Supporting Information

Synthesis and Evaluation of Phosphorodithioate-based Hydrogen Sulfide Donors

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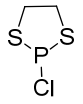
Materials and Methods

***Instrumentations*:** ^{1}H NMR spectra, ^{13}C NMR, and ^{31}P NMR were recorded at 300 MHz (Varian, VX 300) and are reported in parts per million (ppm) on the δ scale relative to residual $CHCl_3$ (δ 7.25 for ^{1}H, δ 77.0 for ^{13}C). NMR experiments were performed at room temperature. All reported melting points for solid materials were measured by Fisher-Johns melting point apparatus and not corrected. Mass spectra were recorded using an electrospray ionization mass spectrometry (ESI, Thermo Finnigan LCQ Advantage). Mass data were reported in units of m/z for $[M]^+$, $[M+Na]^+$, or $[M\text{-}DBU]^-$ (where, DBU is 1,8-Diazabicyclo(5.4.0)undec-7-ene). Infrared spectra were recorded on a Thermo Scientific Nicolet iS10 (Thin film) and reported in units of cm^{-1}. All microscopy images were taken on an EVOS fluorescence microscope from Advanced Microscopy Group (AMG). A microplate reader (Infinite M1000, TECAN Group Ltd.) was used to obtain the optical density (absorbance) from cells at 450 nm. A cell viability analyzer (Vi-Cell Cell Viability Analyzer, Beckman Coulter) was used to count cells. Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies) was used to measure the concentrations of H_2S released from donors.

***Solvents and Reagents*:** Reagents and solvents employed were of the highest grade available. Reagent grade solvents were used for either chromatography or extraction without further purification before use. Dichloromethane (DCM) was directly used from a solvent purifier (Pure Solv, Innovative Technology, Inc.). Carbon disulfide (CS_2) and acetonitrile (MeCN) were purchased from Fisher Scientific and used as received. Elemental Sulfur (S_8) was purchased from Alfar Asar and used directly. All alcohols, amines, and 1,2-ethanedithiol were purchased from Aldrich and used without purification. Phosphorous trichoride (PCl_3) was purchased from Aldrich and freshly distilled before use. Re-distilled diisopropyl ethylamine (DIEA) was purchased from Aldrich and used without further purification. Bio-grade dimethyl sulfoxide (DMSO) and hydrogen peroxide (H_2O_2, 3% solution) was purchased from biomedical LLC and J.T. Baker, respectively and used without further purification.

***Chromatography*:** The progress of the reactions was monitored by analytical thin layer chromatography (VWR, TLC 60 F_{254} plates). Plates were visualized first with UV (254 nm) and then illuminated by CAM stain (2.5 g of ammonium molybdate tetrahydrate and 1 g of cerium ammonium sulfate in a solution of 10% sulfuric acid in water) or ninhydrin solution (0.3 % ninhydrin in a solution of 3 % acetic acid in ethanol). Flash column chromatography was performed using silica gel (230-400 mesh). The solvent compositions for all separations are on a volume/volume (v/v) basis.

Synthesis of H_2S donors



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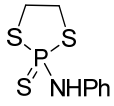
2-chloro-1,3,2-dithiaphospholane 2: 1,2-Ethanedithiol (3.56 mL, 42.5 mmol) was added to PCl_3 (11.1 g, 127 mmol) at room temperature. The mixture was stirred for 3 h (HCl bubbles were observed during this reaction) and then the excess of PCl_3 was distilled out by a simple distillation to yield the product as pale yellow oil (6.4 g, 95 %). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.77 – 3.67 (m, 2H), 3.62 – 3.52 (m, 2H); ^{31}P NMR (122 MHz, $CDCl_3$) δ 168.39; ^{13}C NMR (75 MHz, $CDCl_3$) δ 43.09; MS (ESI) *m/z* calcd for $C_2H_4ClPS_2$ $[M]^+$ 158.0, found 158.0.



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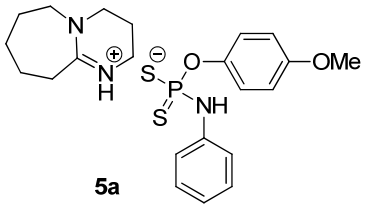
2-(phenylamino)-1,3,2-dithiaphospholane 2-sulfide **4**: 2-Chloro-1,3,2-dithiophospholane (1.1 g, 6.94 mmol) in deoxygenized MeCN (10 mL) was added dropwise to a stirred solution of aniline (0.63 mL, 6.94 mmol) and DIEA (1.33 mL, 7.63 mmol) in dry, degassed MeCN (4 mL) at -40 °C. After stirring for 1 h at the same temperature, the reaction mixture was warmed to room temperature and stirred for an

additional 1 h. A solution of S_8 (667 mg, 7.63 mmol) dissolved in CS_2 (10 mL) was added, and the resultant light yellow heterogeneous mixture was stirred vigorously overnight. The reaction mixture was filtered with EtOAc, and the filtrate having precipitate was filtered again with EtOAc to remove excess sulfur. The filtrate was concentrated under reduced pressure (solid formed during evaporation was filtered with EtOAc), and the residue was purified by flash chromatography on pre-neutralized (1 % TEA in hexanes) silica gel eluting with 2:1 Hex/EtOAc to give the desired product as a white solid (1.04 g, 61 %). mp 95 ^oC; ^1H NMR (300 MHz, $CDCl_3$) δ 7.47 – 7.01 (m, 5H), 5.52 (d, J = 9.5 Hz, 1H), 3.75 – 3.28 (m, 4H); ^{31}P NMR (122 MHz, $CDCl_3$) δ 96.91; ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.11, 129.44, 124.91, 124.89, 123.22, 123.14, 42.09, 42.07; IR (thin film) 3247.0, 2963.6, 1596.5, 1494.6, 1374.5, 1273.0, 1219.8, 1029.6, 912.2, 747.0 cm^{-1}; MS (ESI) *m/z* calcd for $C_8H_{10}NNaPS_3$ $[M+Na]^+$ 270.0, found 270.1.

General Procedure for donor synthesis:

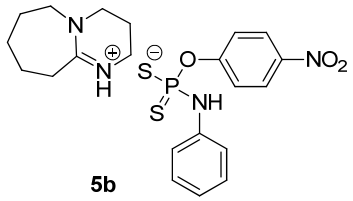
1,8-Diazo[5.4.0]-bicycloundec-7-ene (DBU) (1 equiv) was added to a solution of the compound **4** (1 equiv) and R-OH (1 equiv) in MeCN (0.1 ~ 0.2 M) at room temperature, and the reaction was stirred for 1 h at rt. The solvent was removed under reduced pressure, and the crude product was cooled to - 78 ^oC and then the solid was rinsed with cold diethyl ether and decanted to obtain the desire product as a DBU salt.



5a

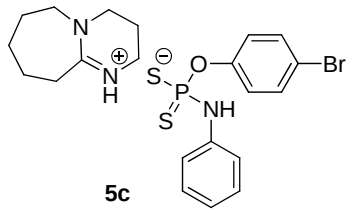
Compound **5a** was obtained as white solid (96% yield): mp 110 ^oC; ^1H NMR (300 MHz, $CDCl_3$) δ 9.60 (s, 1H), 7.34 – 7.04 (m, 6H), 6.83 (t, J = 6.9 Hz, 1H), 6.73 (d, J = 9.0 Hz, 2H), 5.75 (d, J = 10.0 Hz, 1H), 3.70 (s, 3H), 3.34 (m, 6H), 2.78 (, J = 4.9 Hz, 2H), 1.87 (q, J = 5.9 Hz, 2H), 1.72 – 1.50 (m, 6H); ^{31}P NMR (122 MHz, $CDCl_3$) δ 94.95; ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.24, 155.99, 146.67, 142.45, 128.92, 123.46, 123.40, 120.19, 118.20, 118.11, 113.99, 113.96, 55.75, 54.67, 48.88, 38.61, 33.03, 29.12, 26.88, 24.11, 19.64.; IR (thin film) 3224.7, 3100.8, 2924.8, 1637.5, 1601.6, 1575.5, 1503.8, 1438.6, 1389.8,

1282.2, 1190.9, 1102.9, 1034.5, 904.1 871.5, 832.4, 777.0, 679.3 cm^{-1}; MS (ESI, negative mode) *m/z* calcd for $C_{13}H_{13}NO_2PS_2$ $[M\text{-}DBU]^-$ 310.0, found 310.2.



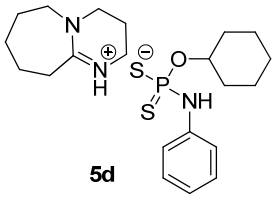
5b

Compound **5b** was obtained in 92% yield: ^1H NMR (300 MHz, $CDCl_3$) δ 9.88 (br-s, 1H), 8.12 (d, *J* = 9.1 Hz, 1H), 8.04 (d, *J* = 9.1 Hz, 1H), 7.55 – 7.41 (m, 1H), 7.32 – 7.05 (m, 4H), 6.99 – 6.78 (m, 2H), 5.85 (d, *J* = 10.3 Hz, 1H), 3.41 (m, 6H), 2.90 – 2.70 (m, 2H), 1.94 (q, 2H), 1.80 – 1.49 (m, 6H); ^{31}P NMR (122 MHz, $CDCl_3$) δ 94.70; ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.42, 166.16, 129.09, 126.48, 125.05, 122.82, 122.75, 120.86, 118.39, 118.30, 116.54, 54.77, 48.92, 38.62, 33.10, 29.14, 26.90, 24.12, 19.65; IR (thin film) 3228.1, 3105.1, 3036.6, 2932.0, 2855.2, 1643.5, 1586.1, 1513.2, 1488.2, 1383.2, 1335.9, 1285.6, 1220.4, 1158.3, 1106.3, 906.3, 872.2, 753.0, 680.1 cm^{-1}; MS (ESI, negative mode) *m/z* calcd for $C_{12}H_{10}N_2O_3PS_2$ $[M\text{-}DBU]^-$ 325.0, found 325.1.



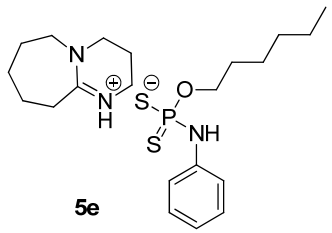
5c

Compound **5c** was obtained as white solid (95% yield): mp 108 °C; ^1H NMR (300 MHz, $CDCl_3$) δ 8.92 (br-s, 1H), 7.45 – 7.03 (m, 8H), 6.86 (t, *J* = 6.9 Hz, 1H), 5.77 (d, *J* = 10.1 Hz, 1H), 3.37 (m, 6H), 2.81 (m, 2H), 1.92 (m, 2H), 1.81 – 1.44 (m, 6H); ^{31}P NMR (122 MHz, $CDCl_3$) δ 94.85, ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.35, 142.12, 131.87, 128.99, 124.71, 124.65, 120.48, 118.29, 118.20, 54.71, 48.92, 38.61, 33.03, 29.17, 26.94, 24.12, 19.67; IR (thin film) 3269.9, 3222.6, 3106.5, 2939.2, 2857.7, 1640.4, 1597.7, 1578.0, 1479.0, 1388.2, 1319.8, 1288.9, 1200.7, 1158.7, 1066.3, 1009.2, 907.7, 866.3, 838.2, 767.7, 723.5, 699.8, 690.9 cm^{-1}; MS (ESI, negative mode) *m/z* calcd for $C_{12}H_{10}BrNOPS_2$ $[M\text{-}DBU]^-$ 357.9, found 358.0.



5d

Compound **5d** was obtained as sticky white solid (94% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.41 (br-s, 1H), 7.33 – 7.00 (m, 4H), 6.74 (m, 1H), 3.61 – 3.19 (m, 8H), 3.19 – 2.48 (m, 5H), 2.44 – 2.08 (m, 1H), 1.83 (m, 3H), 1.71 – 1.41 (m, 9H), 1.48 – 0.97 (m, 2H); ^{31}P NMR (122 MHz, $CDCl_3$) δ 91.02; ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.14, 142.50, 128.79, 128.52, 119.99, 118.85, 118.07, 70.38, 54.63, 48.87, 42.20, 41.13, 38.49, 35.75, 32.84, 31.83, 29.15, 26.93, 25.68, 24.23, 19.68; IR (thin film) 3219.4, 3091.3, 3035.4, 2928.8, 2856.4, 1642.0, 1598.2, 1495.7, 1444.4, 1382.5, 1321.2, 1285.7, 1204.8, 1105.1, 895.4, 751.5, 692.2, cm^{-1}; MS (ESI, negative mode) *m/z* calcd for $C_{12}H_{17}NOPS_2$ $[M\text{-}DBU]^-$ 286.4, found 286.3.



5e

Compound **5e** was obtained as sticky white solid (93% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25 (m, 1H), 7.21 – 6.97 (m, 3H), 6.73 (m, 1H), 5.54 (d, J = 10.2 Hz, 1H), 3.86 (m, 1H), 3.35 (m, 6H), 2.82 (m, 3H), 2.80 – 2.66 (m, 2H), 1.85 (m, 2H), 1.58 (m, 6H), 1.36 – 0.98 (m, 6H), 0.98 – 0.62 (m, 3H); ^{31}P NMR (122 MHz, $CDCl_3$) δ 95.10; ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.14, 142.60, 128.76, 119.65, 117.67, 117.58, 65.63, 54.63, 48.88, 38.50, 32.84, 31.77, 30.59, 29.14, 26.93, 25.89, 24.21, 22.82, 19.68, 14.29; IR (thin film) 3224.6, 3095.9, 3034.0, 2928.2, 2856.7, 2799.2, 1642.1, 1598.4, 1495.9, 1381.8, 1321.1, 1285.4, 1228.2, 1204.8, 1105.3, 894.8, 750.7, 692.2 cm^{-1}; MS (ESI, negative mode) *m/z* calcd for $C_{12}H_{19}NOPS_2$ $[M\text{-}DBU]^-$ 288.1, found 288.2.

GYY4137 was prepared using the known protocol.[2]

Measurement of H_2S concentrations by a fluorescent probe (DNS-N_3):[1]

(1) Calibration curve of hydrogen sulfide in 1:1 MeCN/NaPi (20 mM, pH 7.4) using a fluorometer: 20 mM of a stock solution of DNS-Azide (mw. 276.3) in absolute ethanol was freshly prepared. In a separate vial, a stock solution of $Na_2S{\cdot}9H_2O$ (mw. 240.2) at 2 mM in NaPi buffer (20 mM, pH 7.4) was freshly prepared, sealed, and kept in a refrigerator. 40 µL of DNS-Az solution (final c = 200 µM) was added into a cuvette containing 3.940 mL of 1:1 NaPi/MeCN solution. The final volume (4 mL) was prepared by adding corresponding Na_2S stock solution to bring the final concentration of 0, 2, 4, 6, 8, and 10 µM H_2S, respectively. The cuvette was immediately closed with a PTFE stopper. The solution was then mixed thoroughly and placed the cuvette into a fluorescence spectrophotometer (Agilent Tech.). The emission intensities was scanned (ranged from 400 - 680 nm) and plotted against sulfide concentrations to obtain a calibration curve.

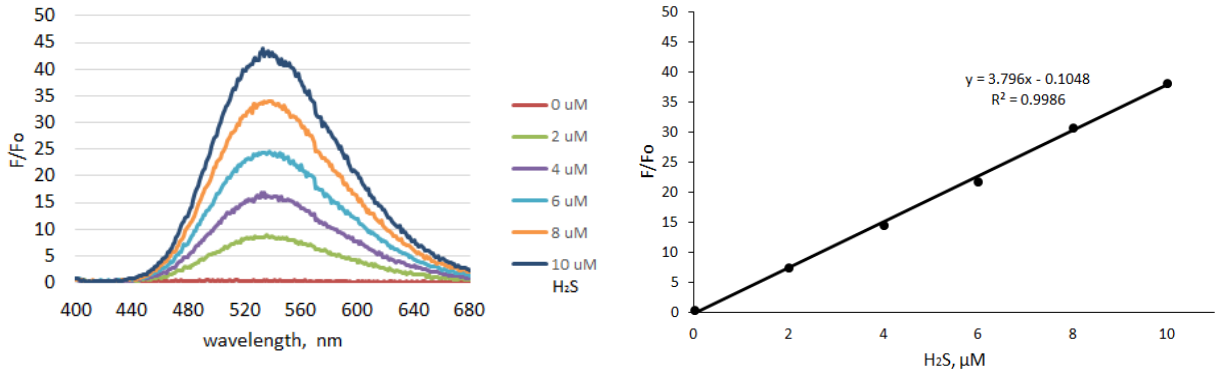


Figure S1. A calibration curve between the emission intensity *vs* H_2S concentrations.

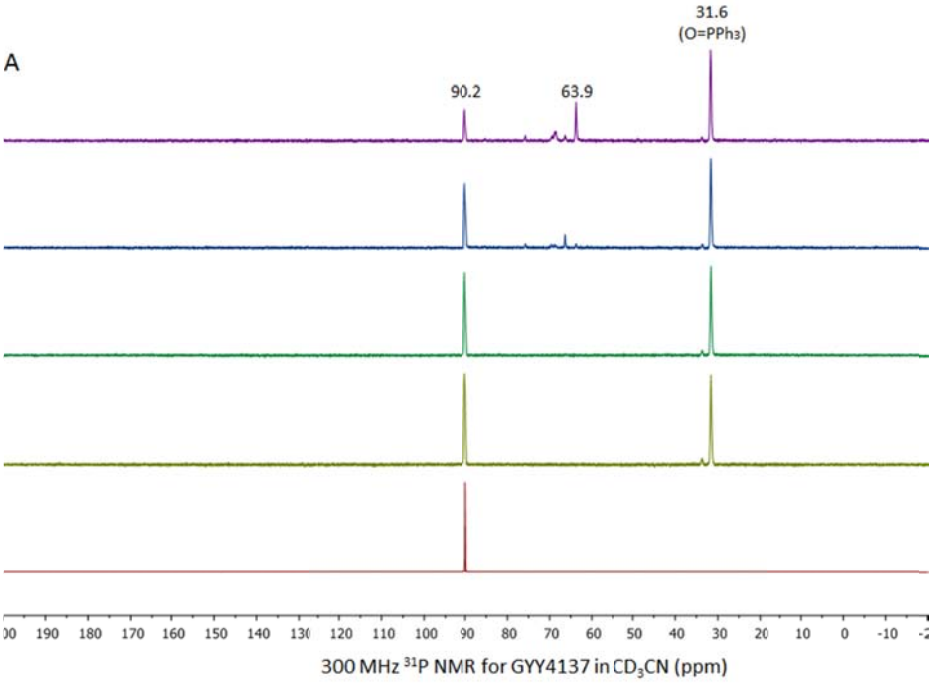
(2) Concentration determination of H_2S released from donors by a fluorescence spectrophotometer:

The time-dependent fluorescence intensity change of each donor (in the presence of DNS-Az) was measured in 1:1 solution of MeCN and 20 mM NaPi buffer (pH 7.4). To a cuvette (max volume = 4 mL) with a PTFE stopper was added 3.940 mL of MeCN/NaPi solution followed by a fresh solution of DNS-Az (40 µL in ethanol, total c = 200 µM). Then a freshly prepared solution of the donor (20 µL in 1:1 solution of MeCN/NaPi, final c = 100 µM) was immediately added into the cuvette. The solution was

mixed in dart for 1-2 min then the time-dependent fluorescence change was measured by a fluorescence spectrophotometer (Ex/Em = 340 nm/535 nm) for 3 h at room temperature.

^{31}P NMR validation of H_2S donors, (A) GYY4137 and (B) 5a

0.3 M solution containing each component (donor, internal standard, and dansyl azide) in 0.5 mL of 1:1 MeCN-*d3*/HEPES buffer (pH 7.1) were prepared. The spectra were continuously recorded on undiluted phases at 22 ^{o}C, 50 ^{o}C, and 65 ^{o}C. Triphenylphosphine oxide (O=PPh_3) was used as an internal standard. In order to validate the donors in a short time, the temperature was increased at a certain time to observe visible changes due to the donor's property of the long-lasting H_2S release. The concentration of each donor was gradually reduced relative to that of an internal standard.



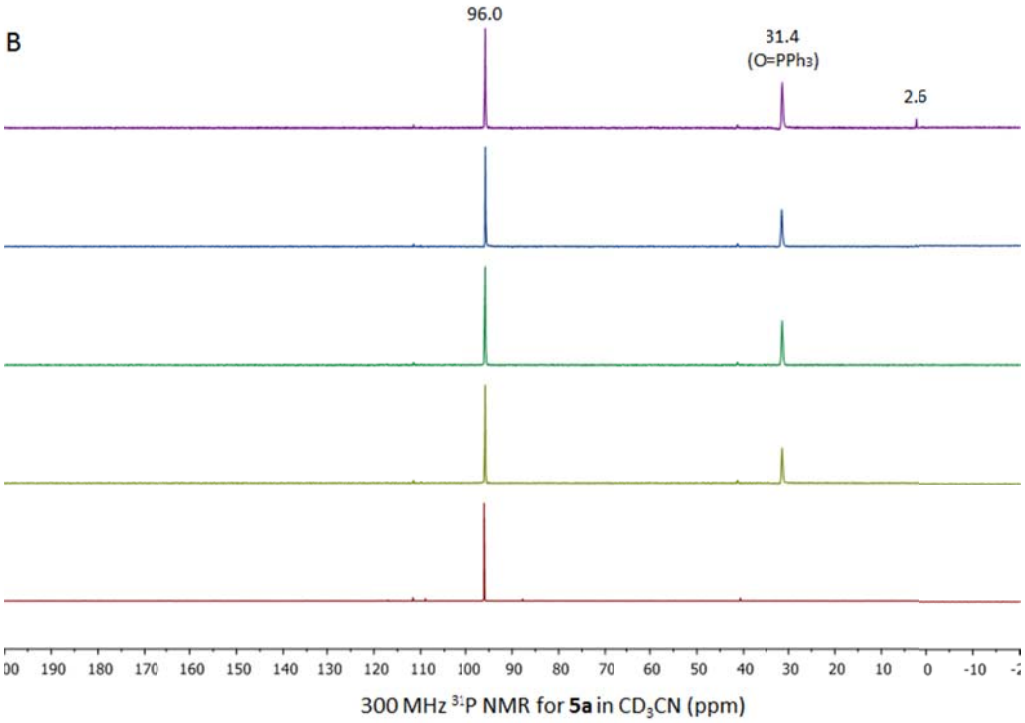


Figure S2. Proton-decoupled ^{31}P NMR spectra for (A) GYY4137 and (B) **5a**: a test without an internal standard and a dye at room temperature (red), a test with an internal standard and a dye at 22 ^{o}C for 2 h (light green), a continuous test at 22 ^{o}C after additional 2 h (green), heating to 55 ^{o}C for 3 h (blue), continuous heating to 65 ^{o}C for 1 h (purple).

Cell viability assay

Rat cardiomyoblasts H9c2 were purchased from the American Tissue Culture Collection and cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 4 mM glutamine, 100 IU/ml penicillin, and 100 ug/ml streptomycin at 37^{o}C and with 5% CO_2.

Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. and 10% CCK-8 solution in FBS-free DMEM with 20 U/mL penicillin, and 20 μg/mL streptomycin was directly added to the cell. The cell viability was measured by a microplate reader (Infinite M1000, TECAN Group Ltd.).

Cell viability was detected using CCK-8. H9c2 cells were cultured in 96-well plates, with 4 duplicate wells in each group. When 80~90% confluence was reached, the cells were treated with H_2S donors at

different concentrations in medium and incubated for 24 h. The CCK-8 solution (10 μL) at a 1:10 dilution with FBS-free DMEM/F12 (100 μL) was added to each well followed by a further 3 h incubation with 5% CO_2 at 37°C. Absorbance was automatically measured at 450 nm with a microplate reader (Infinite M1000, TECAN Group Ltd.). The mean optical density (OD, absorbance) of 4 wells in the indicated groups was used to calculate the percentage of cell viability as follows: percentage of cell viability = $(A_{treatment} - A_{blank}) / (A_{control} - A_{blank}) \times 100\%$ (where, A = absorbance).

***H_2S production in Cells*:** H9c2 cells were incubated in 24-well plates in medium for 2-3 days (>90% confluence). After removing the FBS containing medium, the cells in each well were treated with different concentrations (0, 100, and 200 μM) of H_2S donor **5a** in FBS-free DMEM (0.5 mL) medium and then incubated with 5% CO_2 at 37 ^oC for 24 h. The old medium was removed and then the cells were incubated for 1 h with 0.5 mL of a fresh medium containing WSP-1 (final c = 100 μM) in PBS buffer. After removing the medium, the cells were rinsed with PBS buffer (1 mL × 1). Then to each well was added 0.5 mL of PBS and cell images were taken. This experiment was conducted in triplicate

Cell culture and drug treatment for anti-cancer effects of donors: Mouse melanoma cell line B16BL6 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% penicillin and streptomycin. Culture conditions were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The compounds were dissolved in DMSO to create a concentrate stock solution. Cells (1.5×10^5 or 2×10^5) were seeded in a T25 flask containing 5 ml of DMEM medium. Twenty-four hours after cell seeding, the culture medium was replaced with 5 ml of fresh culture medium containing the indicated compound at the indicated concentration then incubated for 4 days. The cells in the control experiments were cultured with culture medium containing equal volume of DMSO. Cells were harvested with 0.25% trypsin-EDTA solution at the indicated time points after drug exposure. Cell number and viability were determined by Beckman Vi-CELL cell viability analyzer.

Determination of the optimal H_2O_2 concentration

To examine oxidative stress induced injury, the cells were exposed to different concentrations of H_2O_2 (50, 100, 150, 200, 300, 400, 500, 600, 700, 1000 μM) in FBS-free DMEM medium and incubated with 5% CO_2 at 37 ^oC for 5 h. The cells were exposed to highly concentrated H_2O_2 (>400 μM, see graph) were not healthy, detached, and dead. The optimal concentration of H_2O_2 for H_2S protection experiments was decided as 150 μM.

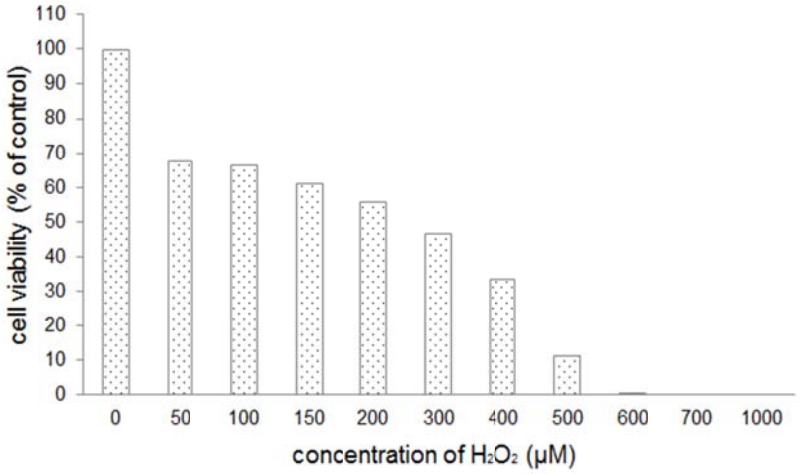


Figure S3. Effects of H_2O_2 on H9c2 cell viability. The cells were treated with increasing concentrations of H_2O_2 (0–1000 μM) for 5 h. Cell viability was measured by CCK-8 assay.

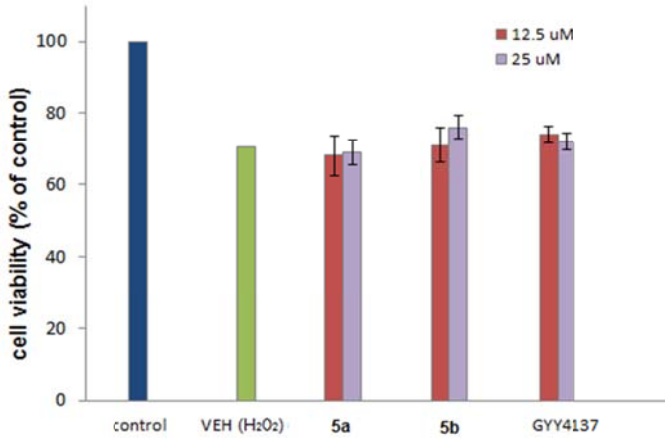


Figure S4. H9c2 cells viability after oxidative injury by H_2O_2 in the presence of donors **5a**, **5b**, and GYY4137 at 12.5 μM and 25 μM. There were no significant cell protective effects at these (or lower) concentrations.

References

1. H. Peng, Y. Cheng, C. Dai, A. L. King, B. L. Predmore, D. J. Lefer and B. Wang, *Angew. Chem. Int. Ed.*, 2011, **50**, 9672-9675.
2. L. Li, M. Whiteman, Y. Y. Guan, K. L. Neo, Y. Cheng, S. W. Lee, Y. Zhao, R. Baskar, C.-H. Tan and P. K. Moore, *Circulation*, 2008, **117**, 2351-2360.