Supplementary Information

For

A Bioorthogonal Fluorescent Probe for Mitochondrial Hydrogen Sulfide: New Strategy for Cancer Cell Labelling

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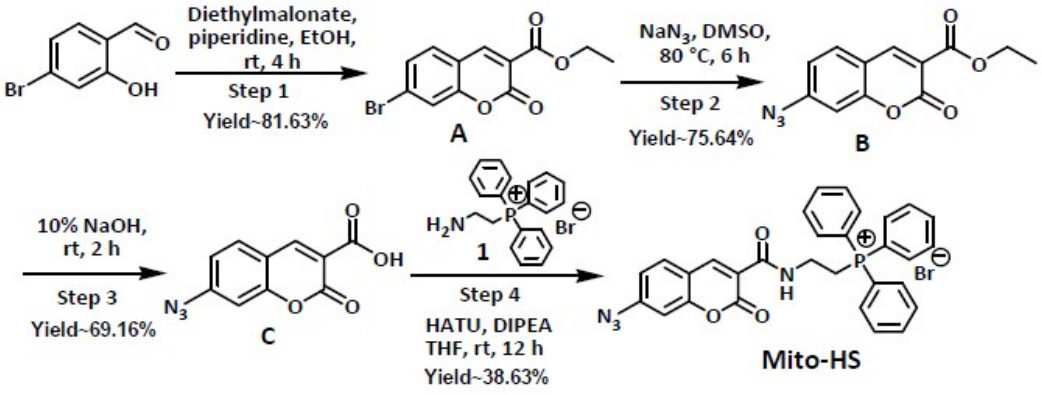
EXPERIMENTAL SECTION

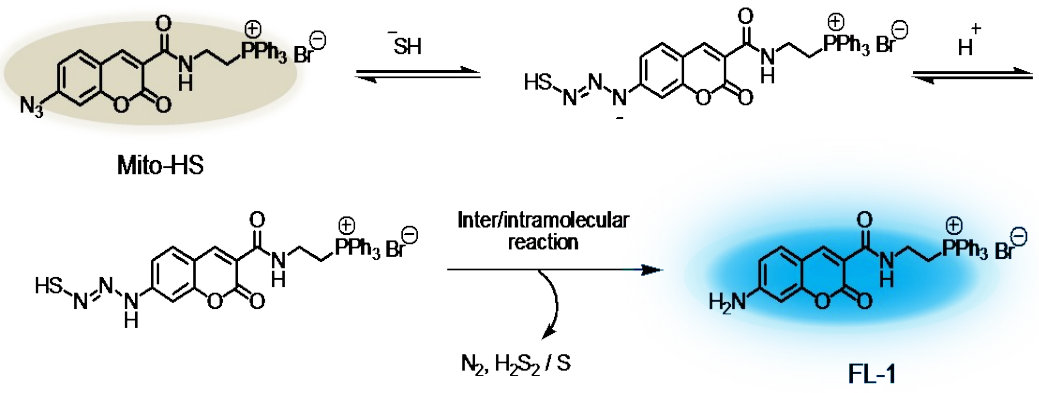
General Information and Methods. Diethylmalonate (Avra, India), 4-bromo-2-hydroxybenzaldehyde (Alfa Aesar, India), piperidine (Avra, India), ethanol (Changshu Yangyuvan Chemical, China), sodium azide (Loba Chem, India), DMSO (Loba Chem, India), sodium hydroxide (Himedia, India), 1-[bis(dimethylamino) methylene]-1*H*-1,2,3-triazolo[4,5-*b*] pyridinium 3-oxid hexafluoro-phosphate,(HATU) (Aldrich), DIPEA (Himedia, India), triphenylphosphine (Avra, India), 2-bromoethylamine.HBr (Avra, India), THF (Merck, India), and sodium sulfate (Loba Chem, India) were purchased and used without further purification. Column chromatography was performed using silica gel (100-200 mesh, Loba Chem) as the stationary phase. Analytical thin layer chromatography was performed using silica gel 60 (precoated sheets with 0.25 mm thickness). UV-vis. spectra were recorded with a Shimadzu UV-1800 spectrophotometer. NMR spectra were collected on a 400MHz spectrometer (Bruker, Germany). Mass spectra were recorded on IonSpec HiResESI mass spectrometer.

Cells and Reagents for Biological Assay. Human cervical cancer cells (HeLa), breast cancer cells (MDA-MB-231), prostate cancer cells (DU145) and fibroblast cells (3T3-L1) were obtained from National Center for Cell Sciences, Pune, India. Cell culture medium DMEM, DMSO (cell culture grade) MTT, PAG and AOAA purchased from Sigma Aldrich. Fetal Bovine Serum (FBS), Penstrep, Amphotericin B and **MitoSOX-Red** purchased from Invitrogen. NEM obtained from Spectrochem.

Synthesis of Probe Mito-HS



Scheme 1. Synthesis of **Mito-HS**



Scheme 2. Reaction between **Mito-HS** and H_2S

Synthesis of compound A: To a solution of 4-bromo-2-hydroxybenzaldehyde (1.0g, 4.97 mmol) in ethanol (20 mL), diethylmalonate (955 mg, 5.97 mmol), piperidine (1.27 g, 14.92 mmol) were added. The reaction was continued to star at rt for 3h. After completion of reaction, ethanol was evaporated. The residue was dissolved in HCl (2N) and extracted with ethyl acetate. The organic layer was washed with water, brine and dried over anhydrous sodium sulfate. The organic layer was concentrated under reduced pressure to obtain compound **A** as white color solid (1.20 g, 81.63%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.76 (s, 1H); 7.86 (d, 1H, *j* = 9.08 Hz); 7.78 (s, 1H); 7.62-7.60 (dd, 1H, *j* = 6.88 Hz); 4.30 (q, 2H); 1.30 (q, 3H); ^{13}C-NMR (100 MHz, DMSO-d_6): 162.34, 157.05, 155.32, 154.74,153.69, 148.09, 147.92, 140.81, 131.55, 130.24, 129.64, 127.95, 127.62,125.39, 125.19, 119.11, 117.4,61.25,47.30, 46.52,44.09, 25.82, 24.81, 23.82, 22.74, 14.11, 13.85.ESI- HRMS m/z (M + K^+): calcd. 336.200, found 336.02; LC-MS purity: 98.10 %.

Synthesis of B: To compound **A** (1.3 g, 4.37 mmol) in DMSO (20 mL), sodium azide (341 mg, 5.25 mmol) was added. The reaction mixture was stirred at 80^{o}C for 6 h. After completion of reaction, ice-cooled water was added and the mixture was stirred for 20 min. The precipitated solid product was filtered, washed with water and dried in vacuo to obtain compound **B** as pale brown solid (0.860 g, 75.64%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.73 (s, 1H); 7.89 (s, 1H); 7.17 (m, 1H); 4.28 (m, 2H); 1.26 (t, 3H, , *j* = 6.18 Hz).^{13}C-NMR (100 MHz, DMSO-d_6): 162.34, 154.73, 147.92, 145.93, 131.55, 127.92, 119.11, 116.49, 115.75, 114.81, 106.32, 61.25,13.85. ESI- HRMS m/z (M + Na^+): calcd. 282.04, found 282.060.

Synthesis of C: Compound **B** (600 mg, 2.30 mmol) was dissolved in NaOH solution (10 mL, 10 %) and stirred for 1 h. After completion of reaction, pH was adjusted to ~3 using HCl (2.0 N). The organic compound was extracted with ethyl acetate. The combined organic layers were washed with water, brine and dried over anhydrous sodium sulfate and evaporated under reduced pressure to afford compound **C** as pale yellow solid (370 mg, 69.16%). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.11 (s, 1H), 8.71 (s, 1H), 7.90 (t, 1H, , j = 6.21 Hz), 7.16 (m, 2H).^{13}C-NMR (100 MHz, DMSO-d_6): 163.84, 156.39, 155.65, 148.01, 145.74, 131.72, 127.89, 116.25, 115.02, 106.35.ESI- HRMS m/z (M-1+2Na^+): calcd. 276.00, found 275.99.

Synthesis of 1: Synthesized according to reported literature.[1]

Synthesis of Mito-HS: To a solution of compound **C** (340 mg, 1.46 mmol) in THF (60 mL) at 0℃, **1** (821mg, 1.78 mmol), HATU (835.0 mg, 2.2 mmol) and DIPEA (1.13 g, 8.8 mmol) were added. The reaction was continued to stir for 12h at rt. After completion of reaction, the reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated in vacuo. The crude product was purified by column chromatography over silica gel (100-200 mesh) using methanol in DCM (0.5: 9.5) as eluent to afford the probe **Mito-HS** as pale yellow solid (295 mg, 38.63%).^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.95 (t, 1H, j = 5.89 Hz), 8.78 (s, 1H), 7.98 (s, 1H), 7.86 (m, 9H), 7.75 (m, 6H), 7.29 (m, 1H), 7.22 (dd, 1H, j_1= 4.89 Hz, j = 12.01 Hz), 3.87 (m, 2H), 3.73 (m, 2H), 1.23 (s, 1H).^{13}C-NMR (100 MHz, DMSO-d_6): 162.47, 155.65, 148.43, 145.92 131.95, 127.92, 119.11, 116.18, 115.89, 114.81, 106.32, 61.08, 13.99. ESI- HRMS m/z (M^+): calcd. 519.159, found 519.158; m/z (M-N_2): calcd. 491.152, found 419.152; HPLC purity: 98.33%

Absorption and fluorescence studies. All fluorescence and UV-vis. spectra were obtained with RF- 6000 FL spectrometer with a 1cm standard quartz cell and UV-1800 spectrophotometer, respectively. Sodium sulfide (Na_2S) was used as the source of H_2S. Stock solutions (600 μM) of various analytes (cysteine, H_2O_2, $NaNO_2$, $Cu(OAc)_2$, $Zn(OAc)_2$, $FeSO_4$, $FeCl_3$, Na_2CO_3, GSH, NO (DEA.NONOate), $Na_2S_2O_3$, K_2S_5, N_2S_4, and ascorbic acid (AA) were prepared in double distilled water. The stock solution of probe **Mito-HS** (20 μM) was prepared in PBS buffer (pH = 7.4) with 0.2% DMSO. Excitation was carried out at 380 nm with excitation and emission slit

widths as 3 nm each. The fluorescence changes of **Mito-HS** (5.0 μM) was recorded in the presence of increasing concentrations of Na_2S (0–40 eq.) in PBS buffer (pH = 7.4) with 0.2% DMSO. **Mito-HS** was incubated with Na_2S for 30 min at 37 °C. The fluorescence quantum yield of **Mito-HS** was measured in the presence and absence of Na_2S in phosphate buffer (pH= 7.4).

The Linear Range and Detection Limit. The detection limit of the probe toward H_2S was calculated by fluorescence titration. The fluorescence emission spectrum of **Mito-HS** (2.0×10^{-6} M) was measured ten times. Also, the standard deviation of the blank solution was also measured. The fluorescence intensity (λ_{em} = 450 nm) of the probe was plotted vs. concentrations of H_2S. The detection limit was calculated by using following equation.

Detection Limit 3σ/k:

Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity versus H_2S concentration.

Determination of the Fluorescence Quantum Yield. The fluorescence quantum yield of **Mito-HS** in the presence and absence of H_2S were determined in PBS buffer (10 mM, pH 7.4), using quinine sulfate (Φ_X= 0.542) as standard. The fluorescence quantum yield was calculated using the following equation:

$$\Phi s = \Phi_X (A_S F_S / A_X F_X)$$

Where, A_S and A_X are the absorbance of the sample and the reference, respectively; at the same excitation wavelength, F_s and F_x are the corresponding relative integrated fluorescence intensities.

Cell Culture, Reagent Preparation and Fluorescence Imaging. Human cervical cancer cells (HeLa), breast cancer cells (MDA-MB-231), prostate cancer cells (DU145) and fibroblast 3T3-L1 cells were cultured in DMEM high glucose media supplemented with 10% fetal bovine serum, 1% Penstrep, 0.2% Amphotericin B. The cells were grown overnight at 37°C incubator with 5% CO_2. HeLa, MDA-MB-231, DU145 and 3T3-L1 cells were seeded at a density of $0.3x10^6$ cells in 35 mm dish and kept overnight. The cells were treated with 5 μM of **Mito- HS** for 15 min. Images were acquired using Zeiss Fluorescence Microscope (A1 Axiovert) with x40 objective lens. The probe **Mito-HS** was dissolved in DMSO to make a stock concentration of 10

mM. All experiments were performed using this stock. Stock solution (500 mM) for Na_2S (fresh) and *N*-propargylglycine (PAG) were prepared in PBS. Aminooxyacetic acid (AOAA) was dissolved in DMSO to make a stock concentration of 1M. NEM stock solution (500 mM) was prepared in ethanol.

Cell Viability Assay. MTT assay was performed to assess the viability of HeLa cells. The cells were seeded at a density of 10,000 cells per well and grown overnight at 37°C incubator with 5% CO_2. The cells were then exposed to different concentrations of **Mito-HS** for 48h. After the stipulated time of probe exposure, 10 μL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] was added at a concentration of 5mg/mL and solubilized using 100 μL of MTT solubilizing agent (DMSO) after 2h. The readings were taken at 590 nm with a reference filter of 620 nm using Synergy HT Multi-Mode Microplate reader (Biotek).The same procedure was followed to assess the viability of probe treated MDA-MB-231 and DU145 cells.

Inhibitor Treatment. Helacells were exposed to 250 μM and 500 μM of *N*-ethylmaleimide (NEM) for 2 hours. 5 μM of **Mito-HS** was then added to the cells and incubated for 15 min. The cell images were taken using Zeiss Fluorescence Microscope (A1 Axiovert). HeLa, MDA-MB-231, and DU145 cells were pretreated with aminooxyacetic acid (2.0 mM) (AOAA) and *N*-propargylglycine (2 mM) (PAG) separately or a combination of them (2.0 mM each) for 1 hour to suppress the activity of cystathionine -β- synthase and cystathionine-γ-lyase respectively. Then pretreated cells were further incubated with **Mito-HS** (5 μM) for 15 min. The medium containing inhibitor and **Mito-HS** was replaced with fresh medium and the cells were observed under the fluorescence microscope (A1 Axiovert).

Co-localization of Mito-HS with MitoSOX Red. Cancer cells were seeded at a density of 0.3 x 10^6 cells in 35 mm dish and kept overnight. The cells were co-treated with both **MitoSOX Red** (250 nM) and **Mito-HS (**5 μM). After 15 min of incubation, the dyes were washed and then images were taken using Zeiss Fluorescence Microscope (A1 Axiovert). The visible light excitation maximum of **MitoSOX Red** and **Mito-HS** is 579 nm and 350 nm respectively and emission maximum is 599 nm and 440 -500 nm respectively.

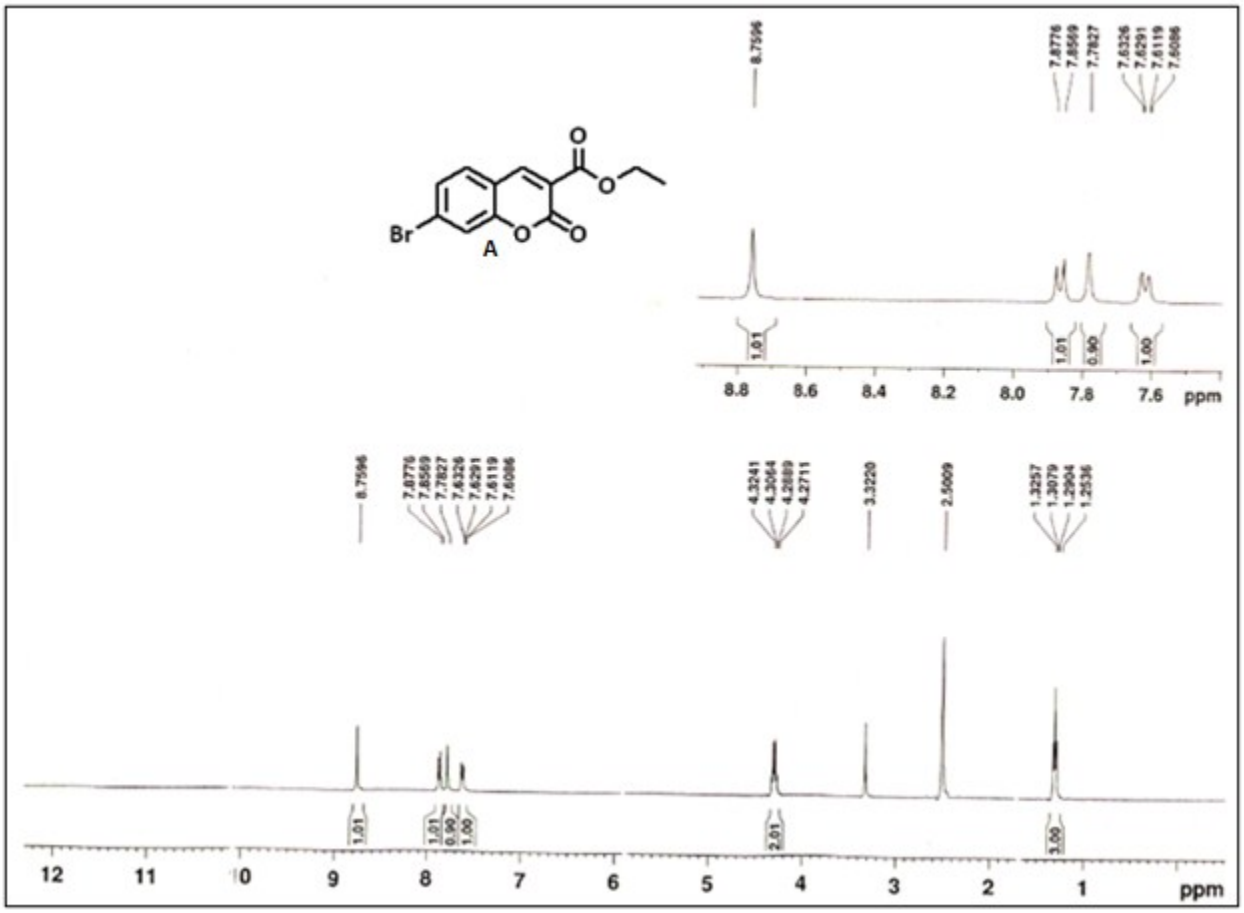


Fig. S1. ^{1}H-NMR of **A** in DMSO.

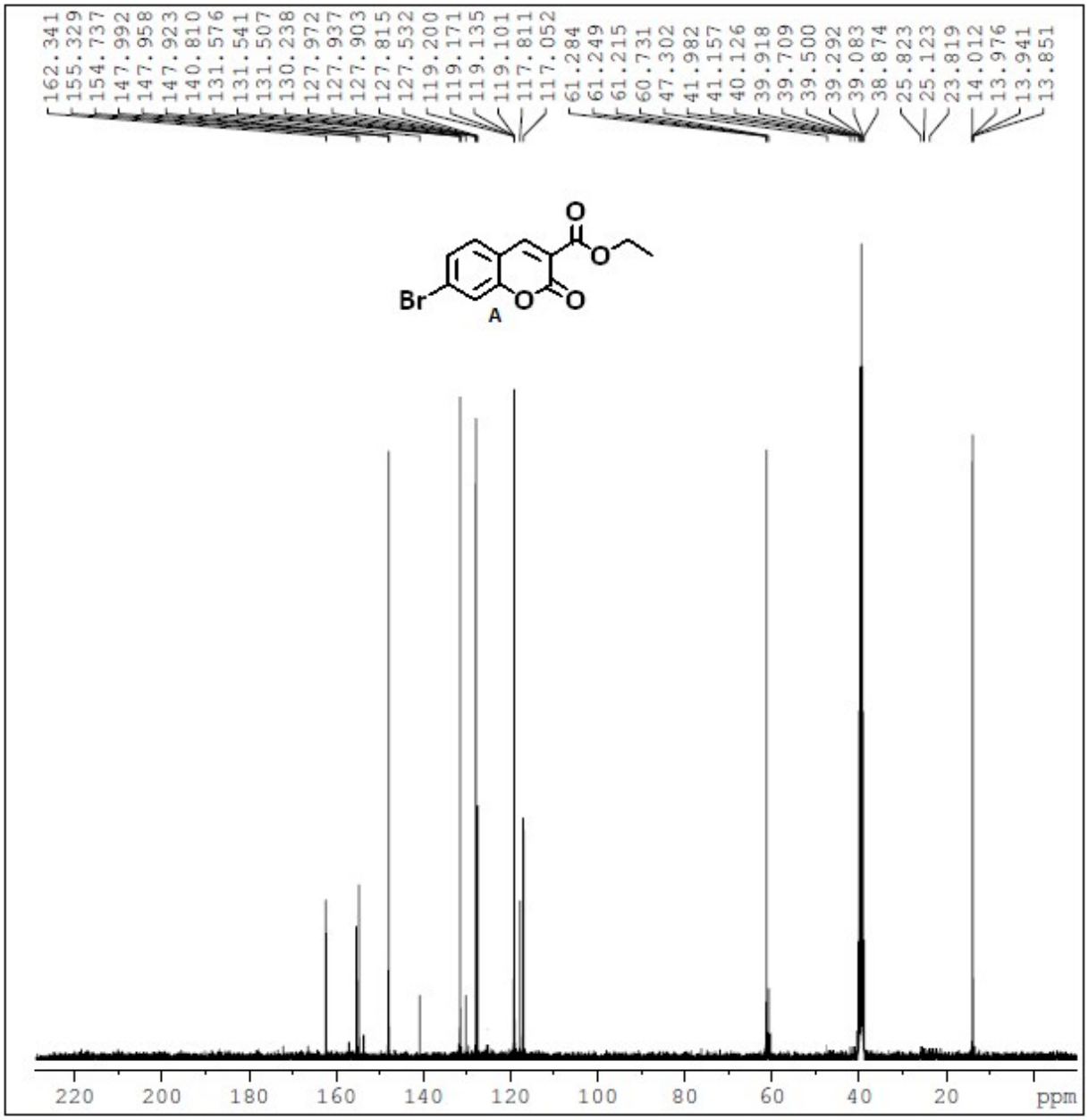
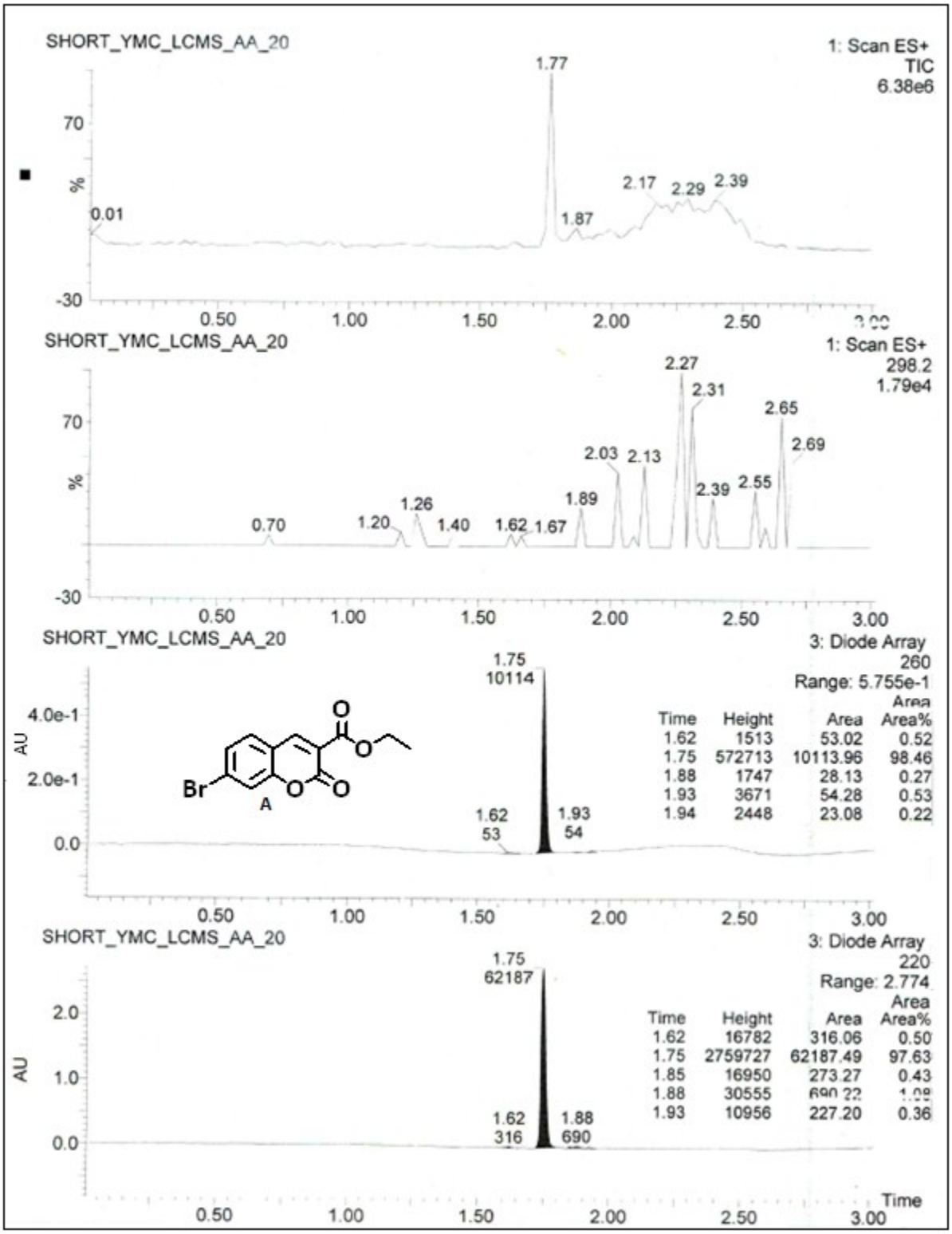


Fig. S2. [13] C-NMR of **A** in DMSO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1.40
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1.93
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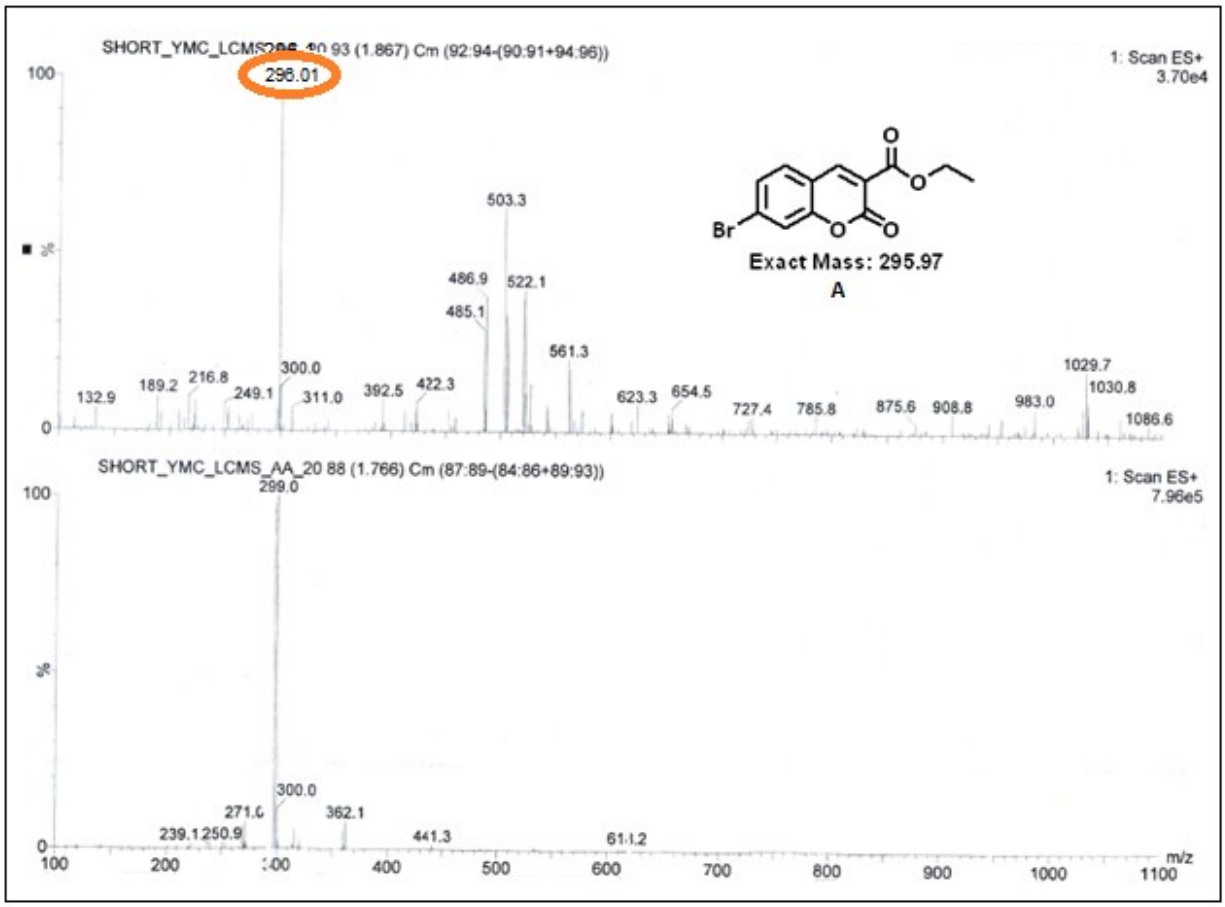

Fig. S3. LCMS of **A**

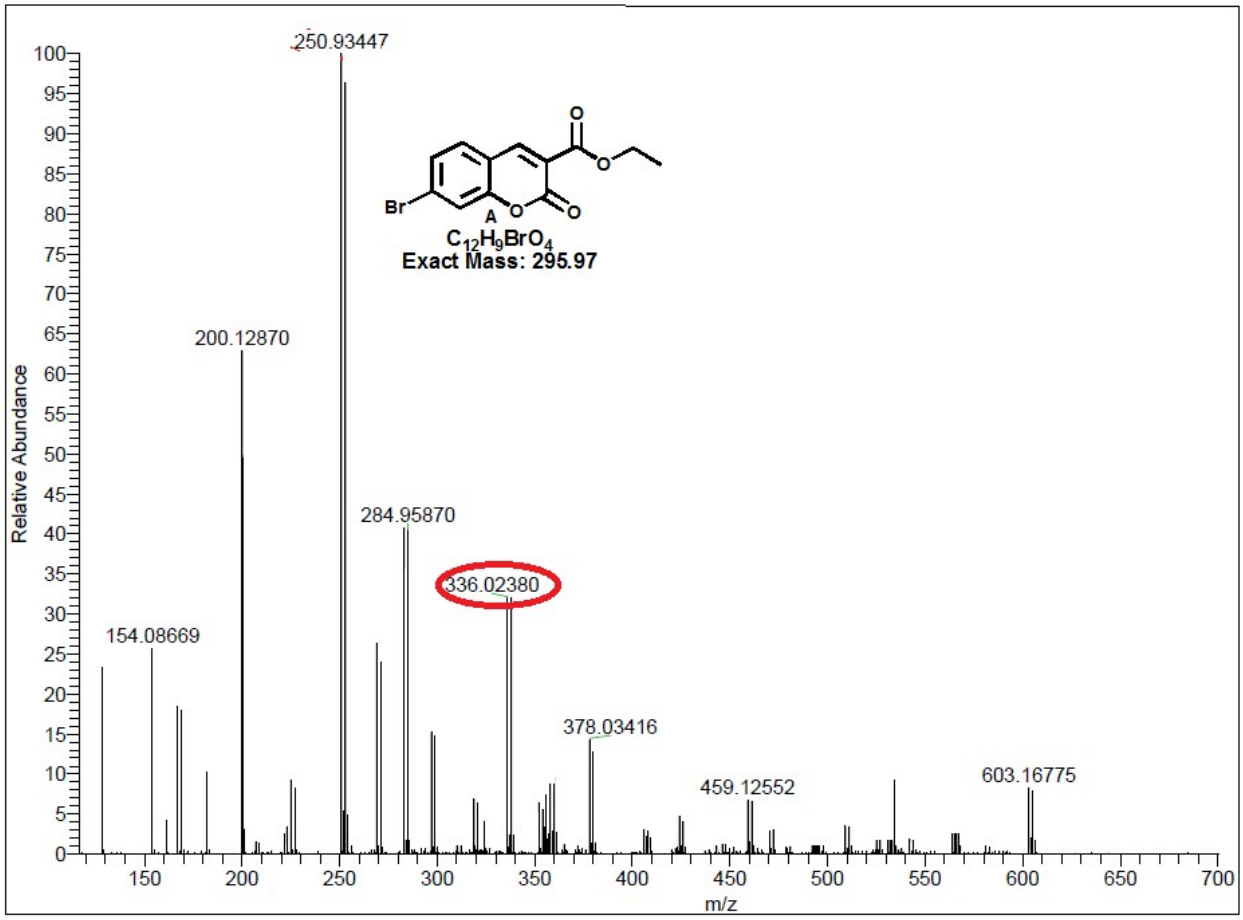

Fig. S4. HRMS of **A**

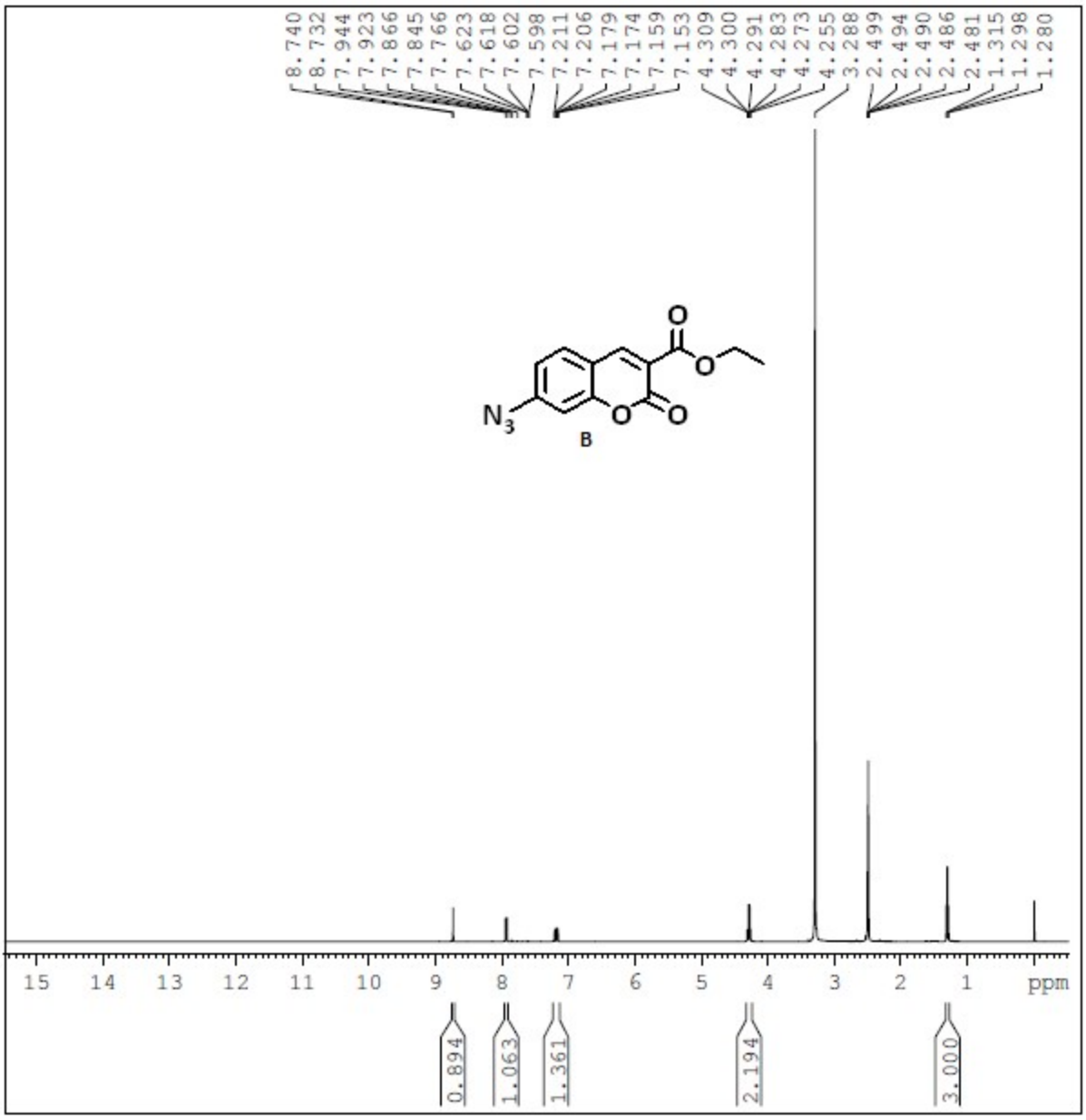

Fig. S5. ^{1}H-NMR of **B** in DMSO.

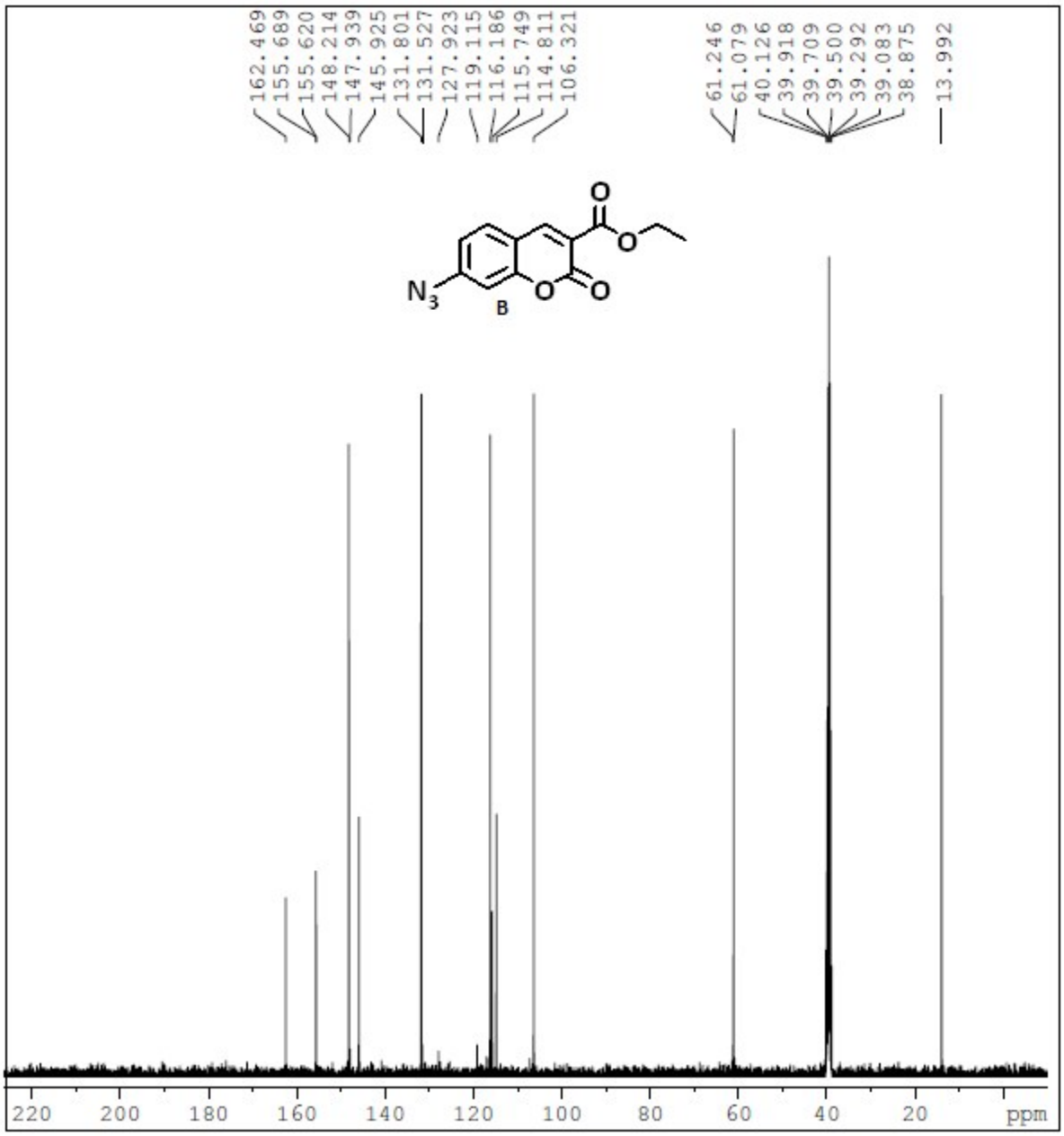

Fig. S6. [13] C-NMR of **B** in DMSO

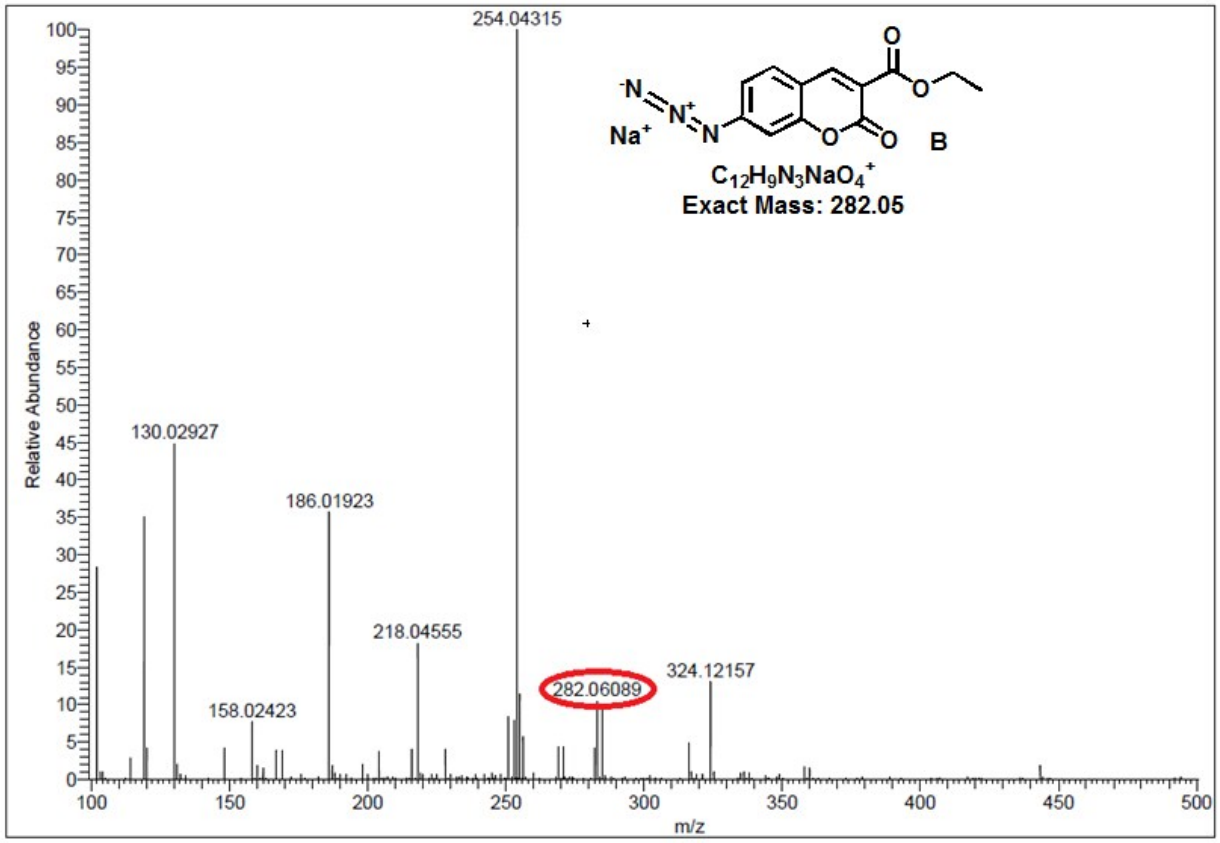

Fig. S7. HRMS of **B**

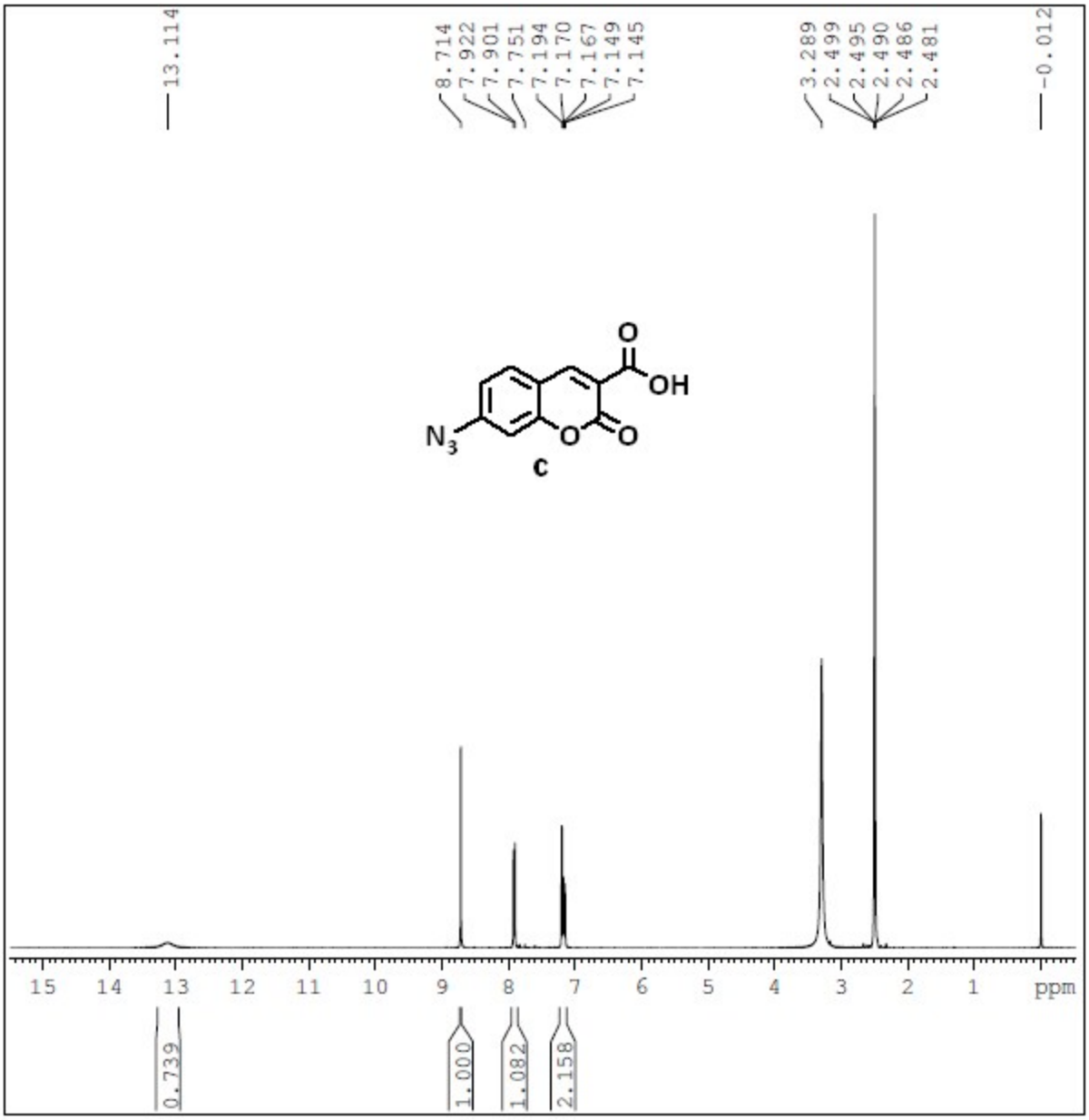

Fig. S8. ^{1}H-NMR of **C** in DMSO.

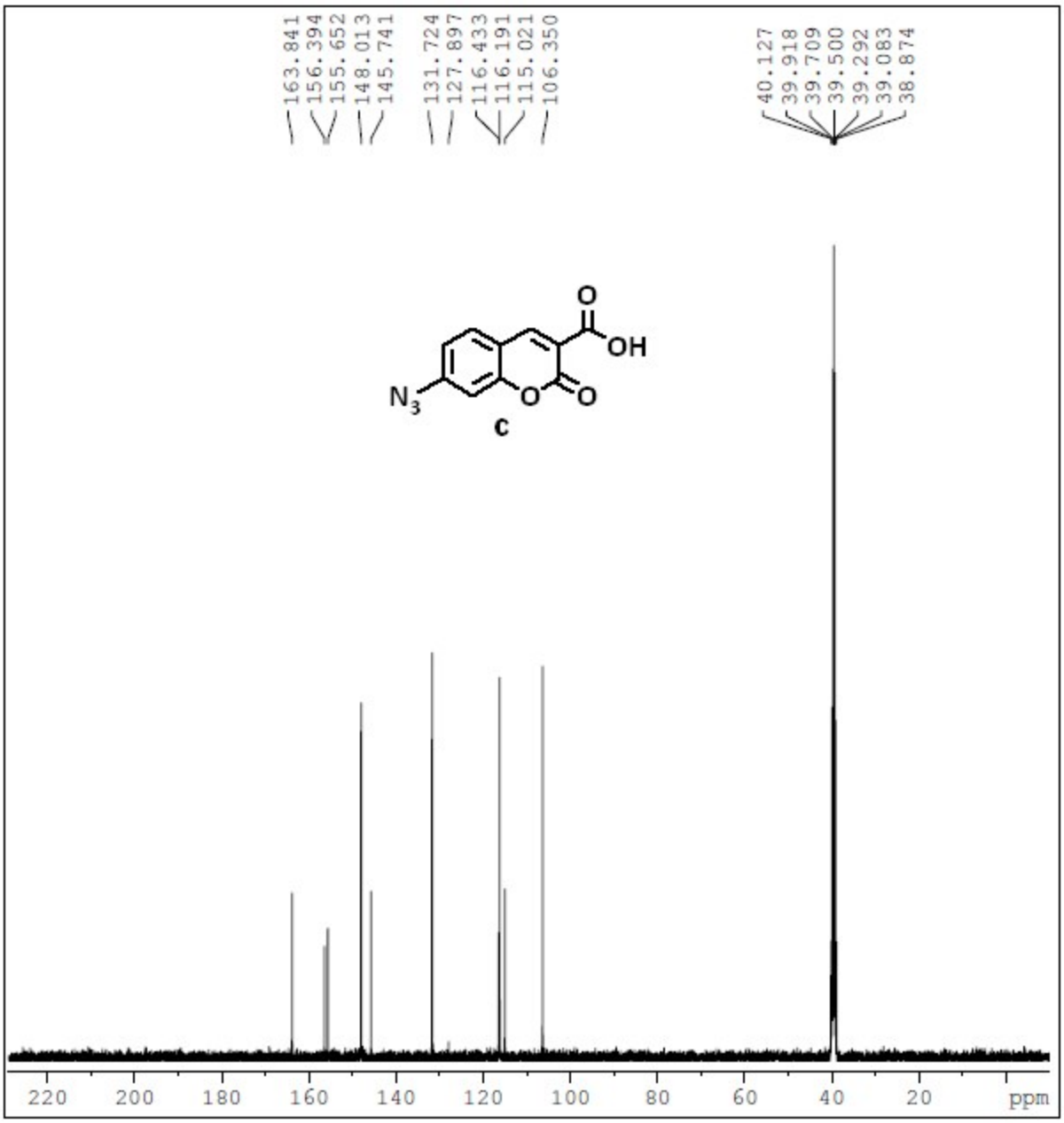

Fig. S9. ^{13}C-NMR of **C** in DMSO

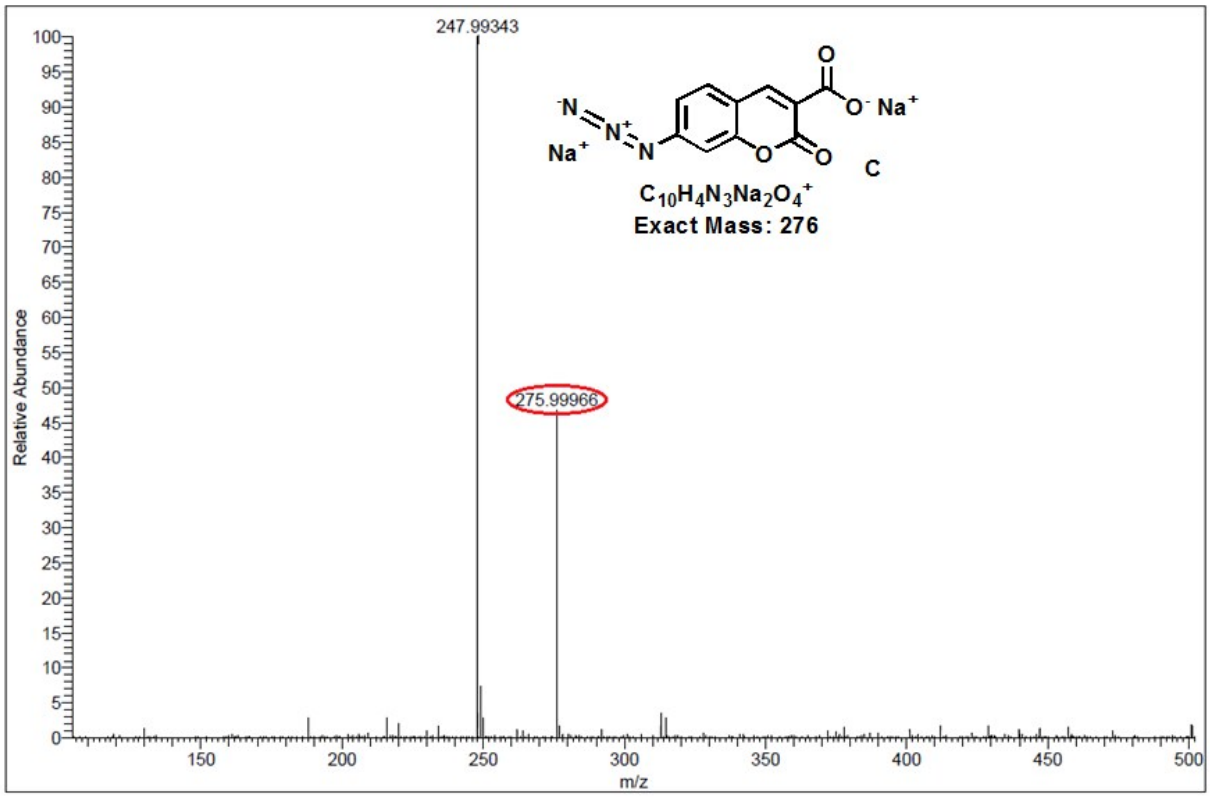

Fig. S10. HRMS of **C**

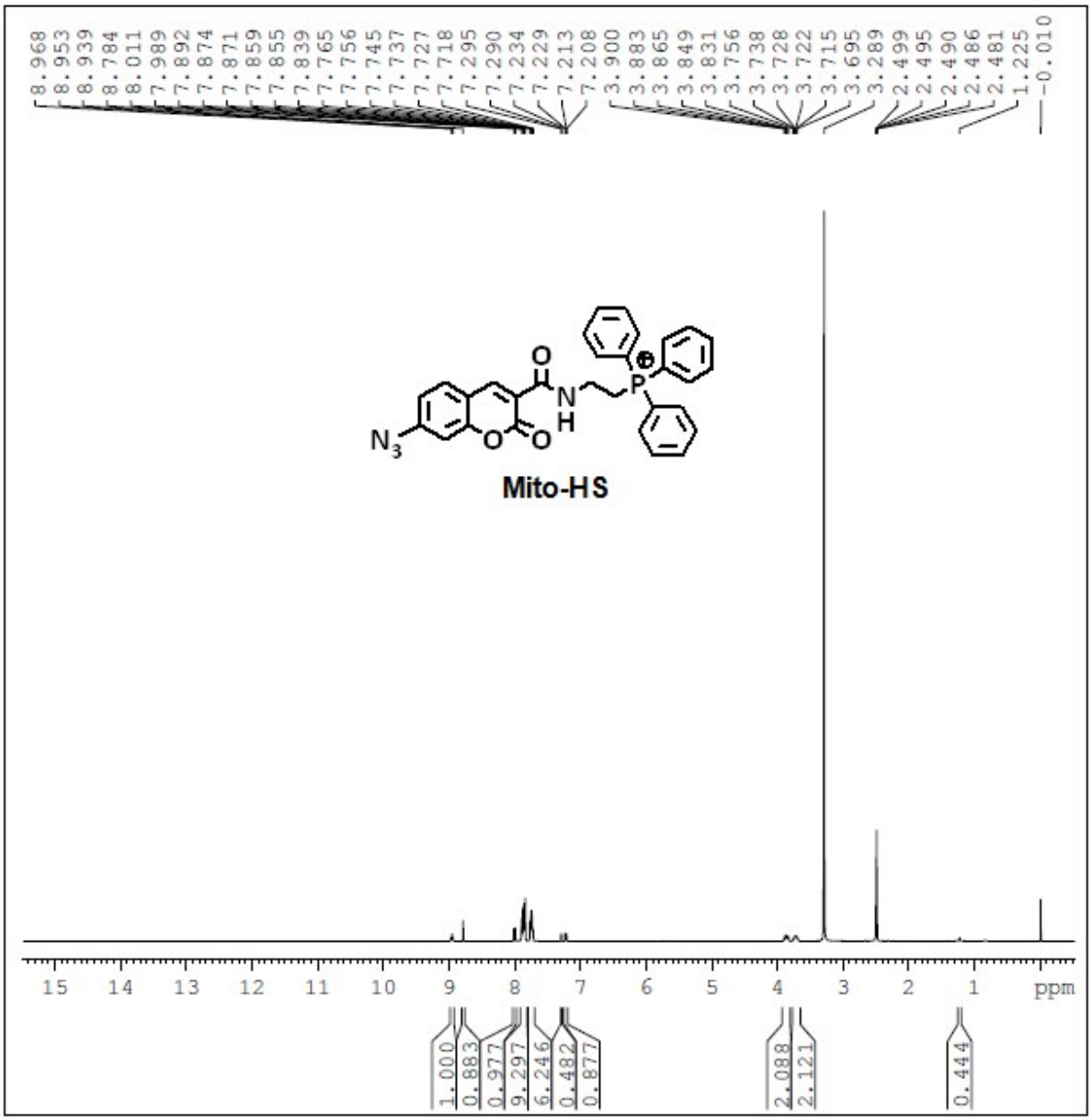

Fig. S11. ^{1}H-NMR of **Mito-HS** in DMSO.

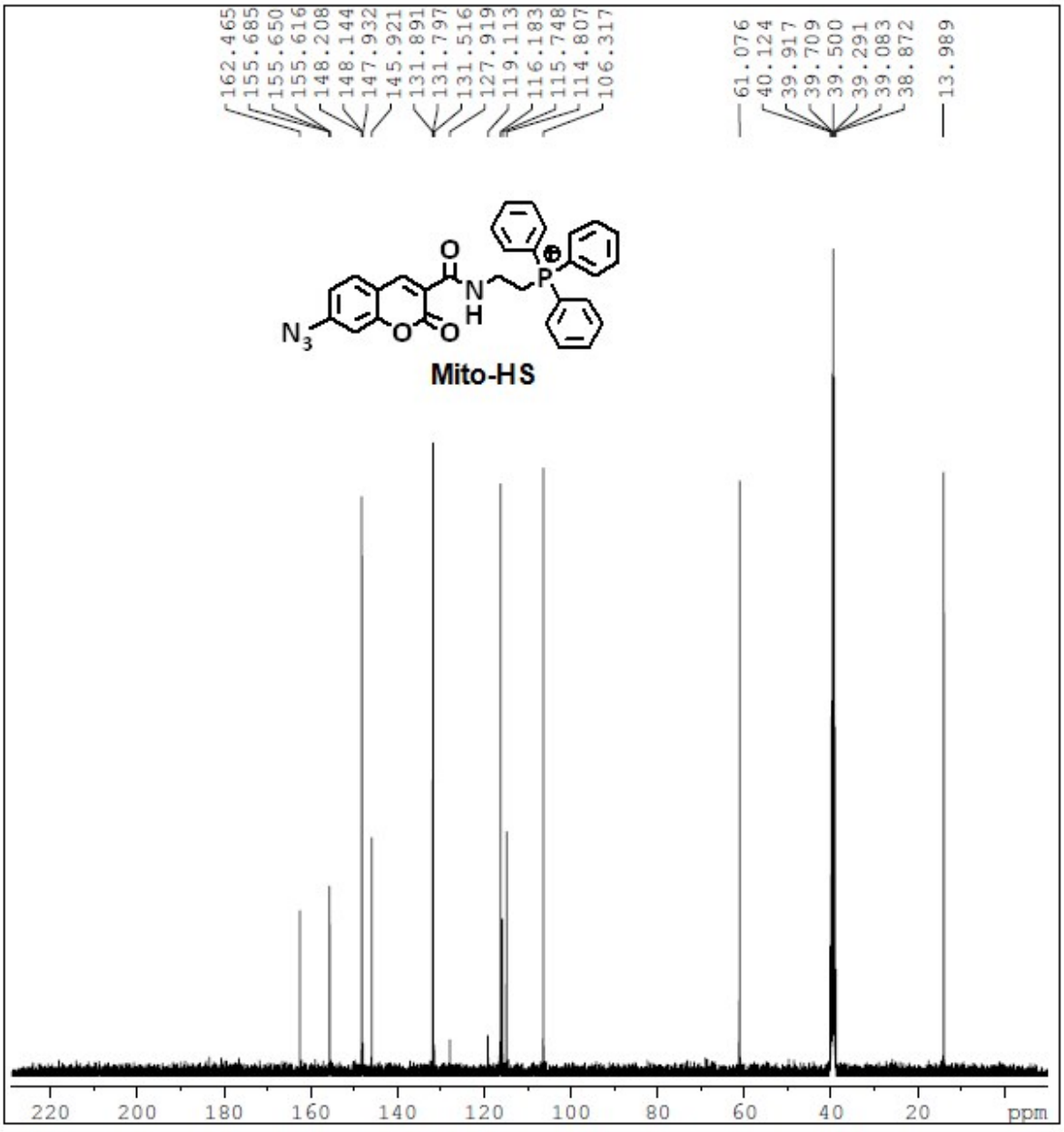

Fig. S12. [13] C-NMR of **Mito-HS** in DMSO

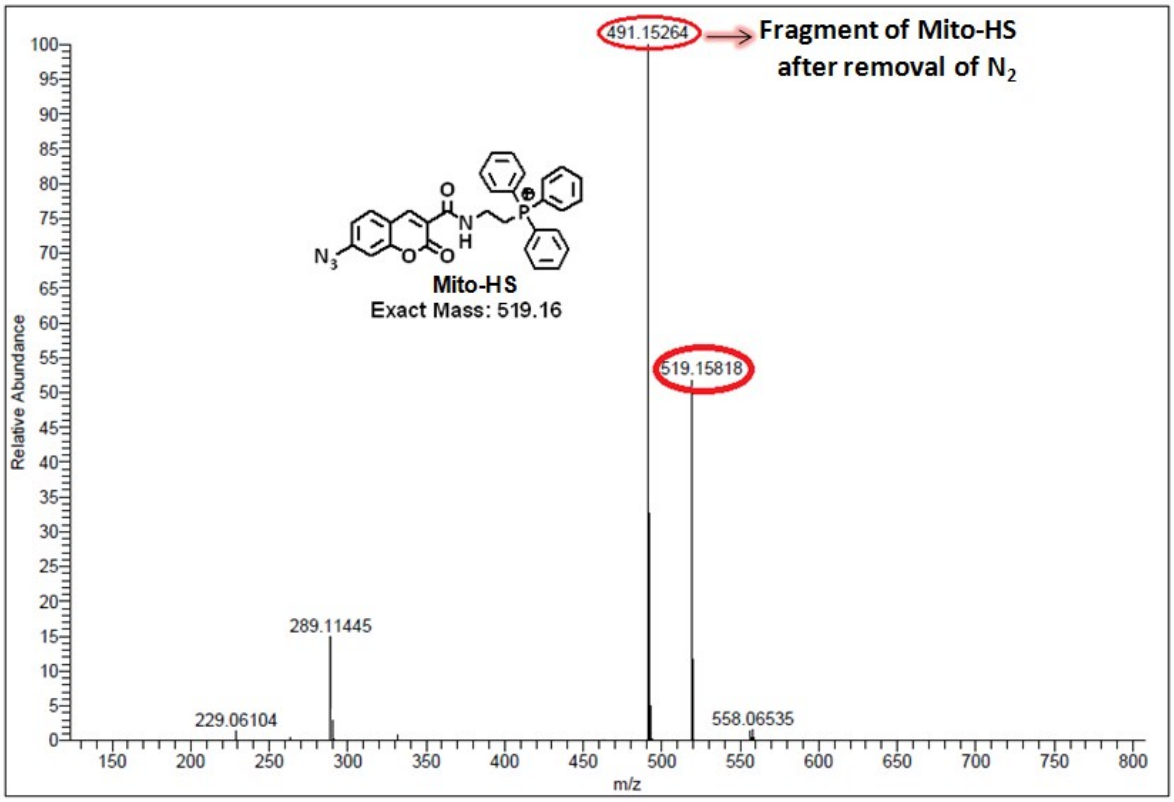

Fig. S13. HRMS of **Mito-HS**

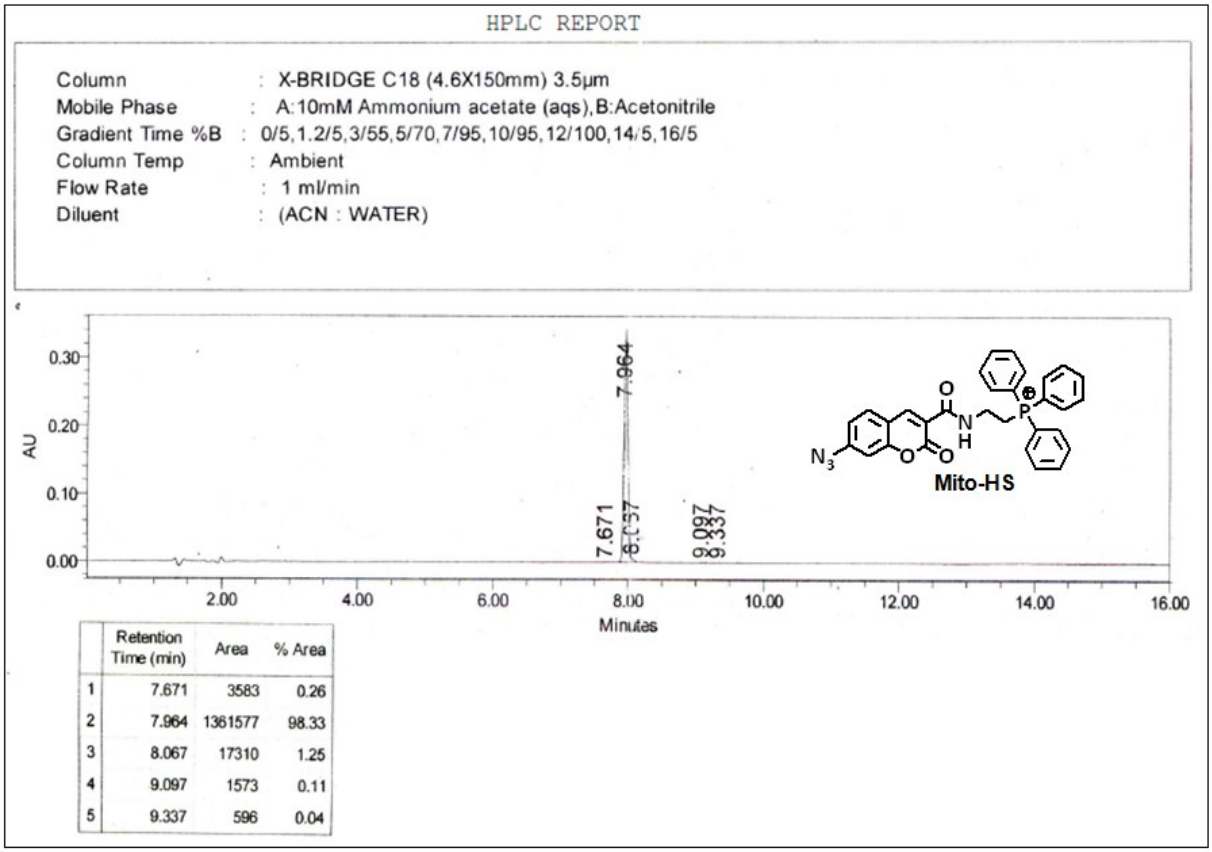

Fig. S14. HPLC of **Mito-HS**.

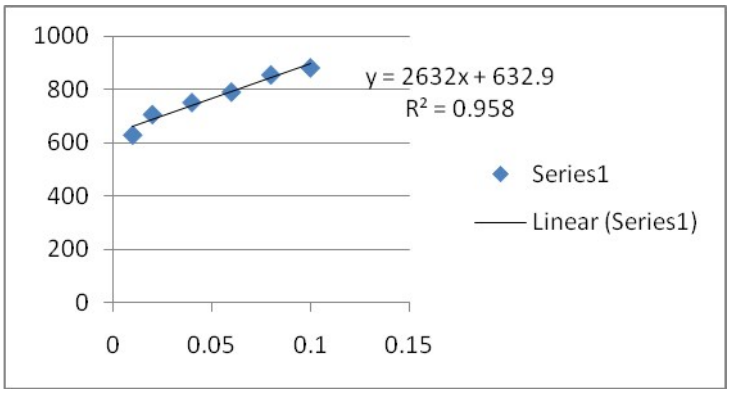

Fig. S15. The changes in the fluorescence intensity of **Mito-HS** (5.0 µM) at 450 nm against varied concentrations of H_2S from 0 to 0.15 µM in DMSO−PBS buffer (0.01 M, pH 7.4) (V/V= 2:98) with the slit width 3/3 nm.

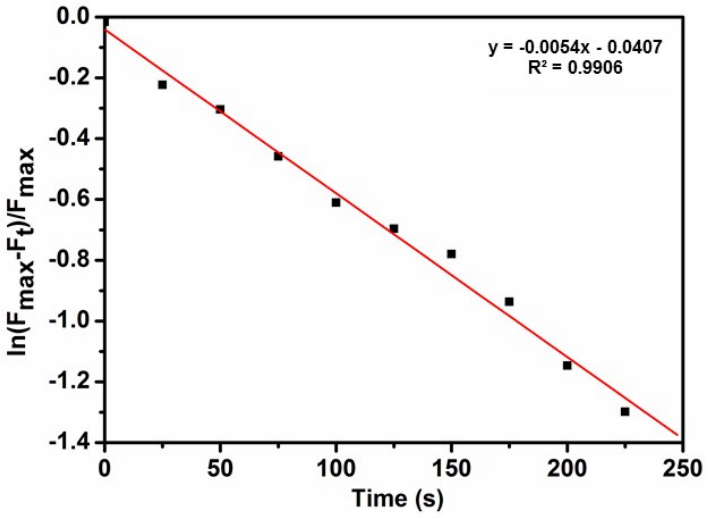

Fig. S16. Kinetic plot of pseudo first order reaction of **Mito-HS** ((5 μM) in presence of Na_2S (100 μM). Slope = 0.0054 S^{-1}

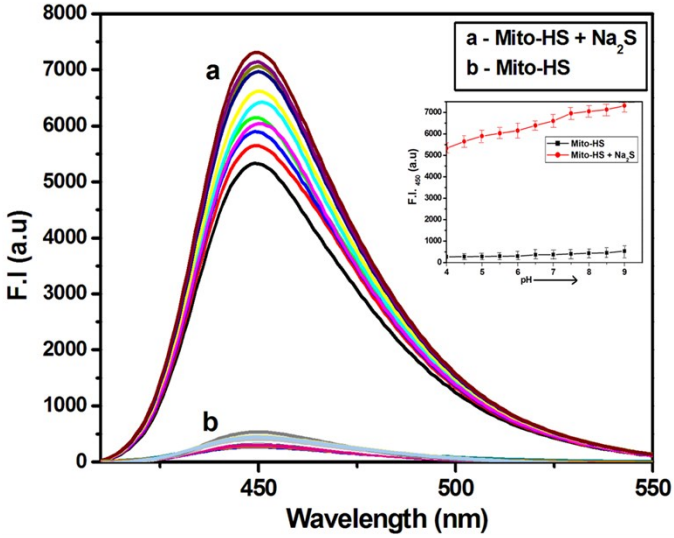

Fig. S17. Fluorescence intensity of **Mito-HS** (5 μM) **(a)** in presence of Na_2S (100 μM) and (**b**) in absence of Na_2S at various pH (pH 4-9) in PBS buffer solution (pH=7.4; 0.2% DMSO) at 37°C. Excitation was effected at 380 nm with the excitation and emission slit widths both set at 3nm

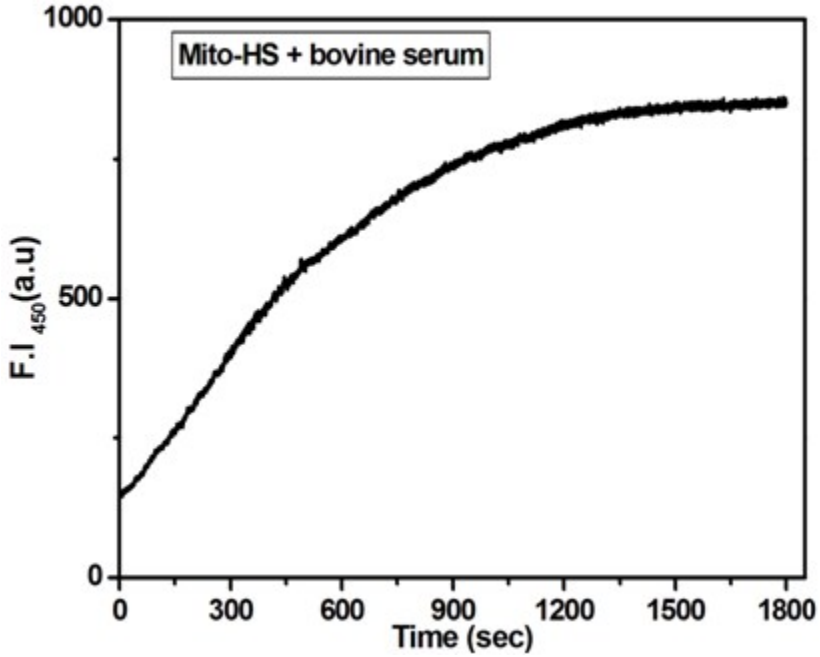

Fig. S18. Time dependent fluorescence changes of **Mito-HS** (5 μM) in fetal bovine serum at 37°C. Excitation was effected at 380 nm and emission at 450 nm with the excitation and emission slit widths both set at 3 nm.

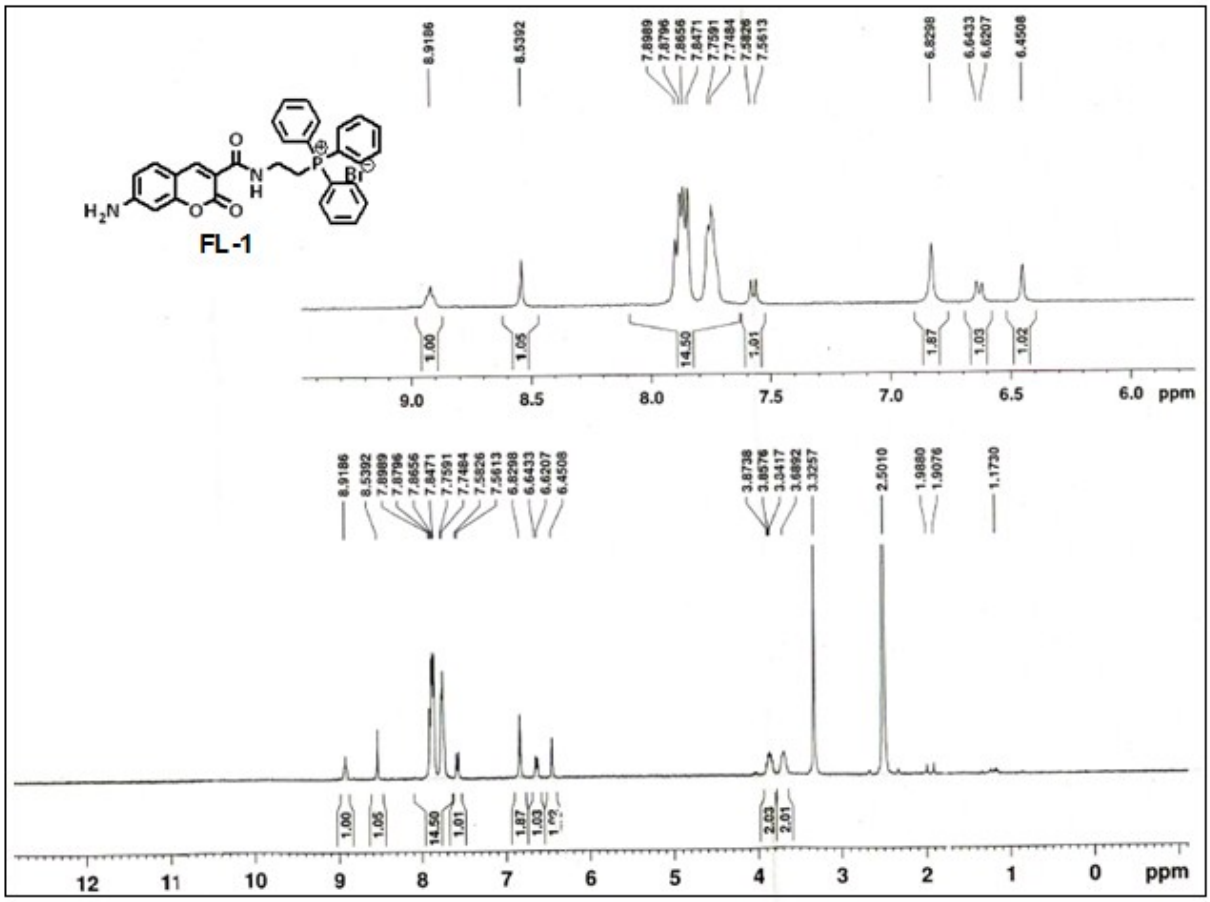

Fig. S19. [1]H-NMR of reference **FL-1** in DMSO.

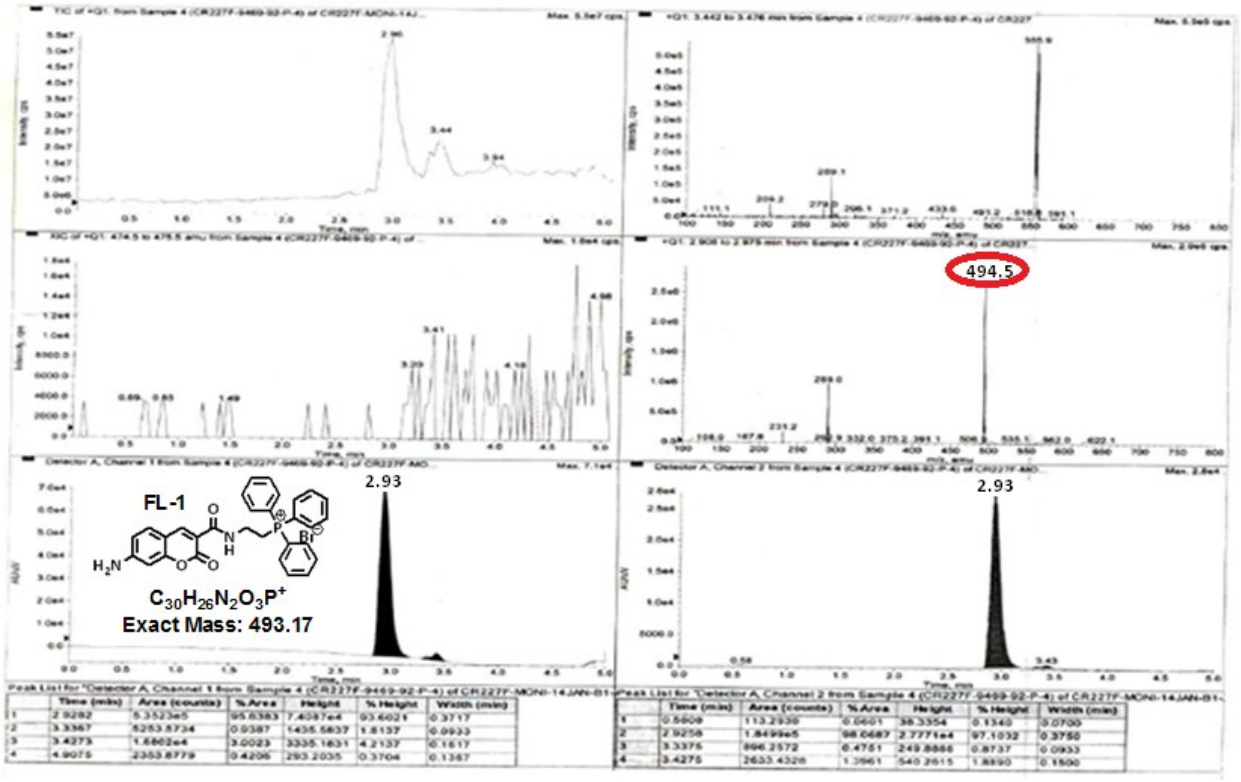

Fig. S20. LCMS of **FL-1**.

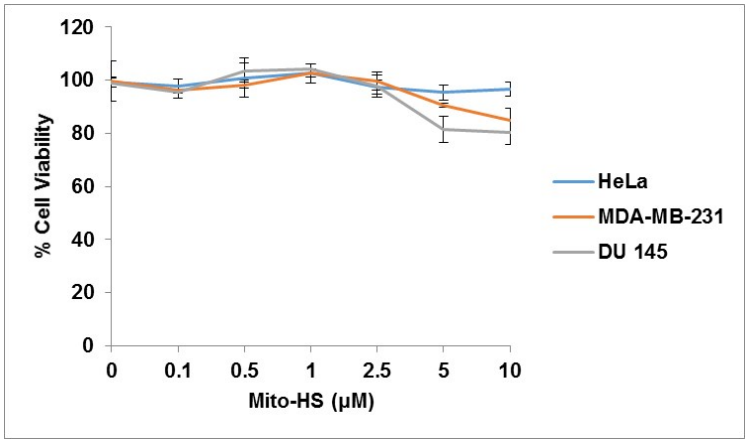

Fig. S21 Cell viability of HeLa, MDA-MB-231 and DU145 cells treated with various concentrations (0, 0.1, 0.5, 1, 2.5, 5 and 10 μM) of **Mito-HS**. The cells were incubated for 48 h after treatment of **Mito-HS**. Cell viability was assessed using MTT assay.

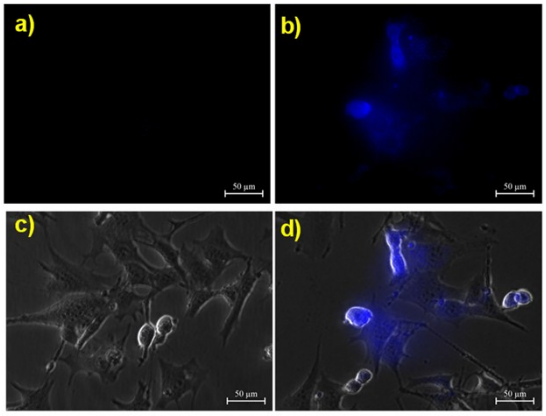

Fig. S22. Fluorescence images of normal 3T3-L1 cells. (a) Cells treated with **Mito-HS** (5 μM) for 1h; (b) cells pretreated with Na_2S (500 μM) for 1h then incubated with **Mito-HS** (5 μM) for 15 min. (c, d) are the bright field images of (a) and (b) respectively. Scale bar, 50 μm. Images were obtained using excitation wavelengths of 390 nm with the emission being monitored over the 440–500 nm.

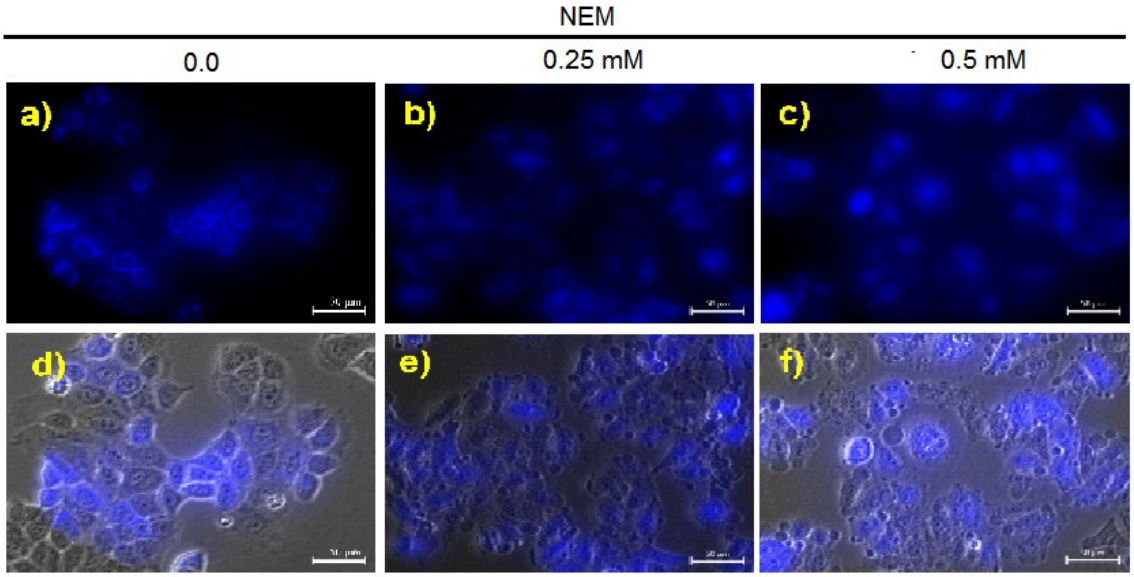

Fig. S23 Fluorescence microscopic images of HeLa cells treated with (a) **Mito-HS** (5 μM) and (b-c) cells pretreated with NEM prior to treatment with **Mito-HS** (5 μM); (d – f) are overlay DIC Bright field images with corresponding fluorescence images. Scale bar, 50 μm. Images were

obtained using excitation wavelengths of 390 nm with the emission being monitored over the 440-500 nm.

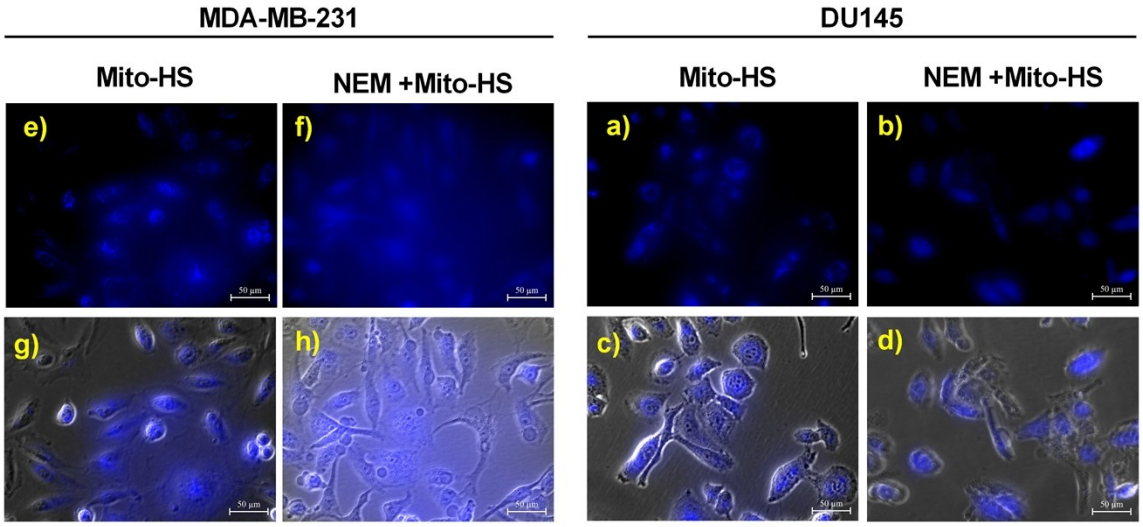

Fig. S24. Fluorescent microscopic images of DU145 (a-d) and MDA-MB-231 (e-h) cells treated with **Mito-HS** (5 μM) (a, e) and (b, f) cells pretreated with NEM (0.25 mM) prior to treatment with **Mito-HS** (5 μM); (c-d, g-h) are overlay bright field images with corresponding fluorescence images. Scale bar, 50 μm. Images were obtained using excitation wavelengths of 390 nm with the emission being monitored over the 440–500 nm.

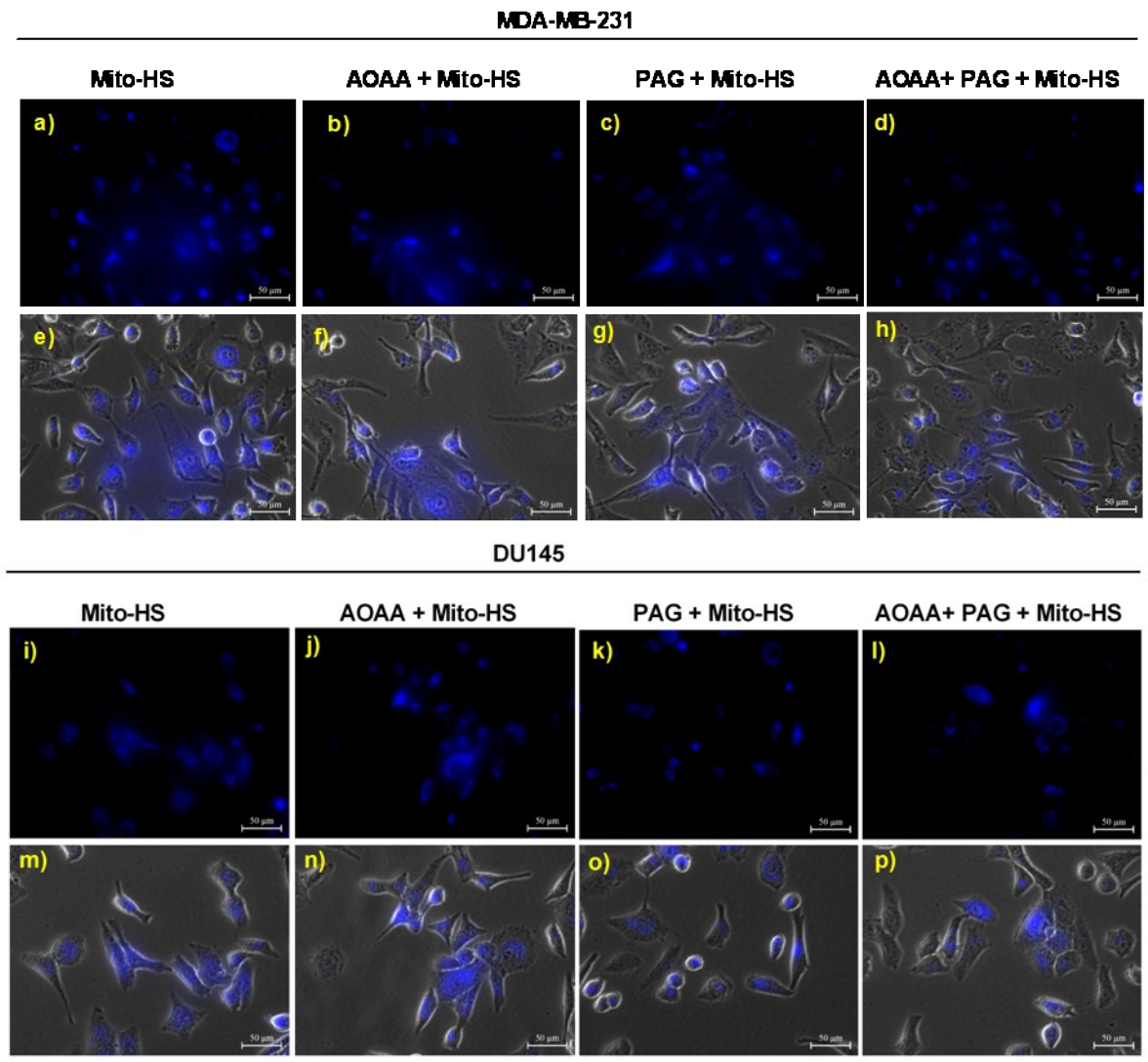

Fig. S25. Cellular fluorescence images of MDA-MB-231, and DU145. **Mito-HS** (5 μM) incubated for 15 min. Cells were pretreated with AOAA (2 mM), PAG (2 mM) separately and with both (2 mM each) for 1h and then incubated with **Mito-HS** (5 μM) for 15 min; (e-h) and (m-p) are overlay bright field images of corresponding fluorescence images (a-d) and (i-l). Scale bar, 50 μm. Images were obtained using excitation wavelengths of 390 nm with the emission being monitored over the 440–500 nm.

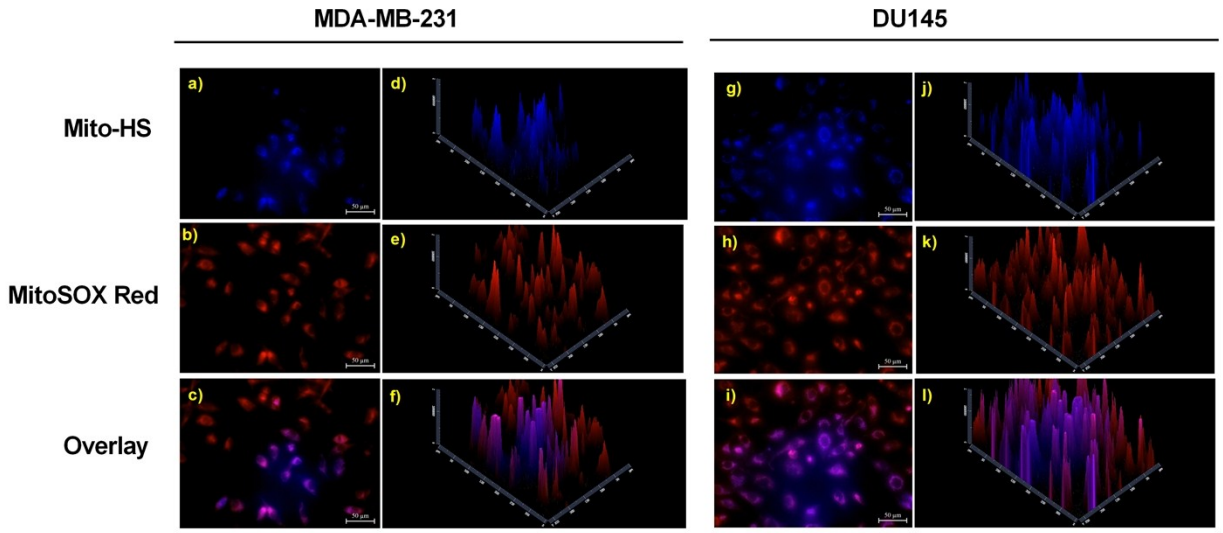

Fig. S26. Fluorescence and co-localization images of **Mito-HS** with **MitoSOX-Red**. The MDA-MB-231 and DU145 cells were co-treated with **Mito-HS** (5 μM) and **MitoSOX-Red** for 15 min. The images were collected at (a, g) ex. 390 nm/ em. 440-500 nm, (b, h) ex.510 nm/ em.580 nm (c, i) overlay image of (a) and (b), (g) and (h) respectively; (d-f) and (j-l) are the 3D images of the corresponding cell pictures (left panel).

References

1. Maryanoff, B. E.; Reitz, A. B.; Duhl-Emswiler, B. A. *J. Am. Chem. Soc.***1985**, *107*, 217–226**.**
2. Fery-Forgues, S.; Lavabre, D. *J. Chem. Educ.* **1999**, *76*, 1260-1264.
3. Chen, B.; Li, W.; Lv, C.; Zhao, M.; Jin, H.; Du, J.; Zhang, L.; Tanq, X. *Analyst* **2013**, *138*, 946-951.