Supporting Information

Enzyme-responsive hybrid prodrug of nitric oxide and hydrogen sulfide for heart failure therapy

Qi Liu, [‡a] Guangbo Ji, [‡a] Yushu Chu,[a] Tian Hao,[a] Meng Qian[*a] and Qiang Zhao[*a]

[a] State key Laboratory of Medicinal Chemical Biology, Haihe Laboratory of Sustainable Chemical Transformations, Key Laboratory of Bioactive Materials (Ministry of Education), Frontiers Science Center for Cell Responses, College of Life Sciences, Nankai University, Tianjin 300071, China.

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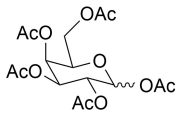
1.General information

All chemicals and reagents were purchased from Sigma Aldrich (China-mainland) and Innochem (China-mainland), and used directly without further purification. Hyaluronic acid hydrogel was synthesized according to the literature[1]. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 400MHz nuclear magnetic spectrometer. Chemical shifts were reported relative to the reference chemical shift of the NMR solvent. The following splitting abbreviations were used: s = singlet, d = doublet, dd = doublet doublet, t = triplet, m = multiplet. High resolution mass spectra (HRMS) were obtained on a Varian QFT-ESI mass spectrometer. HPLC analysis was performed with the Shimadzu LC-20AT system using a Cromasil 5 μm C18 column (250 mm x 4.6 mm). The levels of NO and H_2S in cells were detected by NO fluorescence probe (Beyotime, DAF-FM DA S0019) and H_2S fluorescence probe (maokangbio, WSP-1 MX5301-1MG), respectively. Serum NT-pro BNP and cTnT levels were detected by ELISA kits (Elabscience, E-EL-R0126c E-EL-R0151c). Serum LDH level was detected using microassay activity detection Kit (Solarbio, BC0685). Laser scanning confocal microscopy (Zeiss, LSM710) was used to record cell staining. Echocardiography was recorded using ultra-high resolution small animal ultrasound imaging system (VisualSonics, Vevo 2100). Orthographic microscope system (LeicaDFC420C (CCD)) was used to record histological staining. Immunofluorescence staining was recorded using an orthographic fluorescence microscope (Zeiss, Axio Imager Z1).

2.Synthetic procedure and characterization

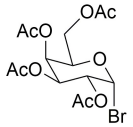
(3*R*,4*S*,5*S*,6*R*)-6-(acetoxymethyl)tetrahydro-2*H*-pyran-2,3,4,5-tetrayl tetraacetate (1):



1

To a solution of D-galactose (2.5 g, 13.88 mmol) in dry DMF (6 mL) was added pyridine (4 mL) and DMAP (339 mg, 2.78 mmol). The resulting solution was cooled to 0°C using an ice-bath, and Ac_2O (1.5 mL) was added dropwise to keep the temperature below 5°C. After addition, the reaction was warmed to rt and stirred overnight. Once the starting material was disappeared, as indicated by TLC, water was added to quench the reaction. The solution was extracted with EA three times and washed with 1 N HCl, saturated $NaHCO_3$ and brine solution. The solvent was dried with Na_2SO_4 and removed in vacuo, and the crude residue was purified by chromatography (PE:EA=4:1) to give the compound 1 (4.3 g) as white solid in 80% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.38 (s, 1H), 5.50 (s, 1H), 5.34 (s, 2H), 4.33 (d, *J* = 6.6 Hz, 1H), 4.10 (dd, *J* = 6.6, 4.6 Hz, 2H), 2.16 (s, 6H), 2.04 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.37, 170.15, 169.89, 168.93, 89.75, 68.79, 67.45, 67.39, 66.47, 61.27, 20.90, 20.68, 20.65, 20.62, 20.56.

(2*R*,3*S*,4*S*,5*R*,6*R*)-2-(acetoxymethyl)-6-bromotetrahydro-2*H*-pyran-3,4,5-triyl triacetate (2):

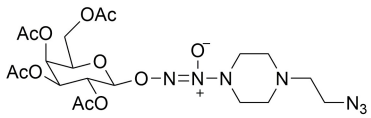


2

Compound 1 (2 g, 5.1 mmol) was dissolved in anhydrous dichloromethane (3.5 mL). The resulting solution was cooled to 0°C using an ice-bath, and 33% HBr in acetic acid (4.5 mL) was added into the reaction to afford an orange solution. After the mixture was stirred for 1 h, the solution was then extracted from a saturated solution of ice-cold $NaHCO_3$, leading to effervescence and a discoloration of the organic phase. The organic phase was then washed with brine solution, dried with Na_2SO_4 and removed in vacuo. The crude product was purified by chromatography (PE:EA= 5:1) to give the desired compound 2 (1.28 g) as pale syrup in 61% yield. This labile intermediate was directly used in next step. ^{1}H NMR

(400 MHz, $CDCl_3$) δ 6.70 (d, *J* = 3.8 Hz, 1H), 5.52 (d, *J* = 2.8 Hz, 1H), 5.41 (dd, *J* = 10.6, 3.2 Hz, 1H), 5.05 (dd, *J* = 10.6, 3.8 Hz, 1H), 4.49 (t, *J* = 6.4 Hz, 1H), 4.15 (m, 2H), 2.15 (s, 3H), 2.11 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.32, 170.07, 169.90, 169.76, 88.16, 71.09, 68.02, 67.80, 67.01, 60.85, 20.76, 20.65, 20.60, 20.57.

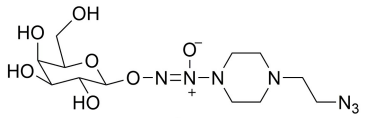
1-(4-(2-azidoethyl)piperazin-1-yl)-2-(((2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-triacetoxy-6-(acetoxymethyl) tetrahydro-2*H*-pyran-2-yl)oxy)diazene 1-oxide (3):



3

PIPERA/NONOate was synthesized according to the reported procedure[2]. To the solution of compound 2 (1 g, 2.56 mmol) in dry DMF (20 mL) was added PIPERA/NONOate (1.21g, 5.12 mmol) under N_2 atmosphere. The resulting mixture was stirred for 24 h at rt. After the reaction was completed, the reaction mixture was quenched by water and extracted three times with EA. The combined organic phase was washed with water, saturated brine solution and dried over Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by chromatography (PE:EA = 1:1), to give the desired compound 3 (1.19 g) as pale solid with the yield of 85%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37 (dd, *J* = 10.4, 8.3 Hz, 1H), 5.29 (d, *J* = 3.4 Hz, 1H), 5.09 (d, *J* = 8.3 Hz, 1H), 4.99 (dd, *J* = 10.3, 3.4 Hz, 1H), 4.04 (d, *J* = 6.6 Hz, 2H), 3.95 (t, *J* = 6.6 Hz, 1H), 3.49 - 3.33 (m, 4H), 3.24 (t, *J* = 5.9 Hz, 2H), 2.56 (dt, *J* = 17.9, 5.4 Hz, 6H), 2.03 (s, 3H), 1.92 (s, 6H), 1.87 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.22, 170.04, 169.88, 168.93, 100.86, 71.35, 70.87, 66.80, 66.70, 61.10, 56.35, 51.38, 50.69, 48.06, 20.59, 20.52, 20.45.

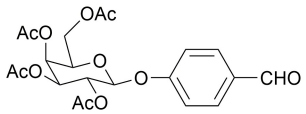
1-(4-(2-azidoethyl)piperazin-1-yl)-2-(((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2*H*-pyran-2-yl)oxy)diazene 1-oxide (4) (NO donor):



4

To the solution of compound 3 (1.19 mg, 2.18 mmol) in dry methanol (10 mL) was added catalytic amount of MeONa. After the reaction was completed, the solvent was removed under reduced pressure. The obtained crude product was purified by chromatography using 10% methanol in DCM as eluent to give the desired compound 4 (578 mg) as white solid in 70% yield. ^{1}H NMR (400 MHz, D_2O) δ 5.25 (d, *J* = 8.1 Hz, 1H), 4.03 (d, *J* = 3.3 Hz, 1H), 3.88 (td, *J* = 8.2, 3.0 Hz, 2H), 3.84 - 3.75 (m, 3H), 3.60 (q, *J* = 6.1, 5.0 Hz, 6H), 2.84 (t, *J* = 5.1 Hz, 4H), 2.74 (t, *J* = 6.1 Hz, 2H). ^{13}C NMR (101 MHz, D_2O) δ 103.67, 76.12, 72.75, 68.83, 68.42, 60.82, 55.17, 50.78, 50.23, 47.67.

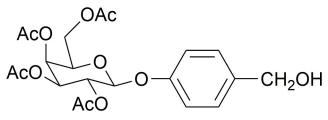
(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-formylphenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (5):



5

A round-bottom flask was charged with compound 2 (3 g, 7.69 mmol), *p*-hydroxybenzaldehyde (1.76 g, 14.36 mmol), caesium carbonate (5.66 g, 17.37 mmol) and anhydrous acetonitrile (50 mL) at rt. After the mixture was stirred overnight, solid impurities were removed by filtration and the solvent was removed under reduced pressure. The obtained crude product was purified by chromatography (PE:EA = 2:1) to give the desired compound 5 (3.02 g) as pale syrup in 87% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.93 (s, 1H), 7.86 (d, *J* = 8.6Hz, 2H), 7.12 (d, *J* = 8.6 Hz, 2H), 5.53 (dd, *J* = 10.4, 7.9 Hz, 1H), 5.48 (d, *J* = 3.2 Hz, 1H), 5.17 (d, *J* = 7.9 Hz, 1H), 5.14 (dd, *J* = 10.4, 3.2 Hz, 1H), 4.24 (dd, *J* = 11.0, 6.9 Hz, 1H), 4.16 - 4.09 (m, 2H), 2.19 (s, 3H), 2.07 (s, 6H), 2.02 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.78, 170.36, 170.20, 170.09, 169.35, 161.34, 131.85, 116.79, 98.60, 71.36, 70.70, 68.46, 66.82, 61.40, 20.71, 20.66, 20.64, 20.57.

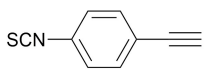
(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-(hydroxymethyl)phenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (6):



6

$NaBH_4$ (417 mg, 11.02 mmol) was added in portions to a stirred solution of compound 5 (3.02 g, 6.68 mmol) in dry methanol (30 mL). Once the starting material was disappeared, as indicated by TLC, the solvent was removed under reduced pressure. The obtained crude product was purified by chromatography using 5% methanol in DCM as eluent to give the desired compound 6 (2.88 g) as white solid in 95% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.29 (d, *J* = 8.4 Hz, 2H), 6.99 (d, *J* = 8.4 Hz, 2H), 5.45 (s, 1H), 5.34 (dd, *J* = 10.2, 7.8 Hz, 1H), 5.29 – 5.21 (m, 2H), 4.54 (s, 2H), 4.29 (t, *J* = 6.0 Hz, 1H), 4.17 (d, *J* = 5.4 Hz, 2H), 2.17 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.96 (s, 3H). ^{13}C NMR (101 MHz, MeOD) δ 170.66, 170.62, 170.09, 169.95, 156.30, 136.15, 128.10, 116.35, 98.94, 70.90, 70.77, 68.91, 67.41, 63.29, 61.27, 19.27, 19.23, 19.14, 19.13.

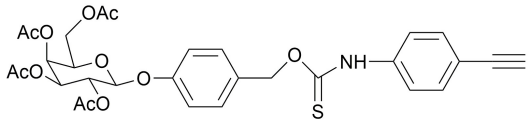
1-ethynyl-4-isothiocyanatobenzene (7):



7

To a mixture of 4-ethynylbenzenamine (2.0 g, 17.1 mmol) and triethylamine (10 mL, 71.6 mmol) in 30 mL CH_2Cl_2. A solution of thiophosgene (3 mL, 38.6 mmol) in 20 mL $CHCl_2$ was added dropwise at 0°C for 1 h, and then warmed to rt. The above mixture was extracted with CH_2Cl_2. The organic layers were washed with H_2O, dried with Na_2SO_4 and concentrated to dryness to give a white residue. This was purified by chromatography on silica gel using PE:EA = 10:1 as eluent. Compound 5 was then obtained as pale white solid in 90% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 8.2 Hz, 2H), 7.17 (d, *J* = 8.2 Hz, 2H), 3.16 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 137.09, 133.40, 131.70, 125.78, 121.18, 82.54, 79.22.

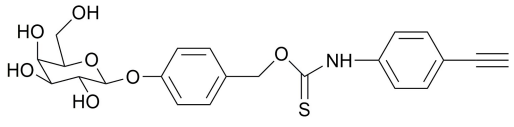
(2*R*,3*S*,4*S*,5*R*,6*S*)-2-(acetoxymethyl)-6-(4-((((4-ethynylphenyl)carbamothioyl)oxy)methyl) phenoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (8):



8

To a solution of compound 6 (3 g, 6.6 mmol) and 7 (1.16 g, 7.26 mmol) in anhydrous tetrahydrofuran (100 mL) being cooled to 0°C was added the DBU (1.2 mL, 7.26 mmol). Water was added to quench the reaction after 24 h, the reaction mixture was extracted with EA three times, and the combined organic phase was washed with water twice, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by chromatography to give the desired compound 8 as white solid (2.05 g) in 58% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63 - 7.22 (m, 5H), 7.01 (d, J = 8.5 Hz, 3H), 5.54 (d, J = 16.8 Hz, 2H), 5.47 (d, J = 4.1 Hz, 1H), 5.12 (d, J = 3.4 Hz, 1H), 5.07 (d, J = 7.9 Hz, 1H), 4.31-4.12 (m, 2H), 4.08 (t, J = 6.6 Hz, 1H), 3.08 (s, 1H), 2.18 (s, 3H), 2.06 (d, J = 6.5 Hz, 6H), 2.02 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.41, 170.28, 170.18, 169.43, 157.13, 132.95, 130.25, 117.03, 99.47, 82.97, 77.65, 77.29, 71.13, 70.84, 68.63, 66.90, 61.40, 20.78, 20.70, 20.63. HRMS (ESI) calculated for $C_{30}H_{31}NO_{11}S$ $[M+Na]^+$: 636.1510, found: 636.1513.

***O*-(4-(((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)benzyl) (4-ethynylphenyl)carbamothioate (9) (H_2S donor):**

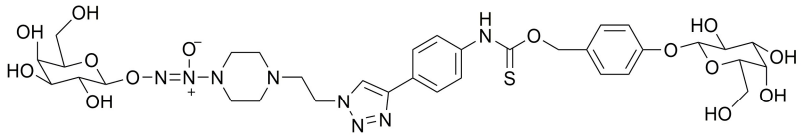


9

To the solution of compound 8 (2.05 g, 3.42 mmol) in dry methanol (10 mL) was added catalytic amount of MeONa. After the reaction was completed, the solvent was removed under reduced pressure. The obtained crude product was purified by chromatography using 15%

methanol in DCM as eluent to give the desired compound 9 (944.59 mg) as yellow solid in 62% yield. ^{1}H NMR (400 MHz, MeOD) δ 7.37 (d, J = 8.4 Hz, 6H), 7.15 - 7.03 (m, 2H), 5.57 - 5.42 (m, 2H), 4.89 (d, J = 7.7 Hz, 1H), 3.90 (d, J = 3.5 Hz, 1H), 3.84 - 3.74 (m, 3H), 3.71 - 3.66 (m, 1H), 3.60 (dd, J = 3.5, 1.3 Hz, 1H), 3.43 (s, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.52, 158.03, 132.62, 130.55, 122.11, 116.70, 101.32, 83.71, 81.00, 75.98, 73.80, 70.75, 68.58, 60.83, 56.50. HRMS (ESI) calculated for $C_{22}H_{23}NO_7S$ [M-H]$^-$: 444.1122, found: 444.1125.

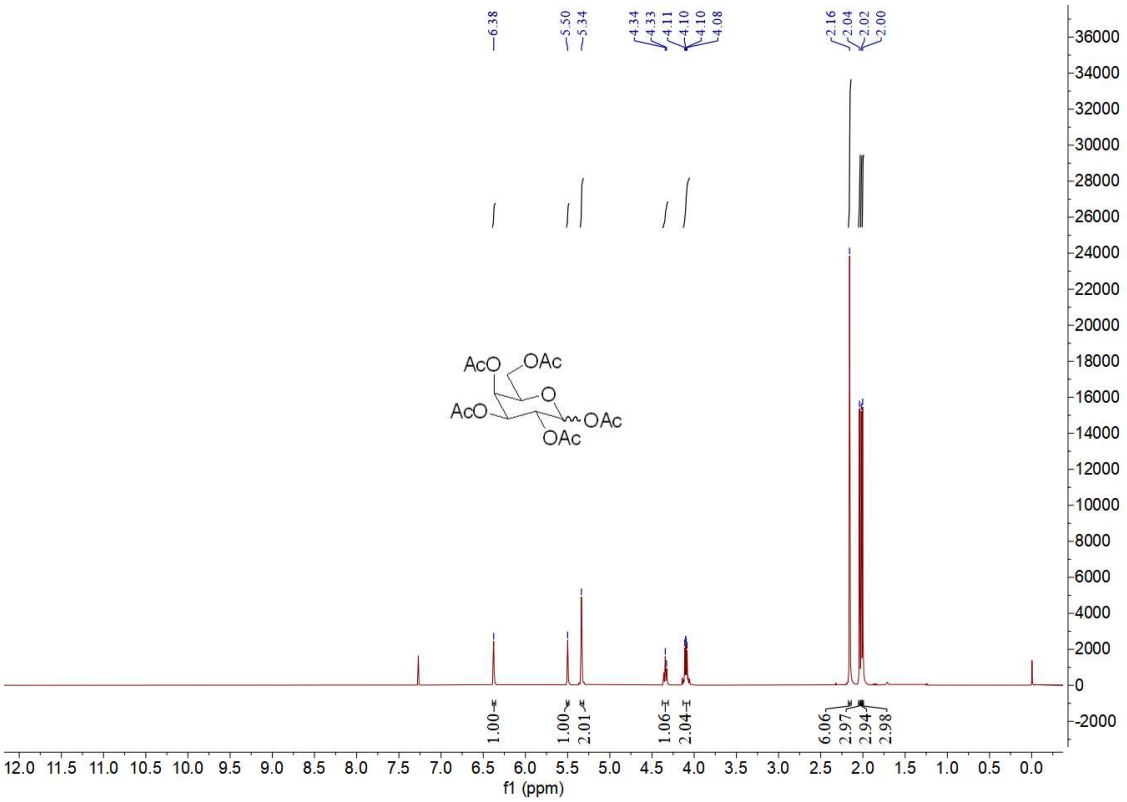
2-(((2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)-1-(4-(2-(4-(4-((((4-(((2*R*,3*S*,4*R*,5*S*,6*S*)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)benzyl)oxy)carbonothioyl)amino)phenyl)-1H-1,2,3-triazol-1-yl)ethyl)piperazin-1-yl)diazene 1-oxide (10) (NO-H_2S donor):

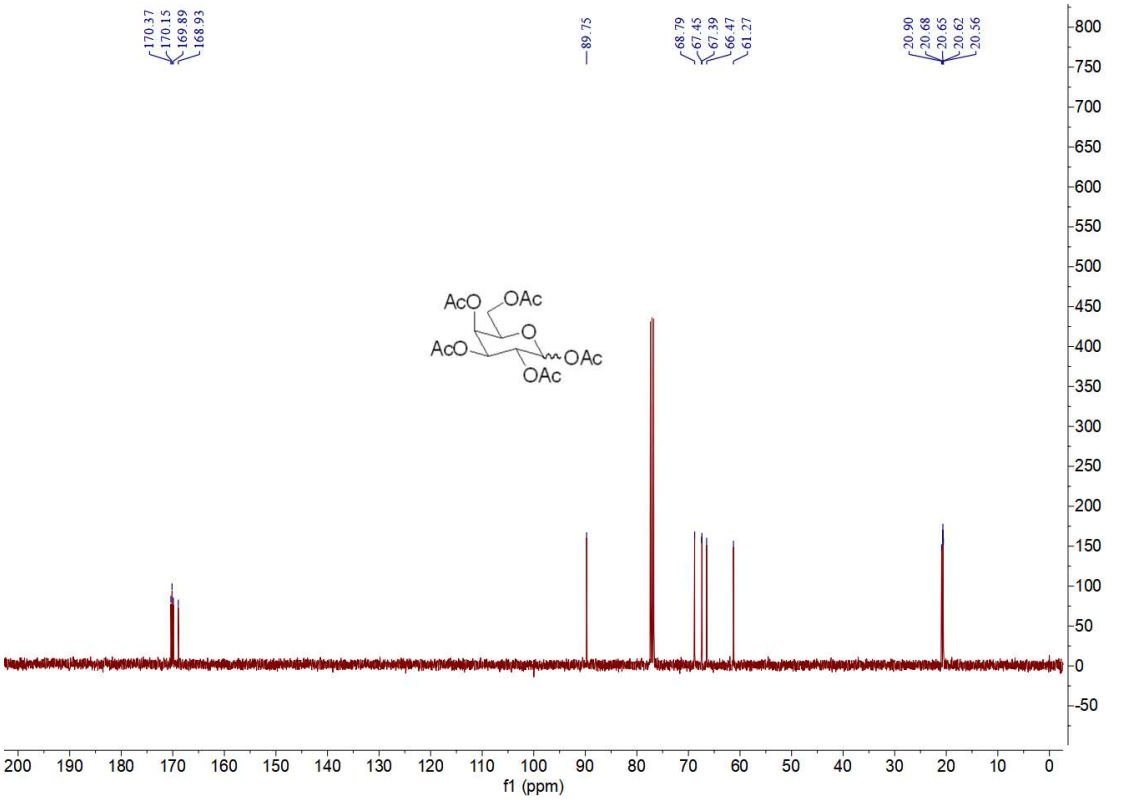


10

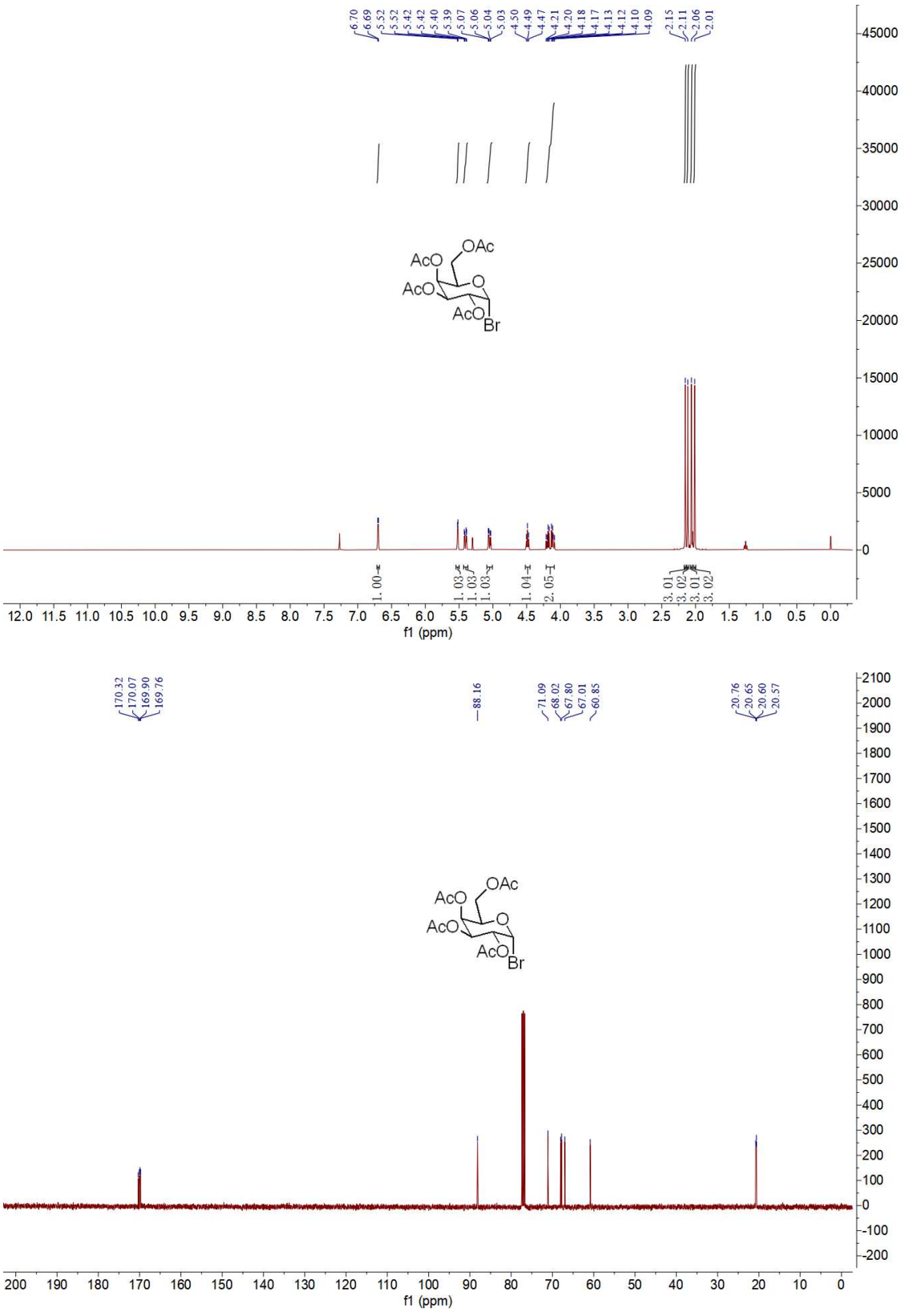
To the solution of compound 4 (588 mg, 1.32 mmol) and 9 (500 mg, 1.32 mmol) in the mixed solvents (5 mL, IPA:DCM:water = 1:1:1) was added sodium ascorbate (94 mg, 0.79 mmol) and $CuSO_4 \cdot 5H_2O$ (65 mg, 0.26 mmol). The resulting mixture was stirred at rt for 24 h. Once the reaction was completed, the solvent was removed under reduced pressure. The crude product was purified by chromatography eluting with 20% methanol in DCM to give compound 10 as a white solid in 42% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.52 (s, 1H), 8.37 (s, 1H), 7.63 (s, 2H), 7.34 (d, *J* = 8.2 Hz, 2H), 7.00 (d, *J* = 8.3 Hz, 3H), 6.74 (s, 2H), 5.15 (d, *J* = 38.9 Hz, 4H), 4.84 (dd, *J* = 13.1, 7.9 Hz, 4H), 4.69 (s, 2H), 4.50 (t, *J* = 6.3 Hz, 2H), 3.68 (dd, *J* = 15.5, 3.2 Hz, 2H), 3.55 (td, *J* = 10.9, 9.6, 7.0 Hz, 4H), 3.49 (d, *J* = 5.5 Hz, 2H), 2.84 (s, 2H), 2.64 (s, 4H). ^{13}C NMR (101 MHz, D_2O) δ 175.72, 171.13, 168.49, 156.48, 152.18, 147.73, 131.89, 129.57, 126.38, 123.81, 123.59, 121.69, 116.50, 104.54, 101.15, 76.22, 75.60, 75.21, 73.81, 73.27, 71.65, 70.98, 69.36, 68.88, 64.89, 63.37, 60.90, 57.45, 55.70, 51.22, 50.87, 50.63, 50.25. HRMS (ESI) calculated for $C_{34}H_{46}N_8O_{14}S$ [M+Na]$^+$: 845.2752, found: 845.2750.

3.NMR spectra of the compounds 1-10 and HRMS of the compounds 8-10

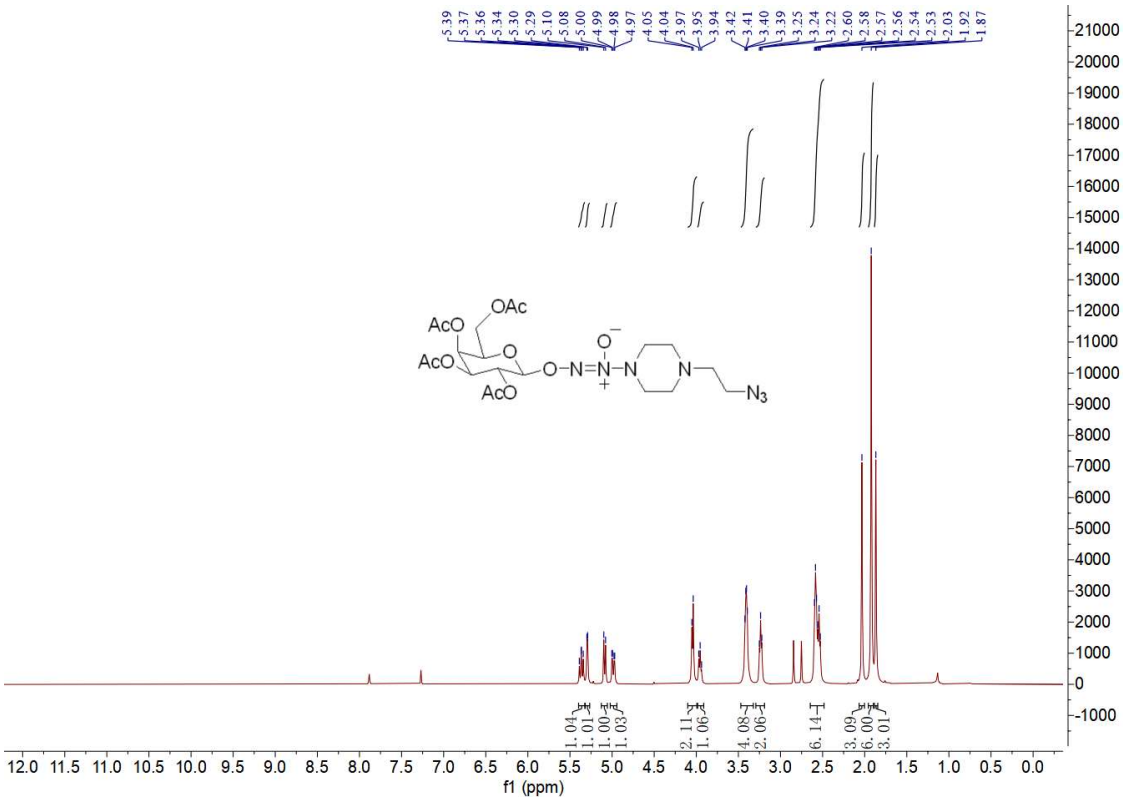


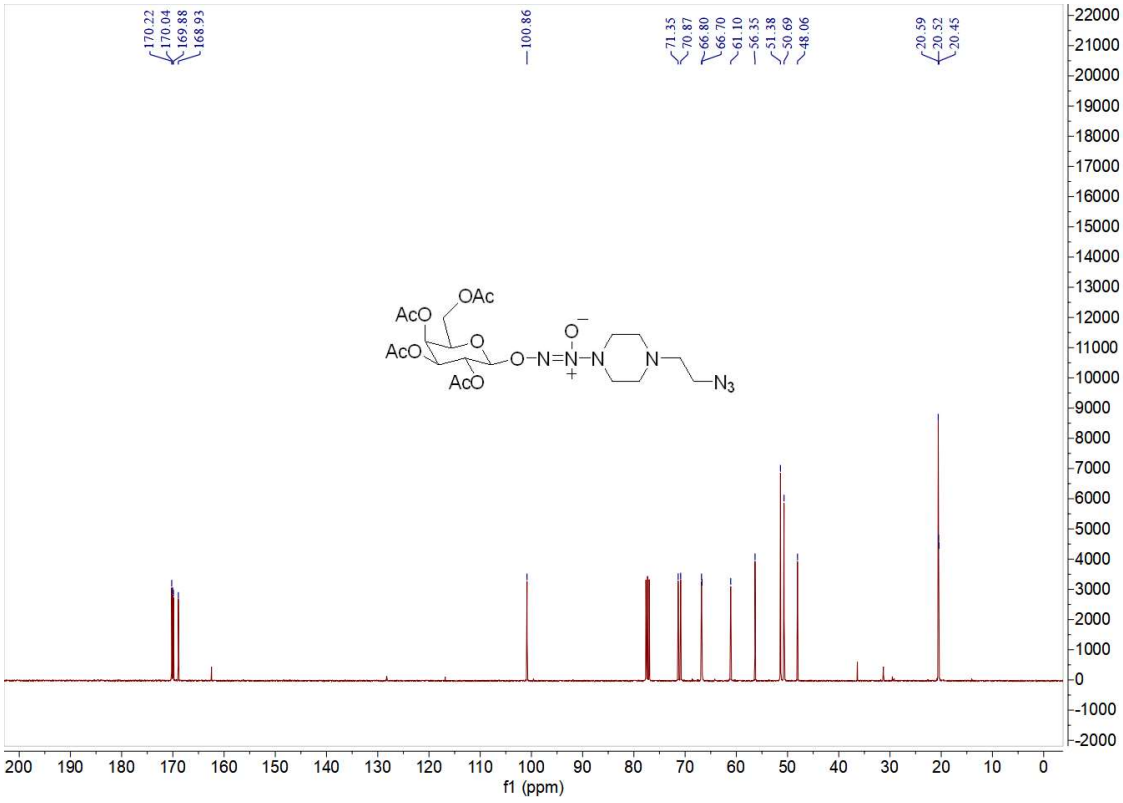


^{1}H NMR and ^{13}C NMR spectra of compound 1

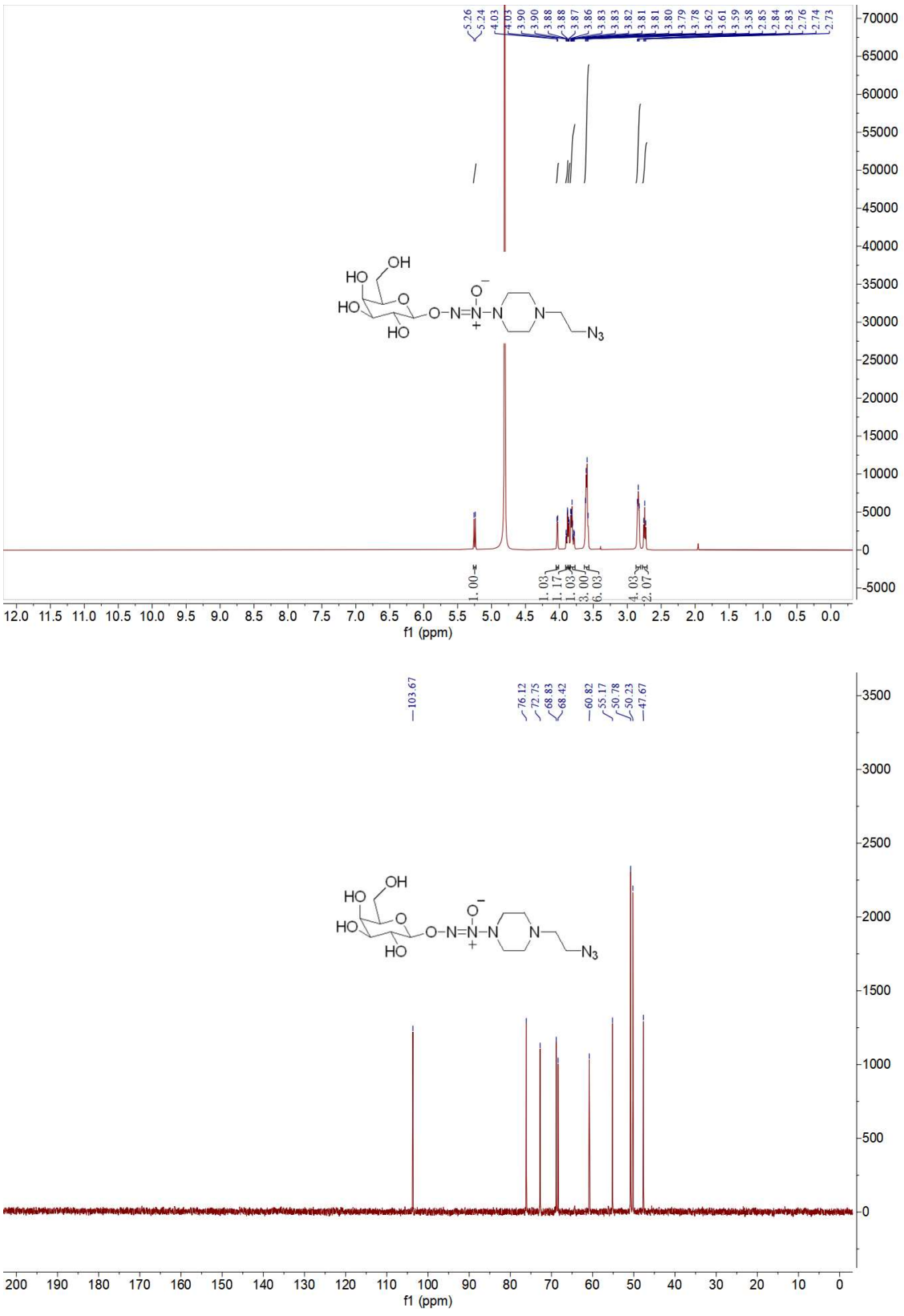


^{1}H NMR and ^{13}C NMR spectra of compound 2

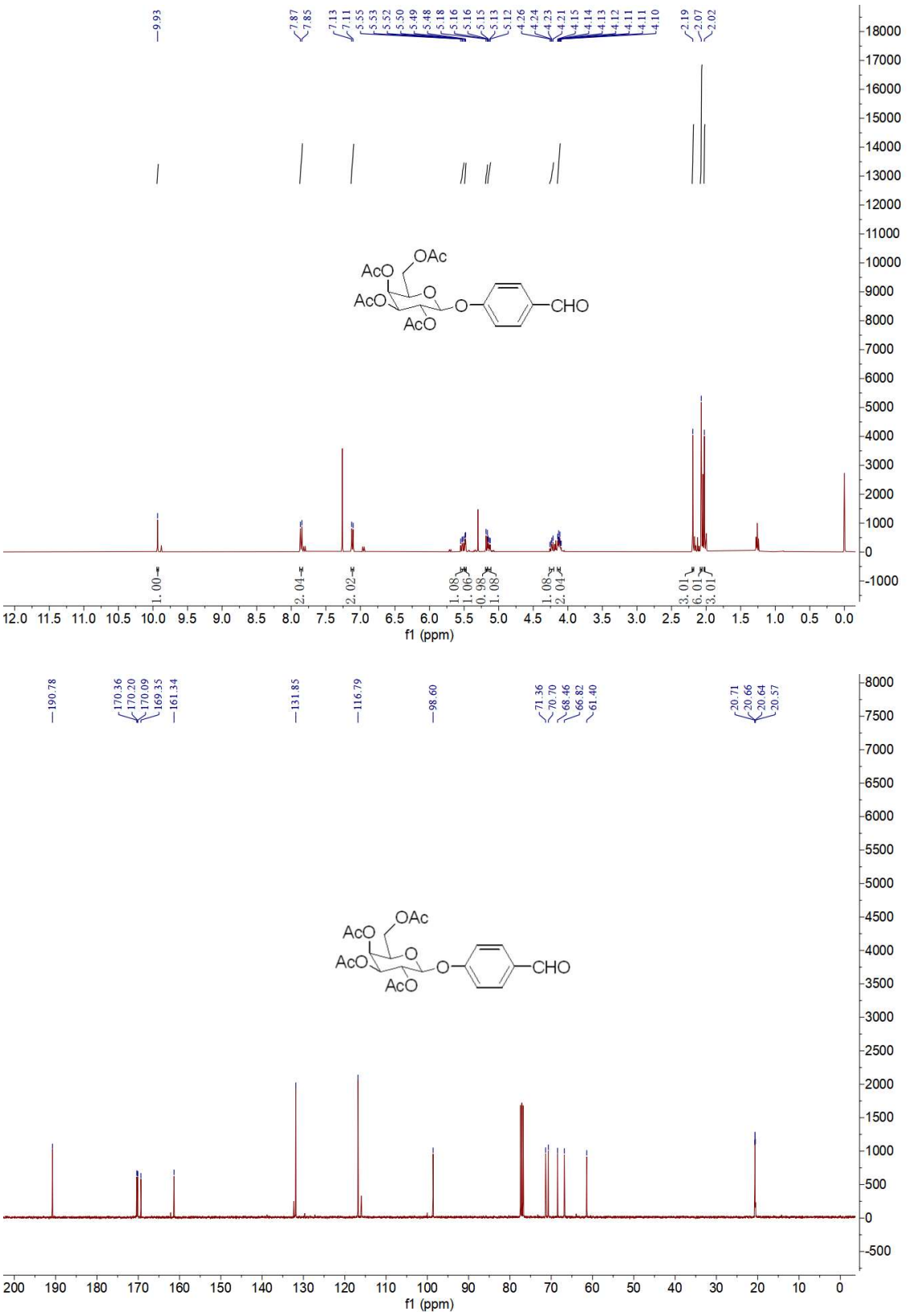




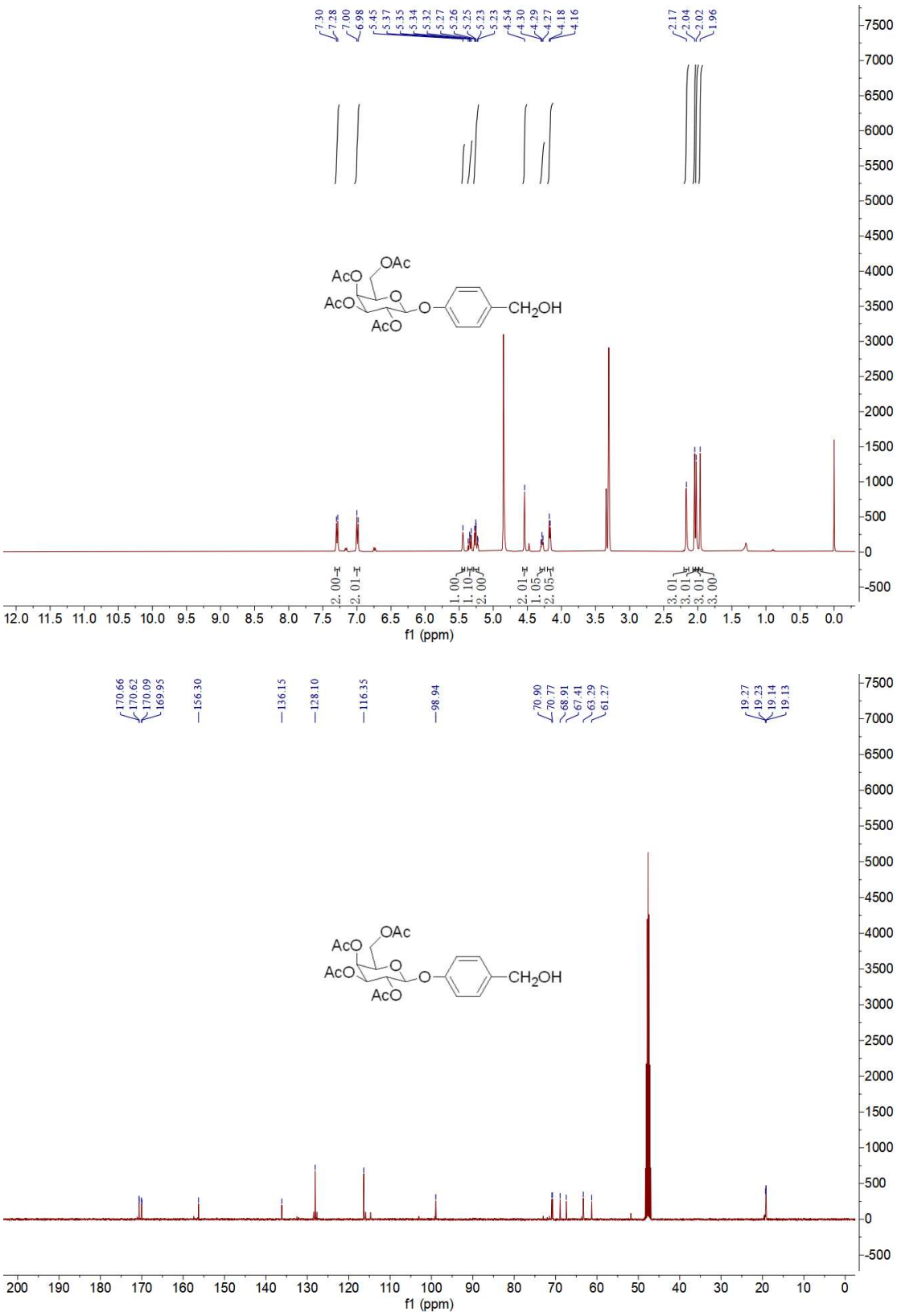
^{1}H NMR and ^{13}C NMR spectra of compound 3



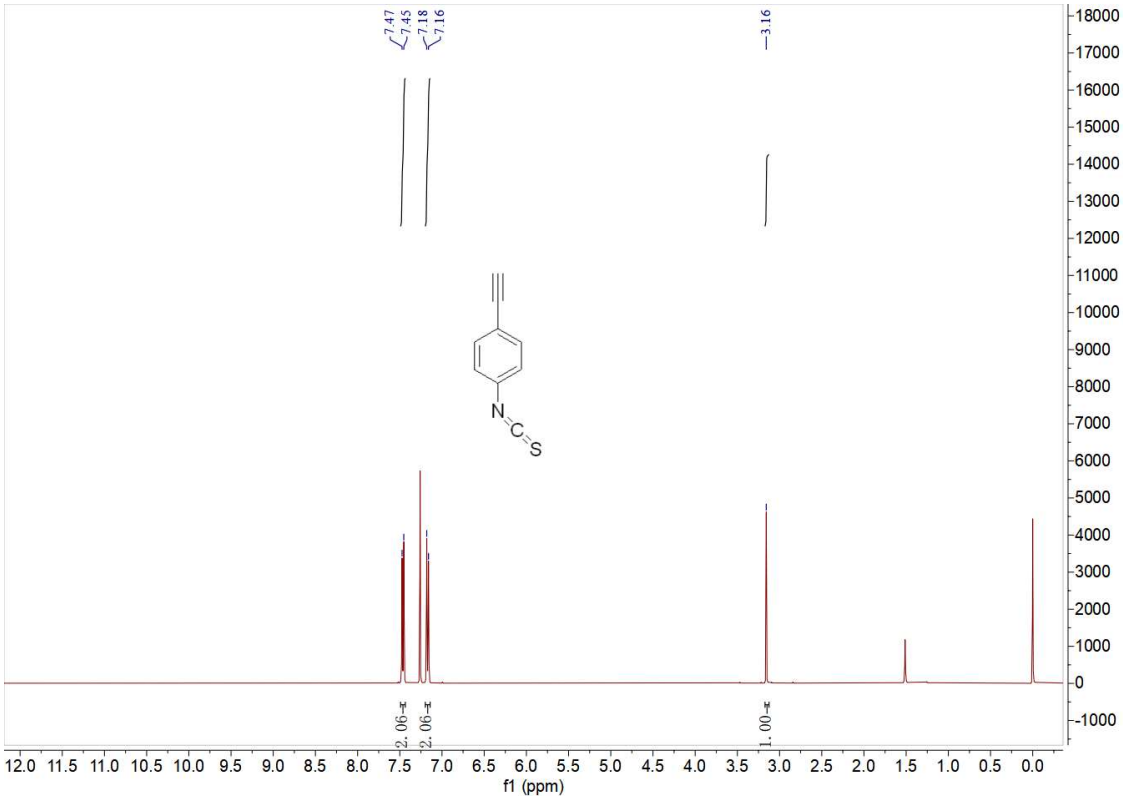
^{1}H NMR and ^{13}C NMR spectra of compound 4

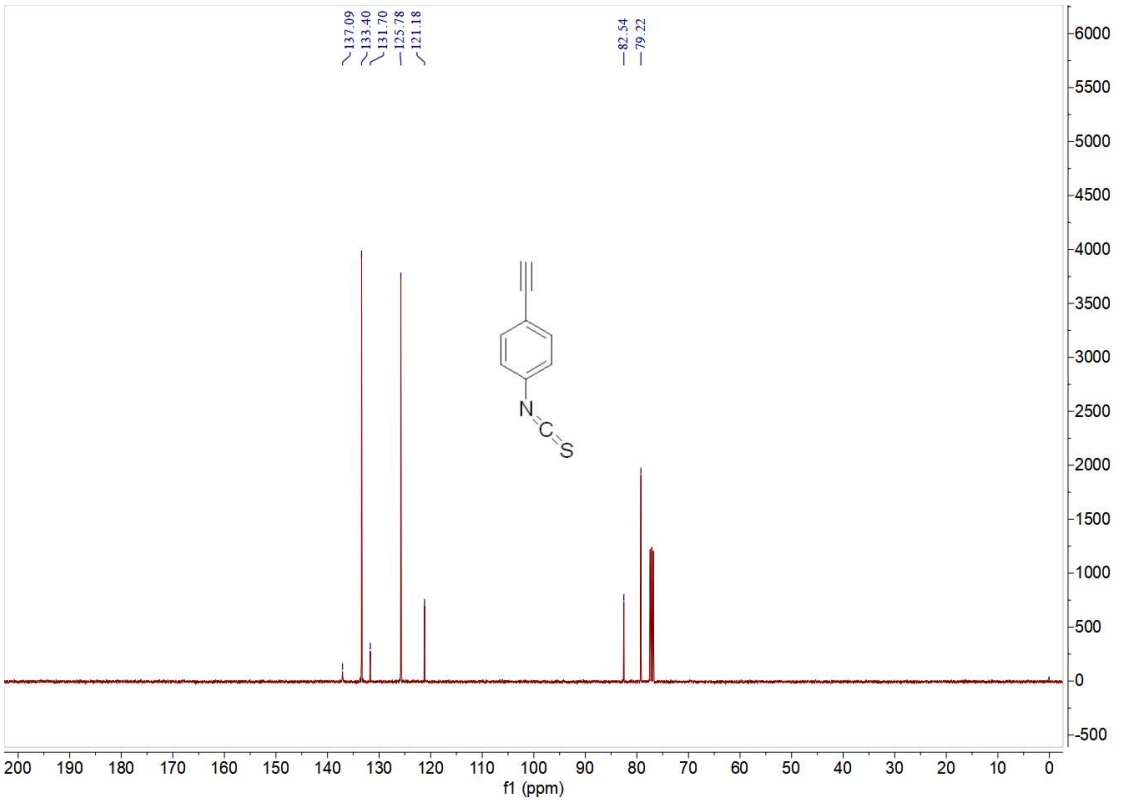


^{1}H NMR and ^{13}C NMR spectra of compound 5

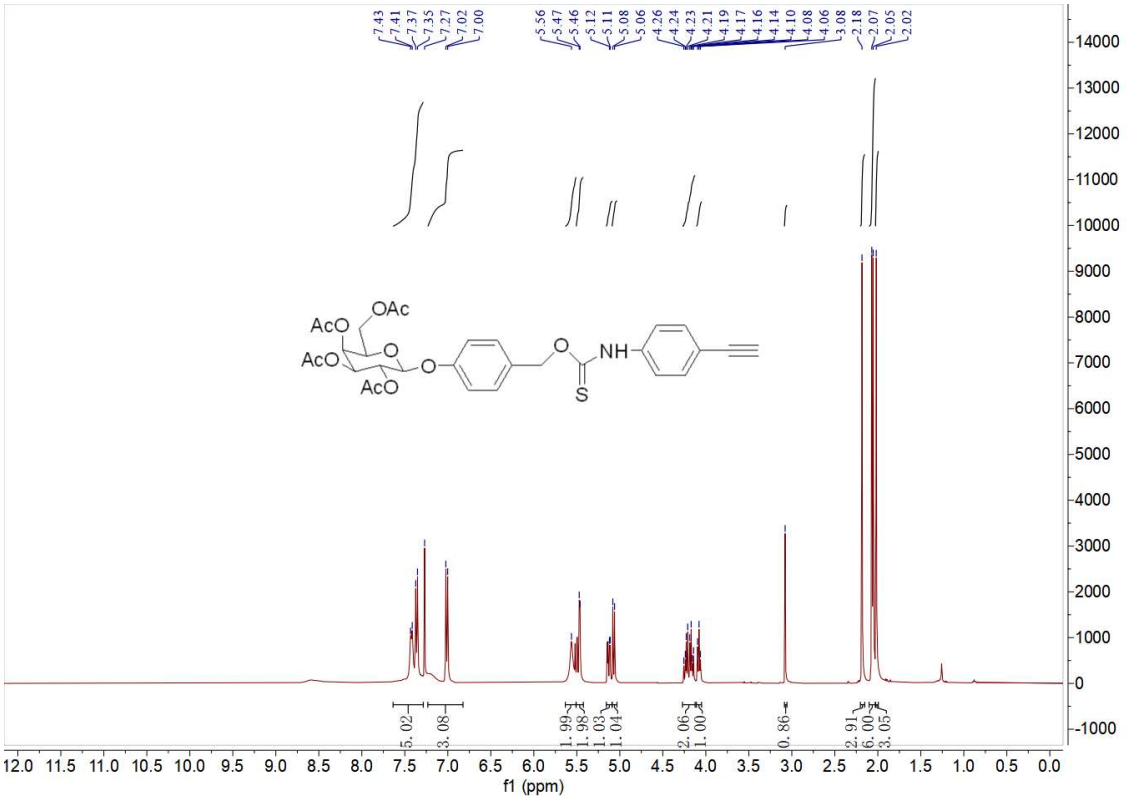


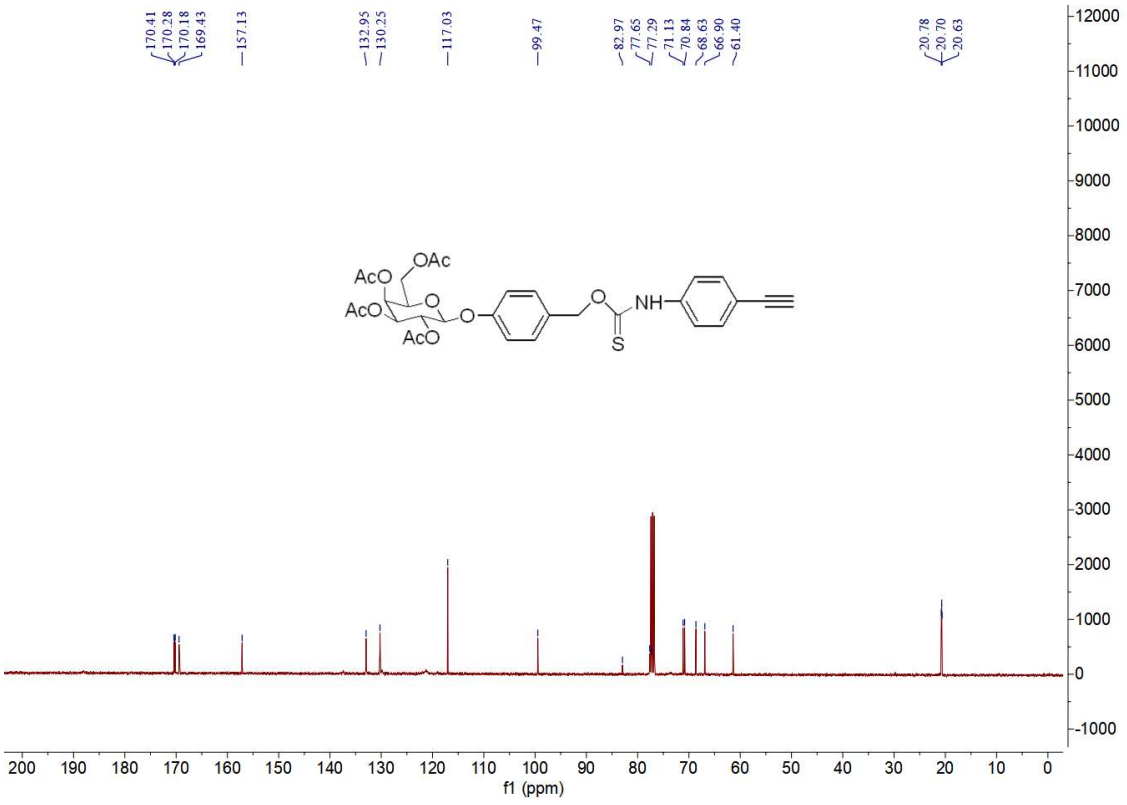
^{1}H NMR and ^{13}C NMR spectra of compound 6



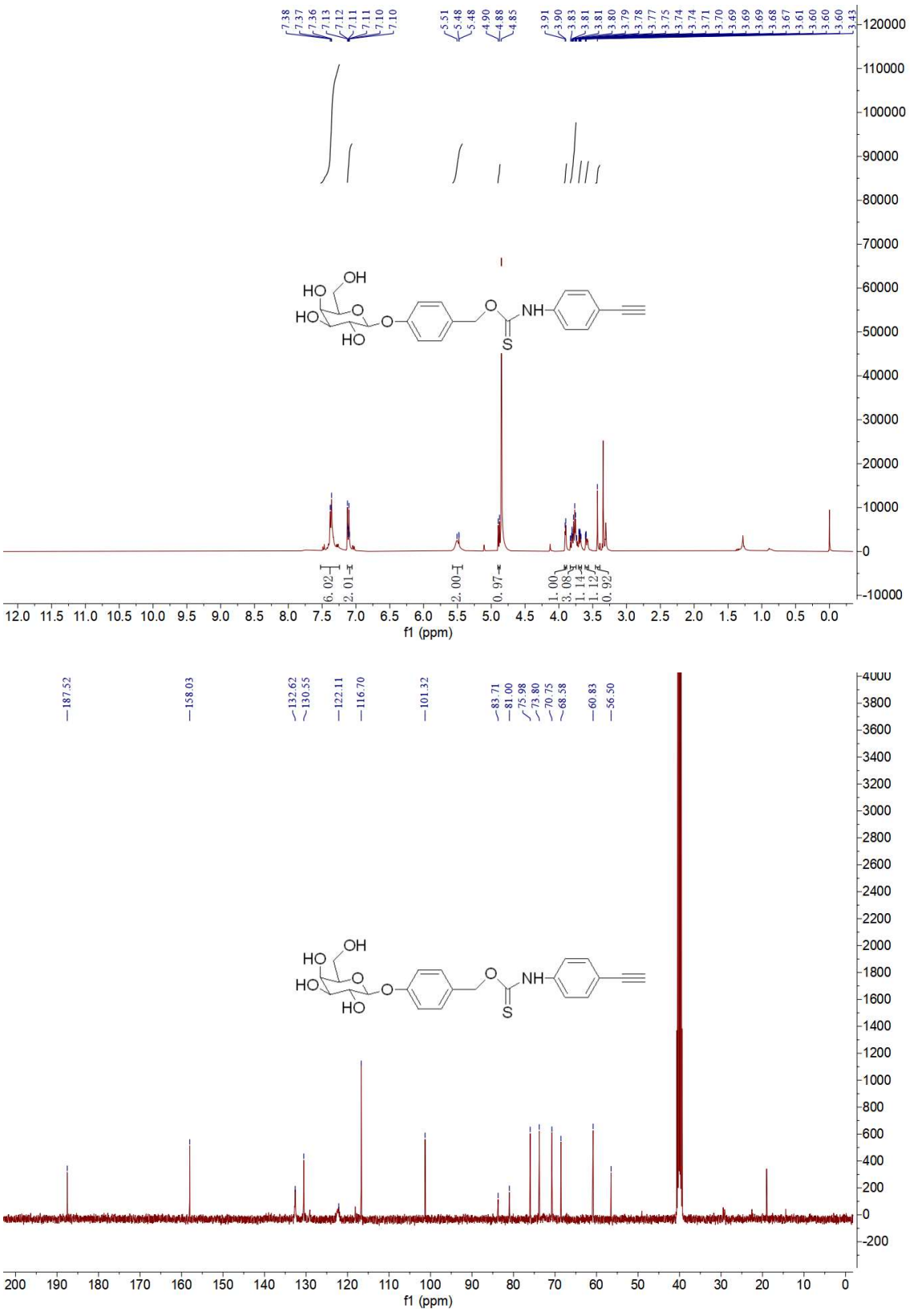


^{1}H NMR and ^{13}C NMR spectra of compound 7

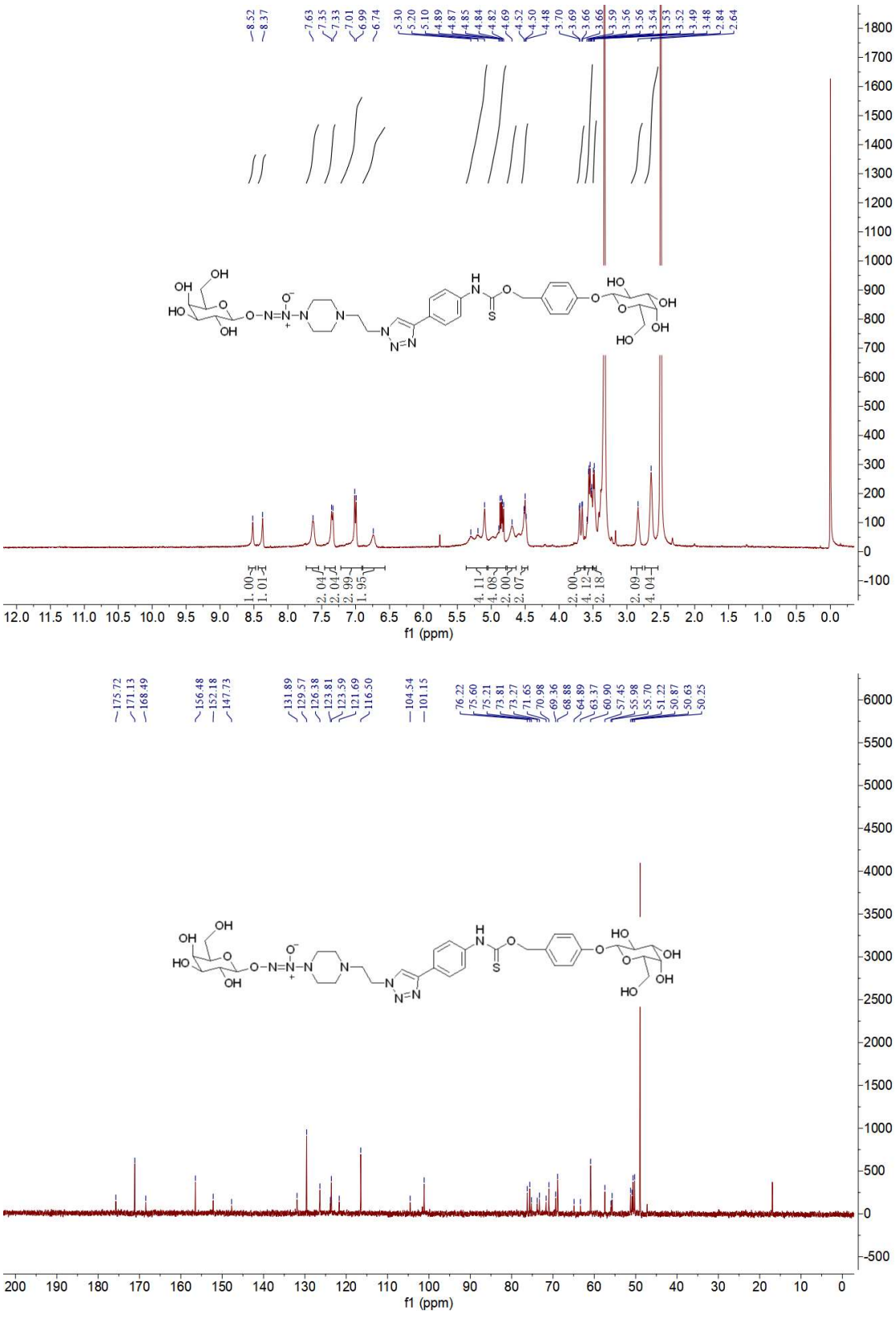




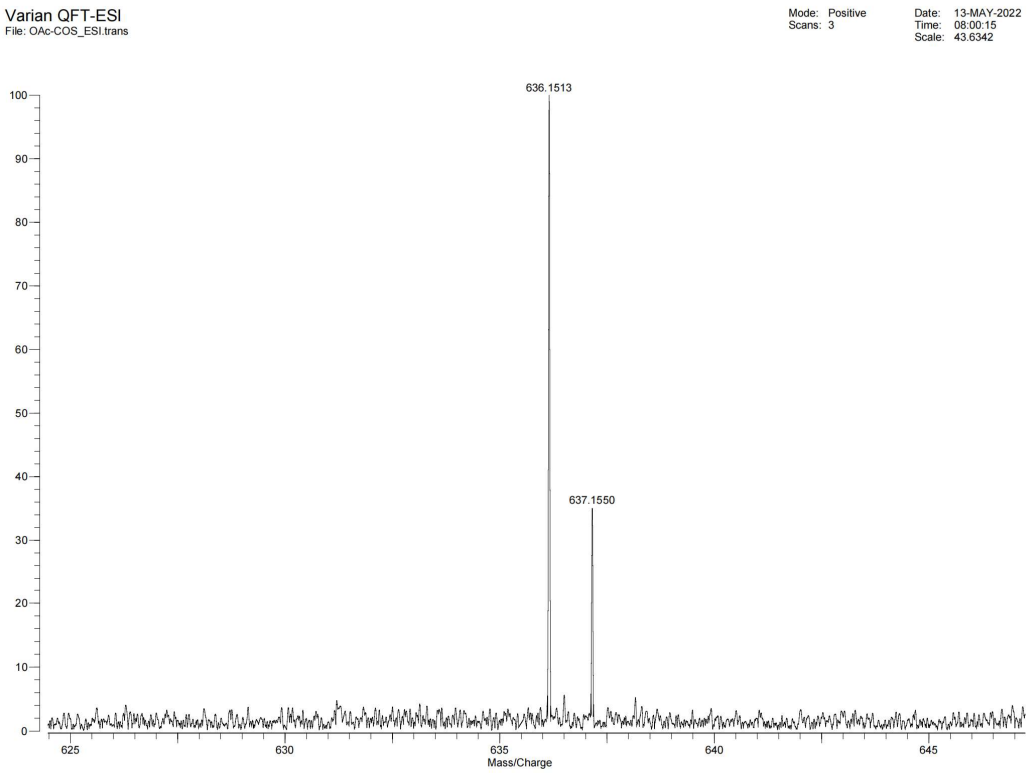
^{1}H NMR and ^{13}C NMR spectra of compound 8



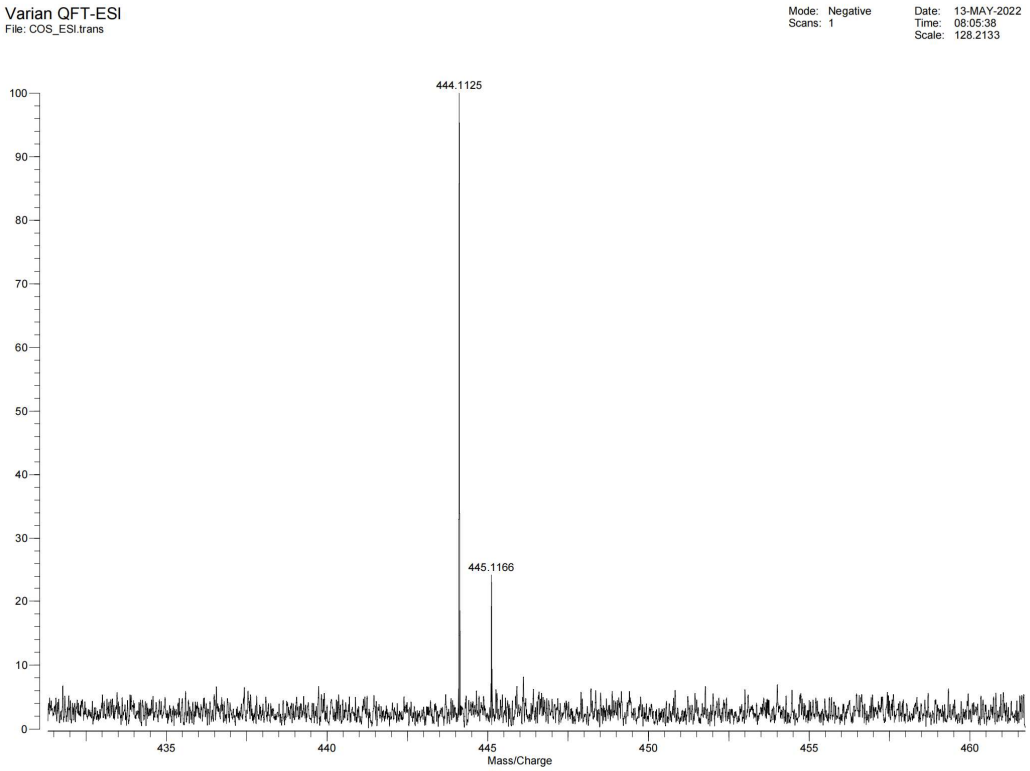
^{1}H NMR and ^{13}C NMR spectra of compound 9



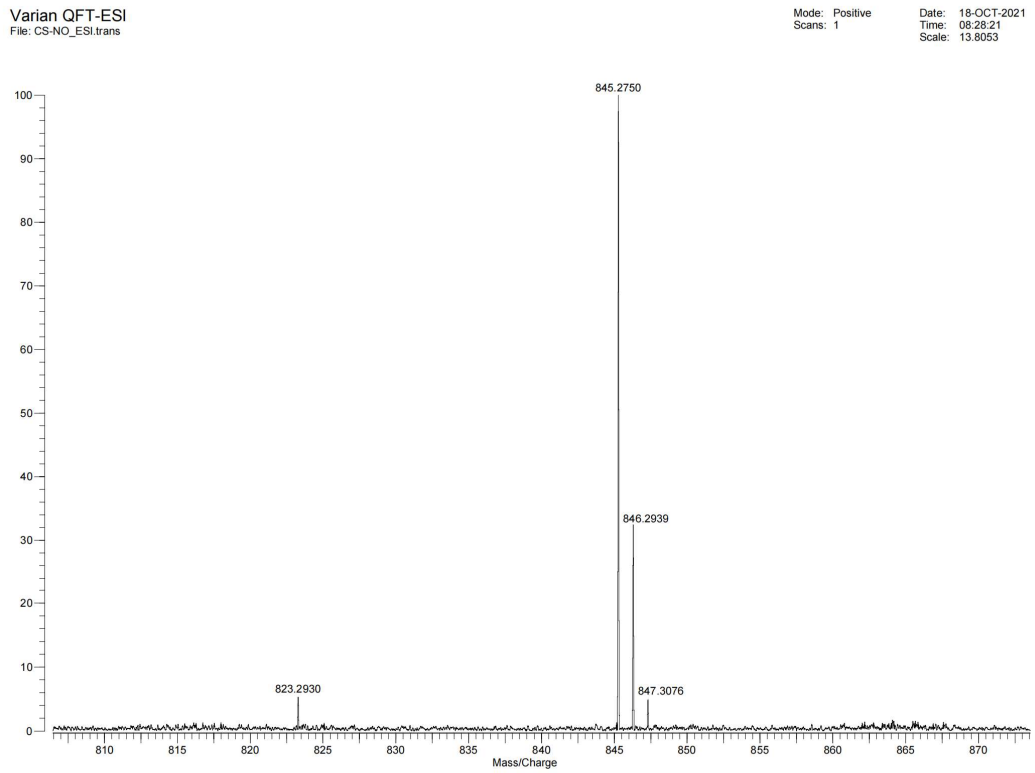
^{1}H NMR and ^{13}C NMR spectra of compound 10



HRMS spectrum of compound 8



HRMS spectrum of compound 9



HRMS spectrum of compound 10

4.NO and H_2S release *in vitro*

Griess assay: 100 μL of donor solution (500 μM) was dissolved in 800 μL of PBS buffer (pH 7.4), and 100 μL of β-galactosidase (20 U/mL) was put into the solution. At different time intervals, 50 μL of reaction solution was transferred into a 96-well plate with 50 μL of Griess A and 50 μL of Griess B. The absorbance was measured at 540 nm and converted to the concentration of NO by the NO standard curve (Fig. S1).

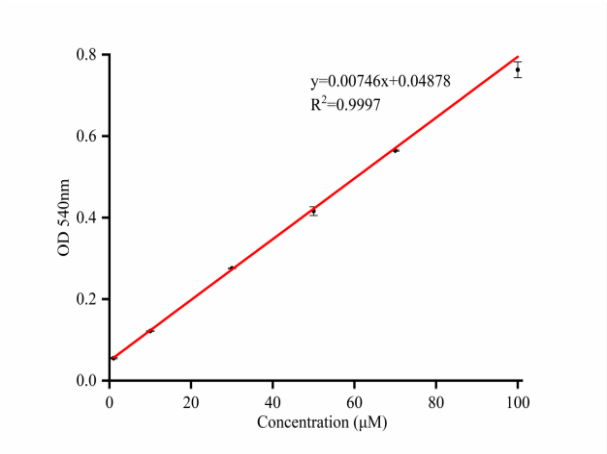


Fig. S1 Standard curve of NO measured by Griess assay. The data was displayed as the mean ± SD (*n = 3*).

Methylene blue (MB) assay: 100 μL of donor solution (500 μM) was dissolved in 700 μL of PBS buffer (pH 7.4), 100 μL of β-galactosidase (20 U/mL) and 100 μL of CA (250 μg/mL) was added into the solution. At different time intervals, 50 μL of reaction solution was added into a 96-well plate containing 50 μL of MB solution (10 μL of zinc acetate (1%, w/v), 20 μL of $FeCl_3$ (30 mM in 1.2 M HCl), and 20 μL of N,N-dimethyl-p-phenylene diamine (20 mM in 7.2 M HCl). The absorbance was measured at 670 nm after 30 min and converted to the concentration of H_2S by the H_2S standard curve (Fig. S2).

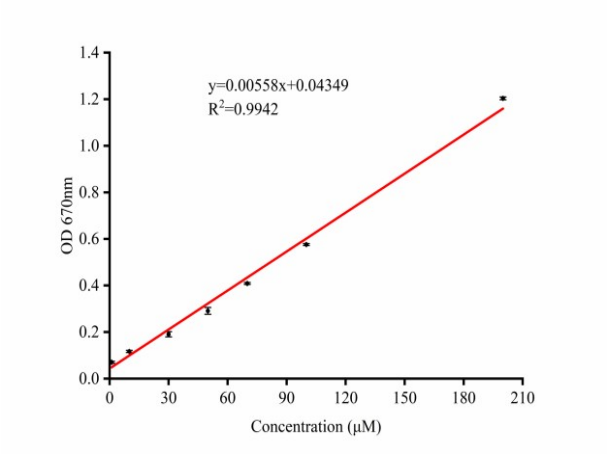


Fig. S2 Standard curve of H_2S measured by the methylene blue assay. The data was displayed as the mean ± SD (*n = 3*).

5.HPLC analysis

The solutions of H_2S donor (50 μM) in PBS (20% MeOH) were incubated with β-galactosidase (2

U/mL), a-glucosidase (20 U/mL), esterase (20 U/mL), cysteine (500 μM) and glutathione (500 μM) at 37°C for 12 h, their stability and specific response were analyzed by HPLC. The HPLC experiments were carried out on Shimadzu LC-20 AT system with a Cromasil C18 column (250 mm x 4.6 mm, 5 μm), UV-Vis wavelength = 220 nm, eluted at 1.0 mL/min with H_2O/CH_3CN, gradient 10% to 80% in 30 min.

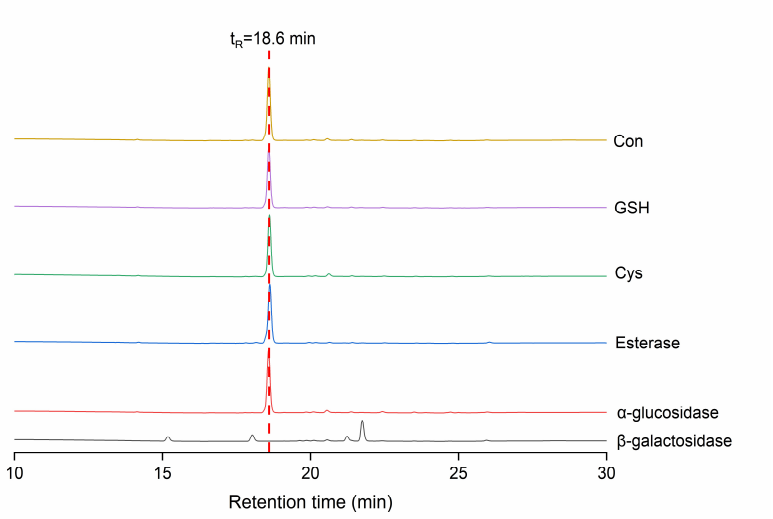


Fig. S3 The specific response of H_2S donor toward various enzymes and the stability of H_2S donor in the present of thiols.

6.Detection of NO and H_2S production in H9c2 cells

About 1×10^4 H9c2 cells were seeded on glass coverslips pretreated with TC (Solarbio, YA0351) in 2 mL DMEM medium with fetal bovine serum (10%) at 37°C in a humidified incubator with 5% CO_2. After incubation for 24h, the medium was removed, and fresh medium containing PBS, NO, H_2S and NO-H_2S donors (1 μM) was added, respectively. After 6h, the medium was removed and washed with PBS for three times. Then the cells were re-incubated with PBS containing DAF-DA FM (2 μM) or WSP-1 (4 μM) for 30 min, respectively. After removing the solution, glass coverslips were washed with PBS. The cells were then fixed with 4% paraformaldehyde solution and washed with PBS. Finally, the cells were stained and mounted with DAPI. Fluorescence was observed with a confocal laser scanning microscope at excitation wavelength of 488 nm.

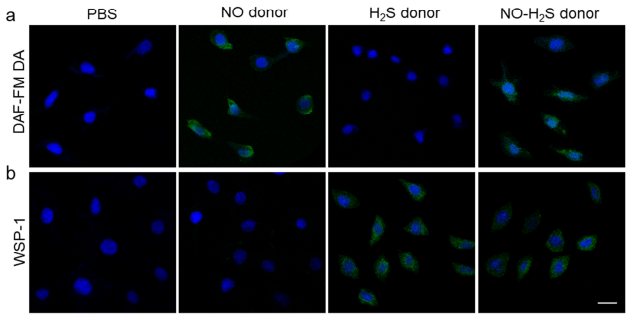


Fig. S4 Intracellular generated NO (a) and H_2S (b) from NO, H_2S and NO-H_2S donors (1 μM) was detected by DAF-FM DA (2 μM) and WSP-1 (4 μM) probes, respectively. Scale bar, 20 μm.

7.Cell viability

The effect of NO, H_2S and NO-H_2S donors on cell proliferation was evaluated by the Cell Counting Kit-8 (CCK-8) (APExBIO, K1018). Briefly, H9c2 cells (2000 cells/well) were cultured in a 96-well plate in the presence of NO, H_2S and NO-H_2S donors at different concentrations for 24 h, respectively. Fresh culture medium containing 10% CCK-8 was added to each well to incubate for 2 h at 37°C. The absorbance at 450 nm was recorded to assess cell proliferation (Fig. S4).

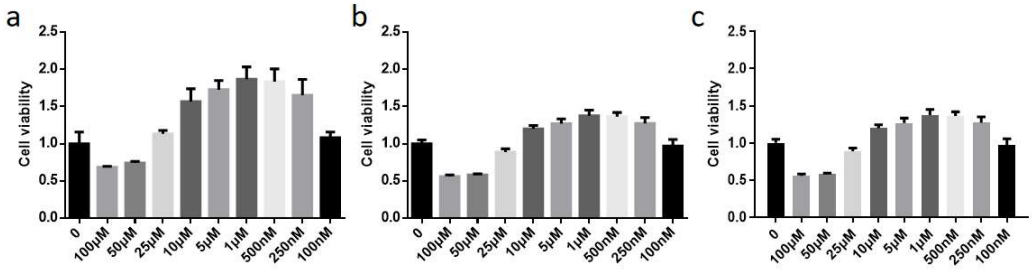


Fig. S5 The proliferation of H9c2 cells treated with different concentrations of (a) NO, (b) H_2S and (c) NO-H_2S donors for 24 hours using cck-8 assay. The data are displayed as the mean ± SD (*n = 3*).

8.The rat model of heart failure post myocardial infarction

All experiments and animal procedures were approved by the Animal Experiments Ethical Committee of Nankai University and carried out in conformity with the Guide for Care and Use of Laboratory. Male Sprague-Dawley (SD) rats (280-320 g) were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg) and then fixed in the supine position on the hot pad (37°C). After endotracheal intubation, the rats were ventilated with a mechanical ventilator (Hallowell EMC Microvent I) with a tidal volume of 6 mL and 110 times per minute. Then the heart was exposed by thoracotomy, and the left anterior descending branch was ligated with 6-0 suture. Ventricular function was assessed by echocardiography two weeks post-surgery, and when the left ventricular ejection fraction (LVEF) dropped to about 45%, heart failure was confirmed. Furthermore, the serum NT-pro BNP was detected to evaluate the degree of heart failure.

The rats were subsequently subjected to secondary thoracotomy by the above-mentioned protocol, and multipoint injection of hyaluronon hydrogel containing different donors was performed in the infarct area.

9.Echocardiographic analyses of cardiac function

Echocardiographic analyses were performed using transthoracic echocardiography (VisualSonics, Inc) to evaluate cardiac function. After inhalation of 2% isoflurane, echocardiography was collected at 1, 14 and 42 days post-surgery. The left ventricular ejection fraction (LVEF), left ventricular shortening fraction (LVFS), left ventricular end systolic volume (LVESV), left ventricular end diastolic volume (LVEDV) and other parameters were analyzed by VEVO 2100 workstation software.

10.Myocardial injury and heart failure assessment

Blood was collected through orbit before and at 14 and 42 days after MI. After standing for 30min

at 37°C, blood samples were centrifuged for 15 min at 3000 rpm, and supernatant (serum) was collected and stored at −80°C before testing. Levels of NT-pro BNP and cTnT in serum were determined by the specific ELISA kits (Elabscience). The concentration of serum LDH was determined by an LDH detection kit (Solarbio).

11.Histological analysis

Hearts were taken from rats at day 42 after MI. The rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (350 mg/kg) and fixed on the operating board in the supine position. Cut the diaphragm upward from the abdomen and expose the heart. Scissor the right atrial appendage, insert the needle tip of the syringe from the apex of the heart, and then fully perfuse with normal saline. The hearts were removed and fixed in 4% paraformaldehyde for 24 hours. Subsequently, the hearts were evenly cut into 4 parts from the ligation point to the apex of the heart, and then cut into 5 μm thick slices after dehydration and embedding. Hematoxylin Eosin (H&E) staining, Masson trichrome staining and Sirus Red staining were performed with paraffin sections according to standard procedures. For immunofluorescence staining, after antigen repair and membrane rupture, the paraffin sections were washed with PBS for three times, and then blocked with goat serum at room temperature for 30 minutes. Antibodies against α-SA (Abcam, ab72592, 1:100) and α-SMA (Abcam, ab7817, 1:100) diluted with goat serum were dropped to cover sections and incubated overnight. Then the sections were washed six times with PBS, followed by incubation with Alexa Fluor™ 488 Goat Anti-Mouse (Invitrogen, A11029, 1:200) and Alexa Fluor™ 594 Goat Anti-Rabbit (Invitrogen, A11037, 1:200) IgG for 2h at room temperature. After washing with PBS for six times, the sections were counterstained and mounted with DAPI-containing fluoromount-G (SouthernBiotech, 0100-20).

12.Statistics

All data were presented as mean SD from at least three independent experiments. Comparisons among more than two groups was performed by one-way ANOVA. Statistical analyses were performed with GraphPad Prism software 7.0, and a statistical significance was accepted at p value less than 0.05.

Reference:

1. A. Takahashi, Y. Suzuki, T. Suhara, K. Omichi, and T.Ito, *Biomacromolecules*, 2013, **14**, 3581-3588.

2. J. Gao, W. Zheng, J. Zhang, D. Guan, Z. Yang, D. Kong and Q. Zhao, *Chem. Commun.*, 2013, **49**, 9173-9175.