Electronic Supplementary Information for

Imaging in living cells and zebrafishes *in vivo* using a ratiometric fluorescent probe for hydrogen sulfide

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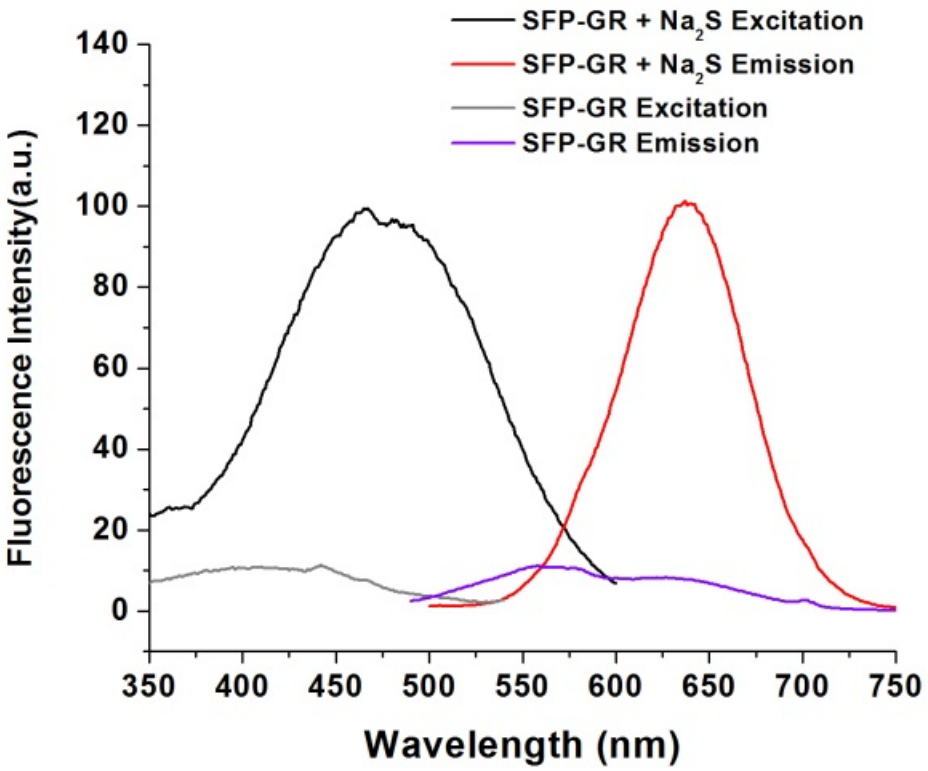


Fig. S1. The excitation and emission of SFP-GR before and after addition of hydrogen sulfide.

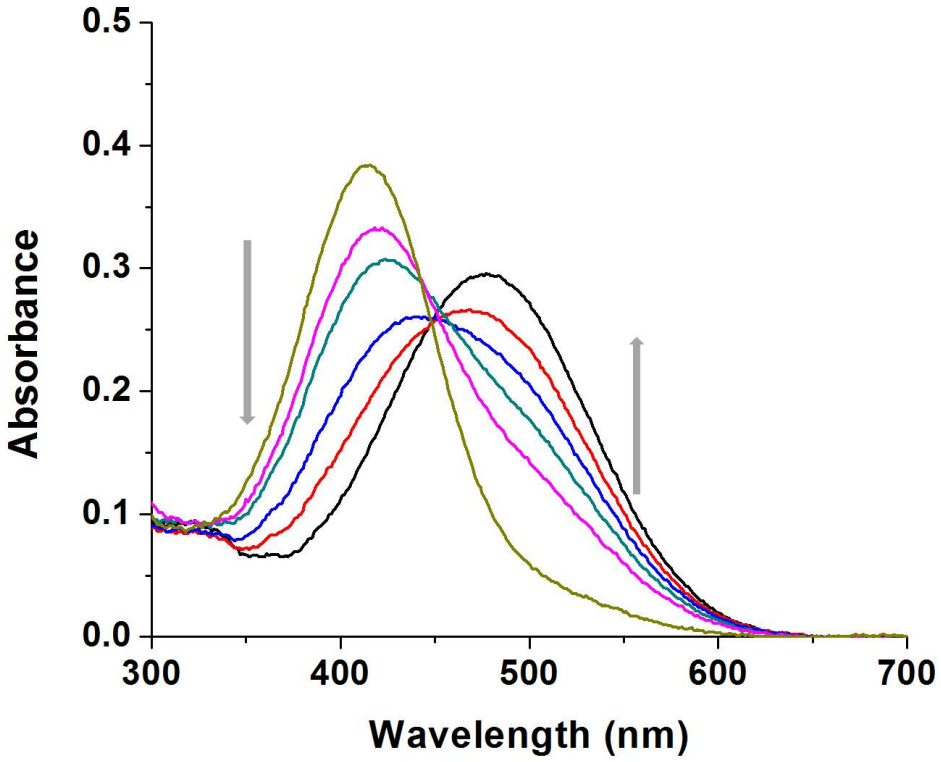


Fig. S2. The absorption spectra of SFP-GR (5 μM) recorded upon the addition of Na_2S (0–20 μM) in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1 mM CTAB.

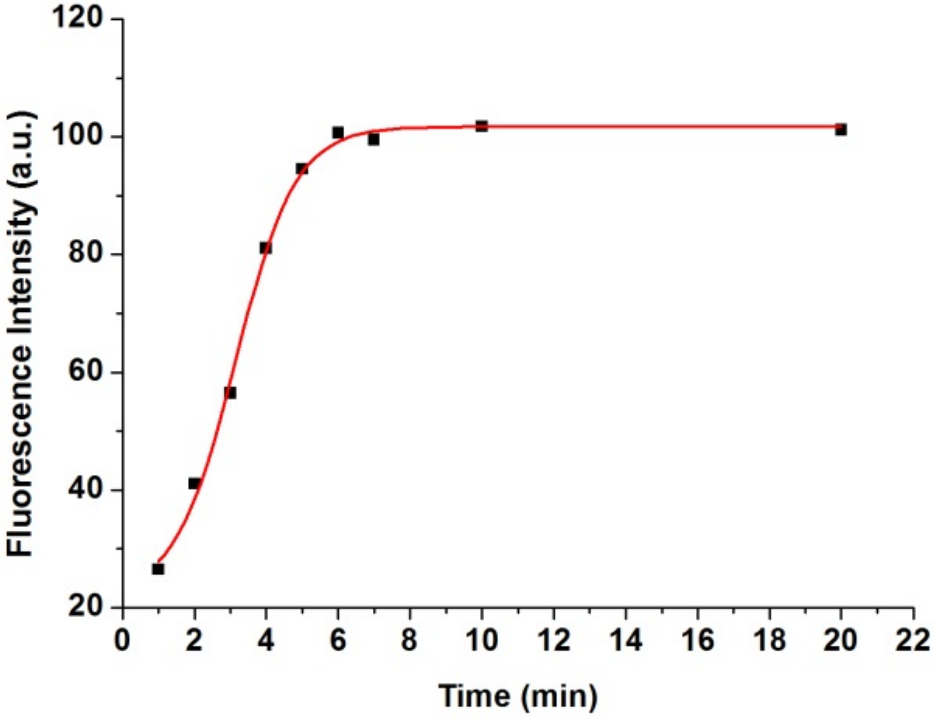
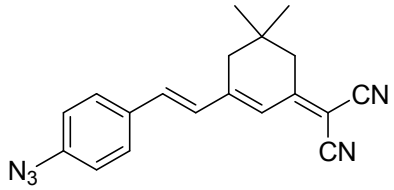
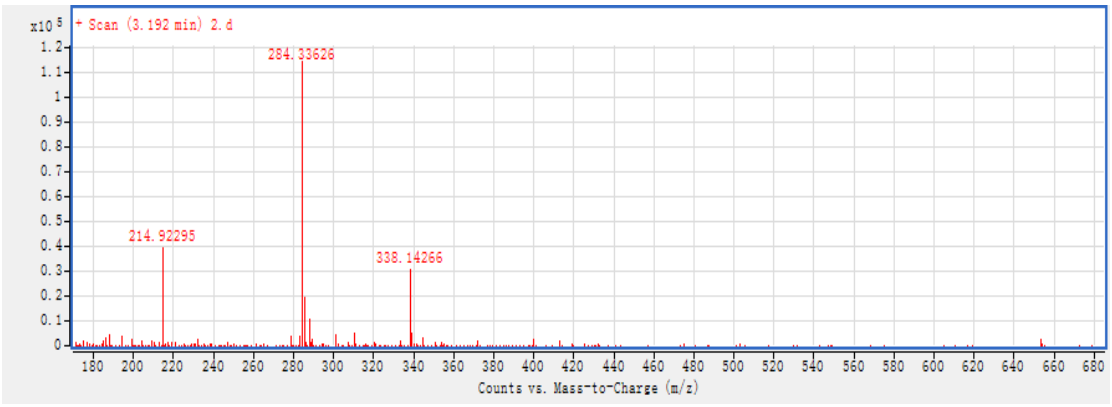
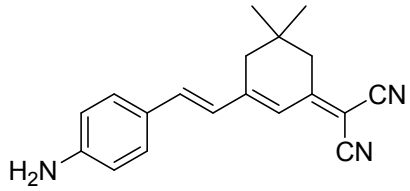


Fig. S3. Fluorescence spectra of SFP-GR (5 μM) in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1 mM CTAB incubated with different time (0-20 min) at 25 °C. Excitation wavelength: 466 nm, emission: 637 nm, excitation and emission slit widths = 5 nm. The data represents the average of three independent experiments.







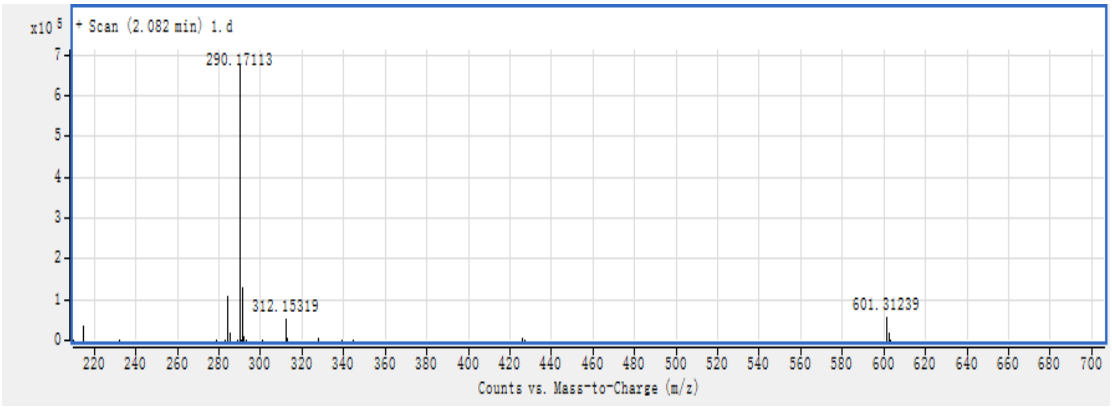


Fig. S4. HPLC-MS of SFP-GR solution before (calculated for $C_{19}H_{17}N_5Na$ $(M+Na)^+$ 338.1382; found 338.1427) and after (calculated for $C_{19}H_{20}N_3$ $(M+H)^+$ 290.1657; found 290.1711) addition of Na_2S (as an aqueous sulfide source).

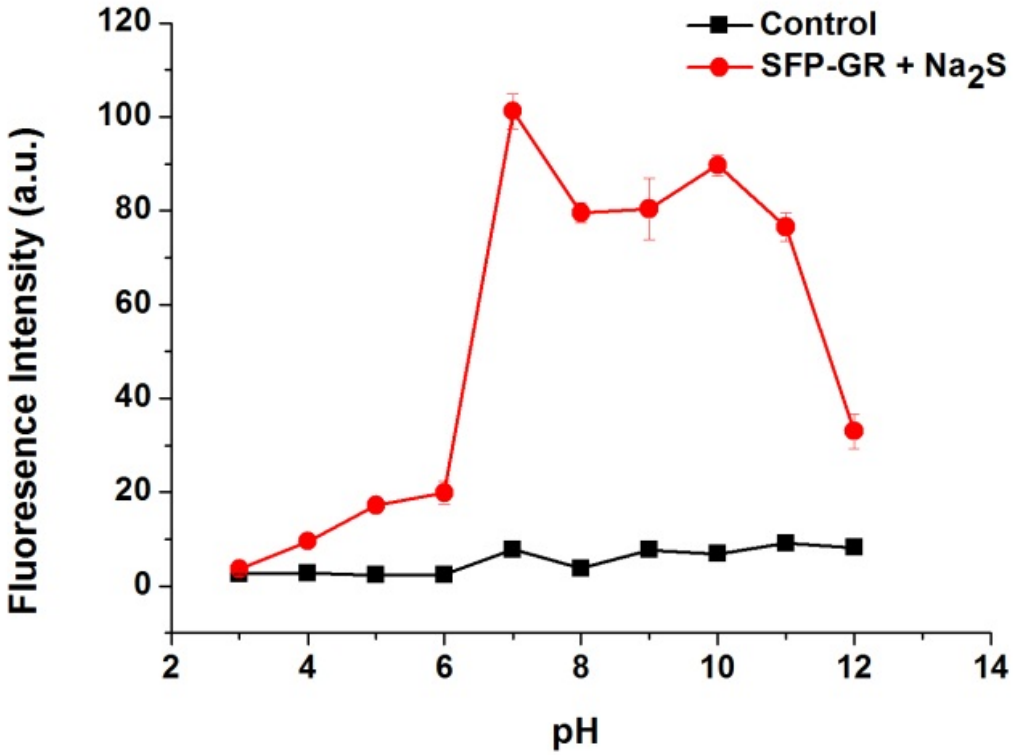


Fig. S5. The fluorescence response of SFP-GR (5 μM) toward Na_2S (20 μM) in the different pH HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1 mM CTAB at 25 °C for 10 min. All the data represent the fluorescence intensity at 637 nm, excitation: 466 nm. The data represent the average of three independent experiments.

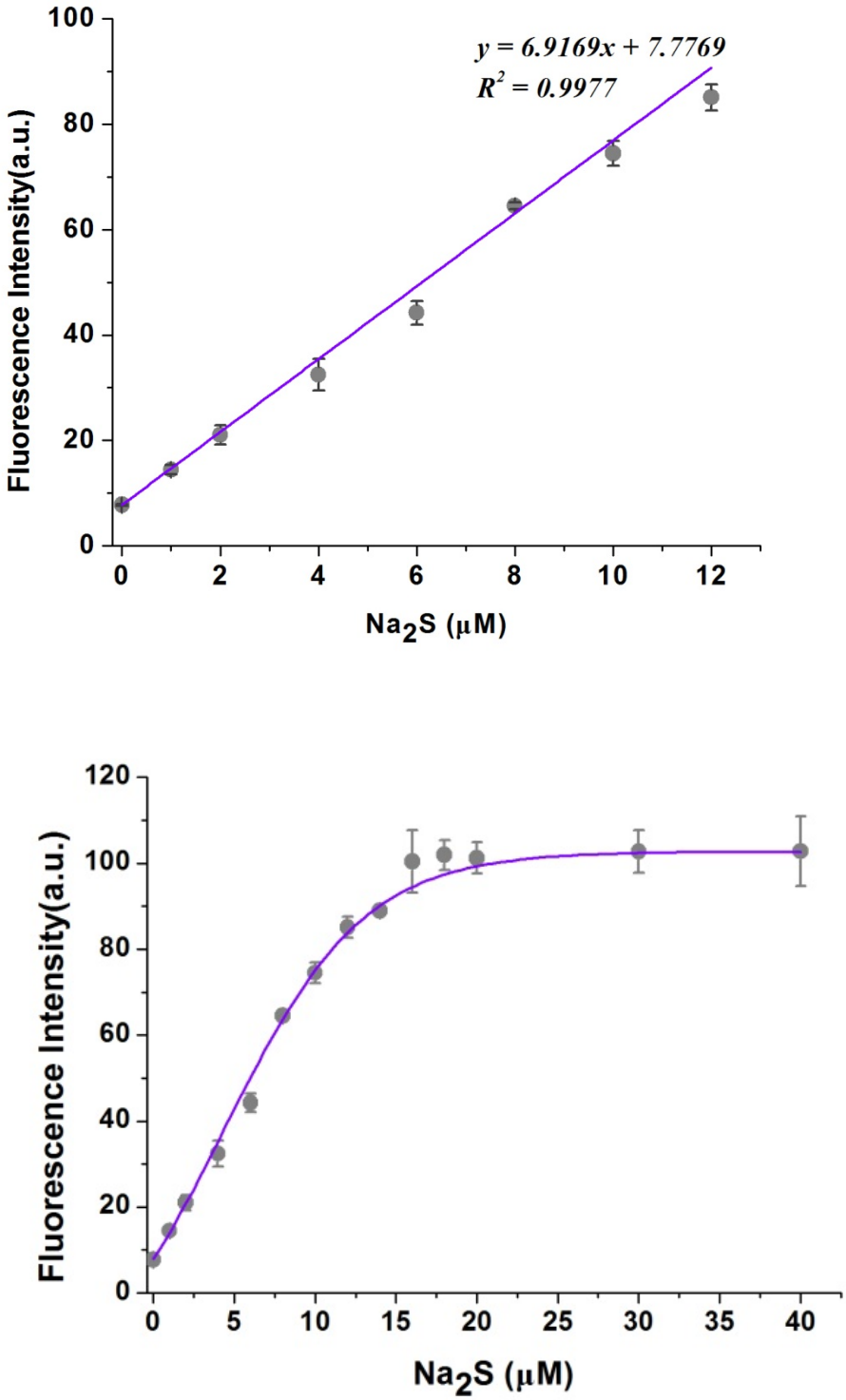


Fig. S6. Fluorescence spectra of SFP-GR (5 μM) in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1mM CTAB, then incubated with different concentrations of Na_2S for 10 min at 25 °C. (up) A linear relationship of the fluorescence intensity (637 nm) toward the concentration of Na_2S (0, 1, 2, 4, 6, 8, 10, 12 μM). (down) fluorescence intensity changes at 637 nm of SFP-GR with the amount of Na_2S. Excitation wavelength: 466 nm, emission: 450–750 nm, excitation and emission slit widths = 5 nm. The data represents the average of three independent experiments.

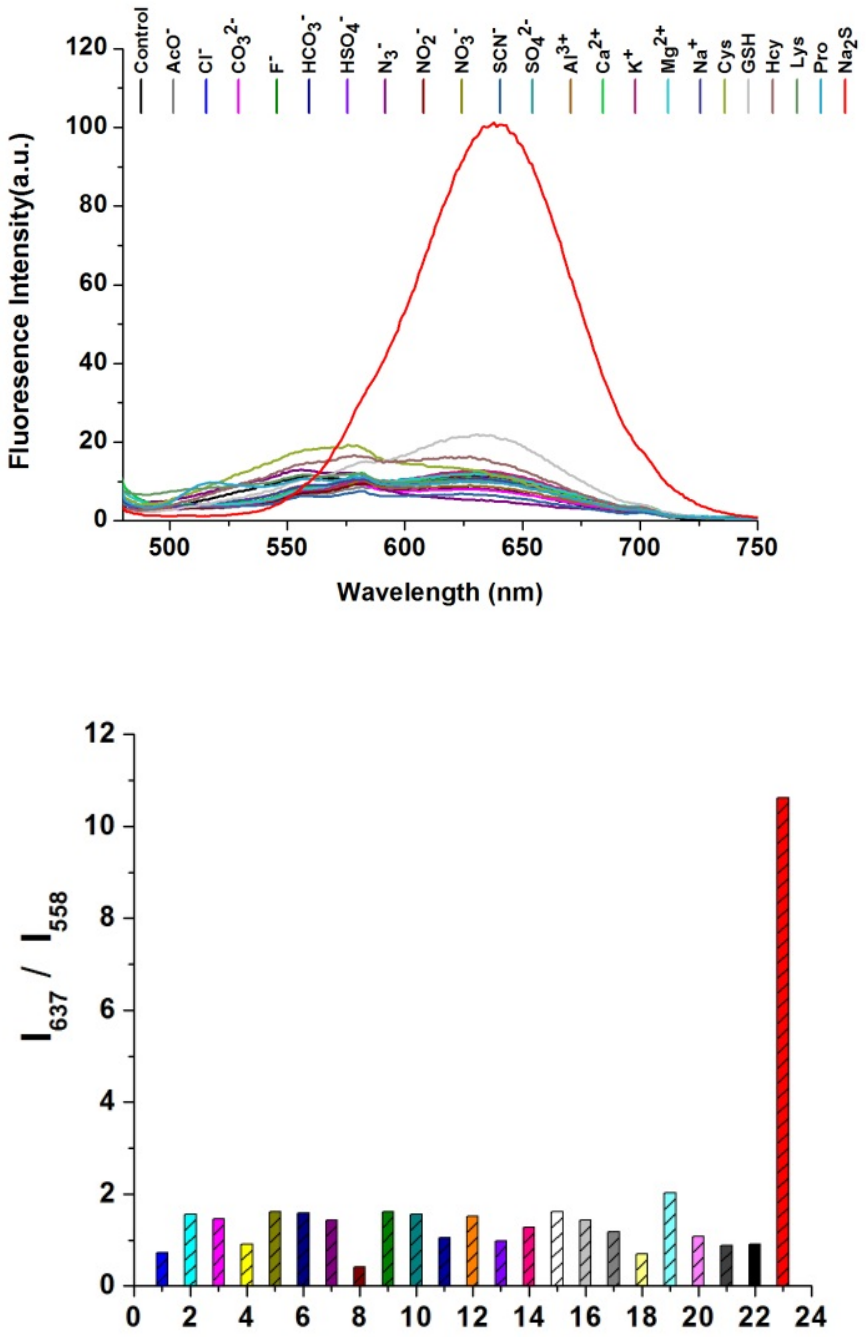


Fig. S7. Fluorescence response of SFP-GR (5 μM) to various biologically relevant species in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1mM CTAB. (a) Fluorescen spectra, (b) I_{637}/I_{558} versus various analytes: Control (probe alone); AcO^- (200 μM); Cl^- (200 μM); CO_3^{2-} (200 μM); F^- (200 μM); HCO_3^- (200 μM); HSO_4^- (200 μM); N_3^- (200 μM); NO_2^- (200 μM); NO_3^- (200 μM); SCN^- (200 μM); SO_4^{2-} (200 μM); Al_3^+ (1 mM); Ca^{2+} (1 mM); K^+ (1 mM); Mg^{2+} (1 mM); Na^+ (1 mM); Cys (1 mM); GSH (1 mM); Lys (1 mM); Pro (1 mM); Hcy (10 mM); Na_2S (20 μM). Excitation: 466 nm; emission: 637 nm.

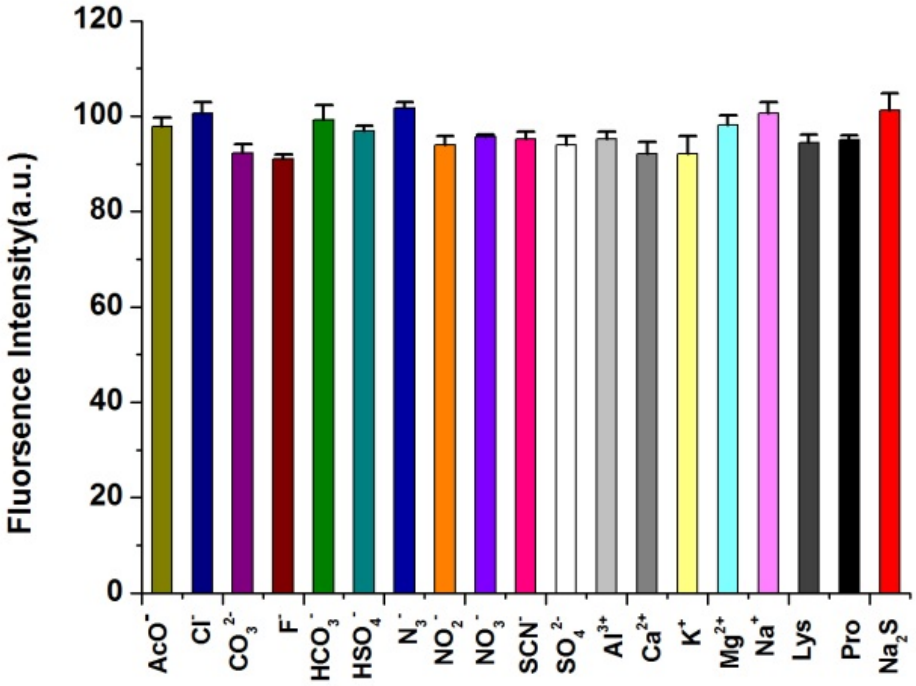


Fig. S8. Fluorescence spectra of SFP-GR in the presence of both Na_2S (20 μM) and the other biological analytes.

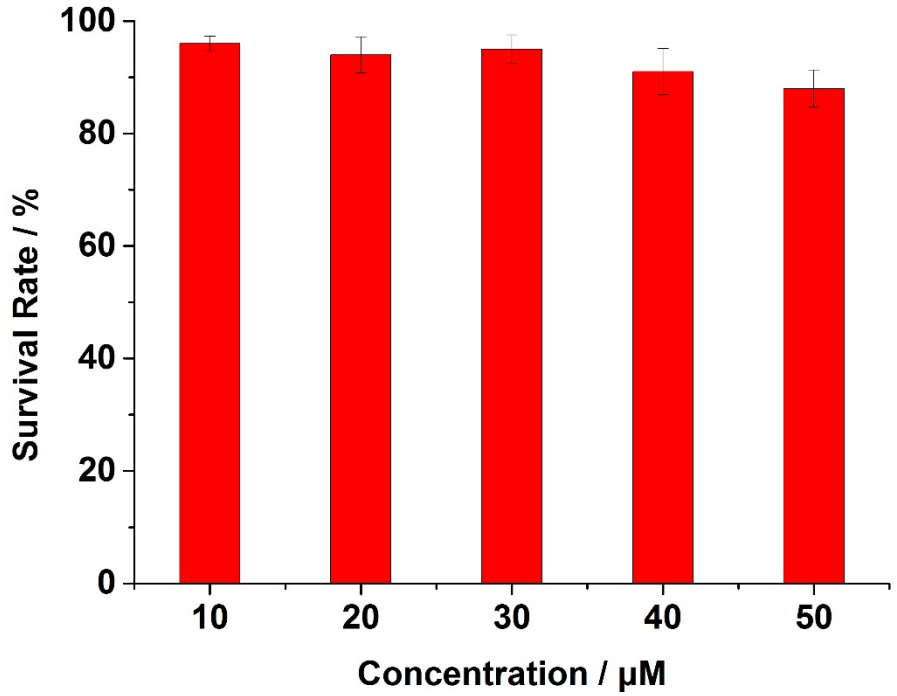


Fig. S9. An MTT assay was performed to evaluate the cytotoxicity of SFP-GR. MCF-7 cells were grown in DMEM (dulbecco's modified eagle medium) and 1% antibiotics at 37 ^{o}C in a humidified environment containing 5% CO2. Before the experiment, the cells were placed in a 96-well plate, followed by the addition of different concentrations of SFP-GR (0.0 to 50.0 μM). The cells were then incubated at 37 ^{o}C in an atmosphere of 5% CO2 for 24 h, followed by analysis *via* MTT assays (n = 10). Untreated assay with Minimum Essential medium (n = 10) was also conducted under the same conditions. As shown in Figure, the percentage of viable MCF-7 cells after treatment with 50 μM of SFP-GR for 24 hours was over 80%, which demonstrated that SFP-GR exhibited low cytotoxicity.

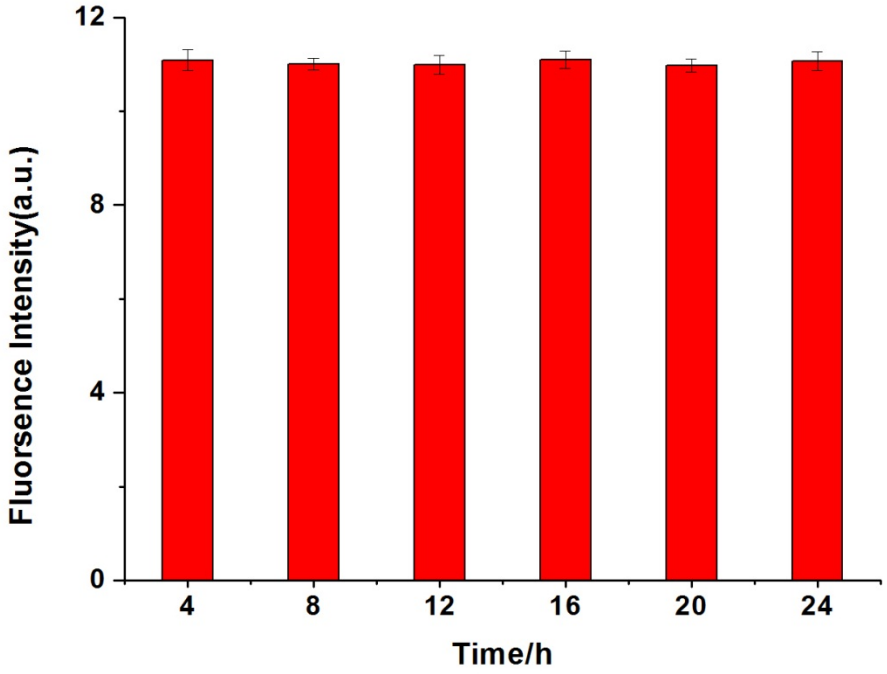


Fig. S10. Fluorescence response of SFP-GR (5 μM) in HEPES buffer (20 mM, pH 7.4, 0.5% DMSO) with 1mM CTAB for different incubation time (0- 24 h).

1. Materials and instrumentations.

All chemicals were purchased from commercial suppliers and used without further purification. Doubly distilled water was used throughout all experiments. HeLa cells were purchased from the Committee on type Culture Collection of Chinese Academy of Sciences. Zebrafish were purchased from the Model Animal Research Center in Nanjing University. Chromatographic purification of products was accomplished by using forced-flow chromatography on silica gel (300-400 mesh). Thin layer chromatography was performed on EM Science silica gel 60 F254 plates (250 μm). Visualization of the developed chromatogram was accomplished by UV lamp. Nuclear magnetic resonance (NMR) spectra were acquired on Bruker DRX-400 operating at 100 MHz for ^{1}H NMR and ^{13}C NMR, respectively; residual protio solvent signals were used as internal standards for calibration purposes. Data for ^{1}H NMR are reported as follows: chemical shift (ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), integration, coupling constant (Hz). High resolution mass spectra was performed by Mass Spectrometry Facility at Nanjing University. All fluorescence measurements were recorded on a Hitachi Fluorescence Spectrophotometer F-7000. All absorption measurements were recorded on a Shimadzu UV-2550 spectrophotometer. All imaging experiments were performed on a fixed cell DSU spinning confocal microscope (Olympus). The pH measurements were carried out on a HI 2221 calibration check pH/ORP meter.

2. Synthesis and Characterization of compounds

(4-azidophenyl)methanol (2)

To a solution of 4-aminobenzyl alcohol (600 mg, 4.88 mmol) ***(1)*** in 10 mL of 10% HCl aqueous solution was added $NaNO_2$ (402 mg, 5.84 mmol) in 6 mL aqueous solution at 0 ^{o}C and stirred for 30 minutes. Then NaN_3 (380 mg, 5.84 mmol) in 6 mL aqueous solution was added at 0 ^{o}C and stirred for another hour. The reaction mixture was warmed to 25 ^{o}C, diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4, concentrated in vacuo and subjected to silica gel chromatography. A yellow oil (620 mg, 85% yield) was obtained by silica gel column chromatography using Petroleum ether/EtOAc (5:1, v/v) as eluent. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, J = 8.6 Hz, 2H), 7.00 (d, J = 8.5 Hz, 2H), 4.63 (s, 2H), 2.14 (s, 1H). ^{13}C NMR

($CDCl_3$, 100 MHz) δ (ppm): 139.3, 137.5, 128.5, 119.1, 64.6.

4-azidobenzaldehyde (3)

A solid of pyridinium chlorochromate (1.73 g, 8.06 mmol) in anhydrous CH_2Cl_2 (10 mL) was added (4-azidophenyl) methanol (600mg, 4.03 mmol) in anhydrous DCM. Then $MgSO_4$ (483mg, 4.03 mmol) was added, and the reaction was stirred for 4 hours under room temperature. Upon completion, it was cooled to room temperature and poured into diethyl ether. The solution was then filtered through a pad of silica and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using Petroleum ether/EtOAc (50:1, v/v) as eluent to afford a yellow oil. Yield: 482 mg (80.9%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.92 (s, 1H), 7.86 (d, *J* = 8.7 Hz, 2H), 7.13 (d, *J* = 8.4 Hz, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 190.6, 146.2, 133.2, 131.5, 119.4.

2-(3,5,5-trimethylcyclohex-2-enylidene) malononitrile (4)

A solution of malononitrile (1.82 g, 27.6 mmol) in dry ethanol (150 mL) was added to a mixture of piperidine (23 mg, 0.276 mmol) and isophorone (3.8 g, 27.6 mmol). The solution was stirred at 60 ^{o}C for 12h. After cooling to room temperature, the black solution was slowly poured into water (200 mL) and the precipitated solid was filtered. Recrystallization from *n*-hexane afforded a brown solid. Yield: 1.2 g (24%). M.p. 72-74 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.60 (d, *J* = 1.36 1H), 2.50 (s, 2H), 2.17 (s, 2H) 2.02 (d, *J* = 0.8, 3H), 1.00 (s, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 170.5, 159.9, 120.6, 113.2, 112.5, 45.7, 42.6, 32.4, 27.8, 25,4.

2-(3-(4-azidostyryl)-5,5-dimethylcyclohex-2-en-1-ylidene)malononitrile (5, SFP-GR)

To a stirred solution of 2-(3,5,5-trimethylcyclohex-2-enylidene) malononitrile (253 mg, 1.36 mmol) and 4-azidobenzaldehyde (200mg, 1.36 mmol) in dry ethanol (5 mL) was added piperidine (11.6 mg, 0.136 mmol). The mixture was stirred at 85 ^{o}C for 12 hours. The resulting mixure was extracted with EtOAc (100 mL×3), and the extract was washed with brine (100 mL). The organic phase was dired over anhydrous Na_2SO_4, filtered and evaporated to dryness *in vacuo*. The residue was purified by

flash column chromatography on silica gel (Petroleum ether/EtOAc, 40:1, v/v) to afford SFP-GR as a solid (124 mg, 28.9%). M.p. 135-137 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.50 (d, *J* = 8.52 2H), 7.05 (d, *J* = 8.68 2H), 6.99 (s, 1H) 6.95 (s, 1H), 6.84 (s, 1H) 2.60 (s, 2H) 1.08 (s, 6H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 169.2, 153.7, 141.4, 135.9, 132.5, 129.1, 128.8, 123.7, 119.7, 113.5, 112.8, 78.8, 43.0, 39.2, 32.1, 28.1. HRMS: $C_{19}H_{17}N_5Na$ $(M+Na)^+$ 338.1382; found 338.1427.

3. General procedure for analysis.

All the solvents were of analytic grade. The stock solutions of all the tested anions were prepared from NaOAc, NaCl, Na_2CO_3, NaF, $NaHCO_3$, $NaHSO_4$, NaN_3, $NaNO_2$, $NaNO_3$, NaSCN, Na_2SO_4 with doubly distilled water. The stock solutions of all the tested cations were prepared from $AlCl_3$, $CaCl_2$, KCl, $MgCl_2$, NaCl with doubly distilled water. The stock solutions of cysteine, glutathione, homocysteine, lysine and proline were prepared in doubly distilled water. The stock solution of $Na_2S{\cdot}9H_2O$ (≥98.0%) was prepared in doubly distilled water, which was freshly prepared each time before use. Stock solution of SFP-GR (10 mM) was prepared in DMSO, which was diluted to the required concentration for measurement. All fluorescence measurements were carried out at room temperature on a Hitachi Fluorescence Spectrophotometer F-7000. The samples were excited at 466 nm with the excitation and emission slit widths set at 5.0 nm. The emission spectrum was scanned from 470 nm to 750 nm at 1200 nm/min. The photomultiplier voltage was set at 650 V.

4. Cell culture and fluorescence imaging.

HeLa cells were grown up in DMEM medium with 10% fetal bovine serum/penicillin/streptomycin in a 5% CO_2 atmosphere at 37 ^oC. Cells were then seeded on Coverglass-Bottom confocaldish and continuously incubated at 37 ^oC in a 5% CO_2 atmosphere for 24 h. After HeLa cells were incubated with **SFP-GR** (10 μM) for 30 min at 37 ^oC, media was removed and cells were washed with PBS buffer three times again to remove any probe left in solution to optimize the background signal. Then, cells were added fresh media and incubated with Na_2S (20 μM) for another 30 min at 37 ^oC before imaging. All imaging experiments were performed on a fixed cell DSU spinning confocal microscope (Olympus). The excitation wavelength was 488 nm, the band path for green channel was 520-560 nm and that for red channel was 620-660 nm. Excitation and emission monitored used Alexa Fluor 488 filters. Imaging performed using oil-immersion objective lens, and captured using Slidebook software. For all experiments, solution of **SFP-GR** was prepared in DMSO (10 mM) and diluted into DMEM to the desired working concentration (10 μM). Na_2S was diluted into DMEM to the desired working concentrations (20 μM) from a 1 M stock solution.

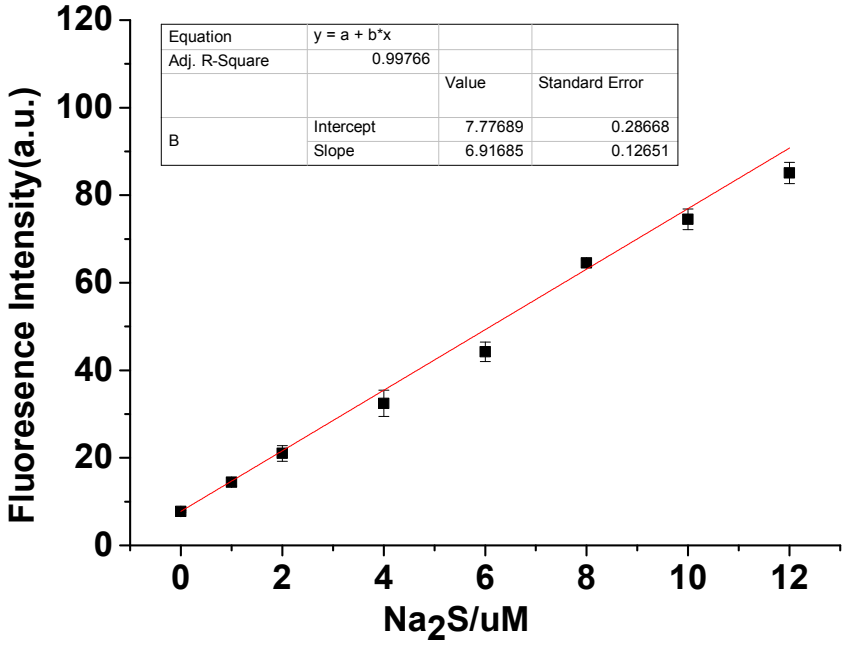
5. Zebrafish cultivation and fluorescence imaging.

All animal procedures are approved by the Institutional Animal Care and Use Committee at Nanjing University. First, male and female fish mating, the next day, collect embryo and grow for five days. After zebrafish were treated with **SFP-GR** (10 μM) for 30 min at room temperature, media was removed and zebrafish were washed with PBS buffer three times again to remove any probe left in solution to optimize the

background signal. Then, zebrafish were added fresh media and treated with Na_2S (20 μM) for another 30 min at room temperature before imaging. All imaging experiments were performed on a fixed cell DSU spinning confocal microscope (Olympus). The excitation wavelength was 488 nm, the band path for green channel was 520-560 nm and that for red channel was 620-660 nm. Excitation and emission monitored used Alexa Fluor 488 filters. Imaging performed using X10 objective, and captured using Slidebook software. For all experiments, solution of **SFP-GR** was prepared in DMSO (10 mM) and diluted into DMEM to the desired working concentration (10 μM). Na_2S was diluted into DMEM to the desired working concentrations (20 μM) from a 1 M stock solution.

6. Detection limit of probe.

The emission spectrum of 5 μM **SFP-GR** in HEPES (20 mM, pH = 7.40, 0.5% DMSO, v/v) was collected for 20 times to determine the background noise σ. Then the solution was treated with Na_2S of concentration from 1 to 40 μM, and all spectra were collected after mixing for 6 min. A linear regression curve was then fitted according to the emission intensity (637 nm) in the range of Na_2S from 0 to 12 μM, and the slope of the curve was obtained. The detection limit (3σ/slope) was then determined to be 0.077 μM.



7. Quantum yield determination of probe.

Fluorescence quantum yield of **SFP-GR** were determined in pure methanol with rhodamine B (Φ = 0.69, λ_{ex}=365 nm) as a reference. The quantum yield was calculated using Eq.1:

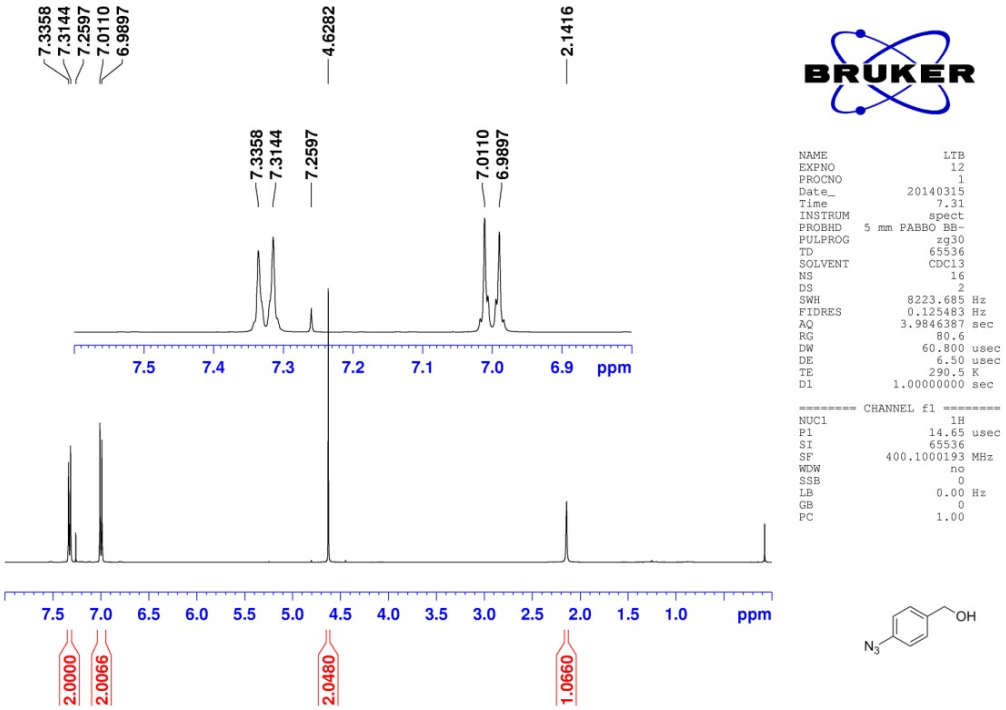
$\Phi u = [(A_s F_u n^2)/ (A_u F_s n_0^2)]\Phi s.$ (Eq.1)

Where A_s and A_u are the absorbance of the reference and sample solution at the reference excitation wavelength, F_s and F_u are the corresponding integrated fluorescence intensity, and n and n_0 are the solvent refractive indexes of sample and reference, respectively. Absorbance of sample and reference at their respective excitation wavelengths was controlled to be lower than 0.05.

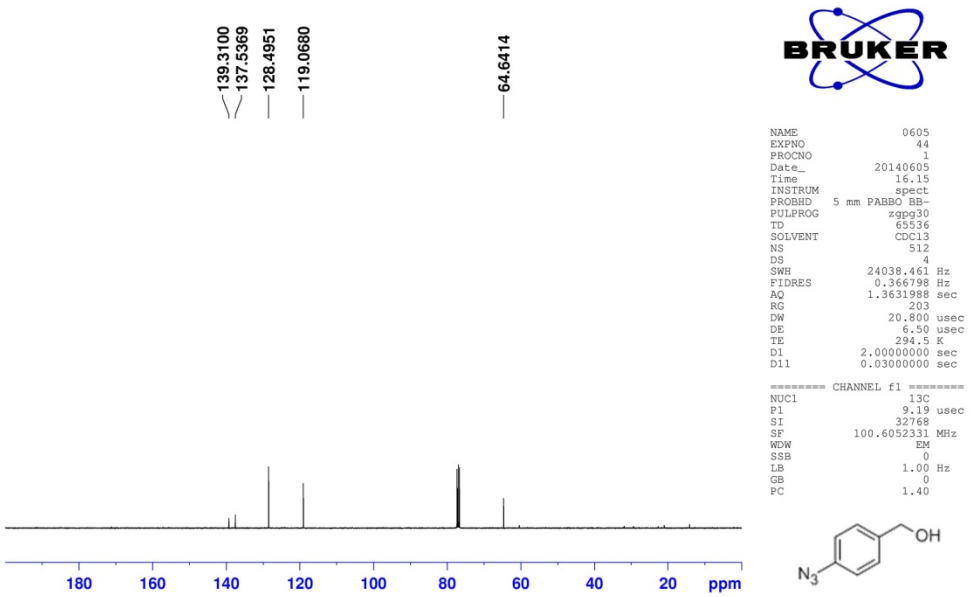
Quantum yield: Φ = 0.4674

NMR spectrum of compounds

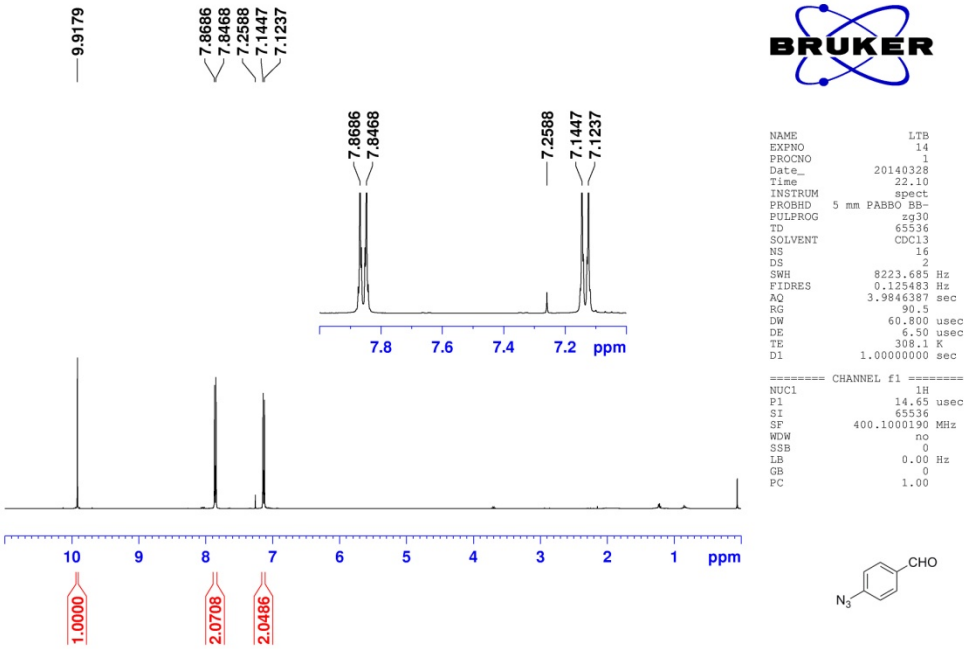
^{1}H NMR of compound 1 (400MHz, in $CDCl_3$)



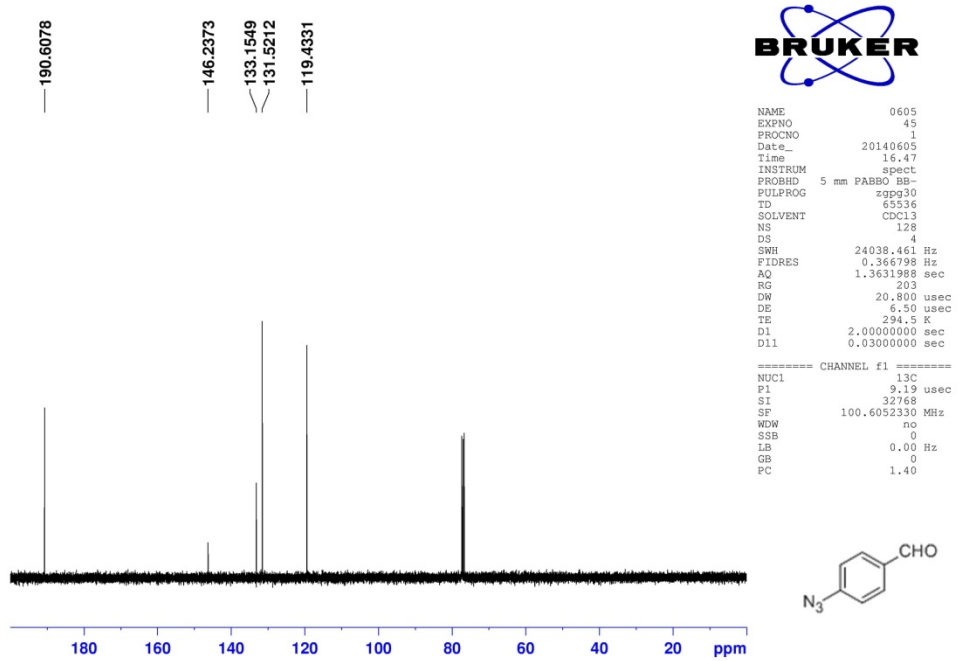
^{13}C NMR of compound 1 (100MHz, in $CDCl_3$)



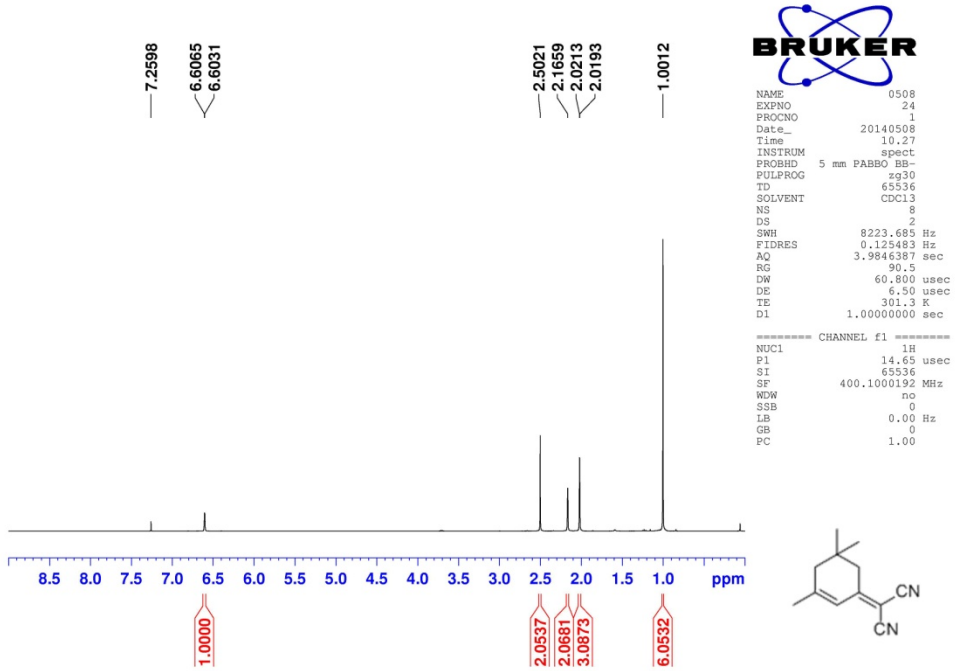
^{1}H NMR of compound 2 (400MHz, in $CDCl_3$)



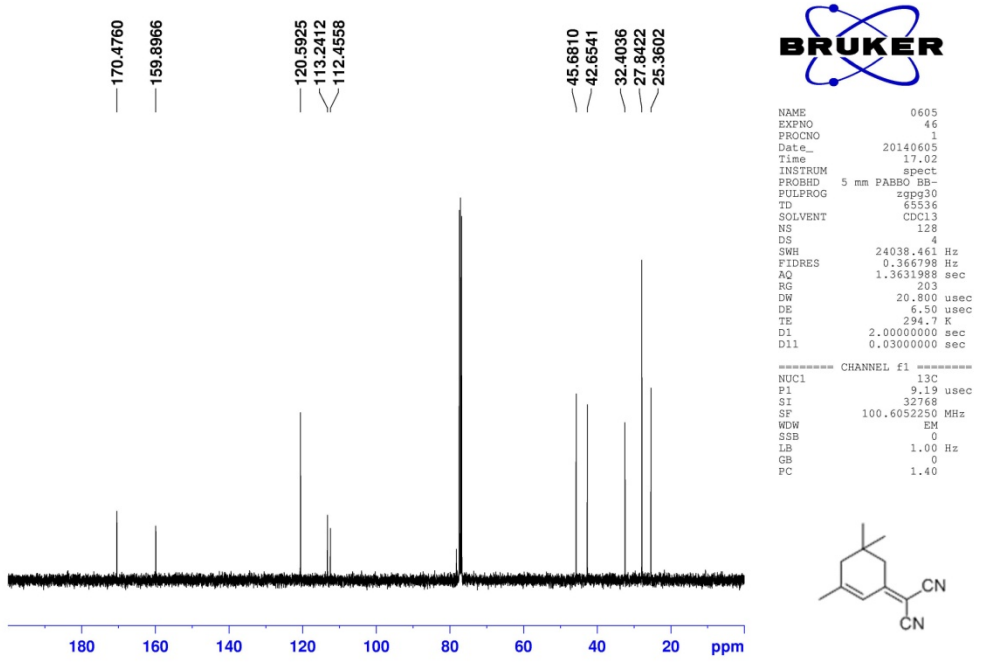
^{13}C NMR of compound 2 (100MHz, in $CDCl_3$)



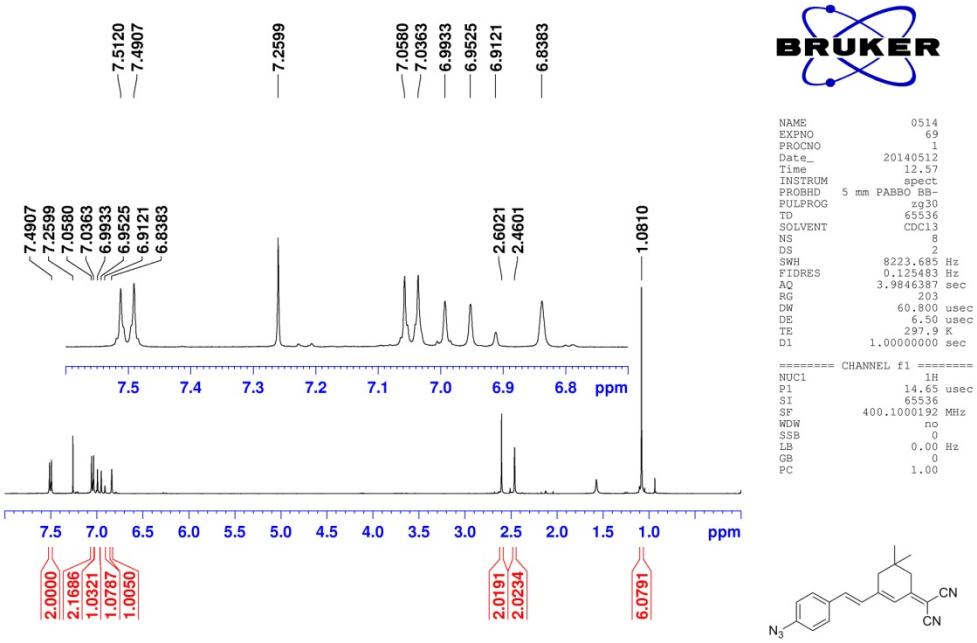
^{1}H NMR of compound 3 (400MHz, in $CDCl_3$)



^{13}C NMR of compound 3 (100MHz, in $CDCl_3$)



^{1}H NMR of compound 4 (400MHz, in $CDCl_3$)



^{13}C NMR of compound 4 (100MHz, in $CDCl_3$)