Supporting Information

Fluorescent Probe for Highly Selective and Sensitive Detection of Hydrogen Sulfide in Living Cells and Cardiac Tissues

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Abbreviations

hydrogen sulfide, (H_2S); reactive sulfur species, (RSS); reactive nitrogen species, (RNS); reactive oxygen species, (ROS); phosphate buffer solution, (PBS); reduced glutathione, (GSH); L-cysteine, (L-Cys); sodium bisulfite, ($NaHSO_3$); sodium thiosulfate, (NaS_2O_3); sodium metabisulfite, ($Na_2S_2O_5$); sodium chloride, (NaCl); sodium bromide, (NaBr); sodium iodide, (NaI); sodium fluoride, (NaF); sodium azide, (NaN_3); sodium nitrite, ($NaNO_2$); sodium sulfate, (Na_2SO_4); sodium hydrosulfide, (NaHS); sodium bicarbonate, ($NaHCO_3$); potassium superoxide, (KO_2); potassium thiocyanate, (KSCN); tert-butyl hydroperoxide, (t-BuOOH); hydrogen peroxide, (H_2O_2); sodium hypochlorite, (NaClO).

Materials

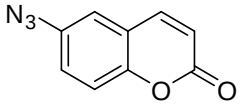
All solvents and chemicals were purchased from Sigma-Aldrich, J&K, or Alfa Aesar. All the buffers used are degassed with N_2.

Instruments

NMR spectra were recorded on a BRUKER AVANCE III 400 at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR, values are in ppm relative to tetramethylsilane. Mass spectra (MS) were measured with a BRUKER APEX IV FT-MS (7.0T). UV-visible spectra were obtained on a DU800 spectrometer and fluorescence data were collected on Cary Eclipse fluorometer or Molecular Devices FlexStation III microplate reader, FTIR were tested on NEXUS-470, pH values of the buffers were obtained on METTLER TOLEDO FiveEasy pH FE20. Tissue images were done on Leica DMIRB Microscope (DC300F). Two-photon confocal laser scanning microscopy was taken on Nikon confocal (A1-RMP-SI)

Synthesis and characterization of coumarin based azide compounds

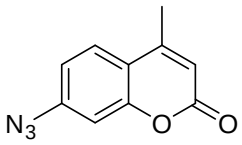
Compound: ***C-6Az***



6-Aminocoumarin (**C-6Am**) hydrochloride（395 mg，2 mmol）was dissolved in 2 mL 5 M hydrochloric acid. To this solution, sodium nitrite （210 mg，3 mL）dissolved in 5mL of water was dropwise added within 30 min. The solution was vigorously stirred in ice-water mixture. Sodium azide （520 mg，8 mmol）was batch added in. The resulting solution was stirred at room temperature overnight. The reaction was monitored by TLC. After the reaction was completed, it was poured into saturated aqueous $NaHCO_3$ and extracted with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, followed by filtration and concentration. The crude product was purified by silica gel chromatography (ethyl acetate: dichloromethane=1:10) to obtain the pure product as yellow solid (310 mg, 83 % yield). R_f=0.7 (ethyl acetate: dichloromethane=1:10); ^{1}H NMR (400 MHz,

DMSO-d6, 298 K) δ 8.17 – 7.84 (m, 1H), 7.52 (s, 1H), 7.46 – 7.05 (m, 2H), 6.70 – 6.30 (m, 1H); ^{13}C NMR (101 MHz, DMSO-d6, 298 K) δ 160.10, 151.16, 143.81, 136.15, 123.34, 120.22, 118.40, 118.36, 117.72; FTIR (Nicolet, KBr, cm^{-1}) 2109.8 (m, N_3), 1716.4 (w, C=O); m/z for $C_9H_5N_3O_2$ (ESI-TOF$^+$) $[M]^+$ calcd 187.15, found 187.04.

*Compound : **C-7Az***



7-Amino-4-methylcoumarin (**C-7Am**, 525 mg, 3 mmol) was dissolved in 4 mL 5 M hydrochloric acid. To this solution, sodium nitrite (210 mg, 3 mmol) dissolved in 8 mL of water was dropwise added within 30 min. The solution was vigorous stirred in ice-water mixture. Sodium azide (780 mg, 12 mmol) was batch added into the reaction mixture. The resulting solution was stirred at room temperature overnight. The reaction was monitored by TLC. After the reaction was completed, it was poured into saturated aqueous $NaHCO_3$ and extracted with ethyl acetate. The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel chromatography (ethyl acetate: dichloromethane=1:10) to obtain the pure product as yellow solid (520 mg, 86 % yield). R_f = 0.7 (ethyl acetate: dichloromethane=1:10); ^{1}H NMR (400 MHz, DMSO-d6, 298 K) δ 7.76 (t, J = 7.2 Hz, 1H), 7.12 (dd, J = 4.8, 2.9 Hz, 2H), 6.32 (d, J = 5.3 Hz, 1H), 2.40 (d, J = 6.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d6, 298 K) δ 159.96, 154.52, 153.33, 143.75, 127.43, 117.24, 116.00, 113.68, 107.26, 18.52; FTIR (Nicolet, KBr, cm^{-1}) 2117.5 (m, N_3), 1724.1 (w, C=O); m/z for $C_{10}H_7N_3O_2$ (ESI-TOF$^+$) $[M]^+$ calcd 201.18, found 201.05.

Reduction of C-6AZ and C-7Az to amine by H_2S

General Methods. C-6AZ or C-7Az (0.1 mmol) was dissolved in 5 mL DMF. To this solution, NaHS (5 equiv) was batch added in. The resulting solution was stirred at room temperature. After the reaction was stopped, it was poured into deionized water

and extracted with ethyl acetate, The combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel chromatography (ethyl acetate: dichloromethane=1:10) to obtain the pure products, which have been proven 6-Amino coumarin and 7-amino-4-methyl coumarin through NMR, MS and FTIR, respectively.

Quantum yield determination of C-6Az, C-7Az and their products

Quantum yields were determined using Quinine Sulfate as a standard according to a published method.[1] The quantum yield was calculated according to the equation: (Φ sample = Φ standard * (I_{sample}/ $I_{standard}$) * (A_{sample}/ $A_{standard}$)); where Φ is the quantum yield, Φ standard = 0.550 in 0.005 M H_2SO_4; I_{sample} and $I_{standard}$ are the integrated fluorescence intensities of the sample and the standard, A_{sample} and $A_{standard}$ are the optical densities, at the excitation wavelength, of the sample and the standard, respectively.

Fluorescence analysis and selectivity tests

PBS buffer (10 mM) used in this study was first degassed with N_2 for 30 min. 50 mM stock solution of C-7Az was first prepared in DMF, which was further diluted to 200 μM in degassed PBS buffer for following measurements. 10 mM NaHS was prepared in degassed PBS buffer, and was further diluted to 200 μM solution for following experiments. For All Other species, 100 mM stock solutions in degassed PBS buffer were prepared, which were further diluted into 2 mM solutions in degassed PBS buffer for use. These above solutions were then immediately used for further fluorescence analysis and selectivity test by mixing with C-7Az and NaHS or other specie working solutions. For each group of experiments, all the solutions were freshly prepared.

Visualization of H_2S in HeLa cells

HeLa cells were cultured according to standard protocols. After removal of cell medium, the cells were incubated with 50 μM 7-azido-4-methylcoumarin (C-7Az) in degassed PBS buffer for 30 min at 37 ^oC, followed by washing the cells with degassed PBS buffer. The treated cells were further incubated for another 30 min with blank

phenol red free media or 100 μM NaHS, 400μM cysteine, 400μM GSH in phenol free media respectively. Fluorescence imaging was then carried out with two-photon confocal laser scanning fluorescence microscopy with the excitation of 720 nm laser (Nikon, A1-RMP-SI).

Animals

Male Sprague-Dawley rats weighing 200-220g were provided by the Animal Research Centre of Peking University First Hospital (Clean grade). In total, 28 rats were randomly divided into 3 groups: the control group (n = 8), the AS group (n = 10), and the AS plus H_2S donor (AS+NaHS) group (n = 10) and were observed for 8 weeks. AS was induced in rats by a single dose of vitamin D_3 (700,000 IU·kg^{-1}) and a high-cholesterol diet containing 3% cholesterol, 0.5% sodium cholate, 0.2% propyl-thyracil, 5% refined sugar, 10% lard, and 81.3% base feed. Food and water were available ad libitum. For rats in the AS+ NaHS group, NaHS was dissolved in physiological (0.9%) saline at 56 μmol/kg body weight, and was injected (i.p.) daily. An identical volume of physiological saline was injected into control group and AS group rats.

Measurement of endogenous H_2S production in myocardial tissues

H_2S production was measured in rat myocardial tissue as described previously.[2] Myocardial tissue was harvested and homogenized with 50 mM potassium phosphate buffer (pH 6.8, 1:10, w/v). The reaction mixture contained 10 mM L-cysteine, 2 mM pyridoxal 5'-phosphate, 100 mM potassium phosphate buffer (pH 7.4), and 10% (w/v) homogenates for a 1 ml total volume. The reactions were done in a 25 ml Erlenmeyer Pyrex flask. Cryovial test tubes (2 ml) containing 0.5 ml of 1 M NaOH were used as the centre well. The flask containing reaction mixture and center well was flushed with nitrogen gas before being sealed with a double layer of parafilm. The catalytic reaction was initiated by transferring the flask from the ice-bath to a shaking water bath at 37°C and stopped after 90 min by adding 0.5 ml of 50% trichloroacetic acid. Reaction mixture was incubated for an additional 60 min at 37°C. Then, the central well was mixed with 0.5 ml of antioxidant solution. The H_2S content in the solution was detected using sulfide-sensitive electrode (PXS-270, Shanghai, China).

Electrodes were rinsed in distilled water, blotted dry and placed into standards and samples. The value in millivolts was recorded when the reading was stable. The H_2S content was calculated from the standard curve and the protein concentration was determined according to Coomassie blue staining in the tissue homogenate. Measurements were done in duplicate for each sample. The H_2S production rate was expressed in nmol/(min · mg protein)

***In situ* fluorescent detection of H_2S in the cardiac tissues**

The heart was rapidly exteriorized through a left thoracotomy and pericardial incision, fixed in 4% paraformaldehyde, embedded in ornithine carbamoyltransferase (OCT), and serially sectioned at 7 μm using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany) for fluorescence detection analysis. After drying for 1 h, the frozen sections were washed for 3 times with 0.01 mol/L phosphate-buffered saline (PBS) for 3 min. The slides were incubated for 30 min at 37°C with C-7Az working fluid (100 μmol/L) and subsequently rinsed for 3 times in PBS for 3 min. 50% buffered glycerol was mounted, and observed under fluorescence microscope in time (blue color). C-7Az working fluid was freshly prepared by a ratio of 2.01 mg C-7Az: 500 μl DMSO: 100 ml 0.01 mol/L PBS. For negative controls, sections were processed as described above, except that the incubation was performed with PBS instead of C-7Az working fluid. In order to exclude the possibility of the penetrability variance of probes in tissues from different groups of rats, we used another type of H_2S-insensitive coumarin 6 (green). The mean fluorescence intensity was obtained with 6-8 slides from different rats for each group of normal rats (6 slides), AS rats (8 slides) and AS rats+NaHS (8 slides) under the same imaging conditions.We calculated the relative change of H_2S-sensitive fluorescence intensity using the ratio of C-7Az (blue) to coumarin 6 (green).

Table S1. Optical properties of C-6Az, C-6Am, C-7Az and C-7Am

	Peak of absorbance (nm)	Molar absorption coefficiency ε (×10^3 $M^{-1}cm^{-1}$)	Fluorescence quantum yield Φ	
			In PBS buffer (pH=7.4)	In DMSO
C-6Az	336	3.7±0.1	0.0012	0.008±0.001
C-6Am	355	2.8±0.2	0.0021	0.053±0.004
C-7Az	325	10.9±0.4	0.0045±0.0015	
C-7Am	340	9.8±0.4	0.88±0.02	

Figure S1a) ^{1}H NMR and ^{13}C NMR of C-6Az (A), its reduced product by H_2S (B) and 6-Aminocoumarin (C); S1b) ^{1}H NMR and ^{13}C NMR of C-7Az (A) its reduced product by H_2S (B) and 7-Amino-4-methylcoumarin (C); S1c) IR spectra changes of C-6Az and C-7Az before or after the reduction by H_2S

Figure S1a

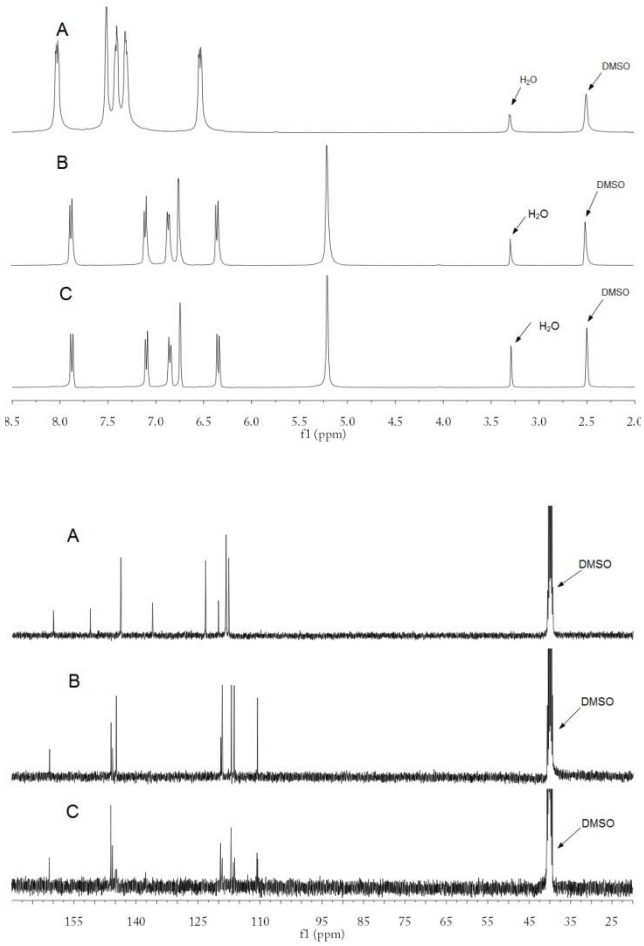


Figure S1b

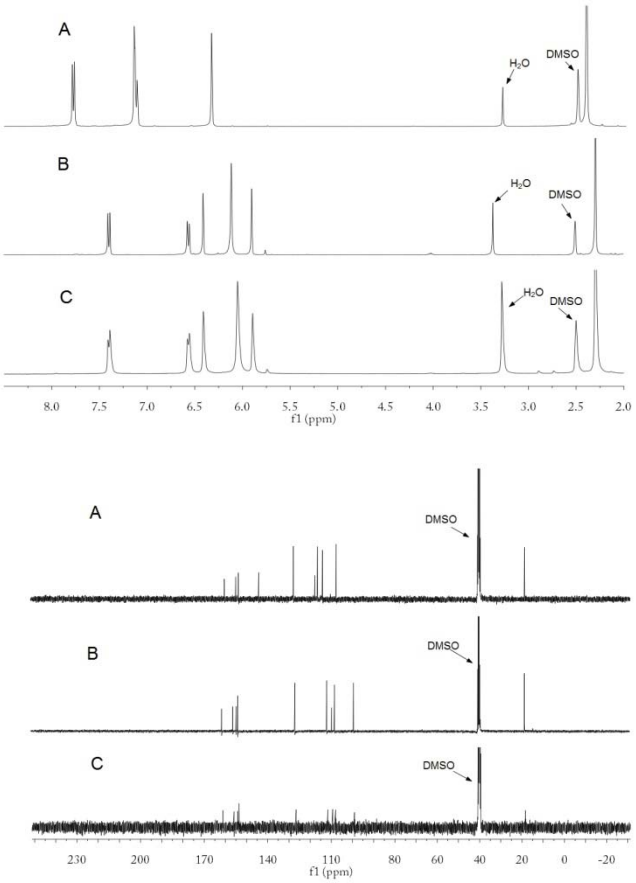


Figure S1c

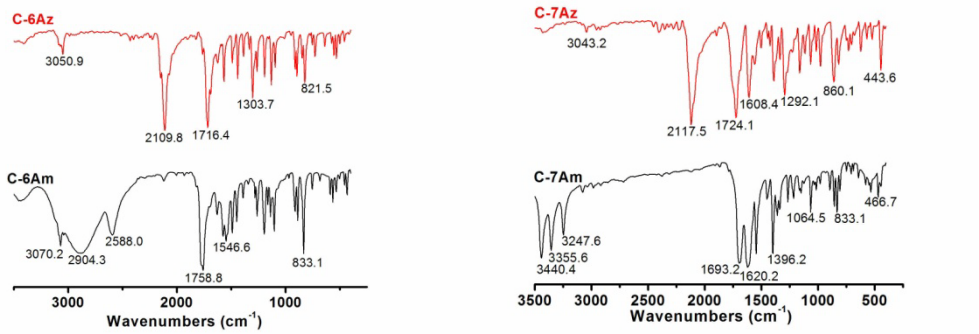
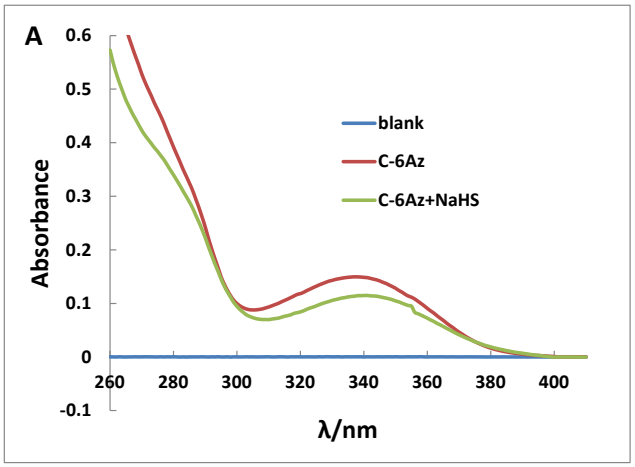


Figure S2. C-6Az and C-7Az: 50 μM, NaHS: 100 μM, tested in 10 mM degassed PBS, pH=7.4, The UV absorption spectra of C-6Az (A) and C-7Az (B) before (1) and after (2) the addition of NaHS for 4 h.



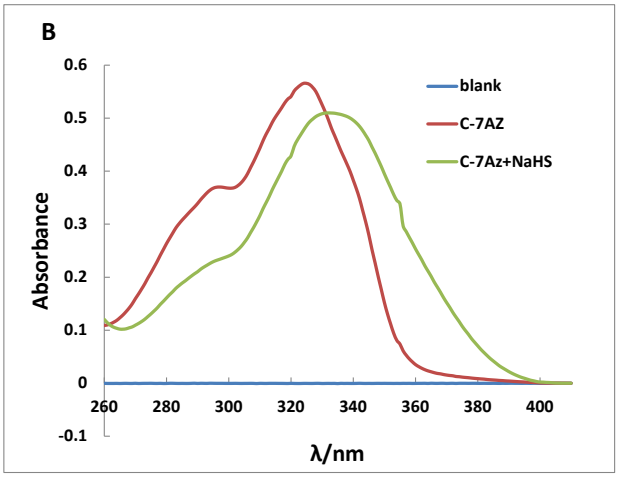
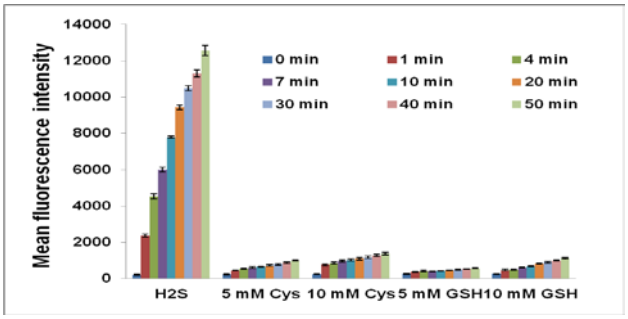
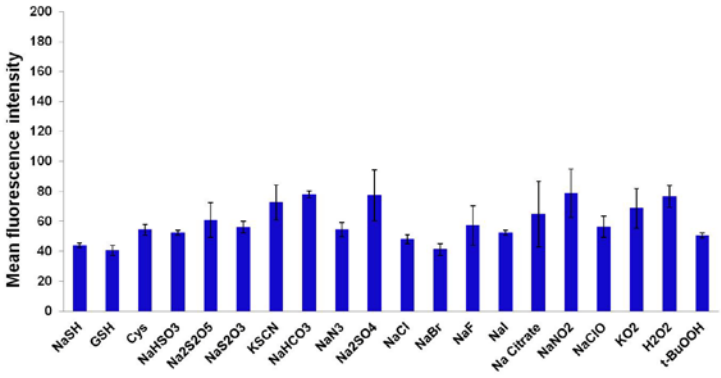


Figure S3. C (A), mean fluorescence intensity of 100μM with the addition of NaHS (100μM), cysteine (5, 10 mM) and GSH (5, 10 mM) at 0, 1, 4, 7, 10, 20, 30, 40, 50 min. (B) 100 μM C-6Az to various anions in degassed PBS after 60 min; (C) fluorescence spectra of 100 μM C-7Az to various anions in degassed PBS after 50 min. Except for NaHS (100 μM), all the following species in B) and C) are included for the study at the concentration of 1 mM in 10 mM degassed PBS (pH=7.4). Selectivity for the probes C-6Az and C-7Az were done in black 96-well plates. Data were acquired in 10 mM degassed PBS at 37 ℃, pH=7.4, Ex/Em = 340 /445 nm (filter: 420 nm, PMT: medium) through top-reading using Molecular Devices FlexStation III microplate reader.

A)



B)



C)

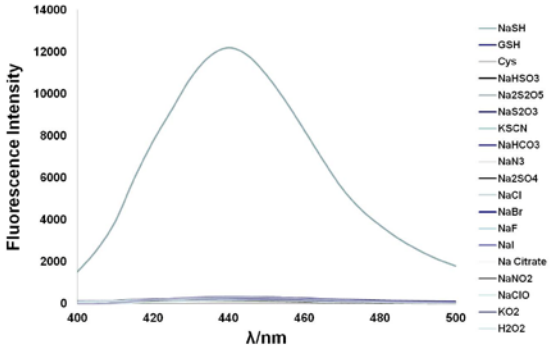
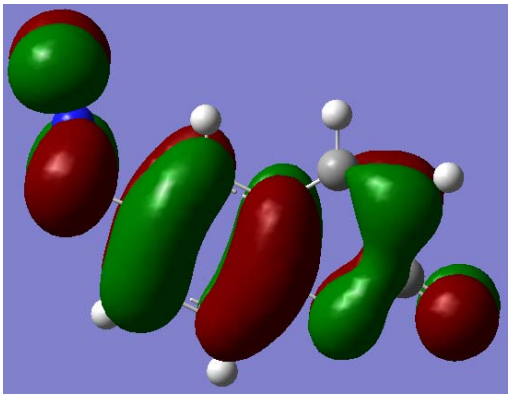
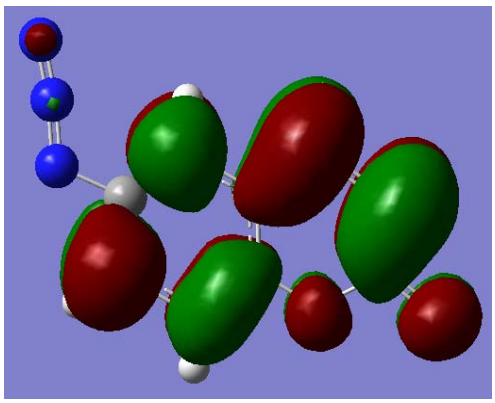


Figure S4. HOMO and LUMO orbits of azido coumarin derivatives C-6Az and C-7Az. HOMO and LUMO wave functions of the optimized structures of C-6Az and C-7Az molecules were calculated with density functional theory (DFT) quantum chemical method by using Gaussian 09 program package[3]. Equilibrium geometries of all molecules were fully optimized at the B3LYP/6-311G (d.p) level[4]. Vibrational frequencies, calculated at the same level, were used to determine the nature of the stationary points.

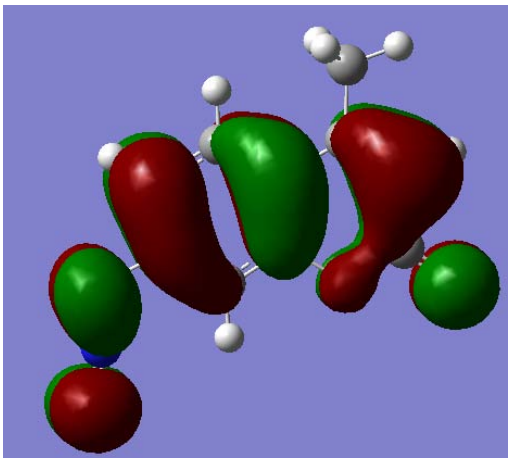
C-6Az HOMO



C-6Az LUMO



C-7Az HOMO



C-7Az LUMO

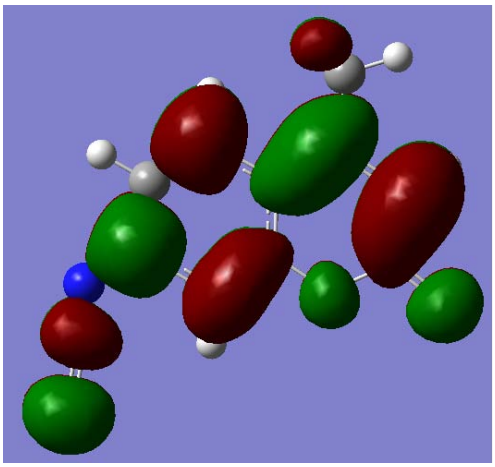
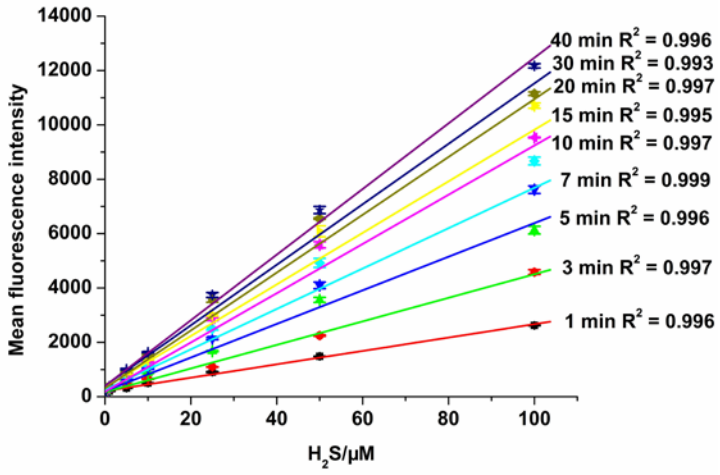


Figure S5. The linear relationship of mean fluorescence intensity and H_2S concentrations for C-7Az (C-7Az: 100 μM, NaHS concentration: 0, 5, 10, 25, 50, 100, 200 μM). (A) C-7Az treated with various concentrations of H_2S in degassed PBS buffer. Emission spectra were collected at 1, 4, 7, 10, 20, 35 and 50 min (B) C-7Az treated with various concentrations of H_2S in fetal bovine serum. Emission spectra were collected at 1, 4, 7, 10, 15, 20 and 30 min The data were acquired at 37 ℃, pH=7.4, Ex/Em = 340 /445 nm (filter: 420 nm, PMT: medium) through top-reading using Molecular Devices FlexStation III microplate reader.

A)



B)

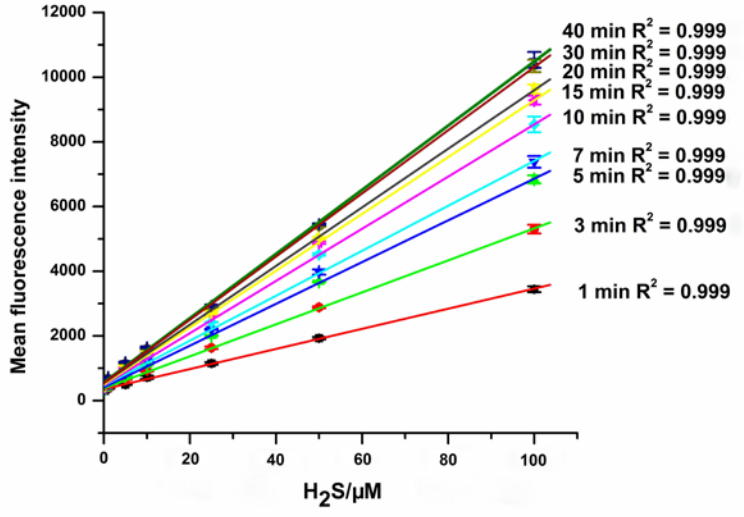


Figure S6. The relationship of mean fluorescence intensity and H_2S concentrations for C-6Az (100 μM). The concentrations of H_2S are 0 μM, 100 μM, 1mM, 10 mM and 100 mM respectively and the fluorescence intensity of each sample was recorded at 1 h, 3 h, 5 h, 7 h, 10 h at 37 ℃, pH=7.4, Ex/Em = 340 /445 nm (filter: 420 nm, PMT: medium) through top-reading using Molecular Devices FlexStation III microplate reader.

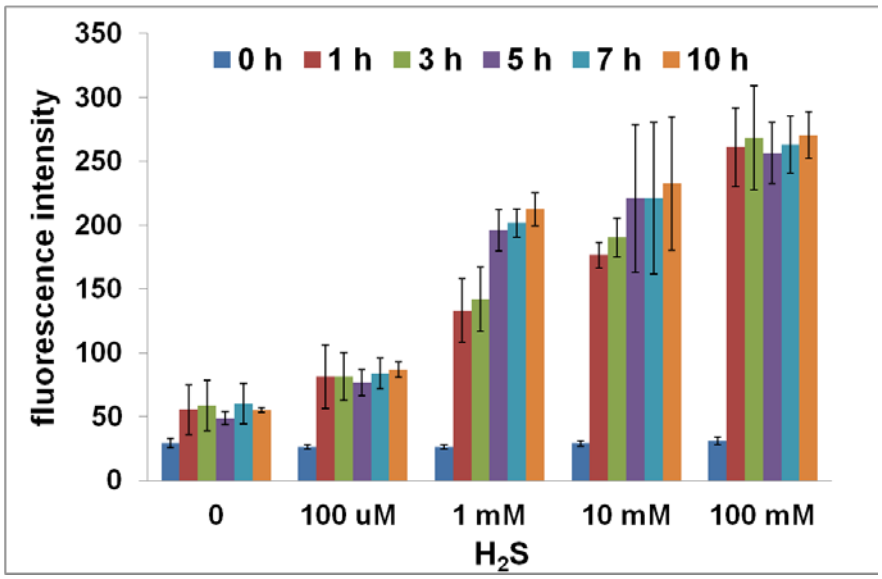


Figure S7. C-7Az (100 μM) treated with 100 μM H_2S in 10 mM degassed PBS with pH at 5.9, 6.4, 7.4, 7.9, Scan time are set 0, 1, 10, 15, 20, 30, 40 min. The fluorescence signals of the probes C-7Az were collected at 37 ℃, Ex/Em = 340 /445 nm (filter: 420 nm, PMT: medium) through top-reading using Molecular Devices FlexStation III microplate reader.

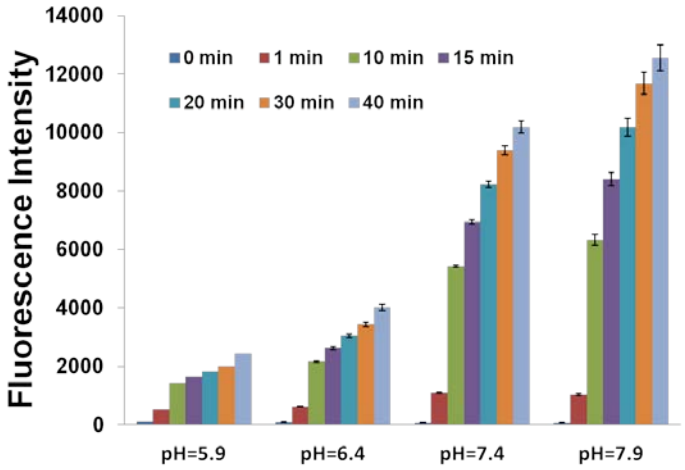


Figure S8. Photostability of C-7Az degassed PBS solution with UV light irradiation and the addition of NaHS (the last black column). The Sample was irradiated at 1, 3, 5, 7, 10, 15, 20, 30 min (365 nm, 11 mW/cm^2) and the fluorescence emission was measured at 37 ℃, Ex/Em = 340 /445 nm (filter: 420 nm, PMT: medium) through top-reading using Molecular Devices FlexStation III microplate reader. The last column is the same solution with the addition of 100 μM C-7Az.

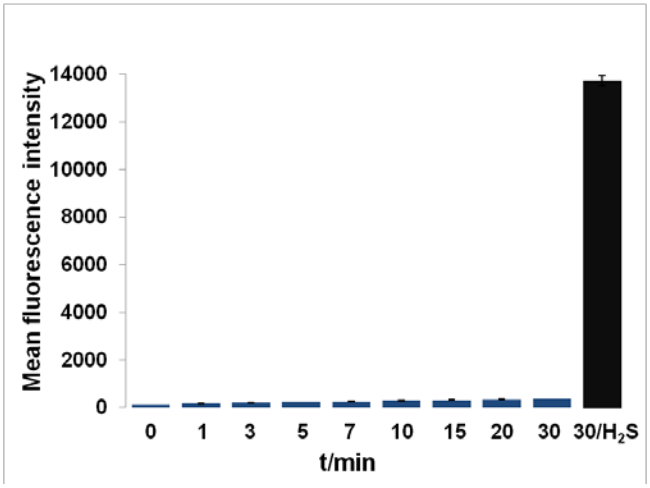


Figure S9. Cell viability of C-7Az for HeLa cells through MTT assay in 24 h at 37 ℃

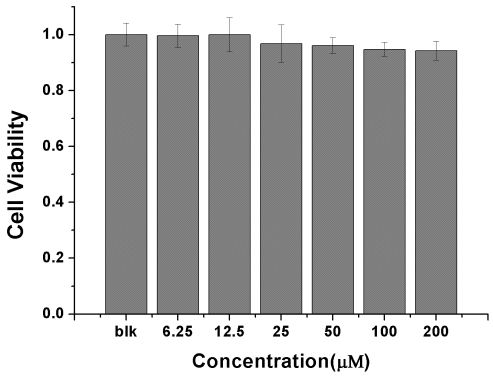


Figure S10. *In situ* visualization of cardiac tissues with H_2S insensitive fluorophore coumarin 6. a) tissue image of control rats with PBS working fluid. b) tissue image of control rats stained with coumarin 6 working fluid. c) tissue image of AS rats stained with coumarin 6 working fluid. d) tissue image of AS rats with exogenous injection of NaHS and stained with coumarin 6 working fluid. All imaging conditions are identical.

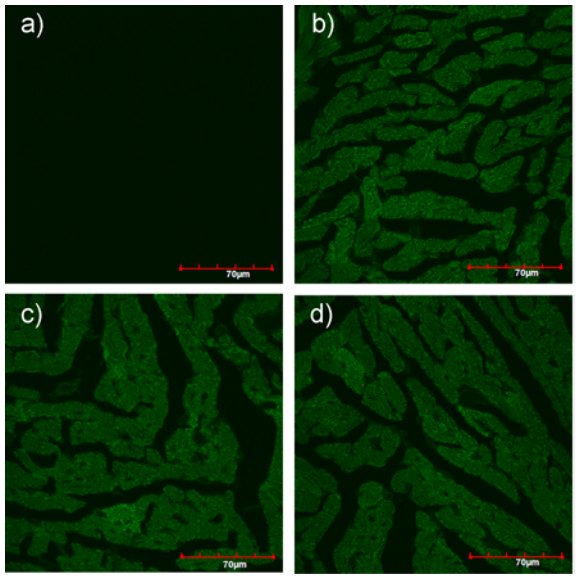
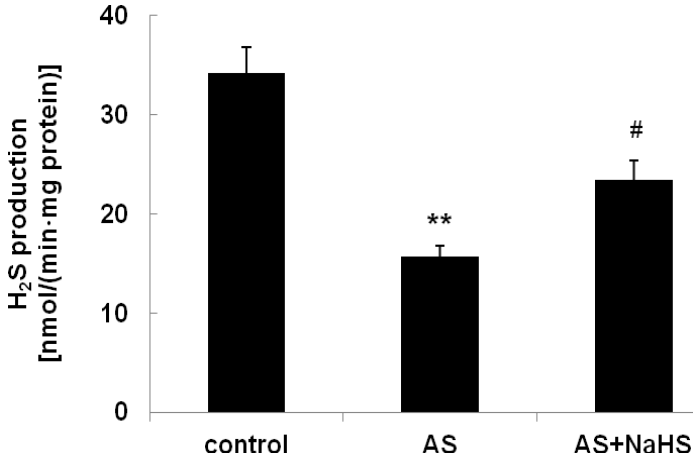


Figure S11. H_2S production in rat myocardial tissue (mean±SE) of three groups of rats (normal control rats, AS rats and AS rats+NaHS). ** P<0.01 vs control group; # P<0.05 vs AS group



References

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