Supplementary Information

for

Biothiol-triggered Organotrisulfide-based Self-immolative Fluorogenic Donors of Hydrogen Sulfide Enable Lysosomal Trafficking

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Author contribution: K.P.B. designed the project and supervised the research work as well as data analysis. S.K.M. optimized the experimental condition, synthesized the probes, purified, characterized and carried out spectroscopic studies and related assays using UV-Vis, Fluorescence spectroscopy and HPLC. D.B. carried out cellular experiments and fluorescent and confocal microscopic experiments. The manuscript was prepared by K.P.B., S.K.M. and D.B.

CONTENT

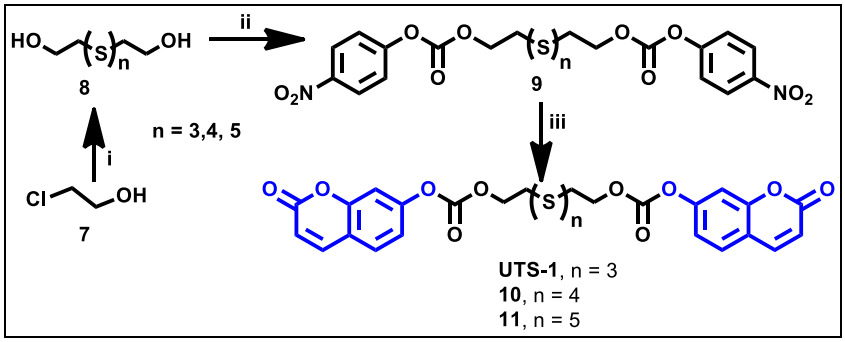
Abbreviations

Cys, L-Cysteine; GSH, Reduced Glutathione; Hcy, D,L-Homocysteine, Met, L-Methionine; Ser, L-Serine; Arg, L-Arginine; Trp, L-Tryptophan; His, L-Histidine; Thr, L-Threonine; Pro, L-Proline; Gln, L-Glutamine; Phe, L-Phenylalanine; Lys, L-Lysine, Asn, L-Asparagine; Tyr, L-Tyrosine; NAC, N-acetyl-L-cysteine; NaHS, Sodium hydrosulfide; NaCl, Sodium chloride; KCl, Potassium chloride; H_2O_2, Hydrogen peroxide; TBHP, *tert*-Butyl hydroperoxide; ME, 2-mercaptoethanol; PhSH, Thiophenol.

Materials and methods

All the reagents were purchased either from Sigma Aldrich or from reputed local suppliers and used without further purification unless otherwise stated. Thin layer chromatographic (TLC) analyses were carried out on pre-coated silica gel on aluminium sheets and the compounds were visualized by irradiation with UV or fluorescent light. Organic solvents used for chromatographic separations were distilled before use. Melting point of the synthesized compounds was recorded in a Büchi B540 melting point apparatus and the values are uncorrected. The NMR spectra were recorded with a Bruker Ascend ^{TM} 400 MHz NMR Spectrometer. Chemical shifts are cited with respect to Me_4Si as internal standard. Mass spectra were obtained using an Agilent 6520 Accurate- Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS spectrometer.

Synthesis of UTS-1



Scheme S1. Synthetic scheme to **UTS-1**. Reagents and condition: (i) Na_2S_n (n = 3,4, 5), H_2O, RT, 2 h; (ii) 4-Nitrophenyl chloroformate, Pyridine, Dichloromethane, 0 °C-RT, 6 h; (iii) Umbelliferone, NEt_3, Dichloromethane, 0 °C-RT, 16 h.

Synthesis of compound 8[1]

To a solution of sodium sulfide nonahydrate (0.7 g, 3.1 mmol) in water (10.0 mL) was added elemental sulfur powder (0.2 g, 0.8 mmol) and the reaction mixture was stirred for 2h at room

temperature during which the solution turned reddish brown indicating the formation of Na_2S_n (n = 3, 4, 5) with Na_2S_3 as the major component. To the solution, 2-chloroethanol (**7**) (0.4 mL, 6.2 mmol) was added and the reaction mixture was stirred for 4h at room temperature. Progress of the reaction was monitored by TLC analysis. Upon completion, the aqueous layer was extracted with ethyl acetate. The combined organic layer was washed with brine solution and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to afford polysulfide **8** as faint yellow oil with almost quantitative yield. The inseparable polysulfide mixture was used for the next step without any further purification. R_f = 0.5 (45% ethyl acetate in petroleum ether). ^{1}H-NMR (polysulfide) (400 MHz, $CDCl_3$) δ (ppm): 4.01 – 3.97 (overlapped triplets from polysulfides, 4H), 3.16-3.13 (t, *J* = 8.0 Hz) and 3.11 - 3.08 (t, *J* = 8.0 Hz) (4H, two triplets from polysulfide).

Synthesis of compound 9

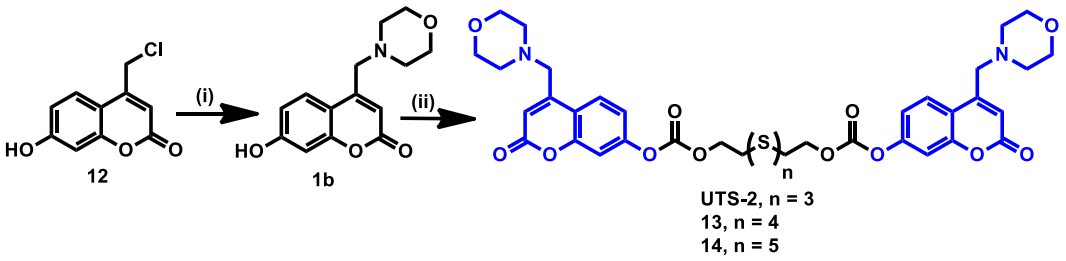
To a solution of polysulfide **8** (0.20 g, 1.0 mmol) in anhydrous dichloromethane (6.0 mL) under inert atmosphere was added pyridine (0.45 mL, 5.3 mmol) and the mixture was cooled to 0 °C with external ice bath. A solution of 4-nitrophenyl chloroformate (0.54 g, 2.6 mmol) in anhydrous dichloromethane (6.0 mL) was added to the above reaction mixture in a dropwise manner at 0 °C. The resulting solution was allowed to attain room temperature and was stirred for another 6 h. The progress of the reaction was monitored by TLC analysis. Upon completion, the mixture was diluted with dichloromethane and sequentially washed with saturated $NaHCO_3$ (15.0 mL), water (15.0 mL), 10% citric acid (15.0 mL) and brine solution (15.0 mL). The organic layer was dried over sodium sulfate and the solvent was evaporated under reduced pressure to afford the crude product **9** as white solid with almost quantitative yield, which was used for the next step without further purification. R_f = 0.5 (20% ethyl acetate in petroleum ether 60 - 80). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 8.30-8.28 (overlapped doublets from polysulfides, 4H), 7.41-7.38 (overlapped doublets from polysulfides, 4H), 4.65-4.62 (overlapped triplets from polysulfides, 4H), 3.33-3.29 and 3.27-3.24 (overlapped triplets from polysulfide, 4H).

Synthesis of UTS-1

To a stirred solution of umbelliferone **1a** (0.2 g, 1.2 mmol) in anhydrous dichloromethane (10.0 mL) at 0 °C under an inert atmosphere was added triethylamine (0.2 g, 1.5 mmol) and the mixture was stirred for 10 min. A solution of compound **9** (0.2 g, 0.4 mmol) in dichloromethane (6.0 mL) was added to the mixture in a dropwise manner and the reaction mixture was allowed

to attain room temperature and stirred for 16h. The progress of reaction was monitored by TLC analysis. Upon completion, the reaction mixture was poured into water and extracted with ethyl acetate. The combined organic layer was finally washed with brine solution, separated, dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford the crude product. R_f = 0.5 (45% ethyl acetate in petroleum ether). The crude compound could not be purified by normal phase silica gel column chromatography and therefore, the inseparable mixture was purified by reverse-phase column chromatographic analysis using semi-preparative HPLC method. HPLC analysis indicated the presence of **UTS-1** as predominant species along with higher homologues **10** and **11** (Scheme S1). However, compounds **10** and **11** could not be separated and isolated completely under the purification process. Yield: 0.06 g (29%), M.P = 135 – 137 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 7.71 – 7.69 (d, J = 8.0 Hz, 2H), 7.51 – 7.49 (d, J = 8.0 Hz, 2H), 7.23 – 7.22 (d, J = 4.0 Hz, 2H), 7.17 – 7.14 (dd, J_1 = 4.0, J_2 = 4.0 Hz, 2H), 6.42 – 6.40 (d, J = 8.0 Hz, 2H), 4.64 – 4.61 (t, J = 8.0 Hz, 4H), 3.28 – 3.25 (t, J = 4.0 Hz, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 160.2, 154.7, 153.2, 152.6, 142.7 128.7, 117.6, 116.9, 116.4, 109.9, 66.4, and 36.6. ESI-MS: *m/z* calcd. for $C_{24}H_{18}O_{10}S_3$ $[M+H]^+$: 563.0140; found $[M+H]^+$: 563.0140.

Synthesis of UTS-2



Scheme S2. Synthetic scheme of **UTS-2**. Reagents and condition: (i) Morpholine, Acetonitrile, 65 °C, 8 h; (ii) Compound **9**, NEt_3, Dichloromethane, RT, 16 h.

Synthesis of compound 12[2]

Resorcinol (4.0 g, 36.3 mmol) was dissolved in conc. H_2SO_4 followed by addition of Ethyl 4-chloroacetoacetate (5.1 mL, 40 mmol) at 0 ºC under constant stirring. The reaction was allowed to continue for 12 hours at room temperature. The progress of the reaction was monitored by TLC method. Upon completion, the reaction mixture was poured into ice-cold water (300 mL) to obtain a white precipitate, which was filtered off and washed with deionized water until the pH became neutral. The residue was then dried to afford the crude product as white solid. R_f =0.5 (40% ethyl acetate in pet ether) Yield: 6.3 g (82%). ^{1}H NMR (400 MHz, DMSO-d_6) δ

(ppm): 10.68 (br, 1H), 7.68 (d, J = 8.8 Hz, 1H), 6.85 (dd, J_1 = 8.7, J_2 = 2.3 Hz, 1H), 6.76 (d, J = 2.3 Hz, 1H), 6.42 (s, 1H), 4.96 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 161.9, 160.6, 155.8, 151.4, 127.0, 113.6, 111.5, 109.8, 103.0 and 41.8.

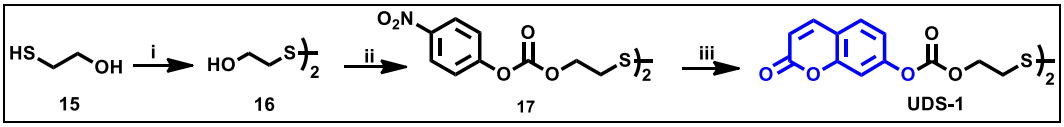
Synthesis of compound 1b[3]

Compound **1b** was prepared following the literature report with minor modifications.[3] To a solution of 4-chloromethyl umbelliferone **12** (0.2 g, 0.95 mmol) in acetonitrile (3 mL), was added morpholine (0.2 mL, 2.09 mmol) and the reaction mixture was allowed to stir at 65 ºC for 8h. The progress of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was evaporated under reduced pressure to afford the crude product as a white solid. R_f = 0.5 (55% ethyl acetate in pet ether 60-80) Yield = (0.18 g) 72 %. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.50 (s, 1H), 7.75 (d, J = 8.8 Hz, 1H), 6.80-6.77 (dd, J_1 = 2.4 Hz, J_2 = 8.0 Hz, 1H), 6.70 (d, J= 2.4 Hz, 1H), 6.23 (s, 1H), 3.61 (s, 2H), 3.60-3.57 (t, J = 8.0 Hz, 4H), 2.49-2.46 (t, J = 8.0, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 161.5, 160.8, 155.6, 152.9, 127.2, 113.2, 111.3, 110.6, 102.6, 66.6, 58.7, and 53.8.

Synthesis of UTS-2

UTS-2 was prepared following the method shown for the synthesis of **UTS-1** by reacting polysulphide linker **9** (0.25 g, 0.48 mmol) with umbelliferone derivative **1b** (0.32 mg, 1.21 mmol) in the presence of triethylamine (0.31 mL, 2.42 mmol). The reaction mixture was allowed to stir for 16 hours at room temperature. The progress of reaction was monitored by TLC analysis. Upon completion, the reaction mixture was poured into water and extracted with ethyl acetate. The combined organic layer was finally washed with brine solution, separated, dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford the crude product. The trisulfide compound was separated using reverse phase HPLC using a 30 min gradient from 50 to 100 % acetonitrile in water (Retention Time = 15.40 min) R_f = 0.4 (55% ethyl acetate in petroleum ether 60 -80). White solid; Yield = 34%; Melting point = 64-66 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.89 (d, J = 8.8 Hz, 2H), 7.23 (d, J = 2.3 Hz, 2H), 7.16 (dd, J_1 = 8.8 Hz, J_2 = 2.3 Hz, 2H), 6.51 (s, 2H), 4.63 (t, J = 6.5 Hz, 4H), 3.73 (t, J = 8.0 Hz, 8H), 3.62 (d, J = 0.8 Hz, 4H), 3.26 (t, J = 6.5 Hz, 4H), 2.55 (t, J = 4.0 Hz, 8H). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm):160.4, 154.4, 153.0, 152.6, 150.9, 126.0, 117.2, 116.9, 114.8, 109.9, 66.8, 66.3, 59.5, 53.8, 36.5. ESI-MS (+ve) *m/z* $[M + H]^+$ calcd. for $[C_{34}H_{36}N_2O_{12}S_3]^+$: 761.1509, found 761.1532.

Synthesis of UDS-1



Scheme S3. Schematic representation for the synthesis of the disulfide probe **UDS-1**. Reagents and condition: (i) NaI, H_2O_2, Ethyl acetate, RT, 30 min; (ii) 4-Nitrophenyl chloroformate, Pyridine, Dichloromethane, 0 °C-RT, 6 h; (iii) Umbelliferone, NEt_3, Dichloromethane, 0 °C-RT, 16 h.

Synthesis of compound 16[4]

To a solution of 2-mercaptoethanol **15** (0.5 g, 6.00 mmol) in ethyl acetate (5.0 mL) was added NaI (5.0 mg, 0.03 mmol) followed by hydrogen peroxide (0.2 mg, 0.70 mL of 30% solution in water). The reaction mixture was stirred at room temperature for 20 min and the colour of the solution turned brownish. The reaction was quenched by addition of saturated $Na_2S_2O_3$ solution (10.0 mL). Upon completion of the reaction, the mixture was poured into a separatory funnel and the organic layer was washed with saturated Na_2CO_3 solution (10.0 mL) followed by brine solution (10.0 mL). The organic layer was collected and dried over anhydrous sodium sulfate. The solvent was evaporated under vacuum to afford compound **16** as colourless oil. R_f = 0.5 (60% ethyl acetate in petroleum ether). Yield: 0.2 g (48%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 3.92 (s, 4H), 2.90 - 2.88 (t, *J* = 4.0 Hz, 4H), 2.34 - 2.33 (d, *J* = 4.0 Hz, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 60.3, 41.2. ESI-MS: *m/z* calcd. for $C_4H_{10}O_2S_2$ $[M+Na]^+$: 177.0020 ; observed $[M+Na]^+$: 177.0036.

Synthesis of compound 17[5]

To a solution of compound **16** (0.2 g, 1.3 mmol) in anhydrous dichloromethane (6.0 mL) was added pyridine (0.5 mL, 6.4 mmol) and the mixture was cooled to 0 °C. A solution of 4-nitrophenyl chloroformate (0.6 g, 3.2 mmol) in anhydrous dichloromethane (6.0 mL) was added to the above reaction mixture in a dropwise manner at 0 °C. The reaction mixture was allowed to attain room temperature and was stirred for another 6h. The progress of the reaction was monitored by TLC method. Upon completion, the mixture was diluted with dichloromethane and the mixture was sequentially washed with saturated $NaHCO_3$ (10.0 mL), water (10.0 mL), 10% citric acid (10.0 mL), water (10.0 mL) and brine (10.0 mL). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure to afford crude compound **10**. The crude residue was purified by silica gel column chromatography using

ethyl acetate and petroleum ether as eluents to obtain compound **10** as white solid powder. R_f = 0.5 (30 % ethyl acetate in petroleum ether 60-80) Yield: 0.4 g (62%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 8.30 - 8.27 (d, *J* = 12.0 Hz, 4H), 7.40 - 7.38 (d, *J* = 8.0 Hz, 4H), 4.59 – 4.56 (t, *J* = 8.0 Hz, 4H), 3.10 – 3.07 (t, *J* = 8.0 Hz, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 155.3, 152.3, 145.5, 125.4, 121.8, 66.7 and 36.8. ESI-MS: *m/z* calcd. for $C_{14}H_{16}N_2O_{10}S_2$ $[M+K]^+$: 522.9883; observed $[M+K]^+$: 522.9885.

Synthesis of UDS-1

To a stirred solution of umbelliferone **1a** (0.2 g, 1.2 mmol) in anhydrous dichloromethane (10.0 mL) at 0 °C under an inert atmosphere was added triethylamine (0.2 g, 1.5 mmol) and the mixture was stirred for 10 min. A solution of compound **17** (0.2 g, 0.4 mmol) in dichloromethane (5.0 mL) was added to the mixture in a dropwise manner and the reaction mixture was allowed to attain room temperature and stirred for 16h. The progress of reaction was monitored by TLC analysis. Upon completion, the reaction mixture was poured into water and extracted with ethyl acetate. The combined organic layer was finally washed with brine solution, separated, dried over anhydrous sodium sulfate and concentrated under reduced pressure to afford the crude product. The crude compound was purified by silica gel column chromatography using ethyl acetate and petroleum ether as eluents to afford pure **UDS-1** as white solid. R_f = 0.3 (35% ethyl acetate in petroleum ether). Yield: 0.1 g (55%), M.P. = 121 - 123 °C. ^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 7.71 – 7.69 (d, *J* = 8.0 Hz, 2H), 7.52 – 7.50 (d, *J* = 8.5 Hz, 2H), 7.22 - 7.21 (d, *J* = 4.0 Hz, 2H), 7.17 – 7.14 (dd, J_1 = 4.0, J_2 = 4.0 Hz, 2H), 6.42 – 6.40 (d, *J* = 8.0 Hz, 2H), 4.58 – 4.55 (t, *J* = 12.0 Hz, 4H), 3.11 – 3.09 (t, *J*= 4.0 Hz, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 160.2, 154.6, 153.2, 152.6, 142.7, 128.7, 117.6, 116.9, 116.3, 109.9, 66.7, 36.8. ESI-MS: *m/z* calcd. for $C_{24}H_{18}O_{10}S_2$ $[M+H]^+$ 531.0420; found $[M+H]^+$: 531.0431.

HPLC Analysis

Purity of the synthesized probes was analysed by analytical high-performance liquid chromatography (HPLC) Agilent 1220 infinity II LC system using reverse-phase C_{18} column (Luna ®, 150 × 4.6 mm, 5 µm). HPLC grade solvents were used as mobile phase and the HPLC profile of compounds were detected by PDA detector at a wavelength of 250 and 323 nm at room temperature at a flow rate of 1.0 mL/min. The final trisulfide probes **UTS-1** and **UTS-2** were separated from the polysulfides using a semi-preparative HPLC system (Thermo Scientific

Dionex, Ultimate 3000 pump with DAD) using a semi-preparative column (Luna ®, polar C18, 250×21.2 mm) using acetonitrile and water as mobile phase with a flow rate of 10 ml/min.

UV-Vis and fluorescence spectroscopic studies

All the stock solutions of biologically relevant analytes were prepared in Milli-Q or double distilled water and the stock solutions of probes and organic compounds were prepared in spectroscopy grade DMSO. All the spectroscopic measurements were performed under physiological conditions (PBS buffer 20 mM, pH 7.4). Samples for absorption and emission measurements were carried out in quartz cuvettes (1.0 mL). UV-Vis spectroscopic measurements were carried out on a Lamda 45 UV-Vis Spectrophotometer and fluorescence emission spectra were recorded on a Fluoromax-4 spectrophotometer (Horiba Jobin Yvon) with an excitation wavelength of 322 nm with a slit width of 3 nm. For the studies with different analytes, emission was measured after incubating **UTS-1** (5 μM) with the analyte (200 μM) for 15 min in PBS. The resulting mixture was further incubated for 15 min with the addition of equivalent amount of Cys (200 μM) to the reaction mixture. The effect of pH on the stability and reactivity of **UTS-1** was studied by the optimized protocol using the PBS (20 mM) of different pH by fluorescence spectroscopic method.

Release profile of H_2S from UTS-1 and UTS-2 (Methylene blue assay)

Release of hydrogen sulfide (H_2S) from **UTS-1** and **UTS-2** (25 μM) in the presence of Cys and GSH (500 μM) was monitored by methylene blue assay using UV-Vis spectrophotometer.[6] Hydrogen sulfide generation was initiated by adding the probes (25 μM) into PBS buffer (pH 7.4, 20 mM) solution containing thiol (500 μM). Formation of methylene blue was monitored at 670 nm in UV-Vis spectrophotometer in different time intervals after adding 500 μl of the above solution to 500 μl of methylene blue cocktail (100 μL of zinc acetate (1% w/v), 200 μL of *N,N*-dimethyl-1,4-phenylenediamine sulfate (20 mM in 7.2 M HCl), and 200 μL of ferric chloride (30 mM in 1.2 M HCl)) in a cuvette. The H_2S concentration of each sample was calculated against a calibration curve, which was obtained using known concentrations of $Na_2S.9H_2O$ solution under identical condition without any thiols.

Detection of Hydrogen sulfide release by Fluorescence Spectroscopic Assay

Generation of hydrogen sulfide by **UTS-1** in the presence of cysteine (Cys) was monitored using a H_2S-sensitive turn-on azide-based fluorescent probe **NAP-1** by fluorescence spectrophotometer. The probe **NAP-1** (25 μM) was pre-incubated with thiol (250 μM) for 90 min in PBS buffer (20 mM, pH 7.4) and the emission spectra was measured to saturate the possible reaction of azide with thiols. The aliquot from the experimental solution of **UTS-1** (25 μM) + Cys (250 μM) was added to the above mixture and measured the emission intensity after an incubation of 90 min to understand the reaction of azide probe with the generated hydrogen sulfide from **UTS-1** (25 μM) in the presence of Cys (500 μM).

Cell Culture

Human cervical cancer cell line HeLa was obtained from the National Centre for Cell Science (NCCS), India. HeLa cells were cultured in DMEM medium (Gibco) supplemented with 10% (v/v) FBS (Gibco) and 1% Pen-Strep (Gibco). Cells were cultured as a monolayer in a humidified incubator at 37 °C in the presence of 5% CO_2 level. Human embryonic kidney cell line (HEK-293) was also cultured in DMEM medium following the similar protocol as used for HeLa cells.

Cell viability studies

The probes **UTS-1** and **UTS-2** along with the released compounds such as umbelliferone derivatives (**1a** and **1b**) and cyclic compound **2** were screened for their anti-proliferative activities using the conventional MTT assay. HeLa cells were seeded in 96-well culture plates at a density of 1×10^4 cells /100 μl/well and treated with the freshly prepared test compounds (2.5 μM to 50.0 μM) for 0 h (control) and 72 h (experimental). At the end of treatment period, 10.0 μL of 5.0 mg/mL of MTT was added to the plate (control) and incubated for 2 h. Following the 2 h incubation, the culture media from the plate was removed and the purple formazan crystals were dissolved using 100 μl of DMSO (HiMedia) and the absorbance at 570 nm was measured using a microplate reader (Multiskan Go microplate reader, Thermo Scientific). In experimental set, similar MTT treatment protocol was followed only after 72 h. The mean ΔOD values were calculated by the subtraction of mean OD values of 0 h plate (control) from the mean OD values of identical wells at 72 h plate (experimental) and the percentage proliferation was calculated keeping the mean ΔOD of untreated control as 100%.

Fluorescence and confocal microscopic studies

HeLa cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO_2 atmosphere. Cells were then plated (0.7×10^4 cells/plate) in 35 mm cell culture petri dishes containing 2.0 mL of DMEM and incubated at 37 ºC under 5% CO_2 for 24 h. The confluent cells were washed with DPBS and finally incubated with **UTS-1** or **UTS-2** (5.0 μM) at 37 ºC under 5% CO_2 for 1 h. After washing with DPBS (5 times), cellular morphology was carefully observed and imaged in a Bio-rad ZOE^{TM} fluorescent cell imager under bright field and suitable fluorescent colored filters. Additionally, the control experiments were designed and performed with the pre-treatment of NEM (2.0 mM) and/or DL-Propargyl glycine (50.0 μM) to affirm the quenching of endogenous bio-thiols and/or H_2S levels, respectively. Initial treatments with **UTS-1** (5.0 μM) or **UTS-2** (5.0 μM) followed by H_2S-sensitive azide-based fluorescence turn-on probes such as **NAP-1** or **NAP-2** (2.0 μM) were performed and treated cells were incubated at 37 ºC under 5% CO_2 for 1 h. Finally, the treated cells were washed with DPBS (5 times) and imaged in a Bio-rad ZOE^{TM} fluorescent cell imager to understand the reactivity of generated H_2S with fluorescent probes. Similarly, a standard Lyso Tracker Red dye (**DND-99**, ThermoFisher) (75.0 nM) was also used in combination with **UTS-2** using the above protocol to confirm the lysosomal selectivity of **UTS-2**.

For the co-localization studies using confocal microscopy, HeLa cells were cultured and grown following the above protocol. The confluent cells were washed with DPBS (5 times), cellular morphology was carefully observed. In order to image the localization property of the probe **UTS-2**, we have treated the cells with the probe with **UTS-2** (5.0 μM) and kept in the incubator for 1h at 37 ºC under 5% CO_2. After washing with DPBS (5 times) a standard Lyso Tracker Red dye (**DND-99**, ThermoFisher) (75.0 nM) was added and kept for incubation at 37 ºC under 5% CO_2 for another 1h. Finally, we washed the cells with DPBS (5times) and fixed with 4% formaldehyde and imaged under confocal microscopy (Zeiss LSM 880) using respective filters for understanding the proper localization behaviour of our compound.

Emission spectral plots of UTS-2, UTS-1 and UDS-1

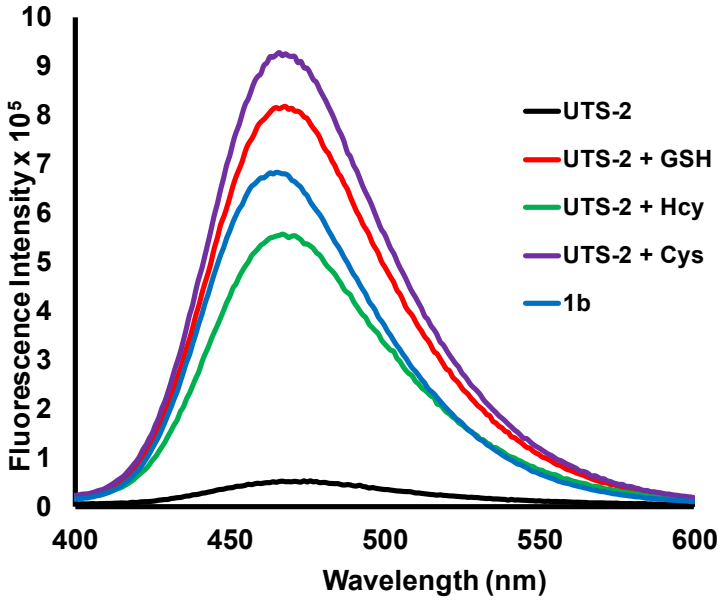


Figure S1. Emission spectra of **UTS-2** (5 μM) in the absence and presence of GSH, Cys and Hcy (200 μM) in PBS buffer (20 mM, 7.4) after 20 min along with the emission spectral pattern of released umbelliferone derivative **1b**.

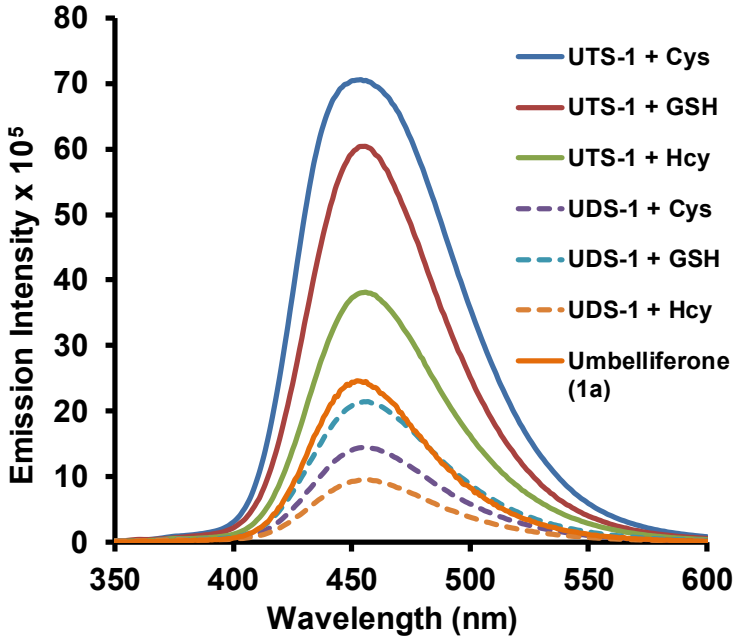


Figure S2. Relative sensitivity/reactivity of **UTS-1** (5 μM) and **UDS-1** (5 μM) towards GSH, Cys and Hcy (200 μM) after 15 min along with the emission spectral pattern of released umbelliferone (**1a**).

H_2S release profiles of UTS-1, UTS-2 and DATS and UV-Vis spectral pattern of MB formation

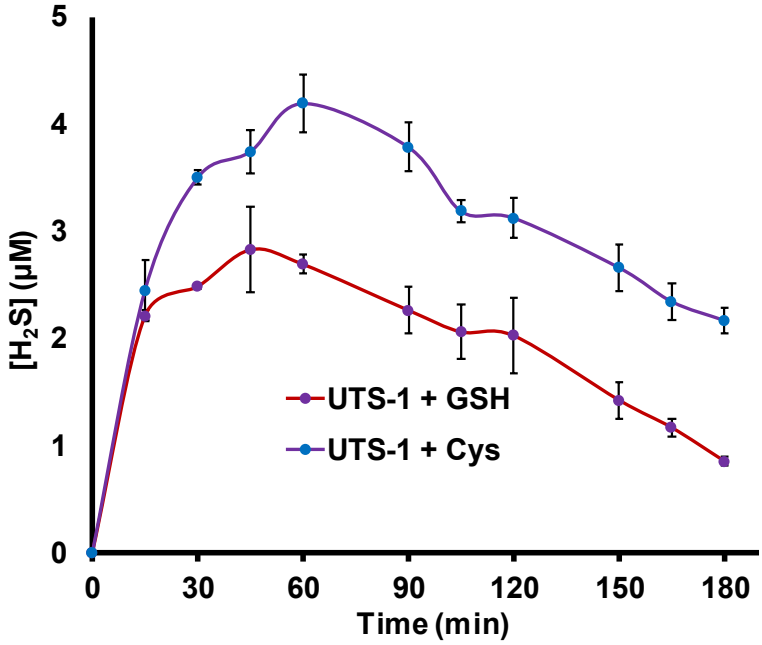


Figure S3. H_2S release profile of **UTS-1** (25 μM) over 180 min in the presence of GSH and Cys (1000 μM).

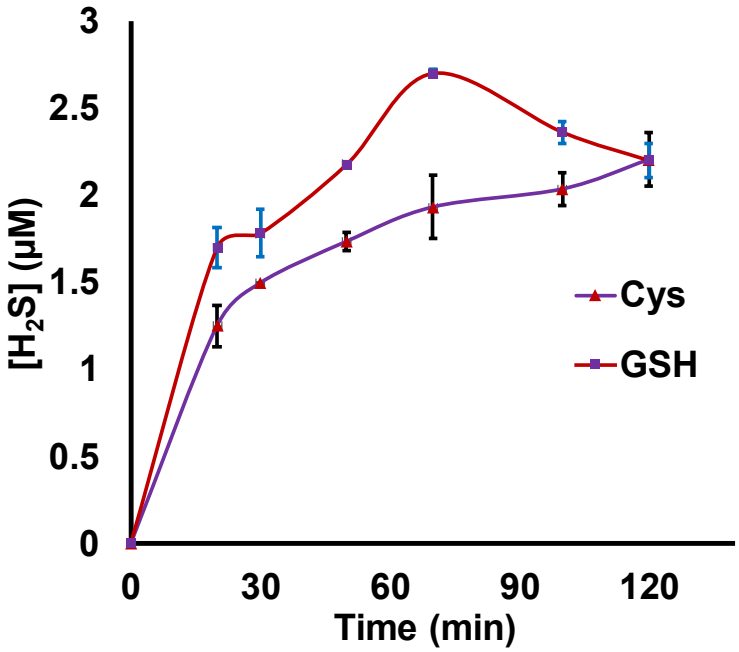


Figure S4. H_2S release profile of **UTS-1** (25 μM) over 120 min in the presence of GSH and Cys (500 μM).

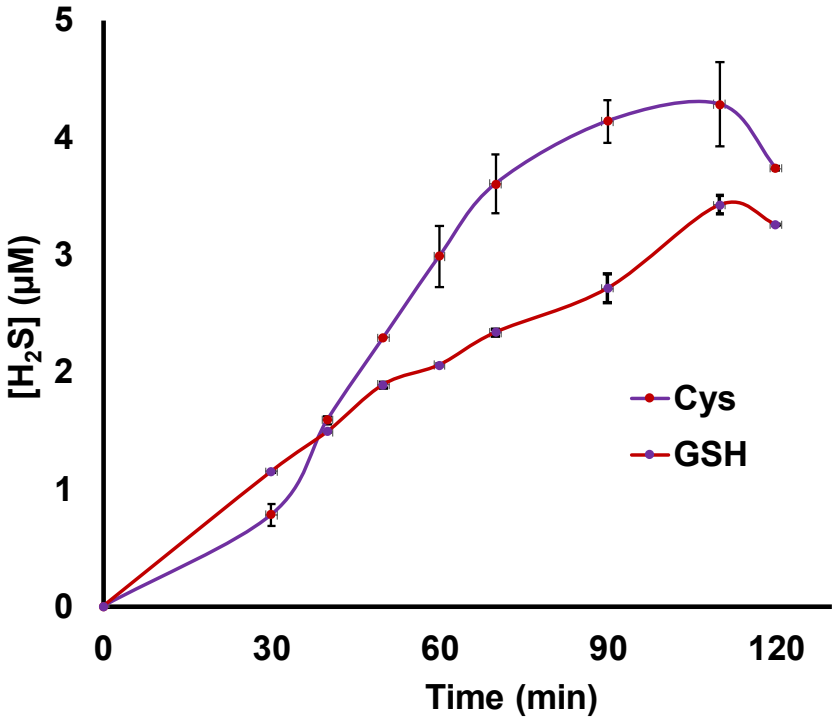


Figure S5. H_2S release profile of **UTS-2** (25 μM) over 120 min in the presence of GSH and Cys (500 μM).

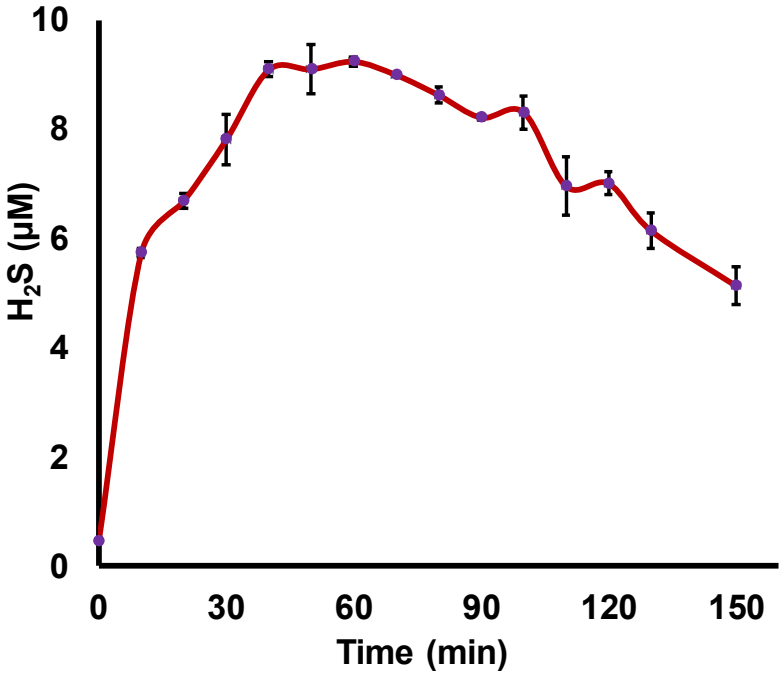


Figure S6. H_2S release profile of **DATS** (25 μM) over 150 min in the presence of L-Cys (500 μM).

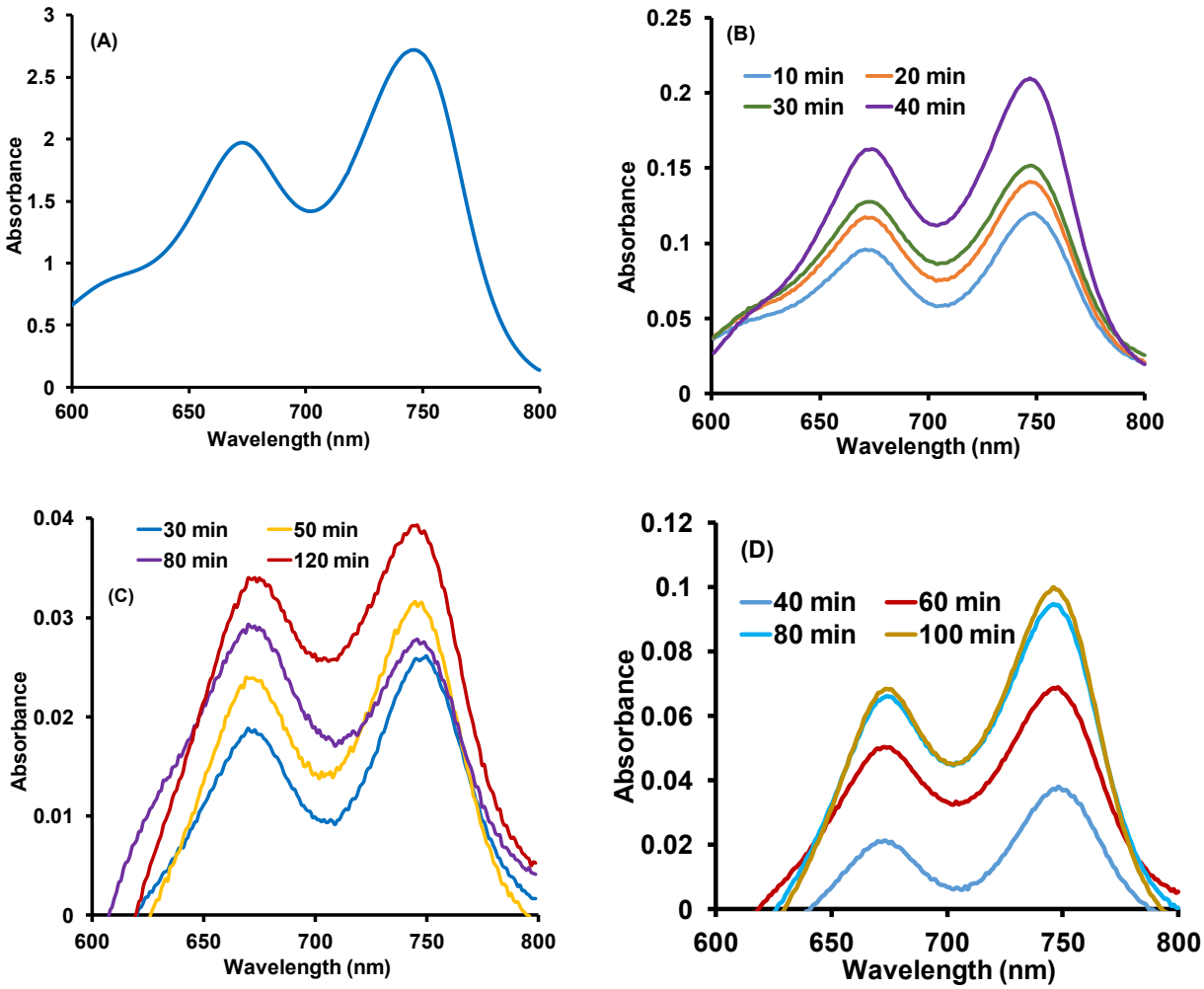


Figure S7. Typical spectral pattern of different reactions in MB assay using UV-Vis spectroscopy. (A) Spectral pattern of pure methylene blue (10 μM) in MB cocktail; (B) Spectral pattern of DATS (25 μM) with L-Cys (500 μM); (C) Spectral pattern of **UTS-1** (25 μM) with L-Cys (500 μM); (D) Spectral pattern of **UTS-2** (25 μM) with L-Cys (500 μM).

HPLC chromatogram for the reaction of UTS-1 and UDS-1 with PhSH

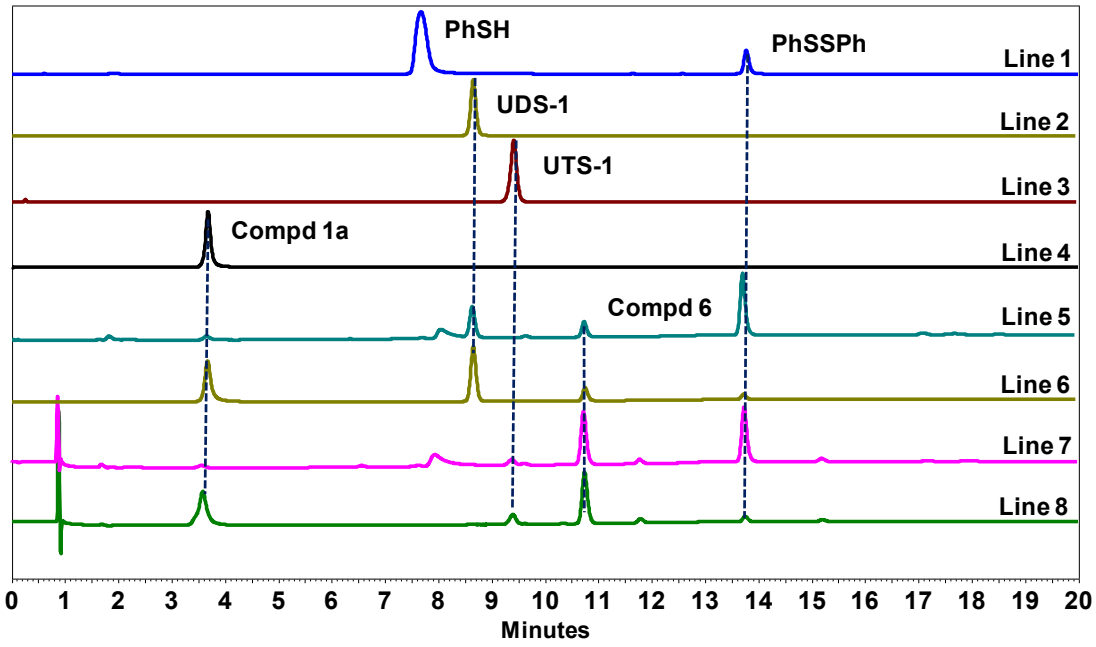
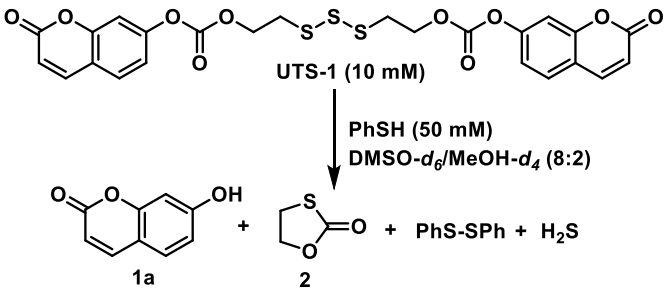


Figure S8. A complete HPLC overlay chromatogram (0-20 min) for the reaction of **UDS-1** and **UTS-1** with PhSH; Lines: (1) Pure PhSH and PhSSPh; (2) Pure **UDS-1**; (3) Pure **UTS-1**; (4) Pure compound **1a**; (5 & 6) **UDS-1** + PhSH; (7 & 8) **UTS-1** + PhSH. All the chromatograms were extracted at 250 nm except lines 4, 6 and 8, which were extracted at 332 nm (to visualize umbelliferone). The chromatograms for the reaction mixtures were recorded after 20 min.

NMR Experiments for the reaction of UTS-1 with PhSH



About the reaction: In this experiment, **UTS-1** (19.5 mM) was reacted with thiophenol (100 mM) in DMSO-d_6/MeOH-d_4 (8:2) mixture at room temperature in the presence of Mesitylene (30 mM) as an internal standard. The final spectrum was recorded after 2h to ensure the completion of expected reaction. $^{13}C\{^1H\}$ NMR spectra of pure **UTS-1**, released umbelliferone (**1a**), diphenyl disulfide (PhSSPh) and thiophenol (PhSH) are included here for spectral comparison. Final spectrum of the reaction mixture clearly demonstrates the formation of umbelliferone (**1a**) as the product along with the cyclic compound (or its hydrolysed analogue), PhSSPh and unreacted PhSH. It further indicates that unreacted **UTS-1** is not present in the reaction mixture. Similar NMR experiment has been reported by Pluth and Co-workers in a recent report for the release of hydrogen sulfide from cyclic sulfenyl thiocarbamates.[7]

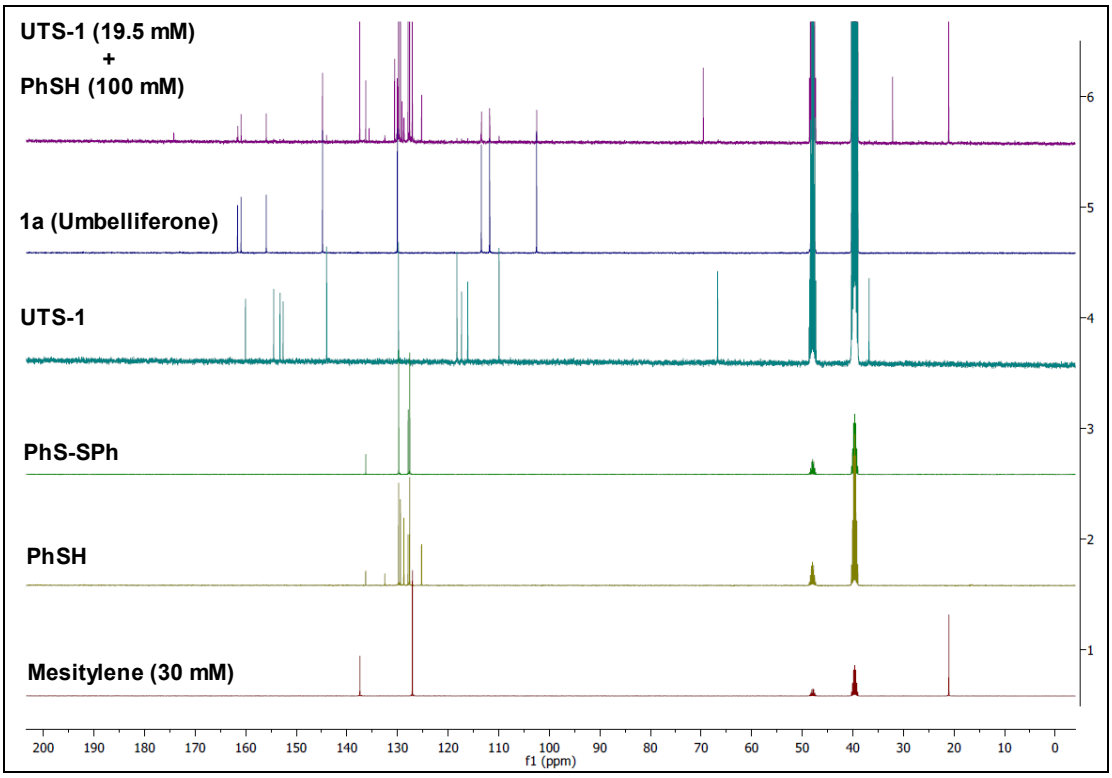


Figure S9. $^{13}C\{^{1}H\}$ NMR spectra of the reaction mixture of **UTS-1** (19.5 mM) and PhSH (100 mM) in DMSO-*d*$_6$/MeOH-*d*$_4$ (8:2) mixture at room temperature confirming the release of umbelliferone (**1a**).

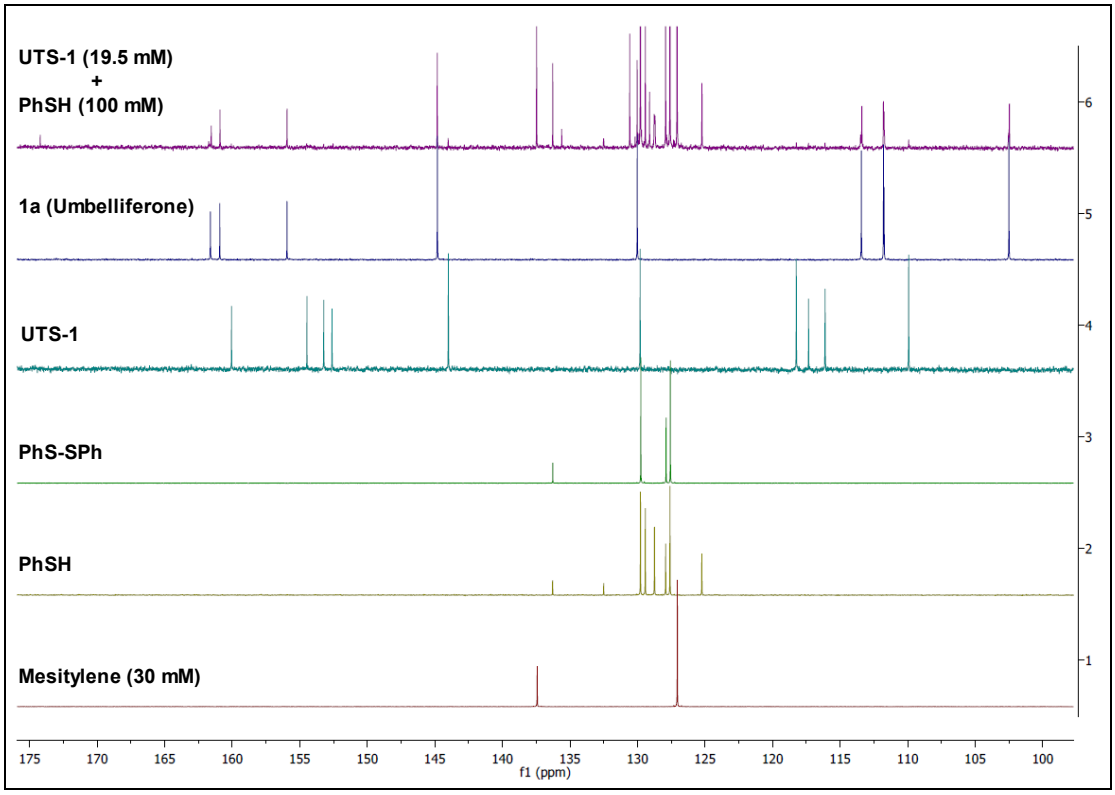


Figure S10. Zoomed version highlighting the aromatic region of the above $^{13}C\{H\}$ NMR spectra of the reaction mixture of **UTS-1** (19.5 mM) and PhSH (100 mM) in DMSO-*d*$_6$/MeOH-*d*$_4$ (8:2) mixture at room temperature confirming the release of umbelliferone (**1a**).

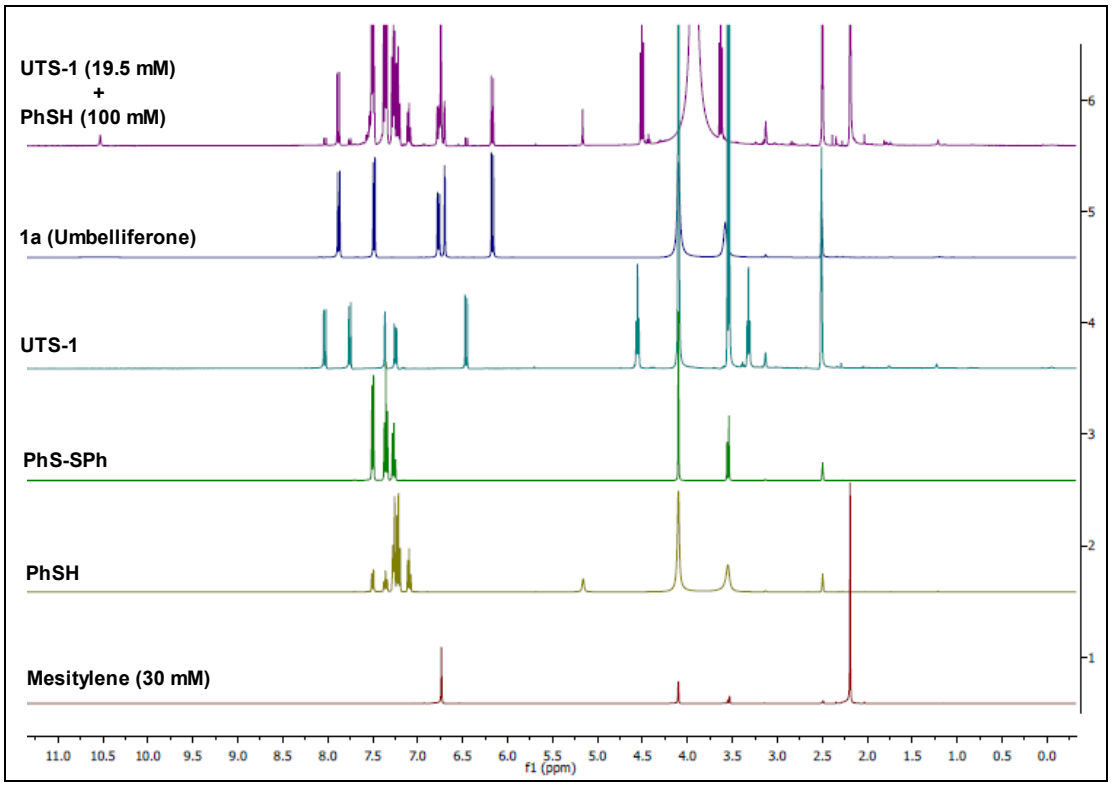


Figure S11. ^{1}H NMR spectra of the reaction mixture of **UTS-1** (19.5 mM) and PhSH (100 mM) in DMSO-d_6/MeOH-d_4 (8:2) mixture at room temperature confirming the release of umbelliferone (**1a**).

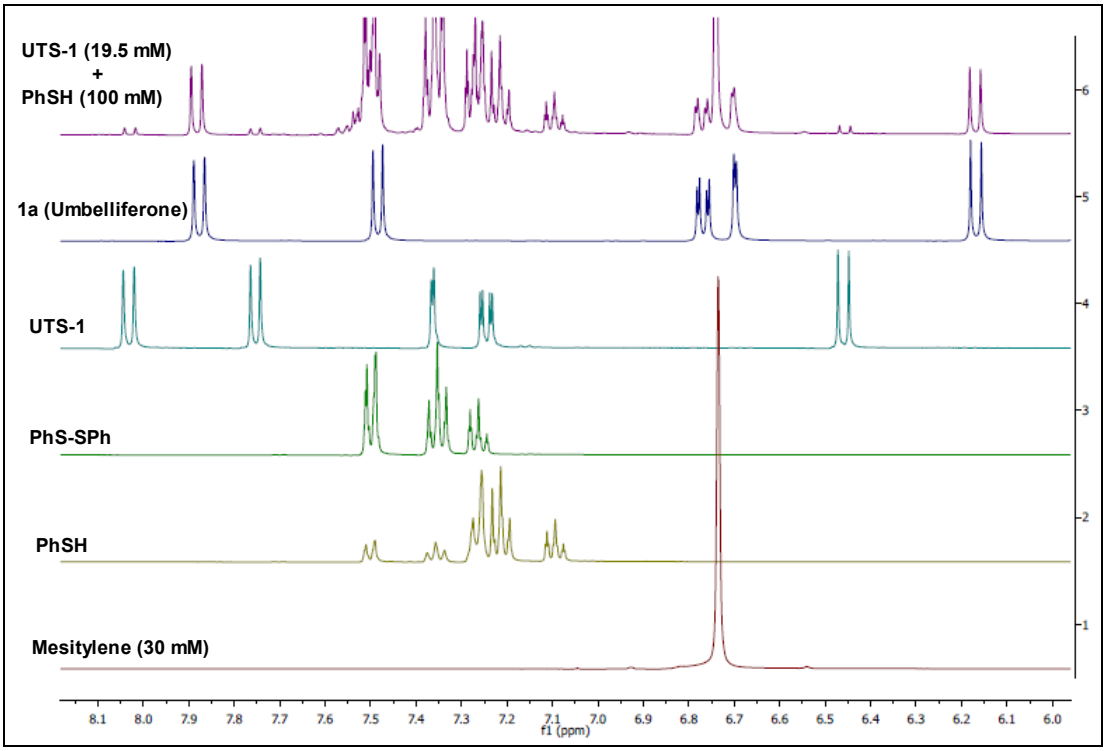


Figure S12. Zoomed version highlighting the aromatic region of the above ^{1}H NMR spectra of the reaction mixture of **UTS-1** (19.5 mM) and PhSH (100 mM) in DMSO-d_6/MeOH-d_4 (8:2) mixture at room temperature confirming the release of umbelliferone (**1a**).

Quantitative ^{1}H NMR Experiment for product yield

The quantitative ^{1}H NMR experiment was carried out with **UTS-1** to determine the yield of released fluorophore (umbelliferone, **1a**) from the probe in the presence of thiophenol (PhSH). The reaction was performed in an NMR tube in DMSO-d_6/MeOH-d_4 (8:2) solvent system using 400 MHz NMR spectrometer. Mesitylene (30 mM) was used as an internal standard to determine the concentration of **UTS-1** and the product (**1a**) during the reaction. As shown in Figure S13, the relative integration ratio of methyl proton (9H) of mesitylene with the olefinic C-H proton (2H, two umbelliferone molecules in a single **UTS-1**) was considered for calculating the actual concentration of **UTS-1** in the solution. Similarly, the concentration of released umbelliferone (**1a**) after 2h was determined from the ^{1}H NMR of the reaction mixture as shown in Figure S14.

$C_{\mathbf{UTS\text{-}1}} = (I_{\mathbf{UTS\text{-}1}} / I_{Mesitylene}) \times (N_{Mesitylene} / N_{\mathbf{UTS\text{-}1}}) \times (C_{Mesitylene})$

$C_{\mathbf{UTS\text{-}1}} = (1.30 / 9.0) \times (9.0 / 2.0) \times (30\ mM)$

$C_{\mathbf{UTS\text{-}1}} = 19.5\ mM$

Similarly, from Figure S14, the relative integration of methyl proton (9H) of mesitylene with the olefinic C-H proton (1H) of free released umbelliferone (**1a**) was considered for calculating the actual concentration of **1a** in the solution.

$C_{\mathbf{1a}} = (I_{\mathbf{1a}} / I_{Mesitylene}) \times (N_{Mesitylene} / N_{\mathbf{1a}}) \times (C_{Mesitylene})$

$C_{\mathbf{1a}} = (0.54 / 9.0) \times (9.0 / 1.0) \times (30\ mM)$

$C_{\mathbf{1a}} = 16.2\ mM$

Therefore,

Yield of **1a** = (16.2 mM / 19.5 mM) $\times$ 100% = **83%**

Additionally, there was a trace amount of an intermediate as evidenced in the ^{1}H NMR spectra of the reaction mixture (Figures S11, S12 and S14).

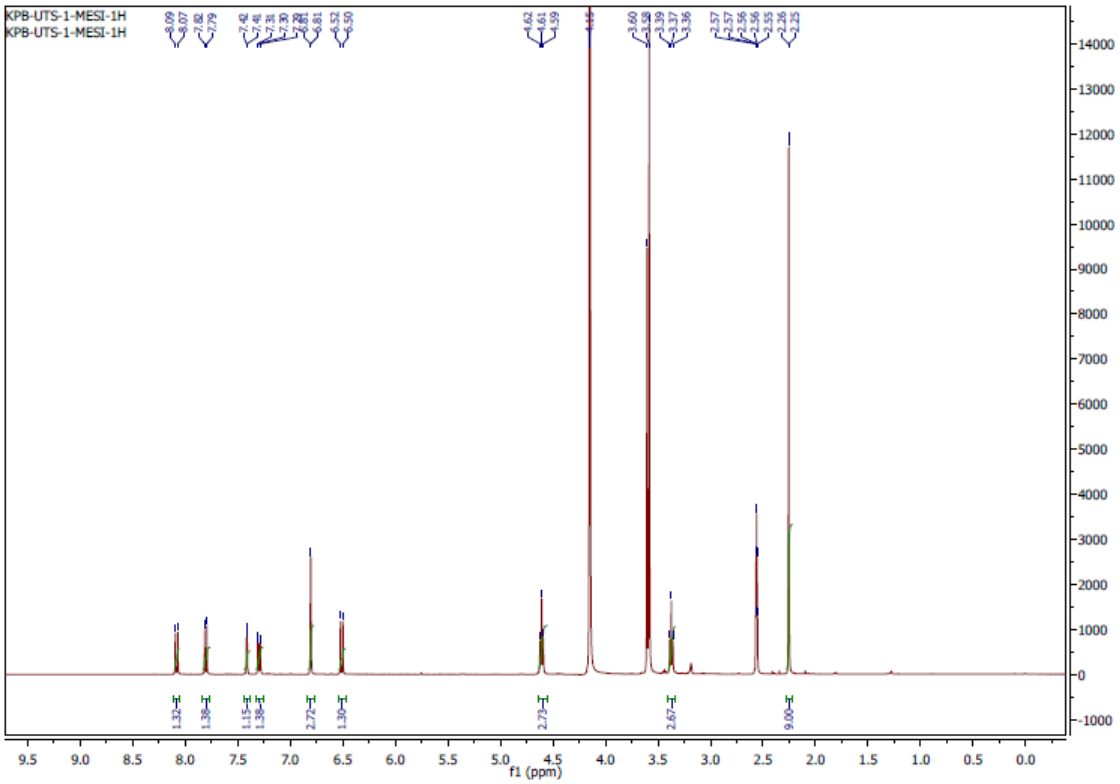


Figure S13. ^{1}H NMR spectrum of pure **UTS-1** (19.5 mM) in the presence of Mesitylene (30 mM) in DMSO-*d*$_6$/MeOH-*d*$_4$ (8:2) mixture at room temperature.

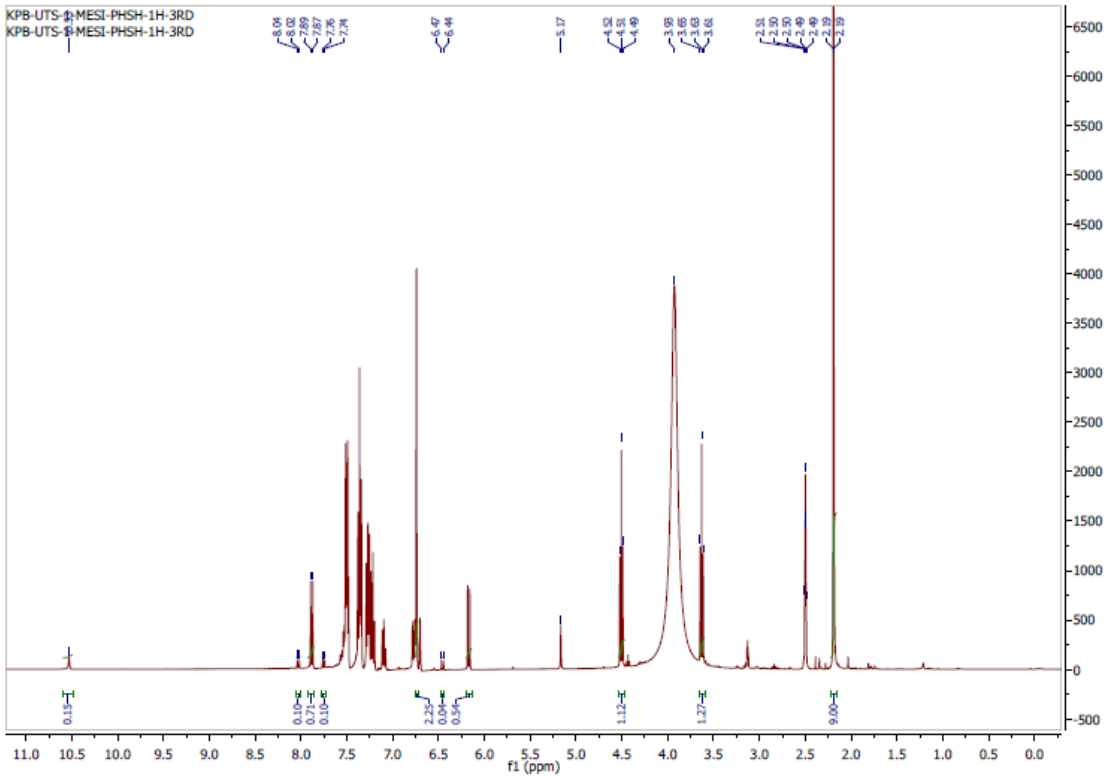


Figure S14. ^{1}H NMR spectrum of the reaction mixture of **UTS-1** (19.5 mM) and PhSH (100 mM) in DMSO-*d*$_6$/MeOH-*d*$_4$ (8:2) mixture at room temperature.

Proposed mechanism of fluorophore release from UDS-1

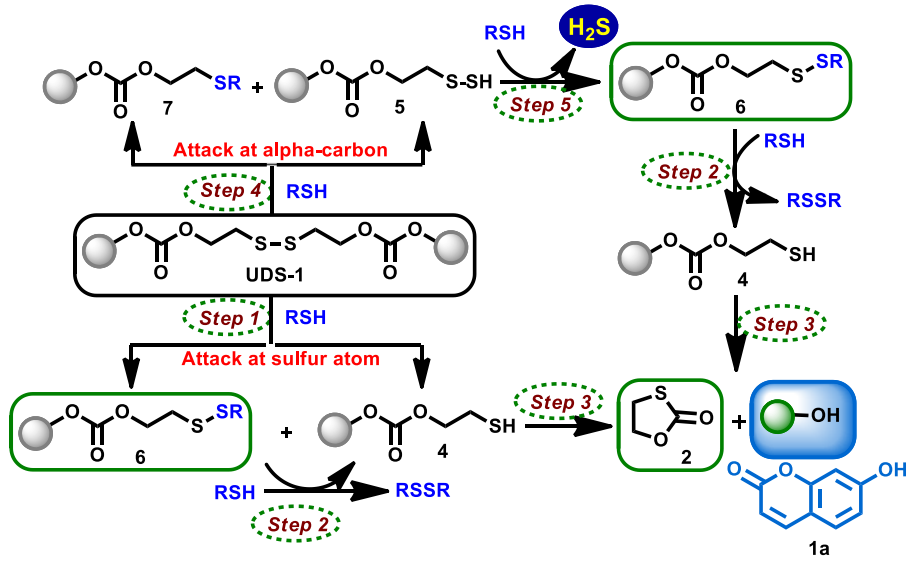


Figure S15. Proposed pathways for the fluorescence emission from the disulfide probe **UDS-1** in the presence of thiols. RSH could be biothiols or PhSH.

Confocal microscopic images for lysosomal localization

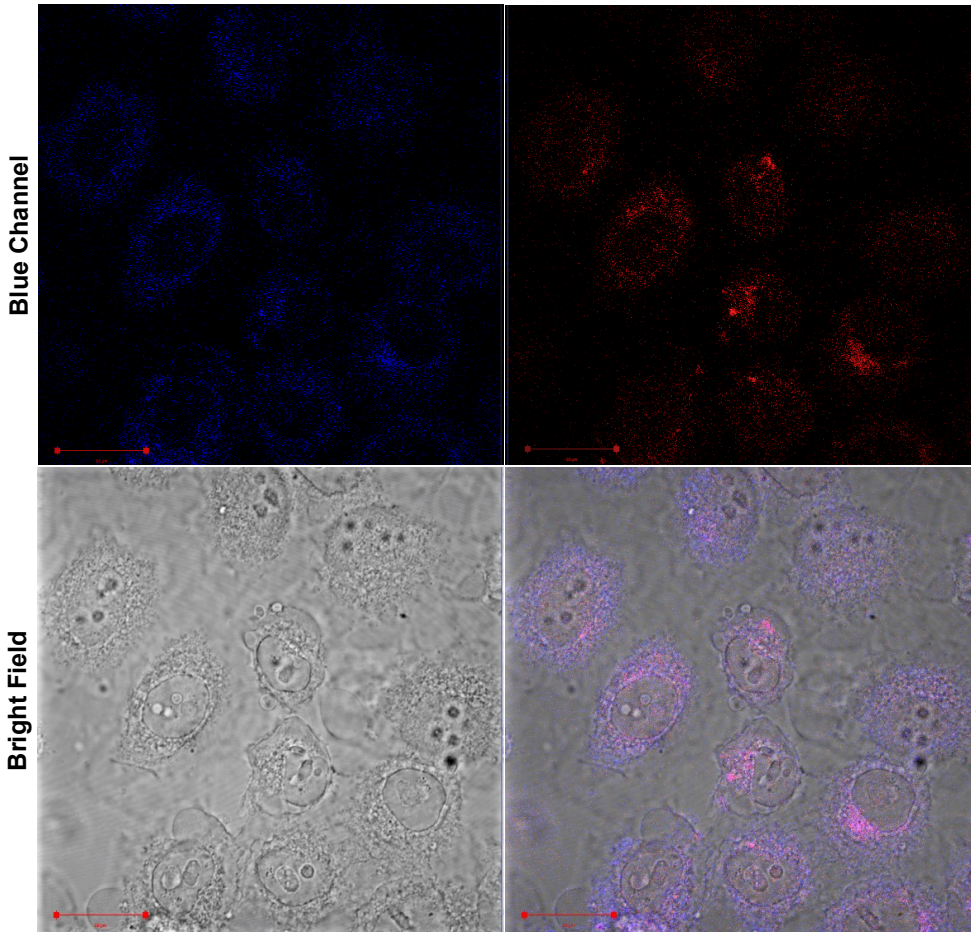


Figure S16. Confocal microscopic images of HeLa cells after the co-treatment of **UTS-2** and **DND-99** (Lyso tracker Red, standard lysosomal probe).

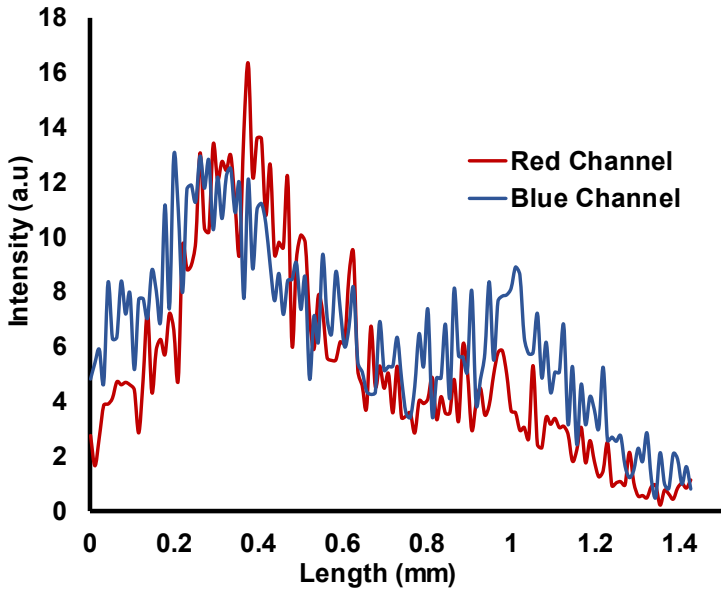


Figure S17. Intensity profiles for the region of interest (ROI) across HeLa cells for red (**DND-99**) and blue (**UTS-2**) channels, indicating significant overlapping profiles.

Fluorescence microscopic images for control experiments

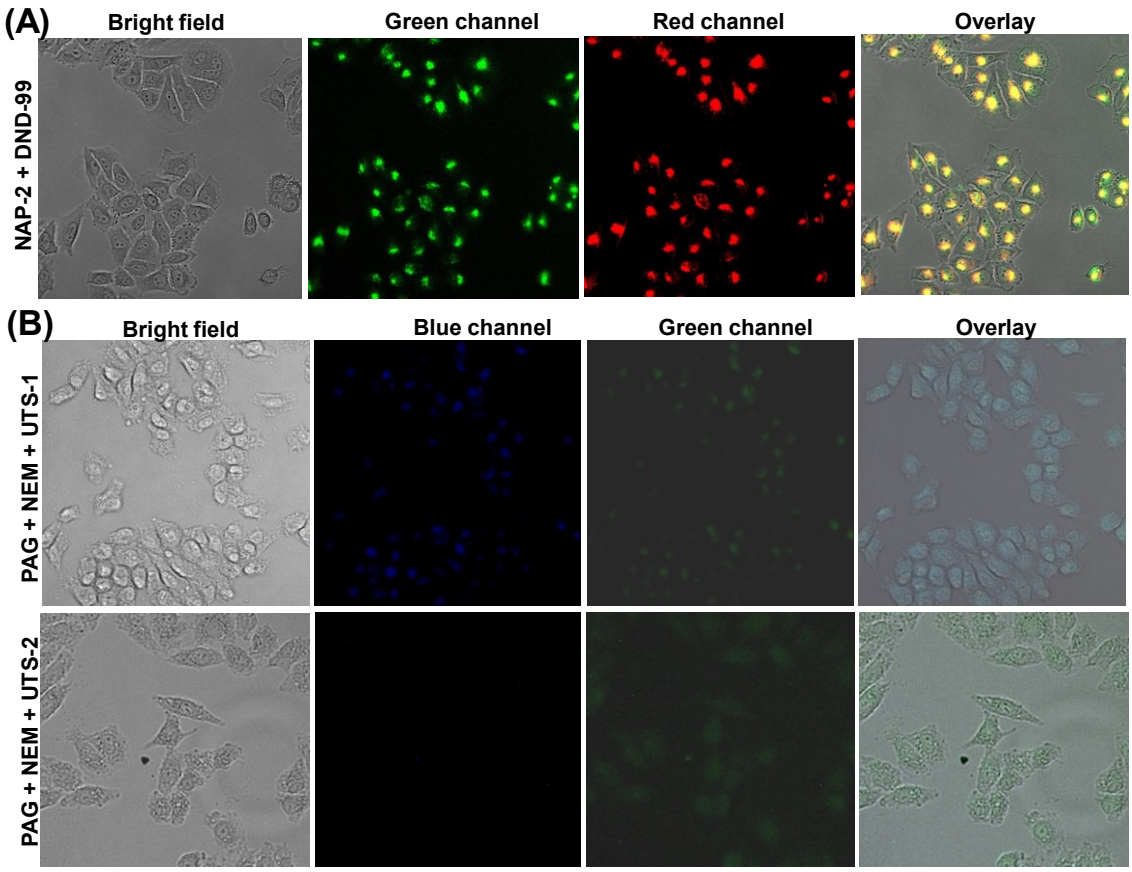


Figure S18. Fluorescence microscopic images of HeLa cells (A) incubated with **NAP-2** + **DND-99** and (B) pre-treated with DL-Propargyl glycine (PAG, 50 μM) and NEM (2.0 mM) before the treatment of **UTS-1** and **UTS-2**.

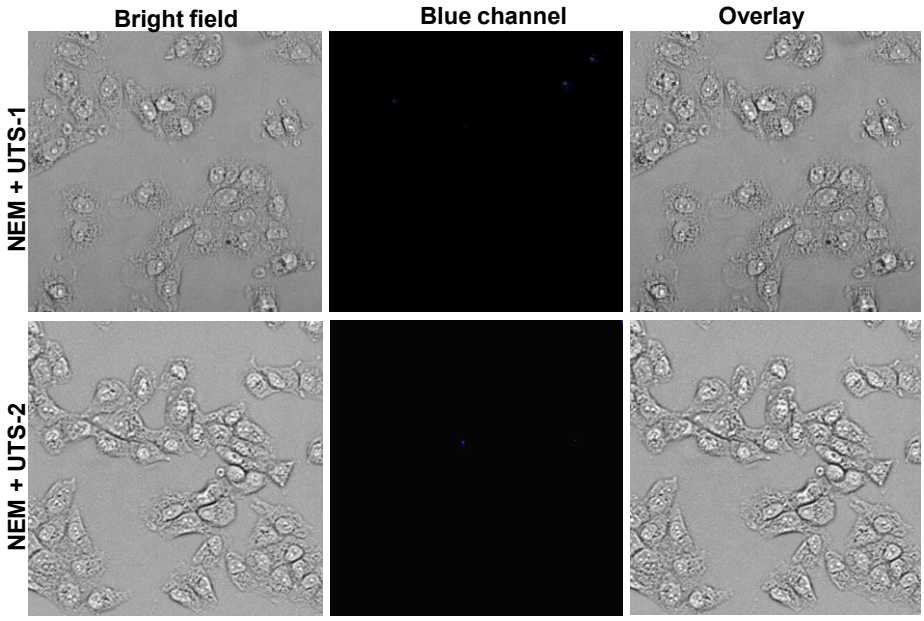
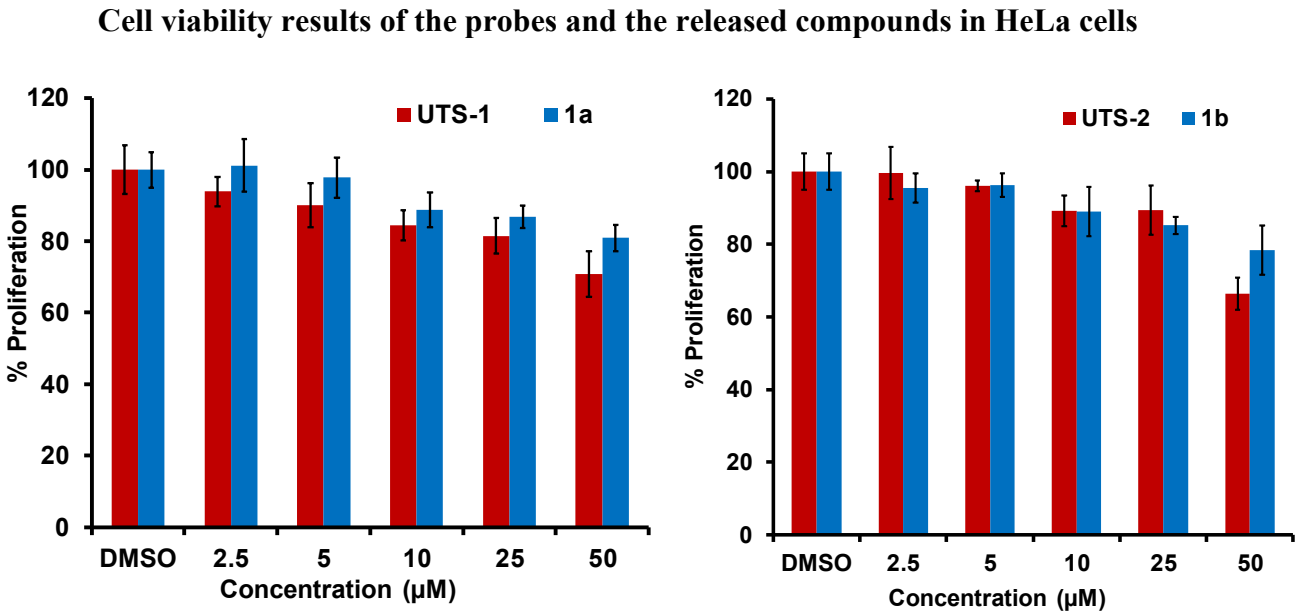


Figure S19. Fluorescence microscopic images of HeLa cells pre-treated with NEM (2.0 mM) before the treatment of **UTS-1** and **UTS-2**.



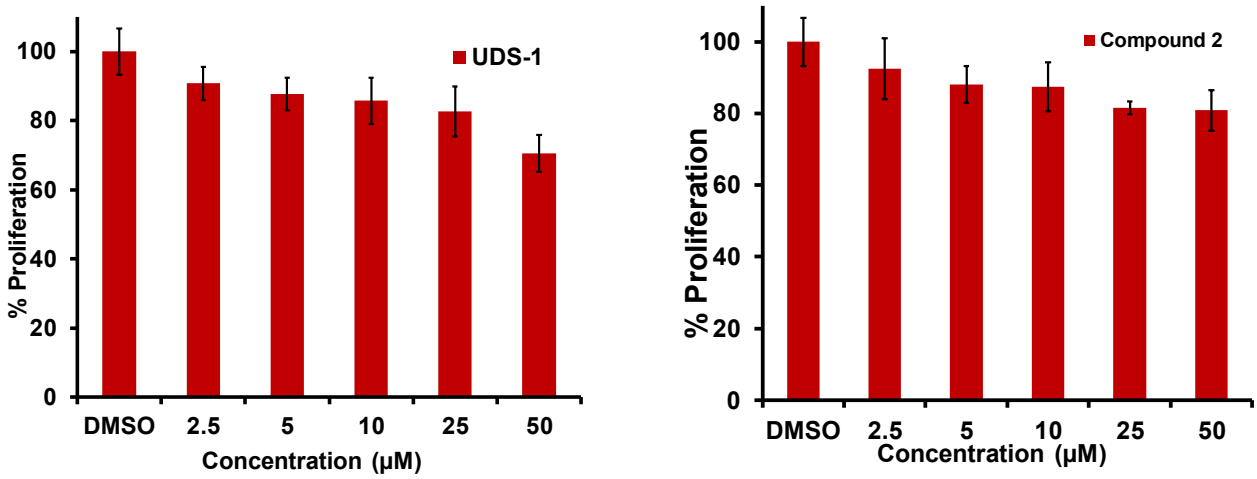


Figure S20. Percentage proliferation of HeLa cells by the test compounds (**UTS-1**, **UTS-2**, umbelliferone derivatives (**1a**, **1b**), **UDS-1** and cyclic compound **2**) in a dose-dependent manner. In addition to control experiments, %proliferation was studied at five different concentrations (2.5 µM, 5.0 µM, 10.0 µM, 25.0 µM and 50.0 µM).

NMR, ESI-MS spectra and HPLC chromatogram of the synthesized compounds

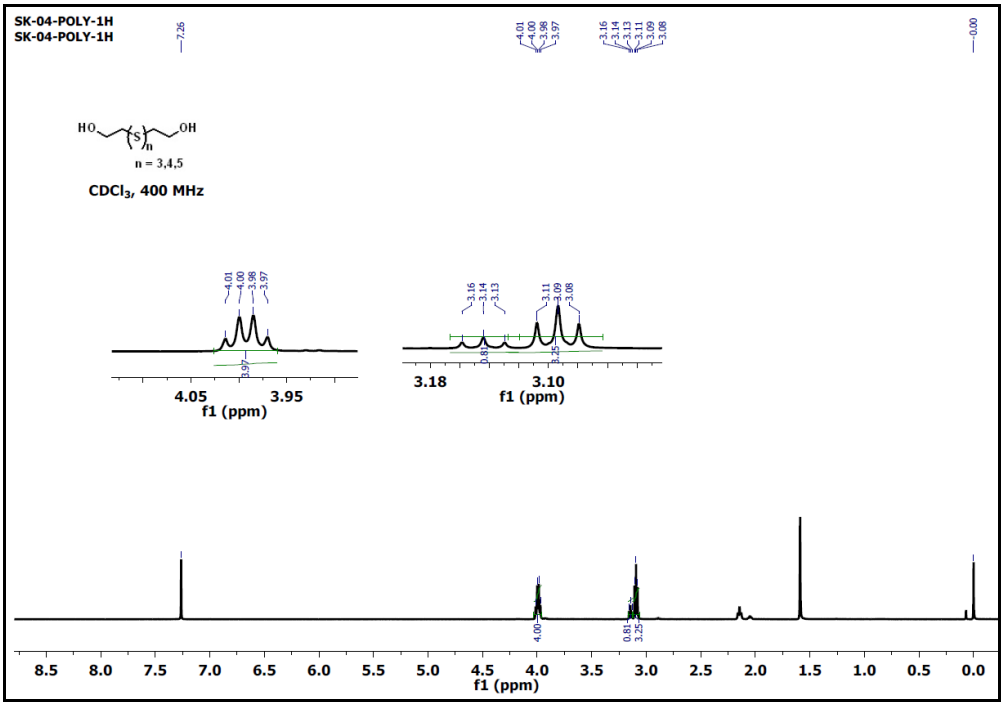


Figure S21. ^{1}H-NMR ($CDCl_3$, 400 MHz, ppm) of compound **8**.

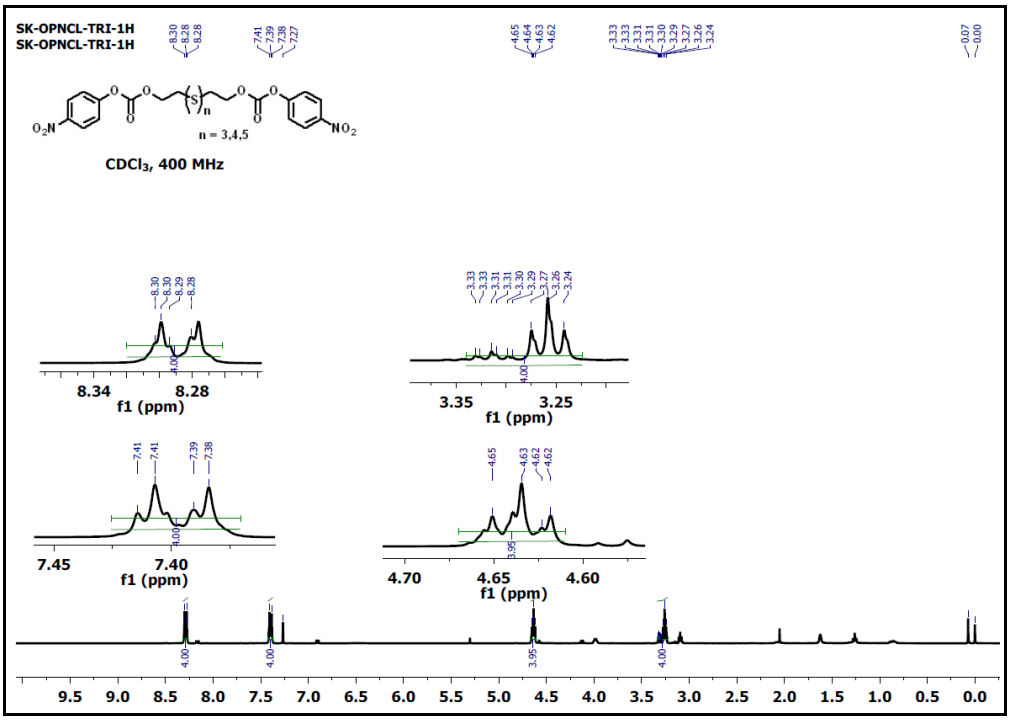


Figure S22. ^{1}H-NMR ($CDCl_3$, 400 MHz, ppm) of compound **9**.

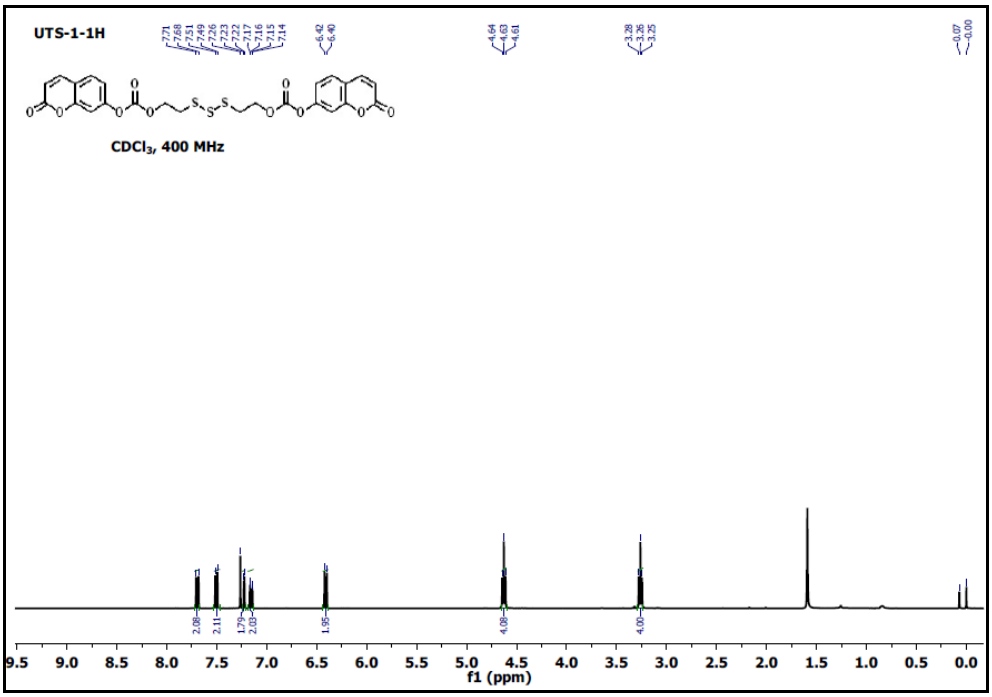


Figure **S23**. ^{1}H-NMR ($CDCl_3$, 400 MHz, ppm) of **UTS-1**.

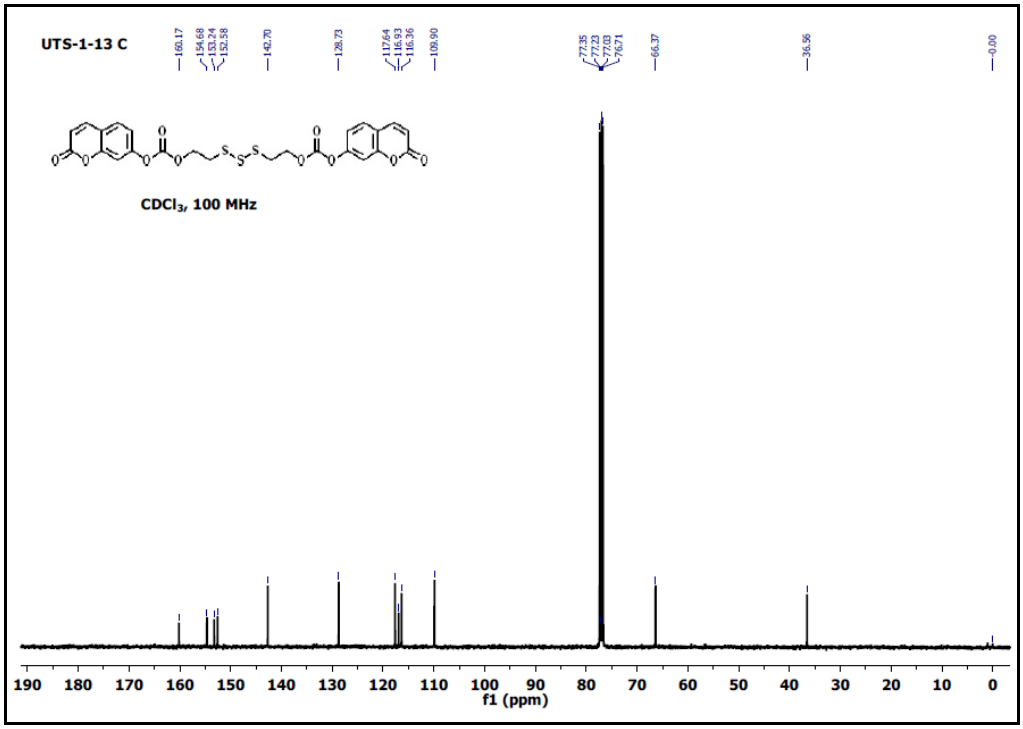


Figure **S24**: ^{13}C-NMR ($CDCl_3$, 100 MHz, ppm) of **UTS-1**.

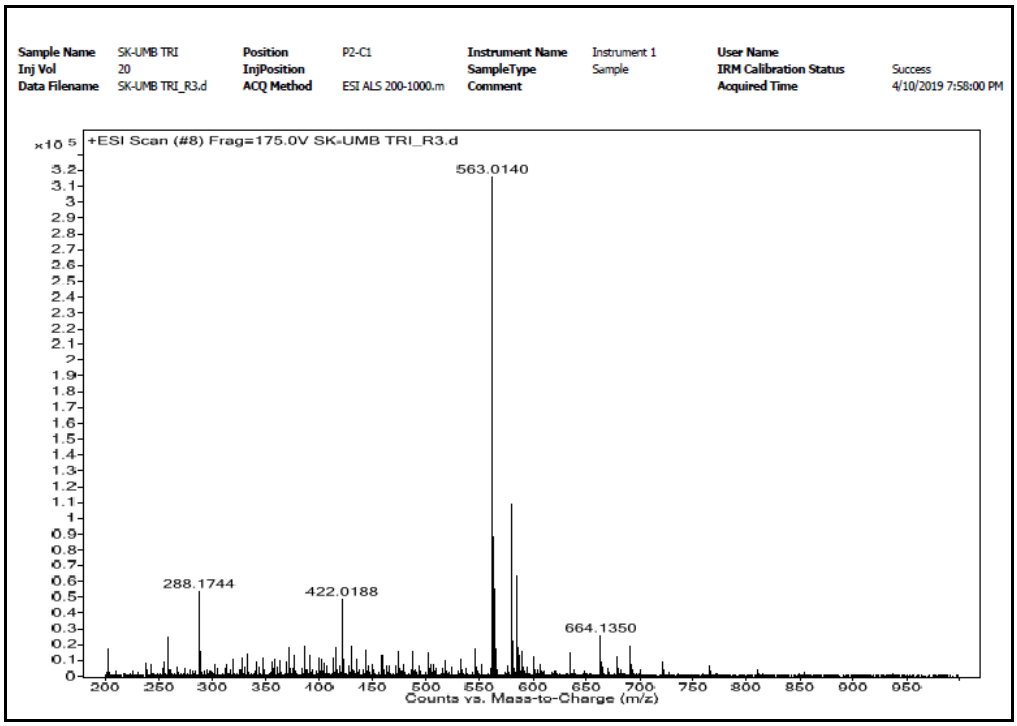


Figure **S25**: ESI-MS spectrum of **UTS -1**. ESI-MS *m/z* calcd. for $C_{24}H_{18}O_{10}S_3$ $[M+H]^+$: 563.0140; found $[M+H]^+$: 563.0140.

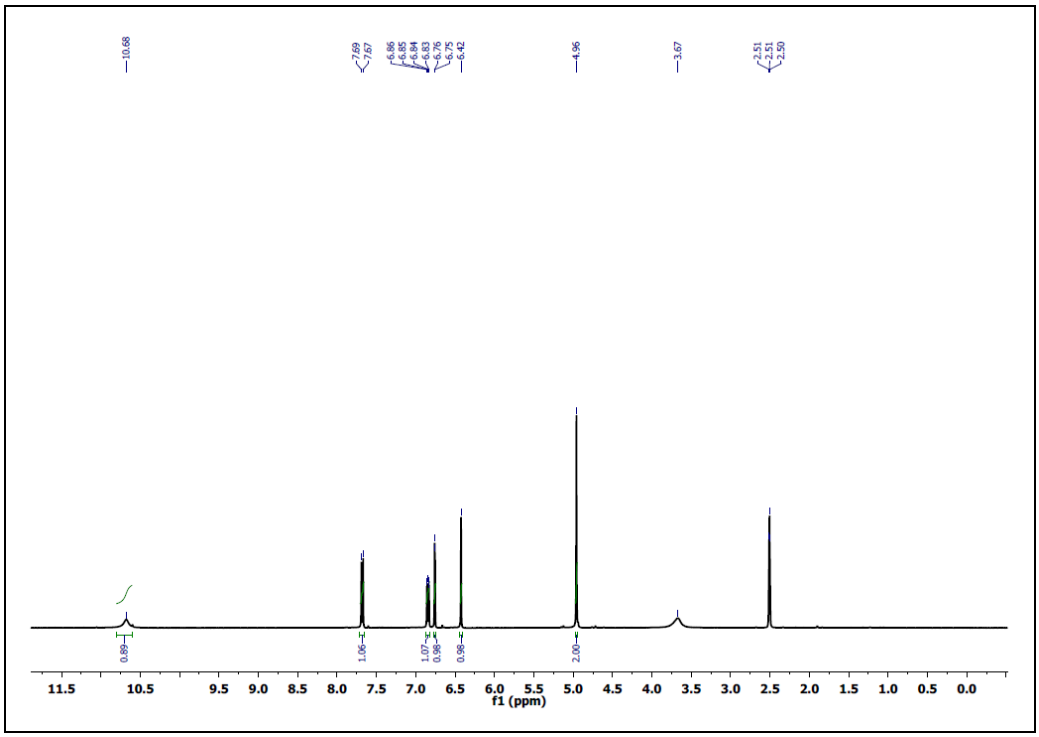


Figure **S26**. ^1H NMR (400 MHz, $CDCl_3$, ppm) spectrum of compound **12**.

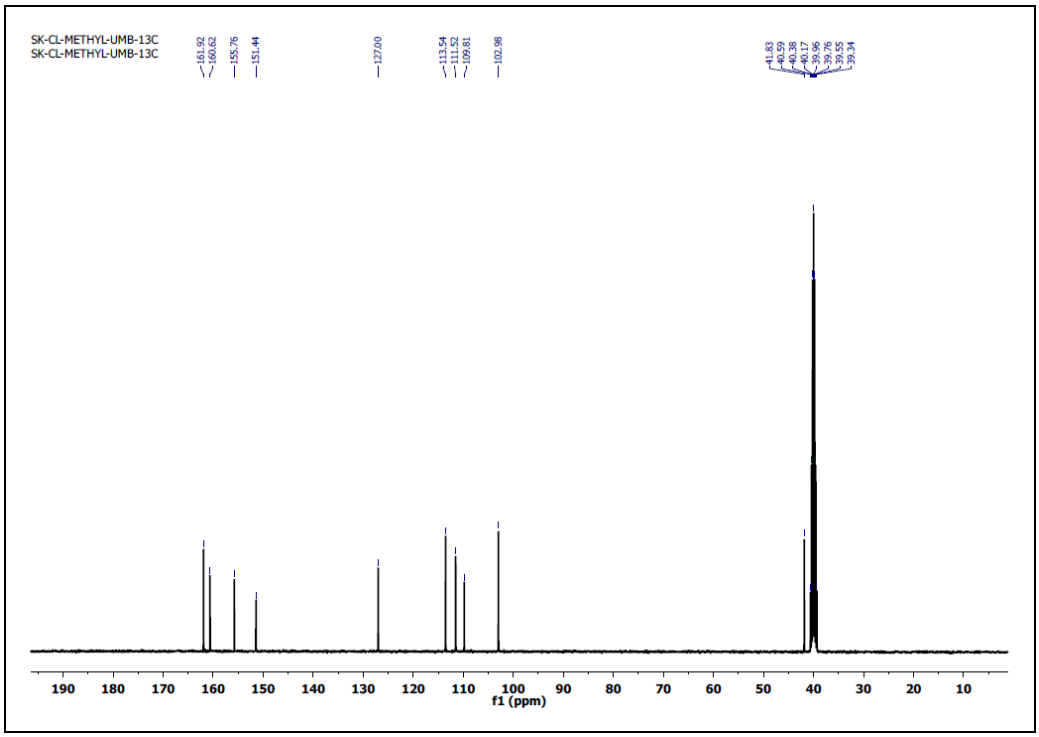


Figure **S27**. ^{13}C NMR spectrum (100 MHz, DMSO-*d*$_6$, ppm) of compound **12**.

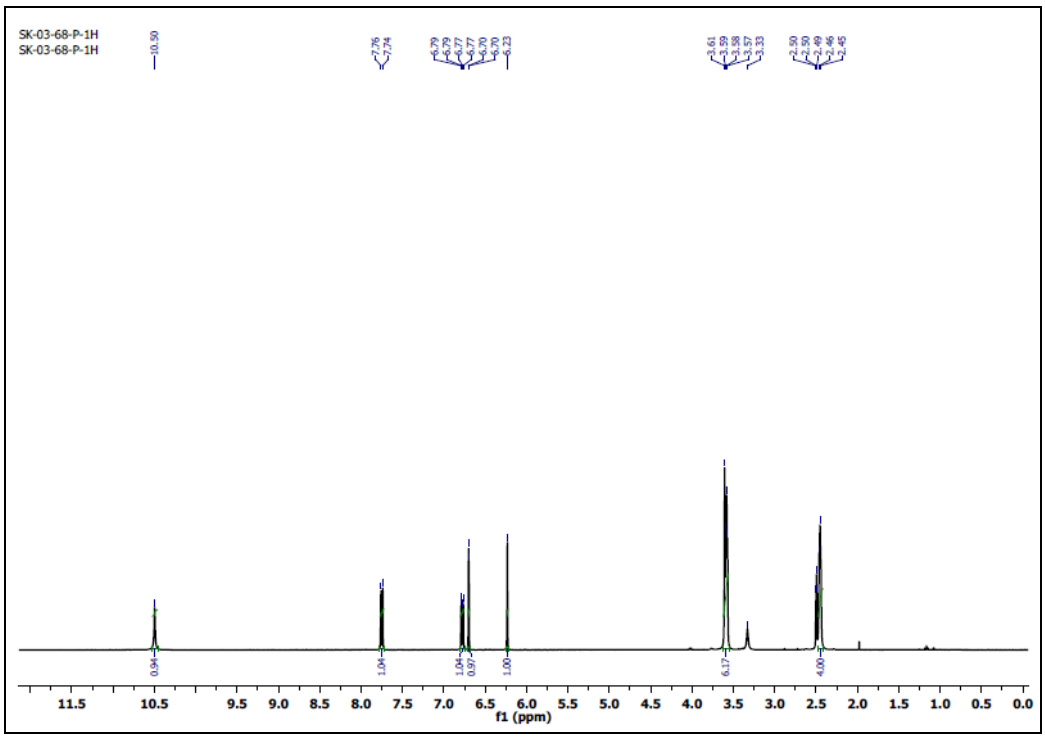


Figure **S28**. ^{1}H NMR (400 MHz) spectrum of compound **1b** in $CDCl_3$.

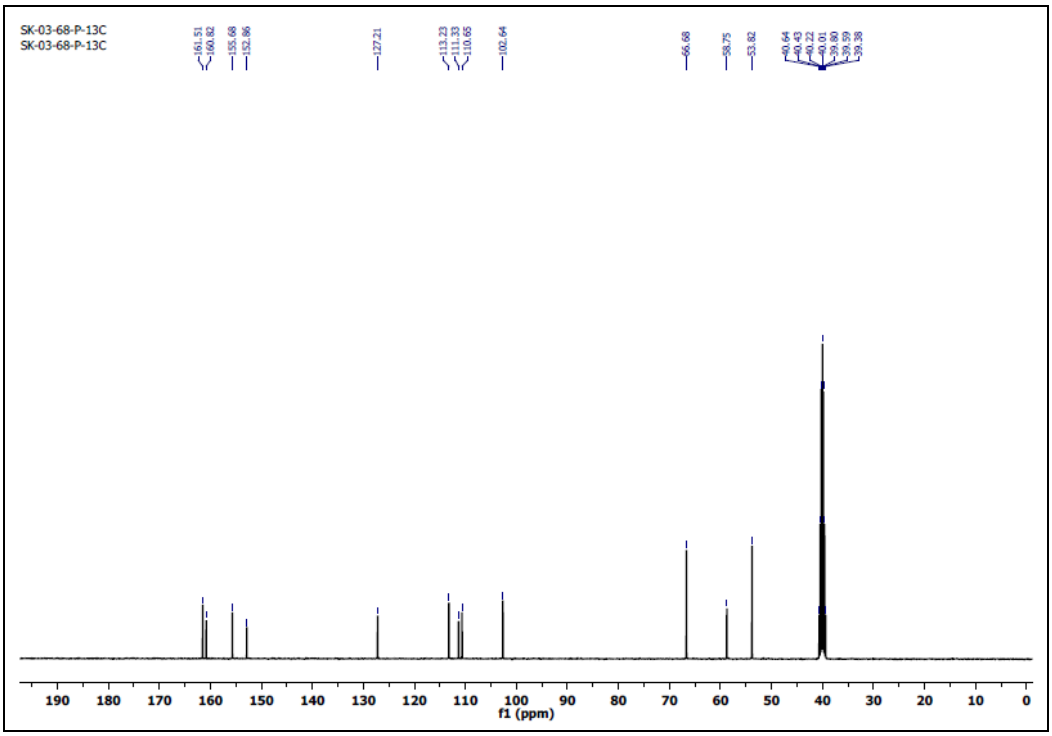


Figure **S29**: ^{13}C NMR (100 MHz, DMSO-*d*$_6$, ppm) spectrum of compound **1b**.

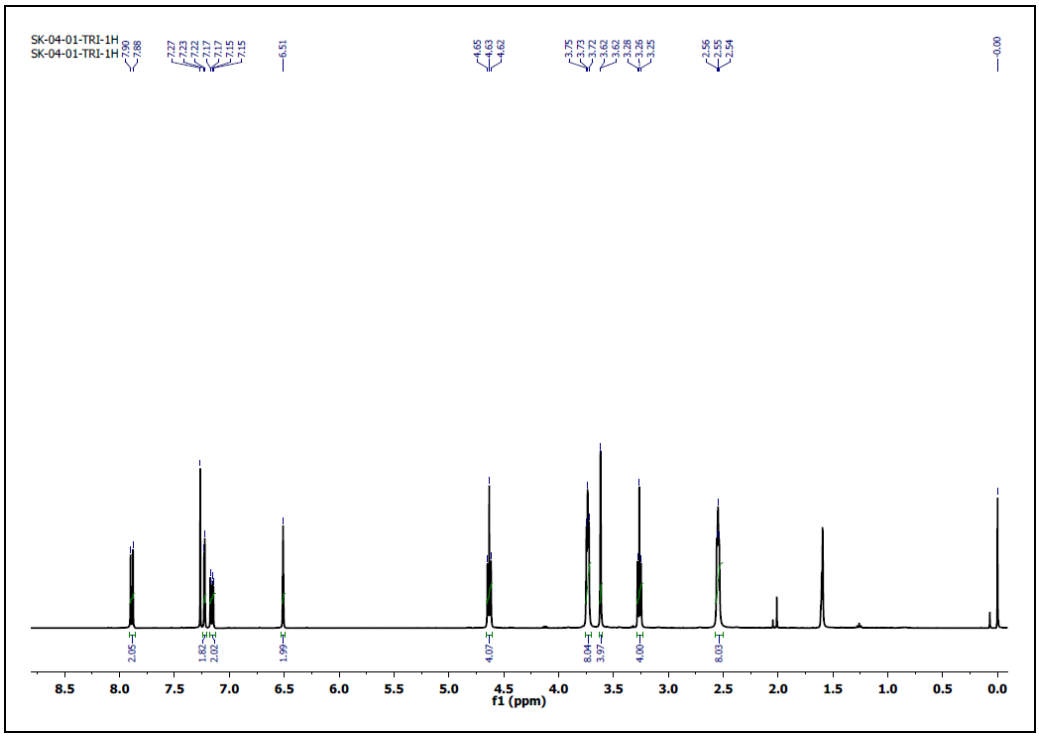


Figure **S30**. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, ppm) of **UTS-2**.

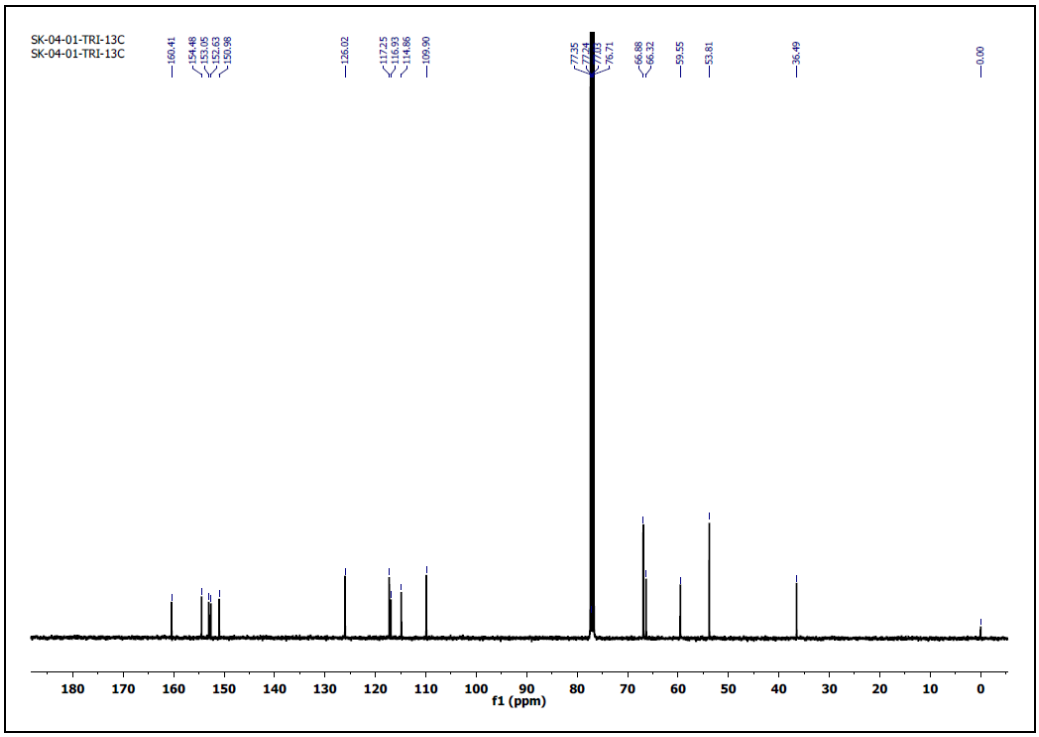


Figure **S31**. ^{13}C NMR spectrum (100 MHz, $CDCl_3$, ppm) of **UTS-2**.

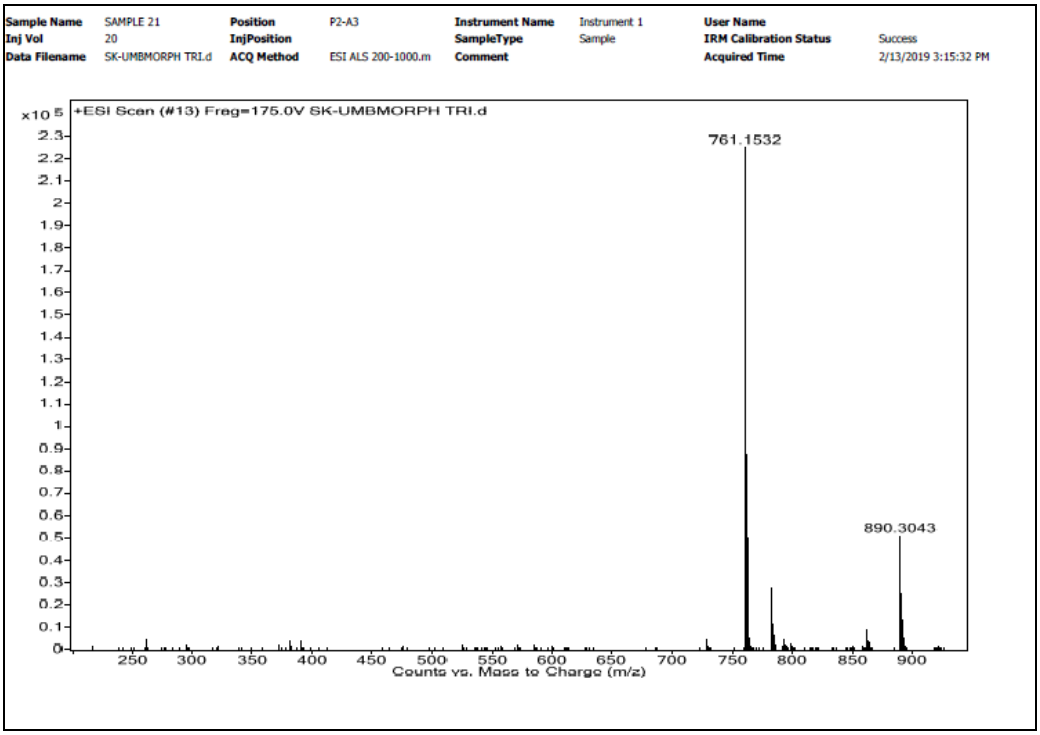


Figure **S32**. ESI-MS (+ve) spectrum of **UTS-2**. ESI-MS (+ve) *m/z* $[M + H]^+$ calcd. for $[C_{34}H_{36}N_2O_{12}S_3]^+$ 761.1509 found 761.1532.

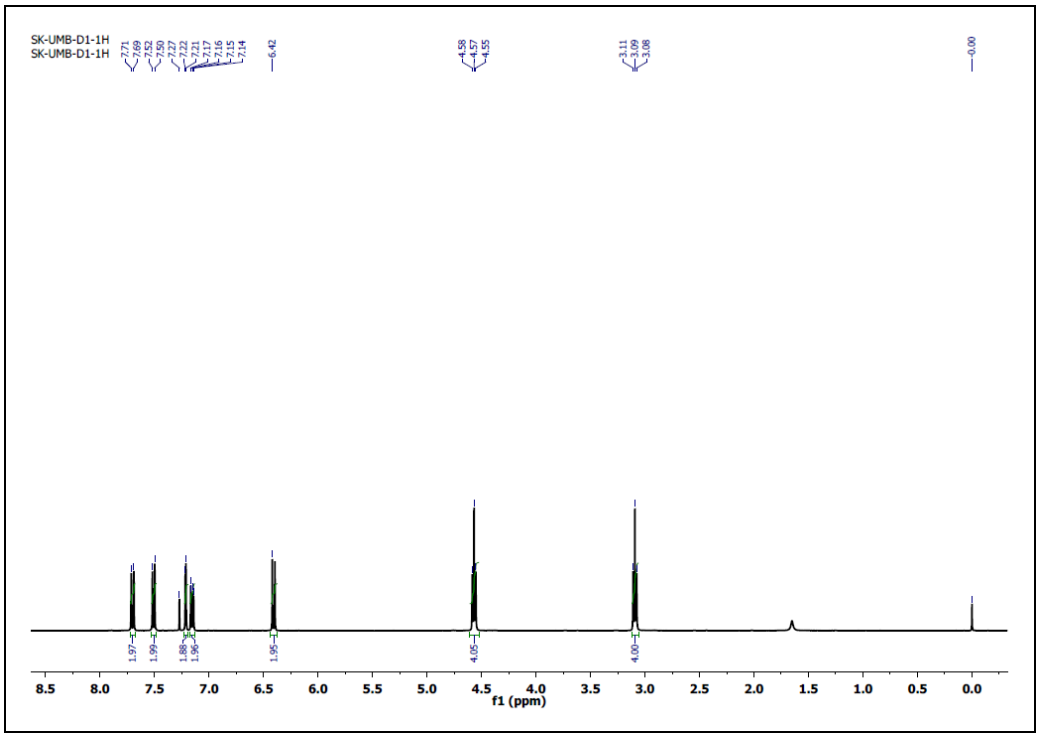


Figure **S33**. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, ppm) of **UDS-1**.

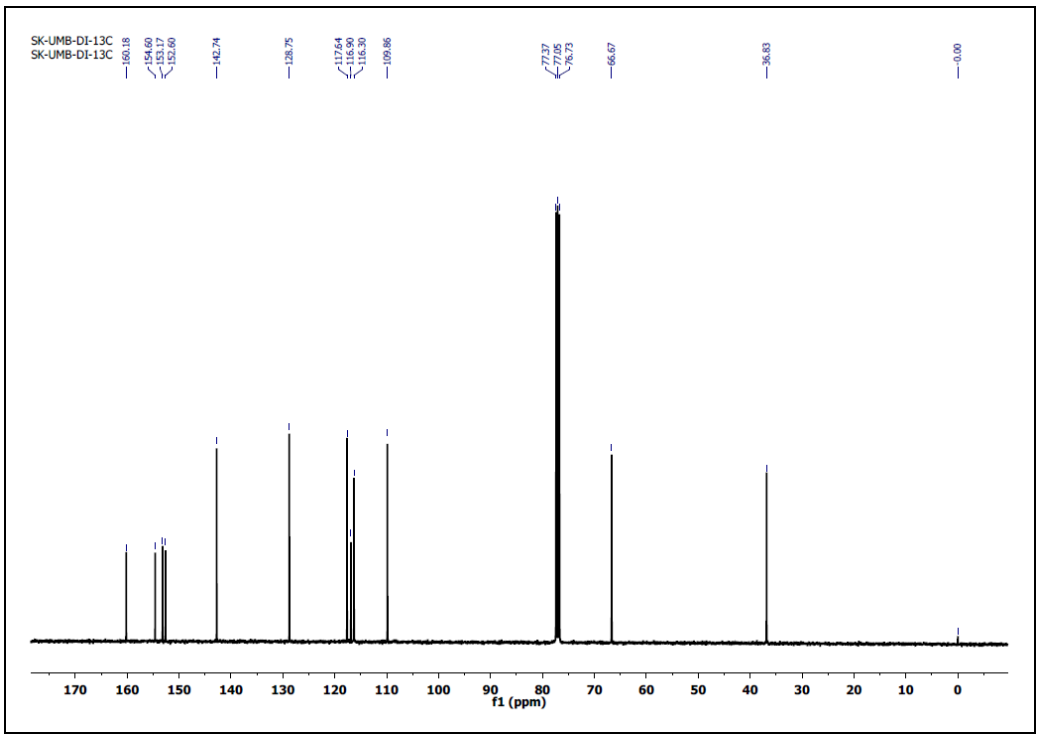


Figure **S34**: ^{13}C NMR spectrum (100 MHz, $CDCl_3$, ppm) of **UDS-1**.

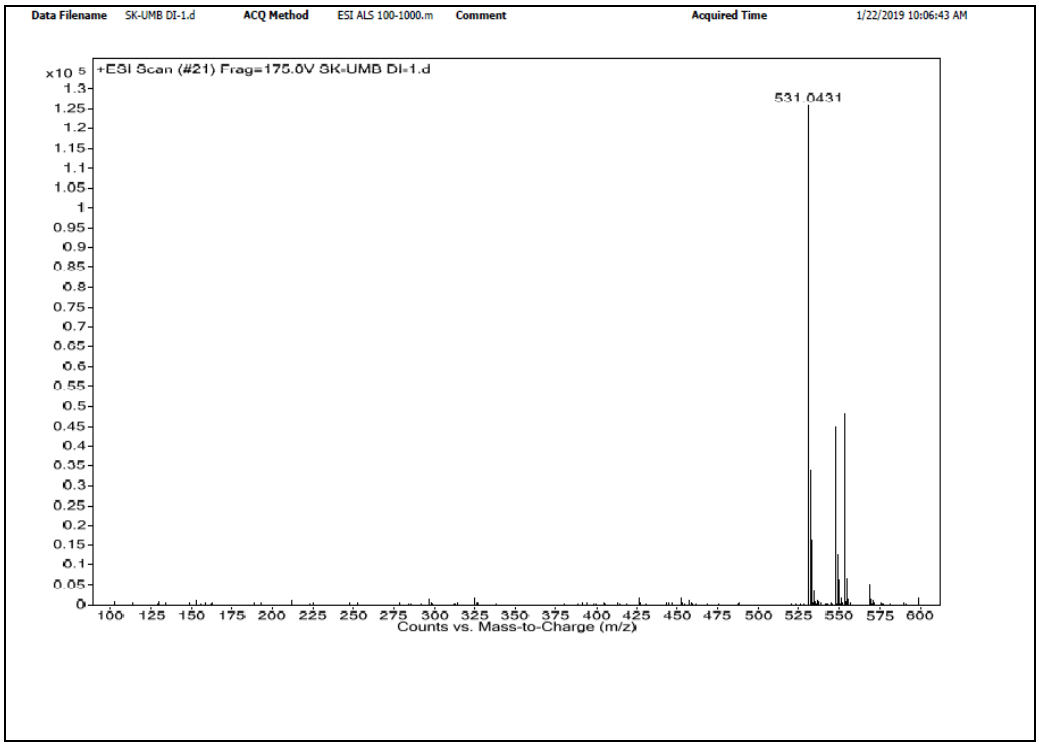


Figure **S35**. ESI-MS (+ ve) spectrum of **UDS-1**. ESI-MS: *m/z* calcd. for $C_{24}H_{18}O_{10}S_2$ $[M+H]^+$ 531.0420; found $[M+H]^+$: 531.0431.

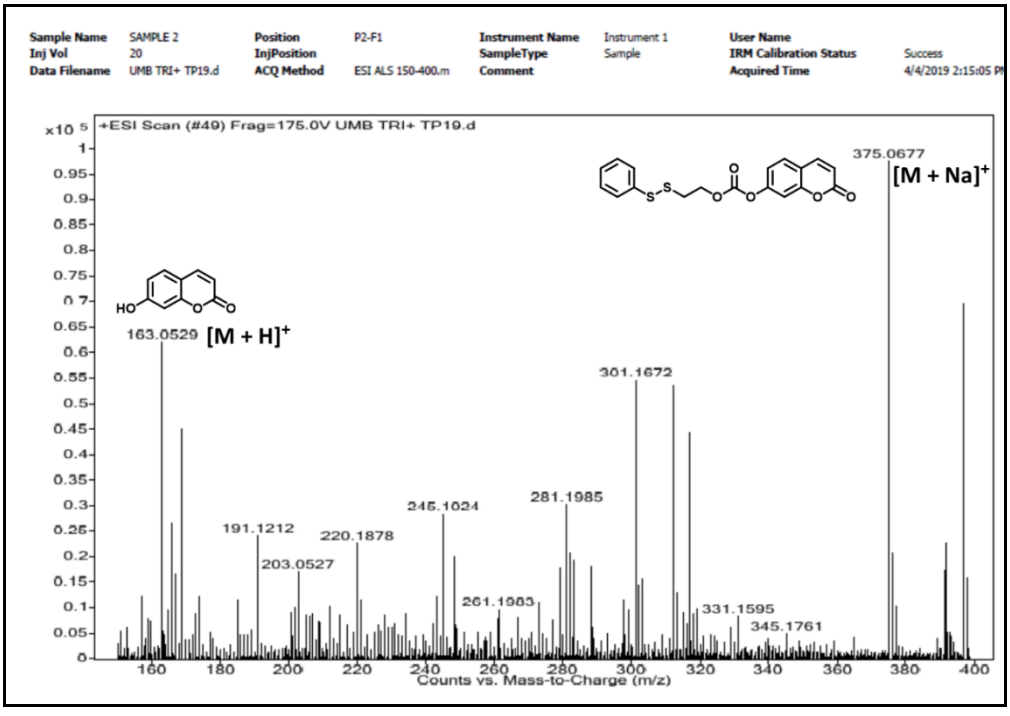


Figure S36. ESI-MS spectrum of the reaction mixture upon the reaction of **UTS-1** with 7.0 equiv of PhSH in acetonitrile. The released umbelliferone **1a** and the key mixed disulfide intermediate **6** could be identified under mass analysis condition.

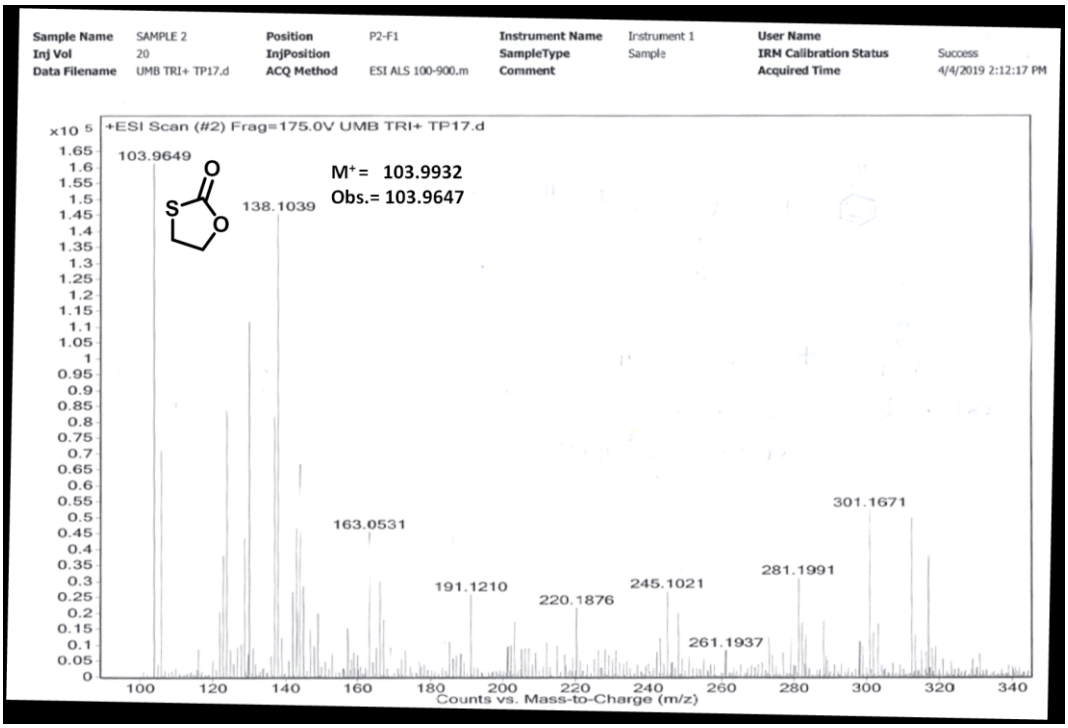


Figure S37. ESI-MS spectrum of the reaction mixture upon the reaction of **UTS-1** with 7.0 equiv of PhSH in acetonitrile. The released cyclic compound **2** could be identified under mass analysis condition.

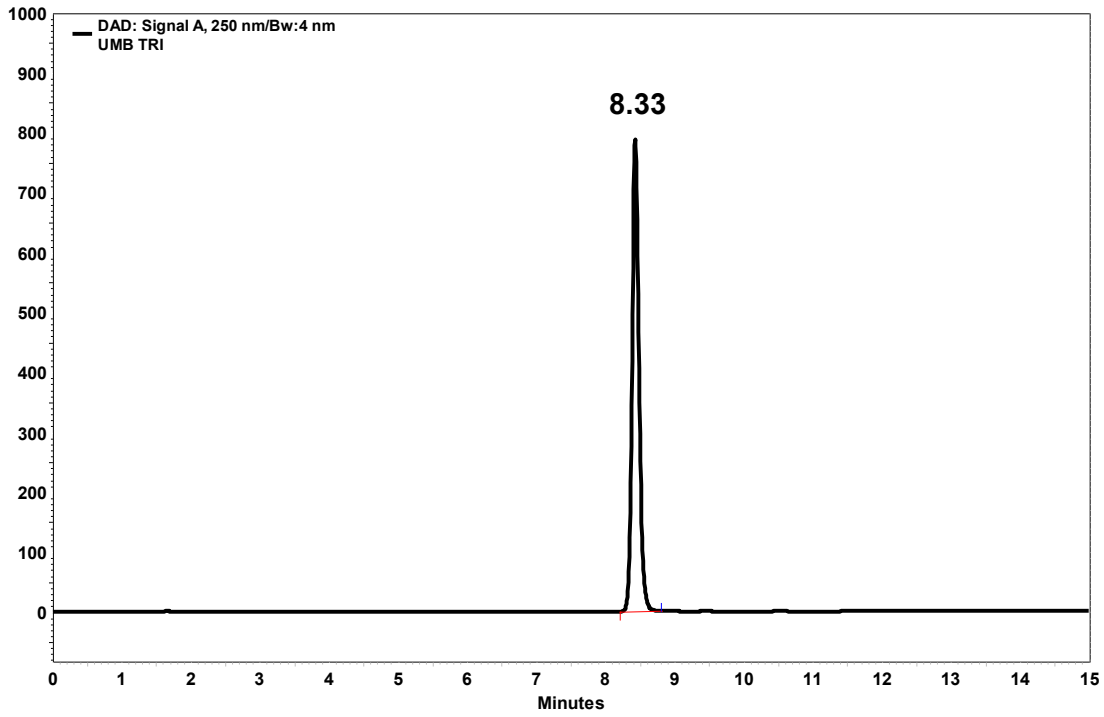


Figure **S38**: HPLC chromatogram of **UTS-1** (retention time: 8.33 min).

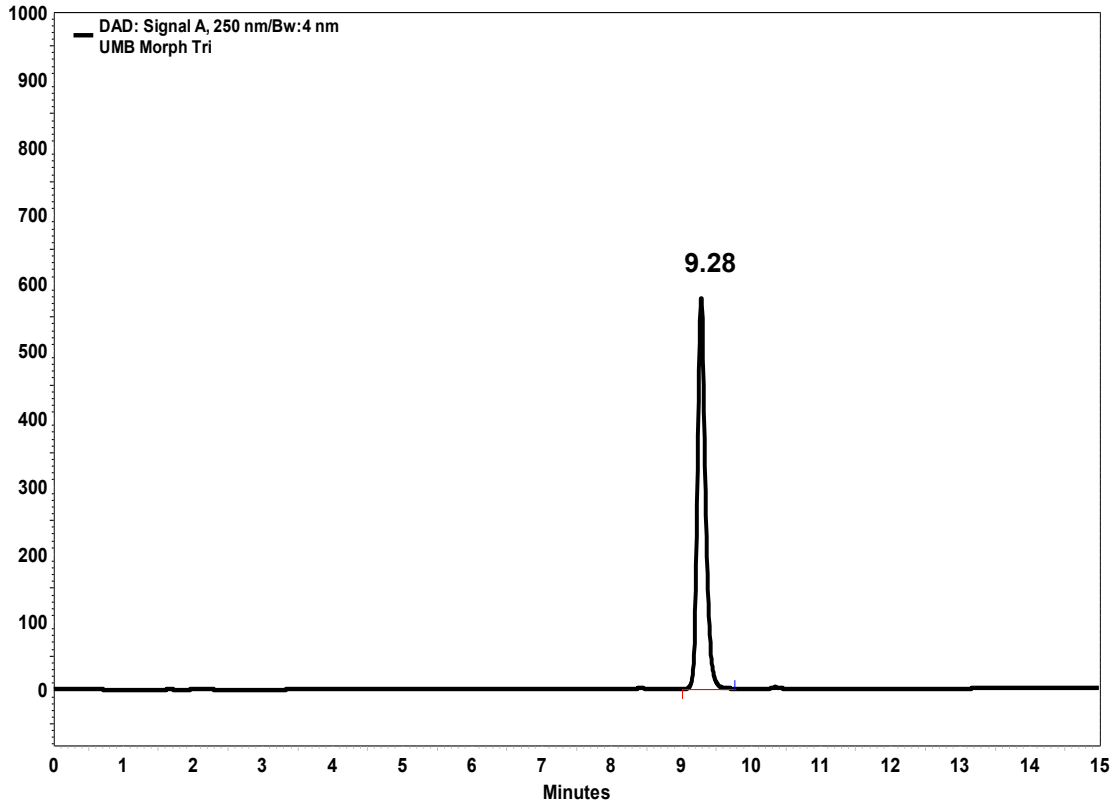


Figure S39: HPLC chromatogram of **UTS-2** (retention time: 9.28 min).

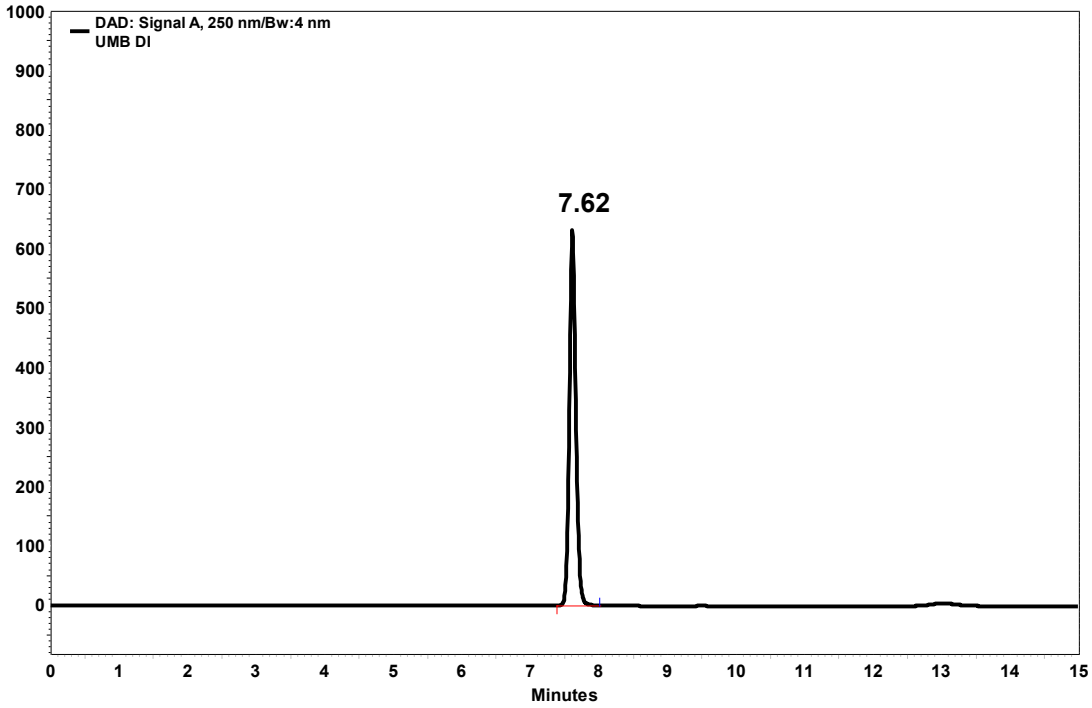


Figure S40. HPLC chromatogram of **UDS-1** (retention time: 7.62 min).

References

1) R. C. Fuson, C. C. Prince, D. M. Burness, R. E. Foster, W. R. Hatchard and R. D. Lipscomb, *J. Org. Chem*. **1946**, *5*, 469-474.

2) Z. Chen, B. Li, X. Xie, F. Zeng and S. Wu, *J. Mater. Chem. B*, **2018**, *6*, 2547-2556.

3) P. Zheng, S.S. Karakaya, S. Lu, J. Roberts, M. Pingle, T. Warrier, D. Little, X. Guo, S. J. Brickner, C. F. Nathan, B. Gold and G. Liu, *J. Med. Chem*. **2014**, *57*, 3755-3772.

4) J. Chen, S. Jiang, Y. Gao and F. Sun, *J. Mater. Sci*. **2018**, *53*, 16169-16181.

5) S. Bhuniya, M. H. Lee, H. M. Jean, J. H. Han, J. H. Lee, N. Park, S. Maiti, C. Kang and J. S. Kim, *Chem. Commun*., **2013**, *49*, 7141-7143.

6) R. R. Moest, *Anal Chem*, **1975**, *47*, 1204-1205.

7) Y. Zhao, A. K. Steiger, M. D. Pluth, *J. Am. Chem. Soc.* **2019**, *141*, 13610−13618.
