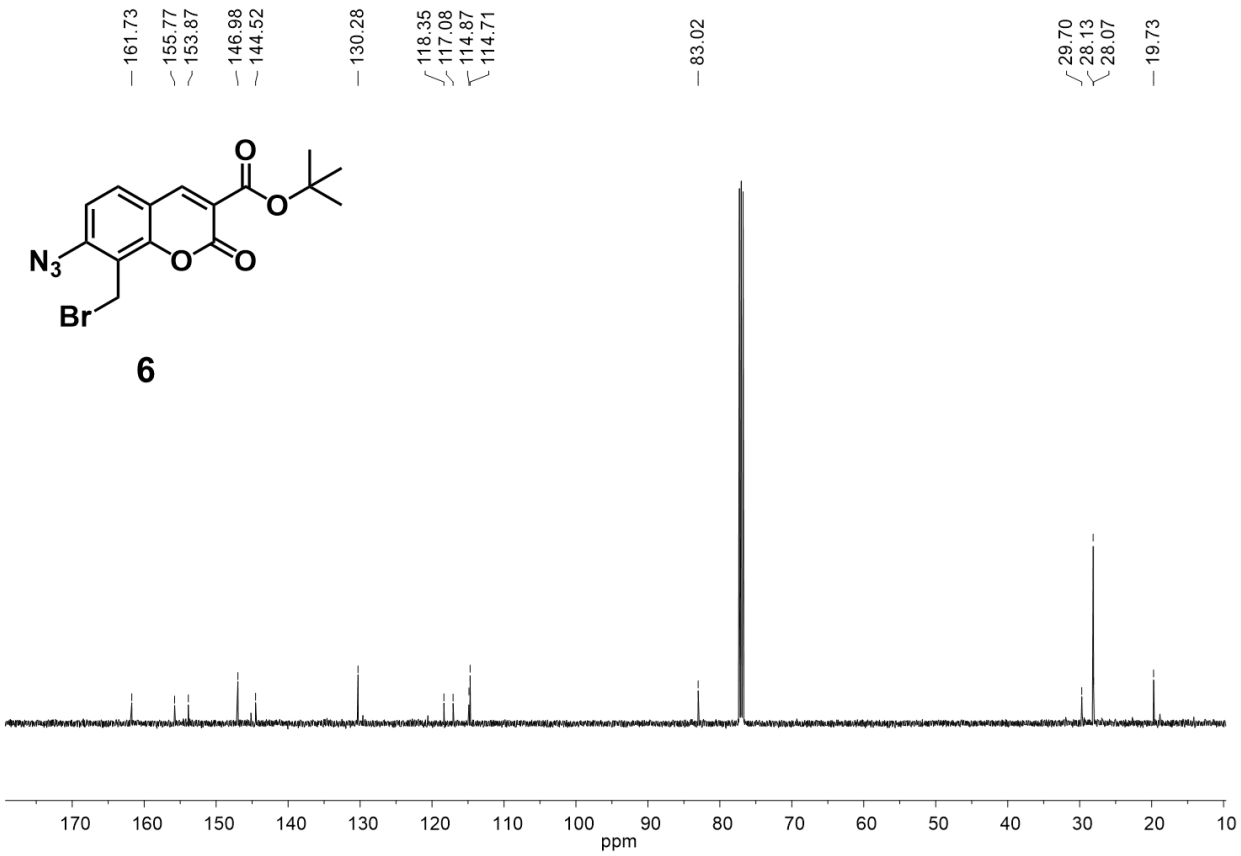


Fig. S17 ^{13}C NMR of compound **6** in $CDCl_3$.

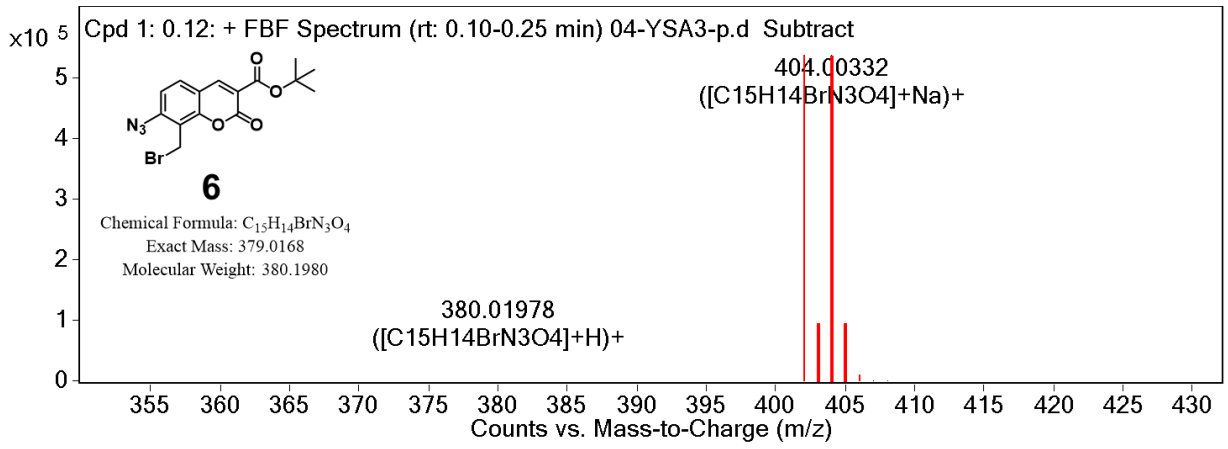


Fig. S18 ESI-MS of compound **6**.

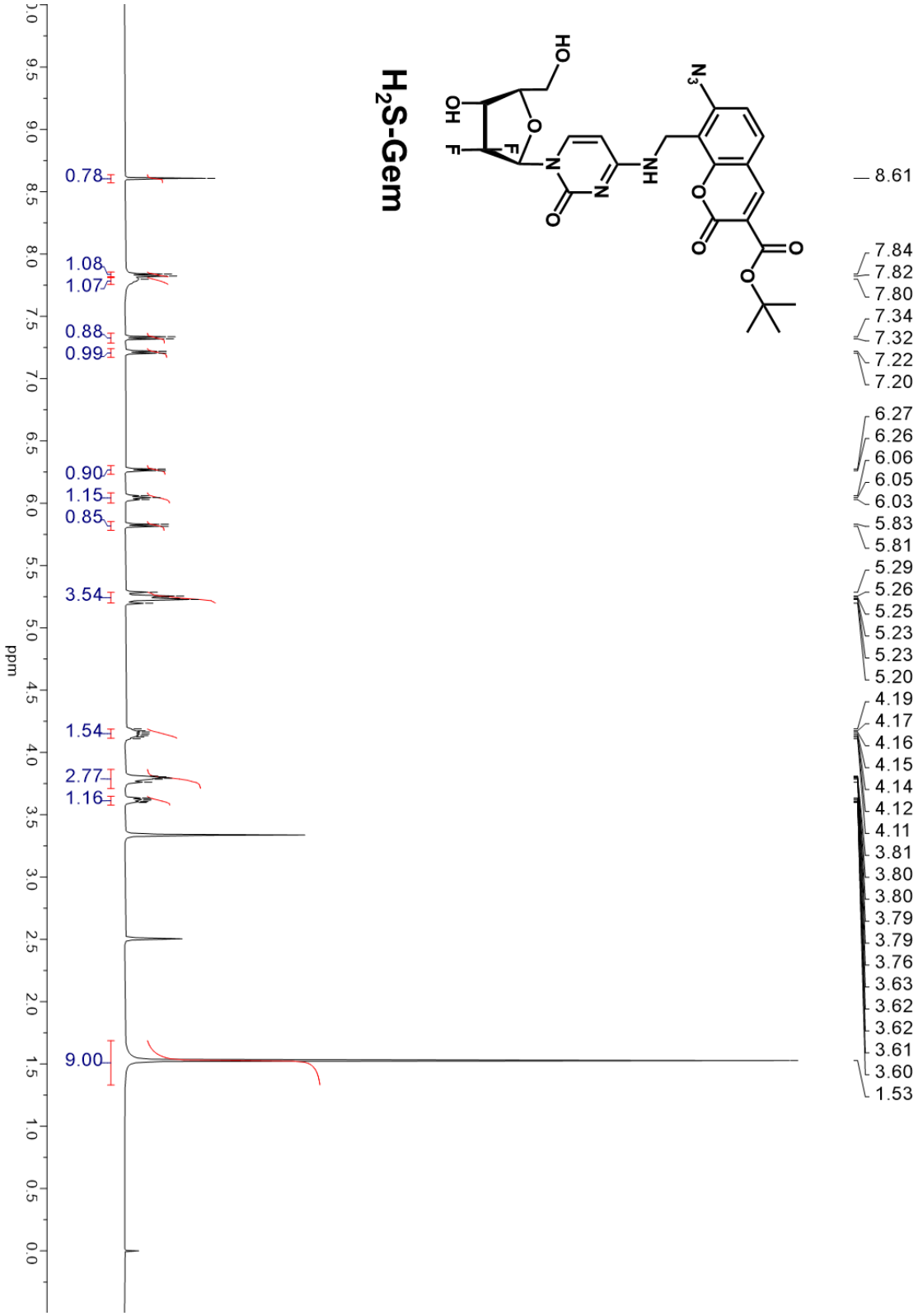


Fig. S19 ^{1}H NMR of **H$_2$S-Gem** in DMSO-*d$_6$*.

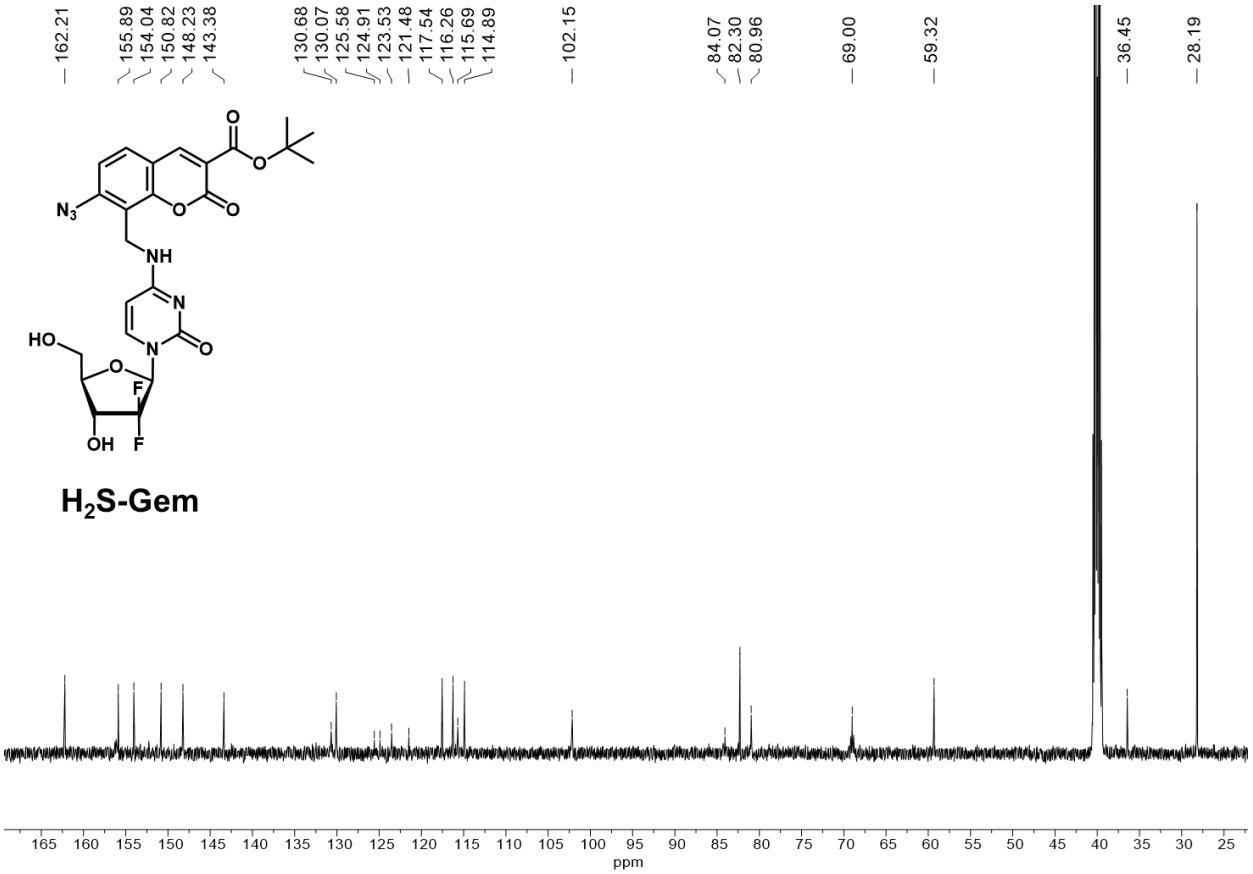


Fig. S20 ^{13}C NMR of **H$_2$S-Gem** in DMSO-*d*$_6$.

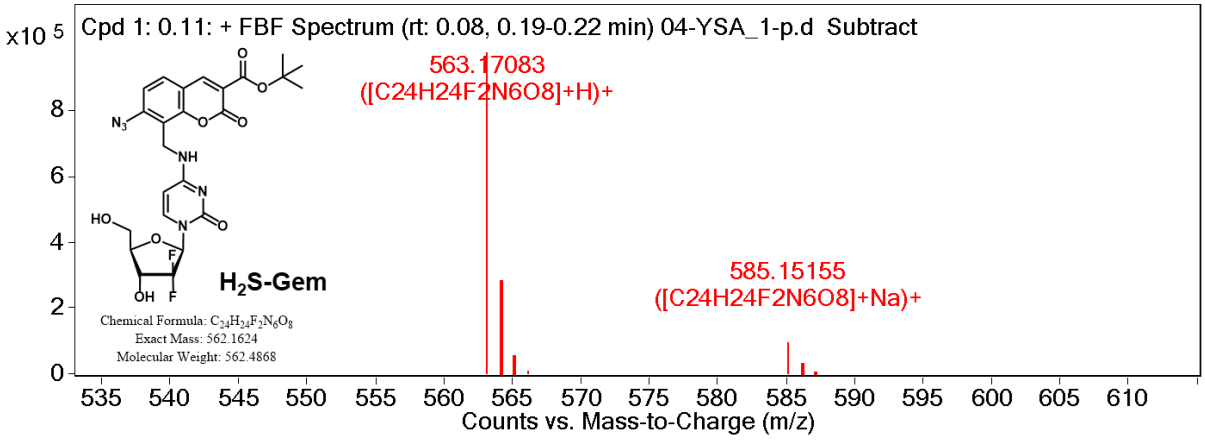


Fig. S21 ESI-MS of compound **H$_2$S-Gem**.

Conditions:

Column: KINETEX C-18,2.1x50mm,1.7µm.
Mobile Phase: A-0.1%FA in ACN B-0.1%FA in WATER
T/%B(min):0/100,1.5/100,2.5/55,3.8/5,5.8/5,6/100
Flow:0.4mL/min, Diluent: ACN

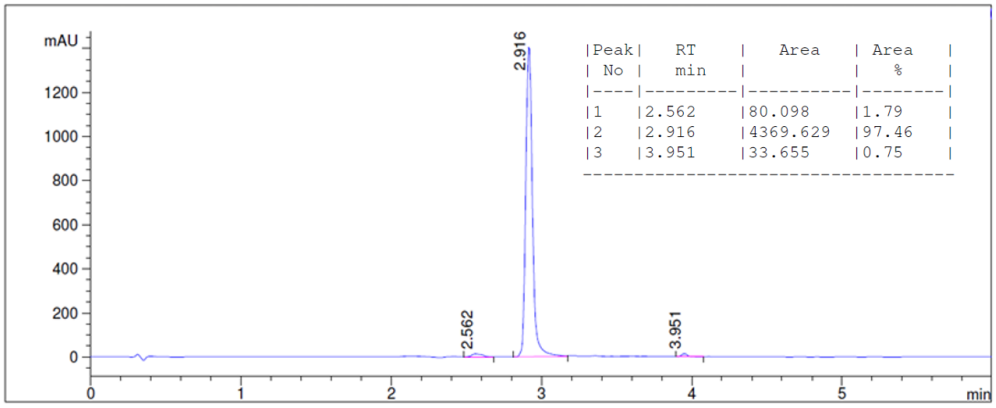


Fig. S22 HPLC chromatogram of **H_2S-Gem**.

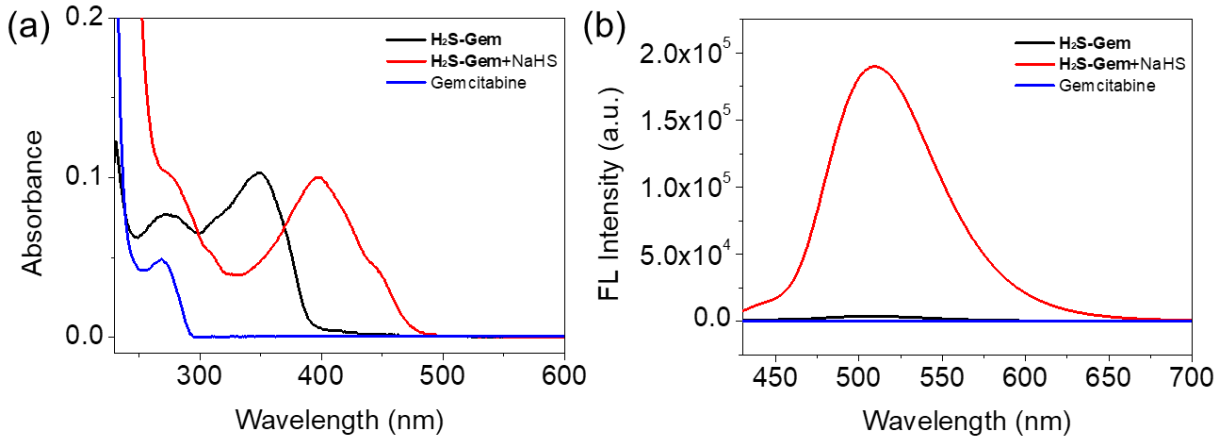


Fig. S23 (a) UV-absorption and (b) fluorescence spectra of **H_2S-Gem** (5 µM), **H_2S-Gem**+NaHS (200 µM) and Gemcitabine (5 µM). All data were obtained in PBS (10 mM, pH 7.4) solution containing 1% (v/v) of DMSO using an excitation at 410 nm.

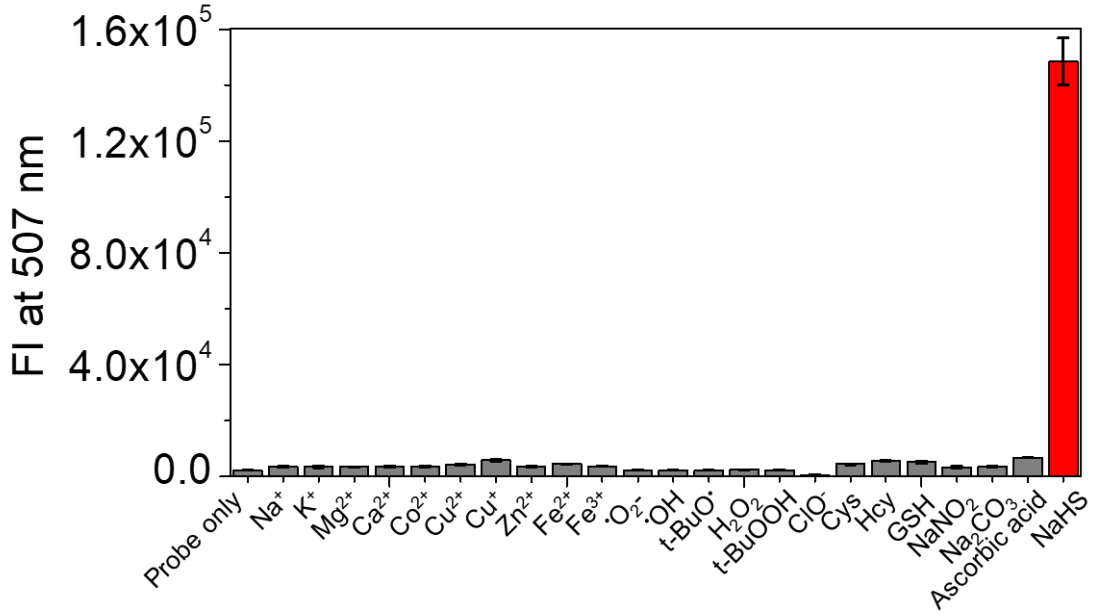


Fig. S24 Fluorescence intensity at 507 nm of **H_2S-Gem** (5 μM) in the presence of various analyte, such as metals (200 μM), ROS (200 μM), GSH (1 mM), Hcy (1 mM), Cys (1mM), $NaNO_2$ (200 μM), Na_2CO_3 (200 μM), ascorbic acid (200 μM) and NaHS (200 μM). All data were obtained in PBS (10 mM, pH 7.4) solution containing 1% (v/v) of DMSO using an excitation at 410 nm after 1 h incubation at 37 ℃.

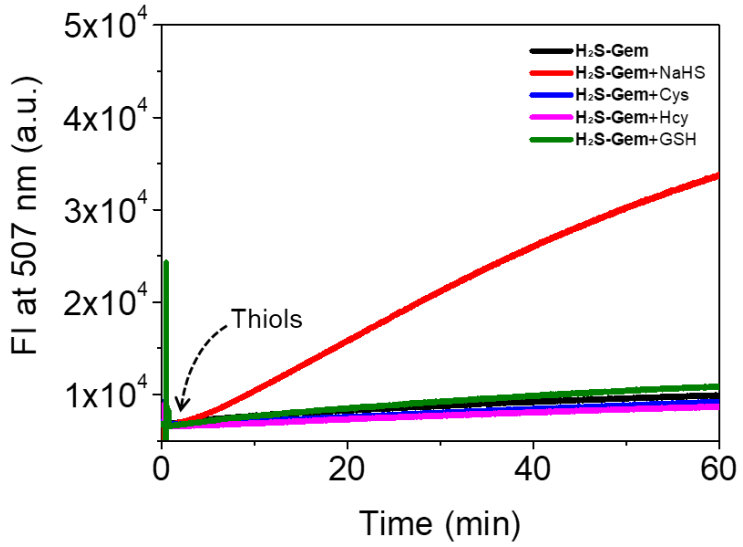


Fig. S25 Time-dependent fluorescence responses of **H_2S-Gem** (5 μM) to NaHS (200 μM), Cys (1 mM), Hcy (1 mM) and GSH (1 mM). All data were obtained in PBS (10 mM, pH 7.4) solution containing 1% (v/v) of DMSO using an excitation at 410 nm.

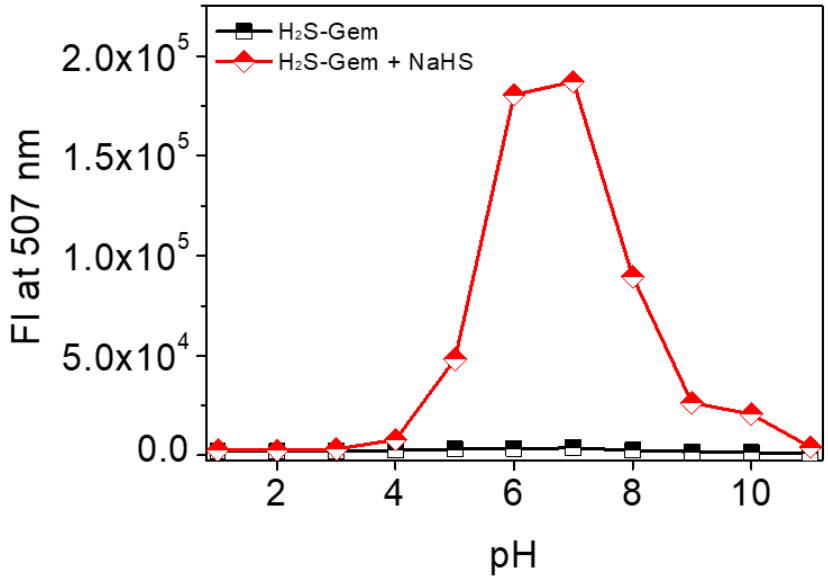


Fig. S26 Fluorescence changes of **H_2S-Gem** (5 μM) with NaHS (200 μM) at different pH solutions. All data were obtained in PBS (10 mM, pH 7.4) solution containing 1% (v/v) of DMSO using an excitation at 410 nm after 1 h incubation at 37 ℃.

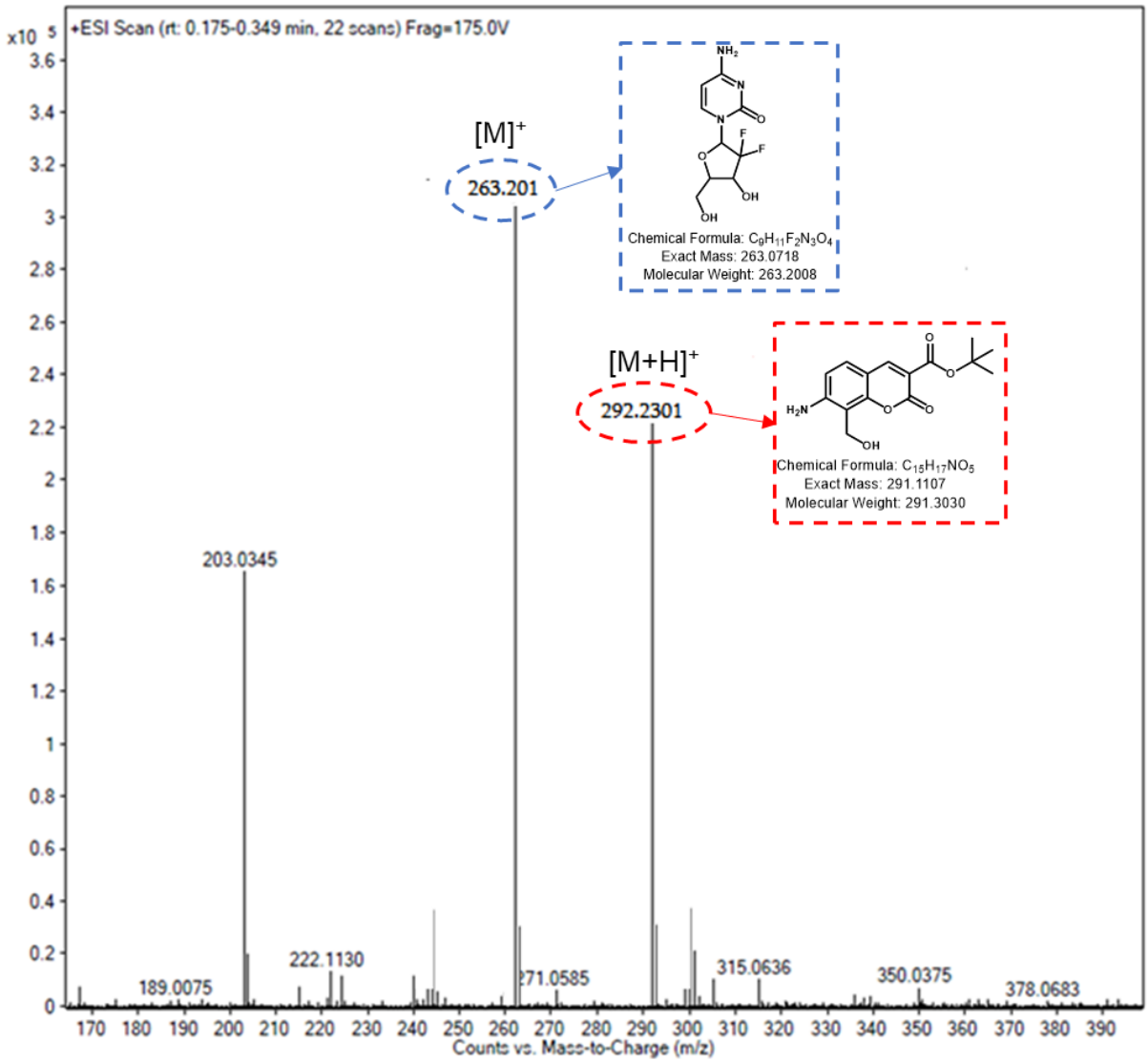


Fig. S27 ESI-MS of H_2S (NaHS) treated **H_2S-Gem**.

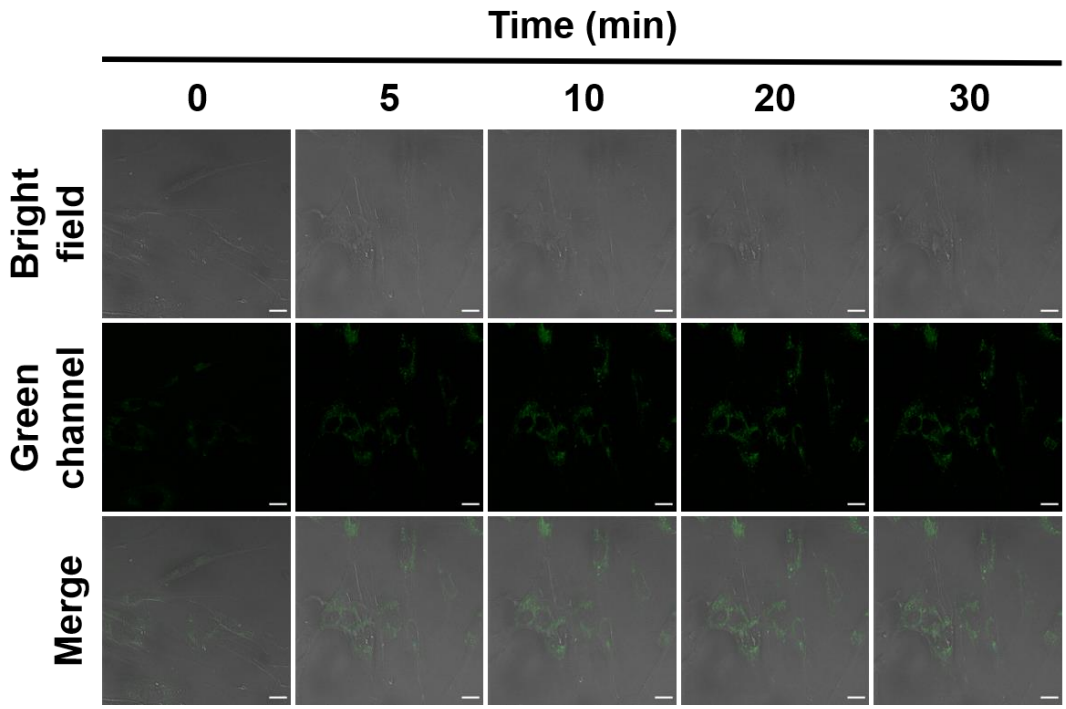


Fig. S28 Confocal microscopy images of **H_2S-Gem** (15 μM) in WI38 cells. Cells were pretreated with **H_2S-Gem** for 1h then incubated with NaHS (600 μM) for 0-30 min.

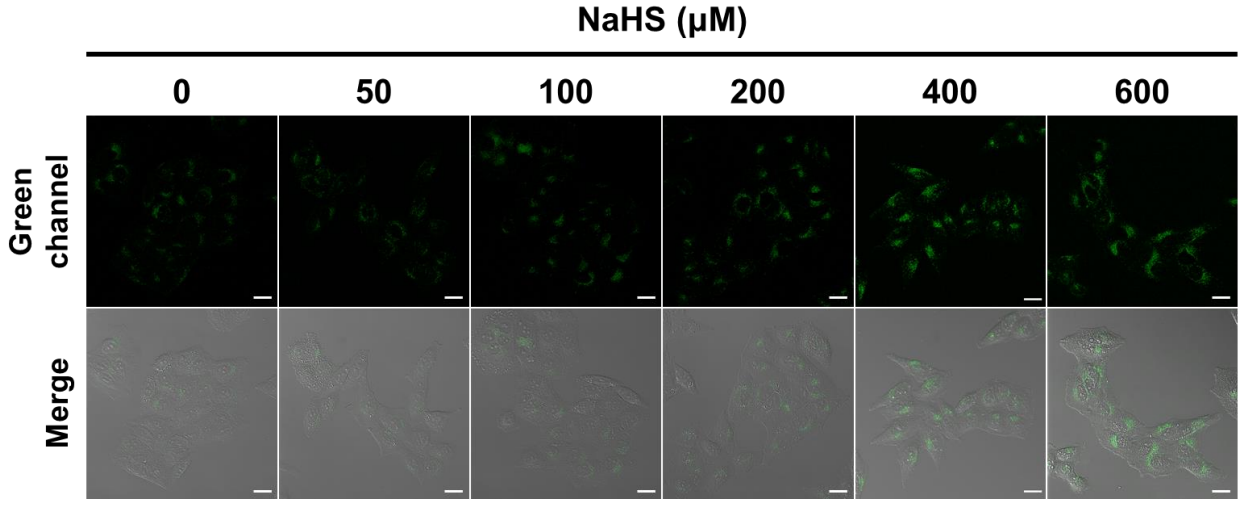


Fig. S29 NaHS concentration-dependent confocal microscopy images. HeLa cells were pretreated with **H_2S-Gem** (15 μM) for 1 h, and then incubated with NaHS (0-600 μM) for 30 min. Emission collected at the 505-600 nm region with excitation set at 405 nm. Scale bar: 20 μm.

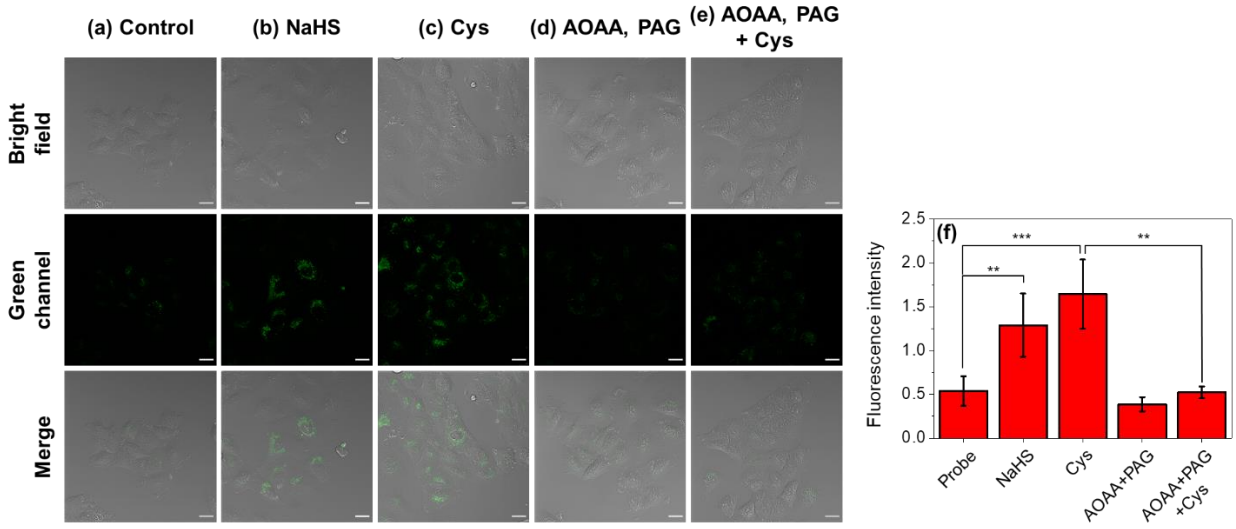


Fig. S30 Confocal microscopy images of **H_2S-Gem** in A549 cells. (a) Cells were incubated with **H_2S-Gem** (15 μM) for 1 h. (b) Cells were pretreated with **H_2S-Gem** for 1h then incubated with NaHS (600 μM) for 30 min. (c) Cells were pretreated with cysteine (200 μM) for 1h then incubated with **H_2S-Gem** for 1 h. (d) Cells were pretreated with AOAA/PAG (2 mM) for 1h then incubated with **H_2S-Gem** for 1 h. (e) Cells were pretreated with AOAA/PAG (2 mM) and cysteine (200 μM) for 1h, respectively, then incubated with **H_2S-Gem** for 1 h. All images were obtained by using an excitation laser of 405 nm and band-path filters for 505-600 nm. Scale bar: 20 μm. (b) Quantification of the fluorescence intensities from each cell. Error bars indicate standard deviation (SD). (***$P \leq 0.001$; **$P \leq 0.01$).

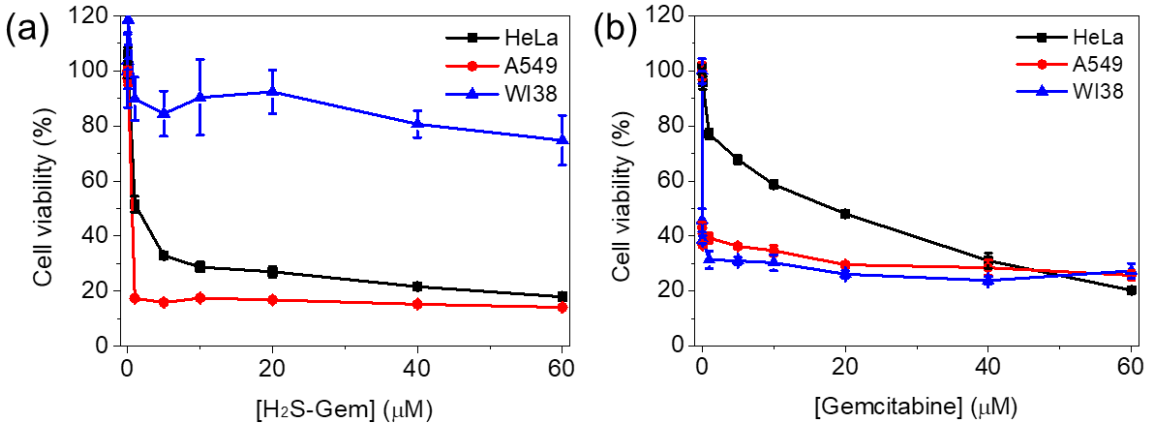


Fig. S31 Cytotoxicity assay of (a) **H_2S-Gem** and (b) gemcitabine for 72 h in various cell lines. Error bars indicate standard deviation (SD, n = 5).

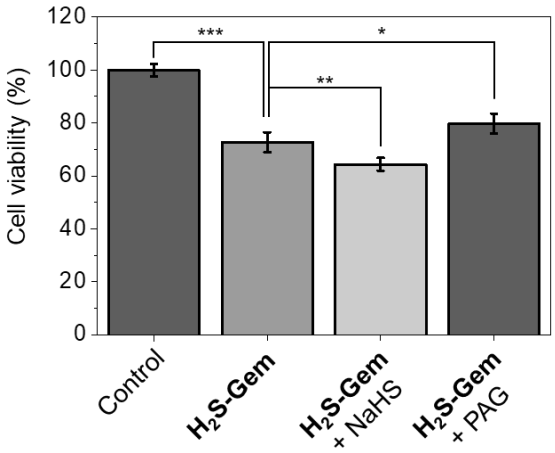


Fig. S32 MTT assay of **H_2S-Gem** in HeLa cells with the presence of NaHS and PAG. Error bars indicate SD. Asterisks indicate statistically significant changes (***$P \leq 0.001$; **$P \leq 0.01$; *$P \leq 0.05$).

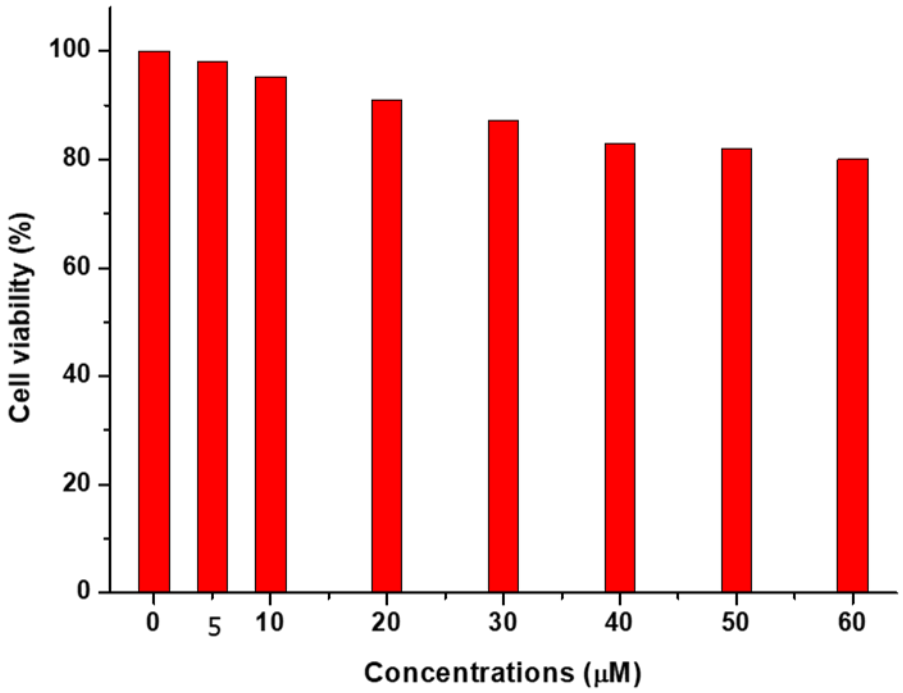


Fig. S33 MTT assay of released fluorophore in HeLa cells after incubation of different doses for 72h.