Supplementary Information

for

Cysteine–responsive Prodrug of the Anti-cancer Drug Amonafide: Fluorogenic Adjuvant Drug Delivery with Hydrogen Sulfide (H_2S)

Sulendar K. Mahato,[a] Pallavi Barman,[a] Md. Badirujjaman,[a] and Krishna P. Bhabak*[a,b]

[a]Department of Chemistry, Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India.

[b]Jyoti and Bhupat Mehta School of Health Sciences and Technology, Indian Institute of Technology Guwahati, Guwahati-781039, Assam, India; Email: kbhabak@iitg.ac.in

Author Contributions

S.K.M. and K.P.B. conceptualized the project. S.K.M. developed the methodology and carried out the synthesis, purification, characterization of the intermediates, final prodrugs and carried out all the spectroscopic and HPLC studies in the aqueous medium. P.B. carried out the cellular viability experiments. P.B. and M.B. carried out the cellular imaging experiments. K.P.B. supervised the overall work. Original draft of the manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Sl. No	Content	Page No.
1.	Literature reported Cys-triggered fluorogenic H_2S donors	S3
2.	Synthetic scheme to the prodrugs	S3
3.	Experimental section for the synthesis of **AM-ITC** and **NB-ITC**	S4-S12
4.	Absorption and emission spectra of **NB-ITC** with Cys	S12-S13
5.	Concentration-dependent emission spectra of **NB-ITC** and **AM-ITC** with Cys	S13
6.	Typical spectral pattern of methylene blue during the reaction of **AM-ITC** and **NB-ITC** with Cys	S14
7.	Detection of released H_2S from **AM-ITC** in the presence of Cys using **WSP2**	S14
8.	Reverse-phase HPLC for the reaction of **NB-ITC** with Cys, GSH and DTT	S15
9.	Reverse-phase HPLC for the reaction of **NB-ITC** with Ala, Gly and Ser	S15
10.	Reverse-phase HPLC for the reaction of **NB-ITC** with Cys at different pH	S16
11.	Dose-dependent anti-proliferative activity of **NB-ITC** and **NAB** in MDA-MB-231 cells	S16
12.	Fluorescence microscopy images of HeLa cells in the presence of **AM-ITC** and **WSP2**	S17
13.	NMR (^{1}H, ^{13}C) and ESI-MS spectra of intermediates and final compounds	S18-S25
14.	ES-MS (+ve) spectrum for the reaction of **AM-ITC** with Cys	S25
15.	ES-MS (+ve) spectrum for the reaction of **NB-ITC** with Cys	S26
16.	References	S26

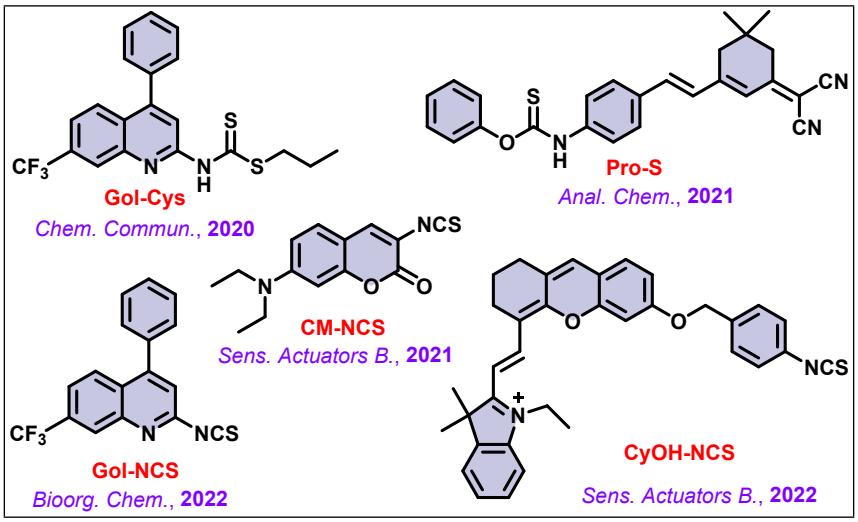
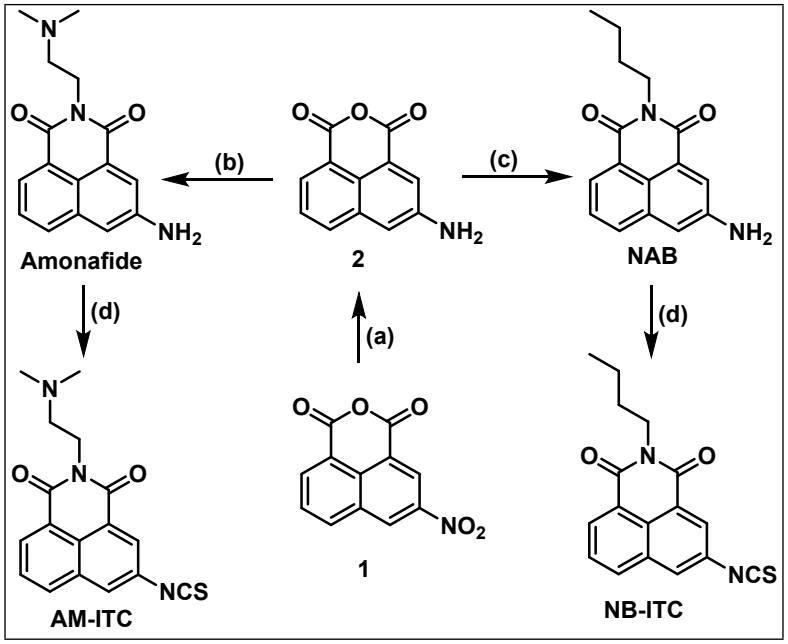


Figure S1. Representative examples of the previously reported Cys-responsive turn-on fluorogenic H_2S donors.



Scheme S1. Synthetic scheme to **AM-ITC** and **NB-ITC**. Reagents and conditions. (a) $SnCl_2 \cdot 2H_2O$, Ethanol, 90 °C, 2.5 h; (b) *N,N*-Dimethyl-1,2-ethylenediamine, Ethanol, 90 °C, 2.5 h; (c) *n*-Butylamine, Ethanol, 90 °C, 2.5 h; (d) Thiocarbonyl diimidazole, dry DMF, 0 °C-RT, 24 h.

Experimental section

Abbreviations

Ala, L-alanine; Val, L-valine; Met, L-methionine; Ser, L-serine; Gly, L-glycine; Glu, L-glutamic acid; Asp, L-aspartic acid; Pro, L-proline; Arg, L-arginine; His, L-histidine; Glu, L-glutamic acid; Gln, L-glutamine; NaCl, sodium chloride; KCl, potassium chloride; H_2O_2, hydrogen peroxide, TBHP, *tert*-butyl hydroperoxide; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; GSH, glutathione; Cys, L-cysteine; Hcy, DL-homocysteine.

Materials and methods

All the reagents were purchased either from Sigma Aldrich or from 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 column chromatographic separations were distilled before use. Melting point of the synthesized compounds was recorded in a Büchi B540 melting point apparatus. The NMR spectra were recorded with a Bruker Ascend^{TM} 600 MHz NMR spectrometers. 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. UV-Vis spectra were recorded on Agilent Cary 100 spectrophotometer. Fluorescence emission were recorded on Horiba Yvon Fluoromax-4 spectrophotometer.

Synthesis of AM-ITC and NB-ITC

Synthesis of Compound 2[1]

To a solution of $SnCl_2$ (4.50 g, 20.56 mmol) in conc. hydrochloric acid (3.50 mL, 0.14 mmol) in a double-necked round bottom flask, was added dropwise a suspension of 3-nitro-1,8-naphthalic anhydride **1** (1.00 g, 4.11 mmol) in absolute ethanol (2.0 mL) and the resulting suspension was refluxed at 90 ºC for 2.5 h. The reaction mixture was cooled to room temperature during which the crude product was precipitated as orange solid. The precipitate was filtered off and washed sequentially with ice-cold water, cold ethanol and diethyl ether and dried under vacuum. The crude product as orange solid was used in the next step without purification. R_f = 0.5 (50% ethyl acetate in pet. ether). Yield = 81% (0.71 g). ^{1}H NMR (600 MHz, DMSO-d_6): δ (ppm) = 8.18 (d, J = 8.3 Hz, 1H), 8.13 (d, J = 7.2 Hz, 1H), 8.02 (d, J = 2.3 Hz, 1H), 7.68 (t, J = 7.8 Hz, 1H), 7.52 (d, J = 2.4 Hz, 1H). ^{13}C NMR (150 MHz, DMSO-

d_6): δ (ppm) = 160.9, 160.7, 133.3, 132.9, 128.0, 127.5, 123.7, 123.6, 119.5, 118.5, 115.09. ESI-MS: *m/z* calc. for $C_{12}H_8NO_3$ $[M + H]^+$ = 214.0504, obs. 214.0503.

Synthesis of Amonafide[2]

To a suspension of compound **2** (3.00 g, 14.08 mmol) in absolute ethanol (10 mL), *N, N*-dimethyl-1,2-ethylenediamine (2.06 g, 28.16 mmol) was added dropwise. The reaction mixture was refluxed at 90 ºC for 2.5 h. The progress of the reaction was monitored by TLC analysis. Upon the completion of reaction, the reaction mixture was allowed to cool to room temperature during which a yellow precipitate was observed, which was filtered off, washed with cold ethanol and pet. ether and dried under vacuum. The residue was purified by basic alumina column chromatography using DCM as eluent to afford the pure product as a yellow fluffy solid. R_f = 0.7 (10% MeOH in DCM, silica TLC). Yield= 63% (2.50 g). ^{1}H NMR (600 MHz, DMSO-*d_6*): δ (ppm) = δ 8.07 (d, *J* = 7.2 Hz, 1H), 8.03 (d, *J* = 8.2 Hz, 1H), 7.95 (d, *J* = 2.3 Hz, 1H), 7.61 (dd, *J* = 8.4, 7.1 Hz, 1H), 7.28 (d, *J* = 2.3 Hz, 1H), 6.01 (s, 2H), 4.12 (t, *J* = 6.9 Hz, 2H), 2.48 (t, *J* = 6.9 Hz, 2H), 2.19 (s, 6H). ^{13}C NMR (150 MHz, DMSO-*d_6*): δ (ppm) = 163.7, 163.6, 147.9, 133.5, 131.5, 126.9, 126.9, 125.4, 122.5, 121.8, 121.7, 120.6, 111.7, 56.5, 45.4, 37.5. ESI-MS: *m/z* calc. for $C_{16}H_{18}N_3O_2$ $[M + H]^+$ = 284.1399, obs. 284.1399.

Synthesis of NAB[2]

To a suspension of compound **2** (1.50 g, 7.00 mmol) in absolute ethanol (2.0 mL), *n*-butylamine (0.56 g, 7.70 mmol) was added and refluxed at 90 ºC for 2.5 h. The progress of the reaction was monitored by TLC analysis. The solution was cooled to room temperature and the precipitate obtained was washed sequentially with ice-cold ethanol and pet. ether. The resulting brown solid was dried under vacuum and purified by silica gel column chromatography using ethyl acetate and pet. ether as eluents to afford the pure product as a yellow solid. R_f = 0.5 (50% ethyl acetate in pet. ether). Yield = 54% (1.02 g). ^{1}H NMR (600 MHz, $CDCl_3$): δ (ppm) = δ 8.62 (d, *J* = 7.3 Hz, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.42 (dt, *J* = 8.4, 1.3 Hz, 1H), 7.77 – 7.71 (m, 1H), 7.46 (d, *J* = 7.9 Hz, 1H), 4.19 – 4.14 (m, 2H), 1.75 – 1.66 (m, 2H), 1.44 (h, *J* = 7.4 Hz, 2H), 0.97 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ (ppm) = 164.6, 164.3, 145.4, 145.3, 133.5, 131.8, 127.6, 127.5, 127.3, 123.7, 122.6, 122.0, 114.0, 40.3, 30.3, 20.5, 14.0. ESI-MS: *m/z* calc. for $C_{16}H_{17}N_2O_2$ $[M + H]^+$ = 269.1290, obs. 269.1293.

Synthesis of AM-ITC

To a solution of amonafide (0.25 g, 0.88 mmol) in anhydrous DMF (3.0 mL) was added triethylamine (0.54 g, 5.28 mmol) at 0 °C under ice-cold condition. The reaction mixture was stirred at 0 °C for 5 min. To the above mixture was added a solution of thiocarbonyl diimidazole (0.31 g, 1.76 mmol) in anhydrous DMF (3.0 mL) dropwise at 0 °C. The reaction was allowed to attain room temperature and continued for 24 h at room temperature. The progress of the reaction was monitored by TLC. Upon completion, the solvent was evaporated and the crude mixture was purified by silica gel column chromatography. R_f = 0.5 (50% ethyl acetate in pet. ether, alumina TLC). Yield = 24% (0.070 g). M.P. = 127-129 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.51 (d, *J* = 7.4 Hz, 1H), 8.28 (d, *J* = 2.2 Hz, 1H), 8.08 (d, *J* = 8.3 Hz, 1H), 7.91 (d, *J* = 2.1 Hz, 1H), 7.74 (t, *J* = 7.7 Hz, 1H), 4.27 (t, *J* = 6.8 Hz, 2H), 2.61 (t, *J* = 6.9 Hz, 2H), 2.31 (s, 6H). ^{13} C NMR (150 Hz, $CDCl_3$) δ (ppm): 163.6, 163.0, 139.1, 133.2, 132.0, 131.6, 130.8, 128.7, 128.6, 128.3, 126.3, 124.5, 122.8, 57.0, 45.9, 38.4. ESI-MS: *m/z* calc. for $C_{17}H_{16}N_2O_3S$ $[M + H]^+$: 326.0963; observed $[M + H]^+$: 326.0971.

Synthesis of NB-ITC

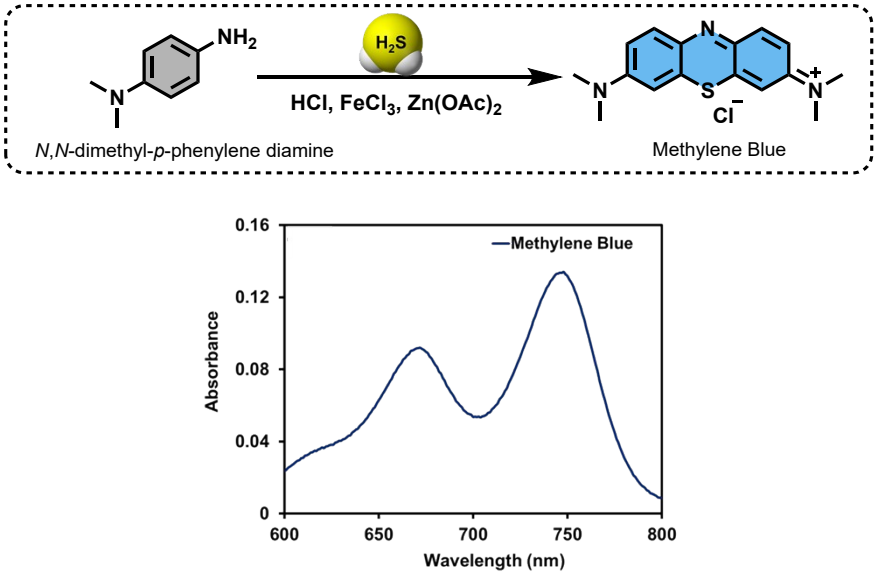
To a solution of NAB (0.15 g, 0.48 mmol) in 1 mL anhydrous DMF was added triethylamine (0.29 g, 2.89 mmol) at 0 °C under ice-cold condition. The reaction mixture was stirred at 0 °C for 5 min. To the above mixture was added a solution of thiocarbonyldiimidazole (0.17 g, 0.96 mmol) in 1 mL anhy DMF dropwise at 0 °C. The reaction was allowed to attain room temperature and then continued for 24 h at room temperature. The progress of the reaction was monitored by TLC. Upon completion, the solvent was evaporated and the crude mixture was purified by silica gel column chromatography to afford the pure product as white solid. R_f = 0.5 (50 % ethyl acetate in pet ether, silica TLC). Yield = 0.10 g (58%). M. P. = 123-125 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.62 (d, *J* = 7.3 Hz, 1H), 8.57 (d, *J* = 8.0 Hz, 1H), 8.42 (d, *J* = 8.2 Hz, 1H), 7.73 (t, *J* = 8.4 Hz, 1H), 7.46 (d, *J* = 7.9 Hz, 1H), 4.16 (t, *J* = 7.8 Hz, 2H), 1.76-1.64 (m, 2H), 1.47-1.41 (m, 2H), 0.97 (t, *J* = 7.4 Hz, 3H). ^{13} C NMR (150 Hz, $CDCl_3$) δ (ppm): 164.0, 163.6, 143.3, 132.2, 131.7, 129.1, 128.7, 126.9, 124.3, 122.7, 119.0, 114.7, 40.2, 30.2, 20.4, 13.9. ESI-MS: *m/z* calc. for $C_{17}H_{14}N_2O_2S$ $[M + H]^+$: 311.0854; observed $[M + H]^+$: 311.0864.

UV-Vis and fluorescence spectroscopic studies

All the stock solutions of the biologically relevant analytes were prepared freshly in Milli Q/double distilled water and stock solutions of fluorogenic probes as well as other analytes were prepared freshly in spectroscopy grade DMSO or in water before carrying out the experiments. All the spectroscopic measurements were carried out at room temperatures in buffered medium (PBS buffer 20 mM, pH 7.4). Samples for absorption and emission spectroscopic measurements were taken in quartz cuvettes (1.0 mL). Fluorescence emission spectra were collected with the excitation wavelength of 415 nm with a slit width of 5 nm. Time-dependent fluorescence experiments were performed by incubating **AM-ITC** (5 μM) or **NB-ITC** (5 μM) with suitable analytes (200 μM) over a period of 60 min.

Measurement of the H_2S release profile from AM-ITC

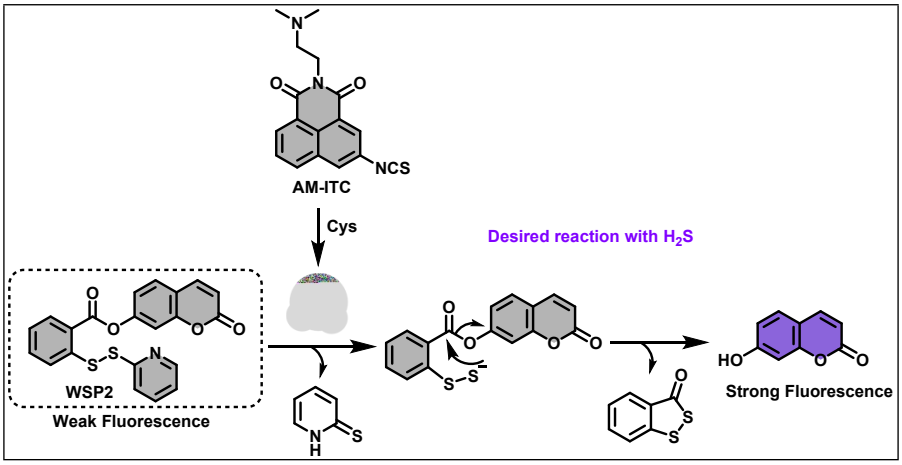
The release of H_2S from **AM-ITC** (5 μM) upon the reaction with Cys (1 mM) was monitored by methylene blue assay (MB assay) using UV-Vis spectrophotometer by measuring the absorbance of methylene blue at 670 nm.[3] The methylene blue dye is produced by the reaction of H_2S with *N,N*-dimethyl-1,4-phenylenediamine sulfate with the formation of methylene blue dye, which exhibits a typical absorption spectral pattern (Scheme S2). The generated H_2S by the reaction of **AM-ITC** with Cys in PBS (20 mM, pH 7.4), and the formation of methylene blue was monitored at specific 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, 7.2 M HCl), 200 μL of Ferric Chloride (30 mM, 1.2 M HCl)] in a 1 mL quartz cuvette. The concentration of H_2S for each sample was calculated against a calibration curve obtained by using the known concentrations of $Na_2S.9H_2O$ without any externally added thiol.



Scheme S2. Reaction scheme for the formation of methylene blue dye and its typical absorption spectral pattern.

Measurement of the H_2S using the turn-on fluorogenic probe WSP2

The release of H_2S from **AM-ITC** (25 μM) in the presence of Cys (1 mM) was measured by a nucleophilic-substitution cyclization-based fluorescence probe **WSP2** (25 μM) using a fluorescence spectrophotometer (Scheme 3).[4] Initially, the probe **WSP2** was pre-treated with Cys (1 mM) in PBS buffer (20 mM, pH 7.4) and then the emission spectra were recorded to saturate the background side-reaction of Cys with **WSP2**. After that, the aliquot from the reaction of **AM-ITC** (25 μM) with Cys (1 mM) was added to the above mixture and the emission spectra were recorded after 60 min of incubation to understand the reactivity of **WSP2** with the H_2S generated by the reaction of **AM-ITC** with Cys. The desired reaction of **WSP2** with the generated H_2S with the turn-on fluorescence is shown below.



Scheme S3. Reaction sequence of H_2S with **WSP2** with the turn-on fluorescence by the released umbelliferone.

Reaction of bio-analytes with AM-ITC and NB-ITC

To understand the reactivity of **AM-ITC** with different analytes, emission profile was monitored using fluorescence spectroscopic studies. The probe **AM-ITC** (5 μM) was incubated with different analytes (200 μM) for 15 min in PBS buffer (20 mM, pH 7.4). The resulting mixture was further incubated for 15 min with the addition of equivalent amount of Cys (200 μM) to the reaction mixture. Excitation was done at 415 nm and the emission was collected in the range 500-740 nm with a slit width of 5 nm.

For the better understanding on the reactivity of the isothiocyanates with different bio-analytes, the reactions of different thiol-based bio-analytes (1250 μM) such as Cys, GSH, DTT and few amino acids such as L-serine, glycine and L-alanine with **NB-ITC** (25 μM) were investigated by HPLC analysis as per the standard HPLC method described in the next section.

pH variation studies

The pH variation studies were performed by incubating **AM-ITC** (5 μM) with Cys (200 μM) at different pH ranges (pH 4 to 10) in PBS (20 mM) and the emission spectrum was measured

after 15 min of incubation. The stability of **AM-ITC** was measured under identical conditions without adding any Cys to understand the stability of the probes at different pH ranges.

To understand the effect of pH on the overall reactivity of **NB-ITC** (25 μM) with Cys (1250 μM) and the product distribution, the reactions were monitored by HPLC as per standard protocol as described below at various pH of the medium (pH: 6.0, 6.5, 7.4, 8.0 and 8.5) and the results were summarized.

Measurement of reaction kinetics using HPLC

First, the purity of synthesized probes was analysed using analytical high-performance liquid chromatography (HPLC) Agilent 1220 Infinity II LC system using a reverse-phase C18 column (Luna, 150 × 4.6 mm, 5 μm). HPLC grade acetonitrile and water (Finar Ltd.) were used as mobile phase and the absorbance profile of compounds were detected using a PDA detector. The stock solutions of the samples were prepared in HPLC grade acetonitrile and were injected into the system using the autosampler at a flow rate of 1.0 mL min^{-1} using an acetonitrile/water system as a mobile phase [0–10 min (linear gradient): 75% acetonitrile in water to 95% acetonitrile in water; 10–15 min (isocratic): 95% acetonitrile in water].

Cell culture

The triple-negative breast cancer (TNBC) cells (MDA-MB-231), Human cervical cancer cells (HeLa) and Human Dermal Fibroblast cells (HDF) were obtained from the National Centre for Cell Science (NCCS), Pune, India. The 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 ºC in the presence of a 5% CO_2 level.

Cell viability assay

The synthesized prodrugs **NB-ITC** and **AM-ITC** were screened for their anti-proliferative activities using the conventional MTT assay in MDA-MB-231, HeLa and/or HDF cell lines. MDA-MB-231, HeLa and HDF cells were seeded in 96-well culture plates at a density of 2 ×

10^4, 1 × 10^4 and 2 × 10^4 cells/100 μL per well, respectively and treated with the freshly prepared test compounds **AM-ITC** (5, 10, and 25 μM) and **NB-ITC** (5, 10, and 25 μM) for 0 h (control) and 48 h (experimental), respectively. At the end of the treatment period, 100 μL of 5 mg/mL of MTT was added to the plate (control) and incubated for 4 h. Following the 4 h incubation, the reagent from the plate was removed and the purple formazan crystals were dissolved using 100 μL of DMSO (Avra Synthesis Pvt Ltd) and the absorbance at 570 nm was measured using a microplate reader (Thermo Scientific™ Multiskan™ GO microplate reader). In the experimental set, a similar MTT treatment protocol was followed only after 48 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 48 h plate (experimental) and the percentage proliferation was calculated keeping the mean ΔOD of untreated control as 100%.

Fluorescence microscopic studies

HeLa cells were cultured in high glucose DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO_2 atmosphere. Cells were then plated (1.5 × 10^4 cells per plate) in 35 mm cell culture Petri dishes containing 2 mL of DMEM and incubated at 37 °C under 5% CO_2 for 24 h. The confluent cells were washed with DPBS and finally incubated with **AM-ITC** (25 μM) for 1 h. After washing the cells with DPBS (3 times), cellular morphology was carefully observed and imaged using Bio-Rad ZOE™ fluorescent cell imager under a bright field and green channel. A negative control experiment was performed upon the pre-treatment of cells with *N*-ethylmaleimide (NEM, 200 μM) for 30 min to quench the endogenous thiols. The cells were washed with DPBS (3 times) and treated with **AM-ITC** (25 μM). The endogenous level of Cys was increased by incubating the cells with omeprazole (30 μM) for 8 h at 37 °C under 5% CO_2 atmosphere. The cells were washed with DPBS (3 times) and treated with **AM-ITC** (25 μM).

Under the identical condition, imaging of free amonafide (25 μM) was studied as a positive control.

For the detection of the release of H_2S from **AM-ITC**, the HeLa cells were grown similar to the above condition and the cells were treated with **WSP2** (5 μM) for 1 h and cells were imaged under blue channel after washing to understand the endogenous level of H_2S. For studying the released H_2S from **AM-ITC**, the cells were treated with **AM-ITC** (25 μM) + **WSP2** (5 μM) for 1 h. The cells were washed finally with DPBS and imaged under bright field, blue and green channels. The contribution of the endogenous Cys and H_2S towards the triggered H_2S release process, the negative control experiment was carried out by the pre-incubation of cells with *N*-ethylmaleimide (NEM, 200 μM) and DL-propargyl glycine (PAG, 20 μM) for 30 min. The cells were washed and treated with **AM-ITC** (25 μM) + **WSP2** (5 μM) and incubated for 1 h. The cells were washed finally with DPBS and imaged under bright field, blue and green channels.

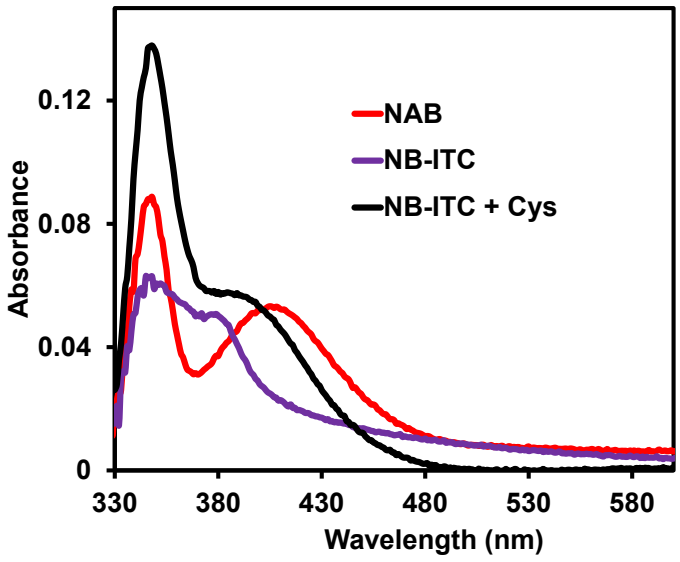


Figure S2. Absorption spectra of **NB-ITC** (25 μM) in the presence of Cys (1 mM) along with the spectra of free amine in phosphate buffer saline (PBS, 20 mM, pH 7.4). Incubation time 30 min.

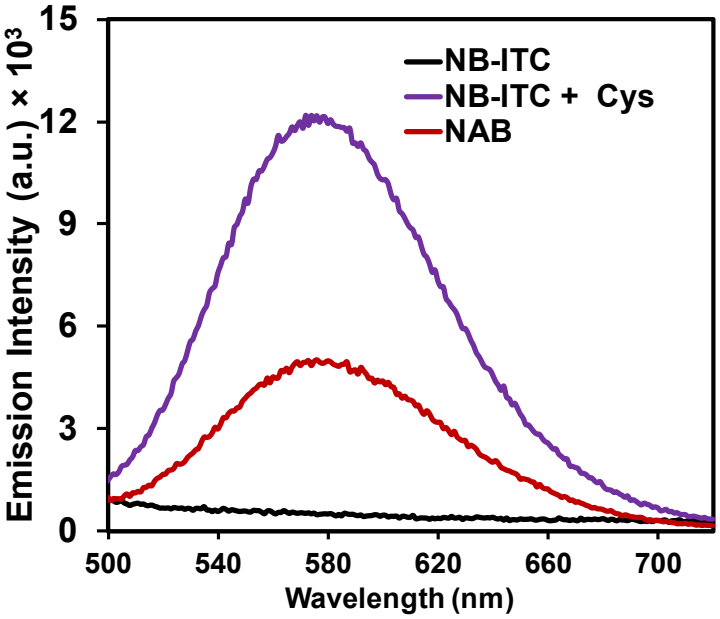


Figure S3. Emission spectrum of **NB-ITC** and (5 μM) with and without Cys (200 μM) along with the spectra of released fluorophore NAB amine in phosphate buffer saline (PBS, 20 mM, pH 7.4). Incubation time 30 min.

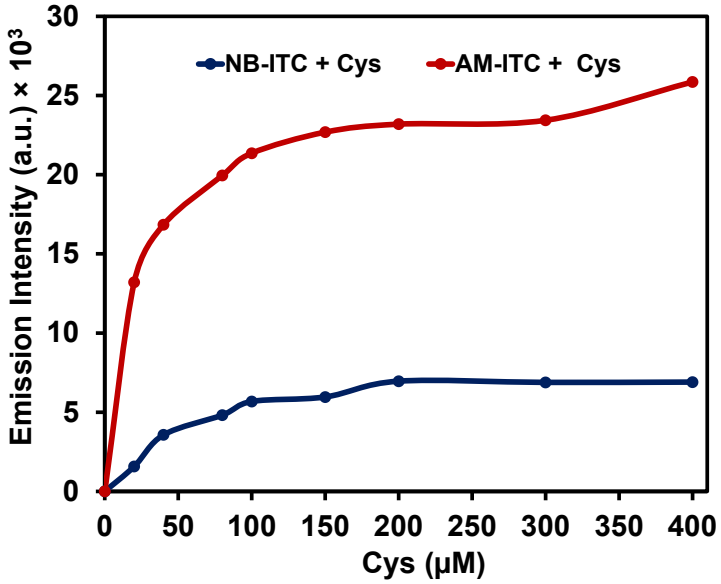


Figure S4. Emission spectra of **AM-ITC** (5 μM) and **NB-ITC** (5 μM) with increasing concentration of Cys (0-400 μM) amine in phosphate buffer saline (PBS, 20 mM, pH 7.4). Incubation time 30 min.

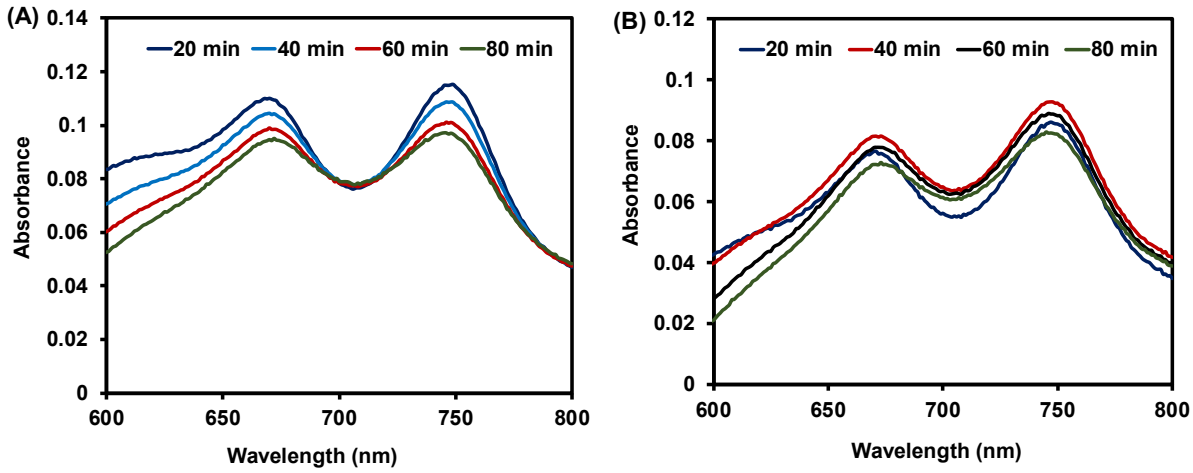


Figure S5. (A) Spectral pattern of the produced methylene blue during the reaction of (A) **AM-ITC** (25.0 μM) and (B) **NB-ITC** (25.0 μM) with Cys (1.0 mM) for the generation of H_2S.

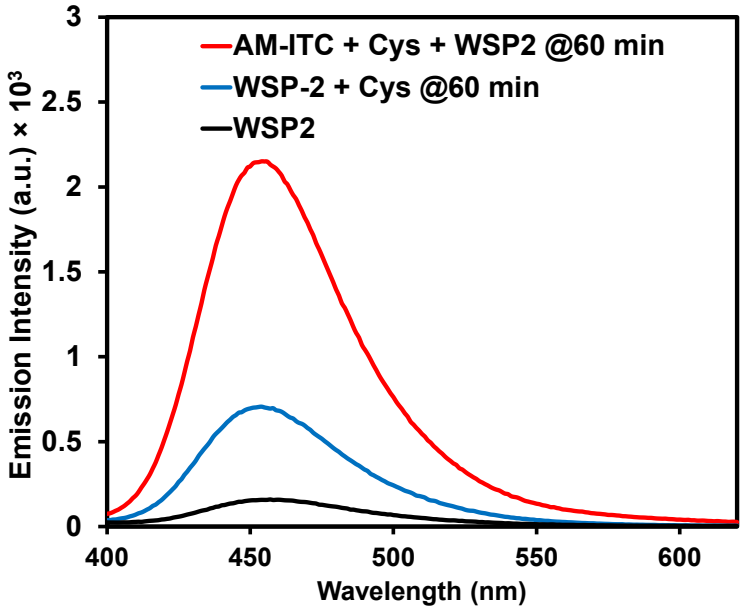


Figure S6. Emission spectra after 60 min from **WSP2** (25 μM) alone, in the presence of Cys (1 mM), and in the presence of **AM-ITC** (25 μM) + Cys (1 mM) in phosphate buffer saline (PBS, 20 mM, pH 7.4).

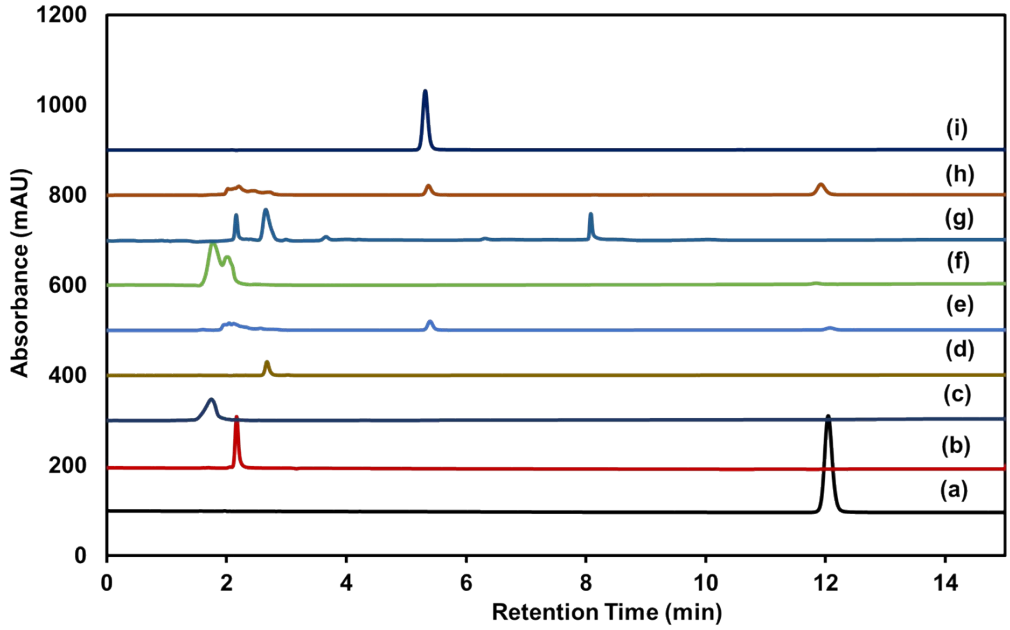


Figure S7. Reverse-phase HPLC chromatogram for the reaction of **NB-ITC** (25 μM) with Cys, GSH and DTT (1250 μM). Legends: (a) pure **NB-ITC** @254 nm; (b) Cys @210 nm; (c) GSH @210 nm; (d) DTT @210 nm (e) **NB-ITC** + Cys @254 nm; (f) **NB-ITC** + GSH @254 nm; (g) **NB-ITC** + DTT @254 nm; (h) **NB-ITC** + GSH + Cys @254 nm; (i) **NAB** @254 nm. The reactions were carried out in PBS (20 mM, pH 7.4) and incubated for 30 min. In lane (h), the GSH and Cys were added together (co-treatment) to **NB-ITC** for the analysis.

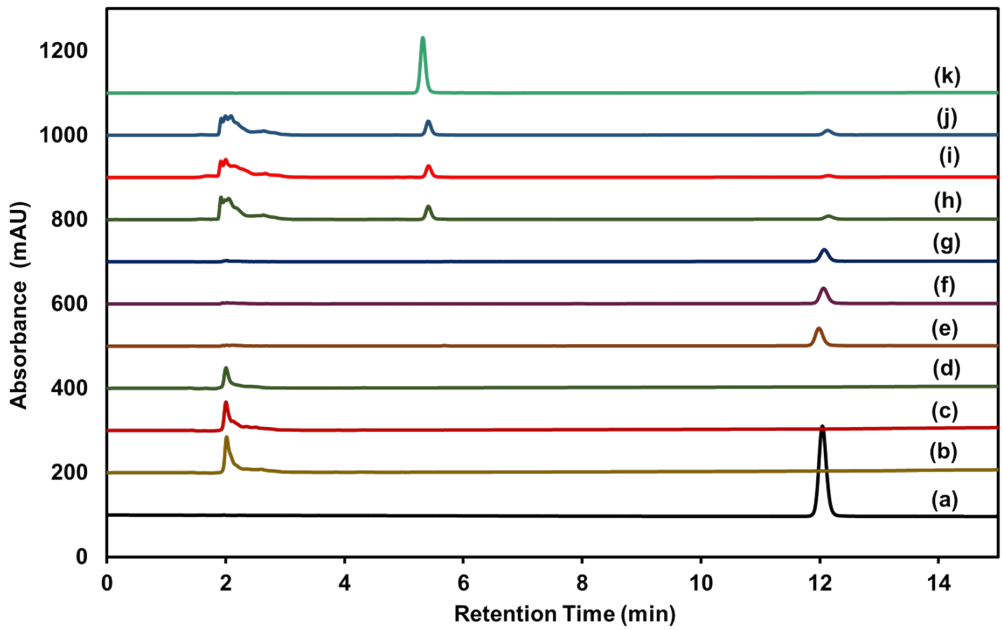


Figure S8. Reverse-phase HPLC chromatogram for the reaction of **NB-ITC** (25 μM) with Ala, Gly and Ser (1250 μM). Legends: (a) pure **NB-ITC** @254 nm; (b) Ala @210 nm; (c) Gly @210 nm; (d) Ser @210 nm; (e) **NB-ITC** + Ala @254 nm; (f) **NB-ITC** + Gly @254 nm; (g) **NB-ITC** + Ser @254 nm; (h) **NB-ITC** + Ala + Cys @254 nm; (i) **NB-ITC** + Gly + Cys @254

nm; (j) **NB-ITC** + Ser + Cys @254 nm; (k) NAB @254 nm. The reactions were carried out in PBS (20 mM, pH 7.4) and incubated for 30 min.

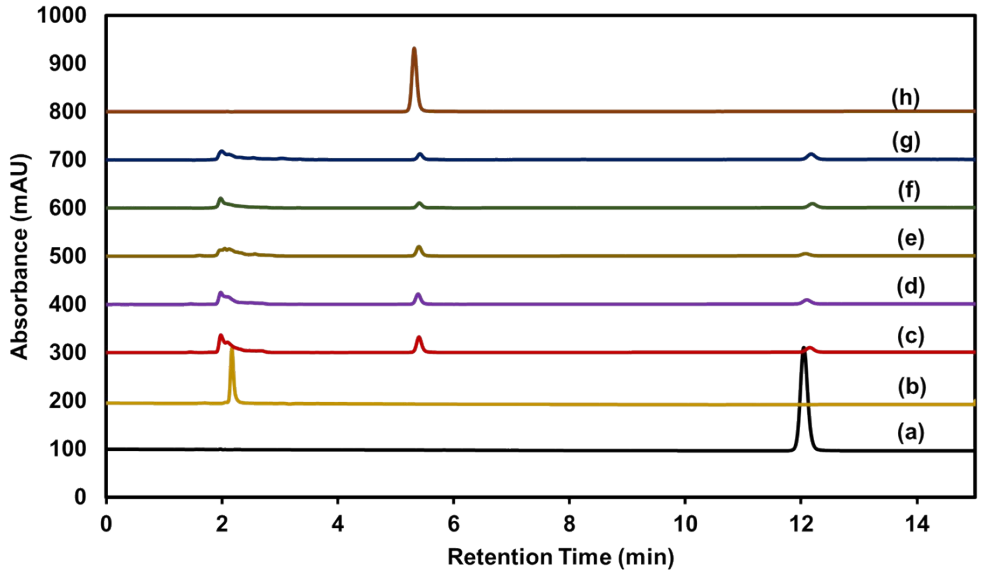


Figure S9. Reverse-phase HPLC chromatogram for the reaction of **NB-ITC** (25 μM) with Cys (1250 μM) at different pH. Legends: (a) pure **NB-ITC** @254 nm; (b) Cys @210 nm; (c) **NB-ITC** + Cys_pH 8.5 @254 nm; (d) **NB-ITC** + Cys_pH 8.0 @254 nm; (e) **NB-ITC** + Cys_pH 7.4 @254 nm; (f) **NB-ITC** + Cys_pH 6.5 @254 nm; (g) **NB-ITC** + Cys_pH 6.0 @254 nm; (h) **NAB** @254 nm. The reactions were carried out in PBS (20 mM, pH 7.4) and analyzed after 2 h.

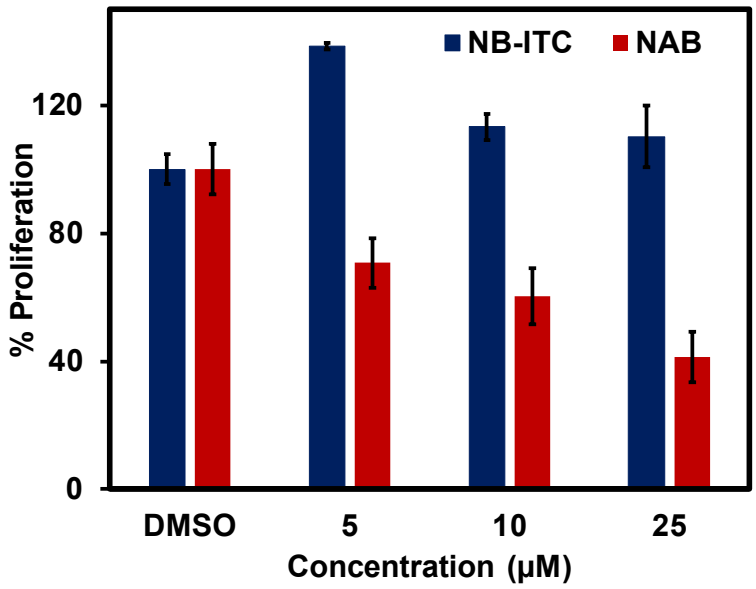


Figure S10. Dose-dependent anti-proliferative activity of **NB-ITC** and **NAB** in MDA-MB-231 cells over a period of 48 h.

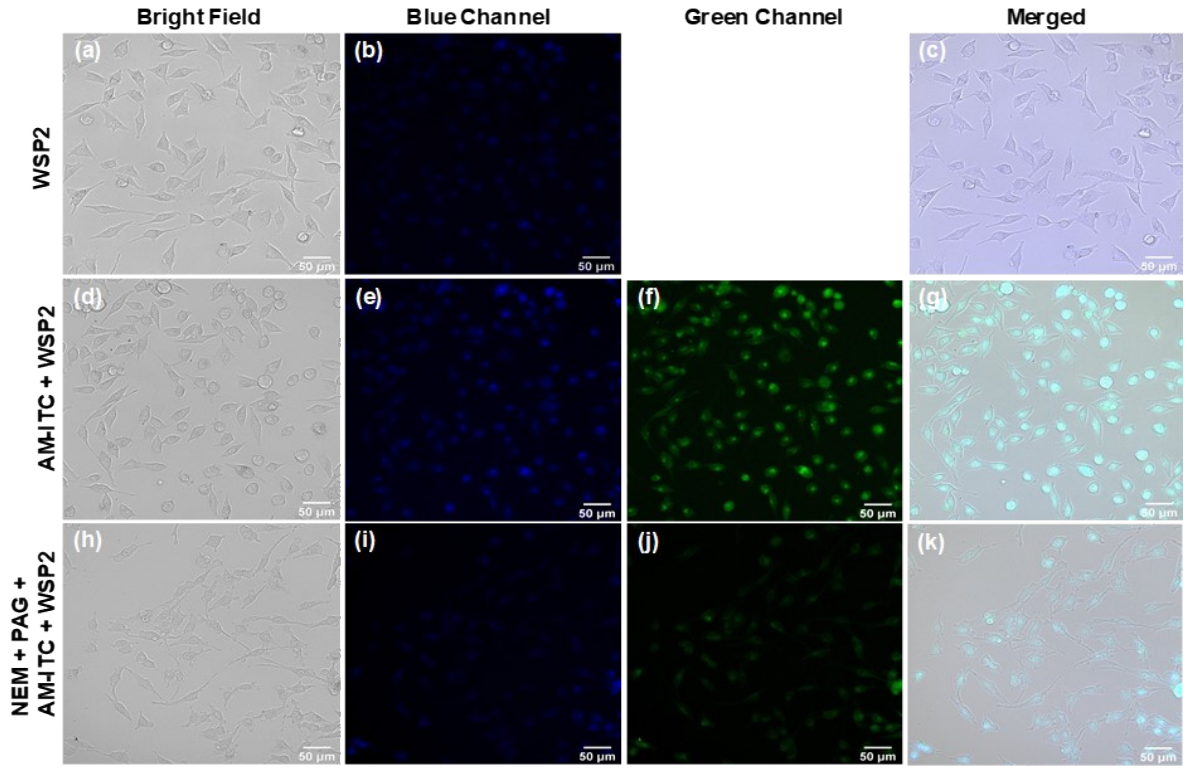


Figure S11. Fluorescence microscopy images (bright field, blue channel, green channel and merged) of HeLa cells in the presence of **AM-ITC** (25 μM) and **WSP2** (5 μM) for confirming the cellular release of H_2S. Scale bar: 50 μm.

NMR (^{1}H and ^{13}C) and ESI-MS spectra of the synthesized compounds

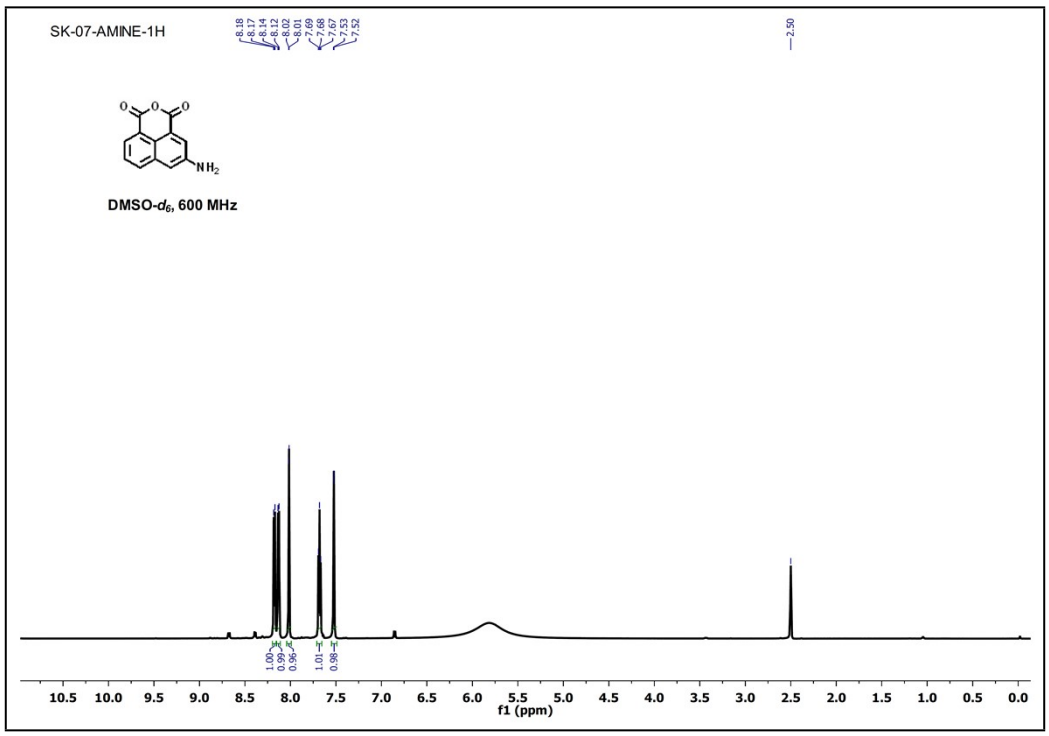


Figure S12. ^{1}H NMR spectrum (DMSO-d_6, 600 MHz) spectrum of compound **2**.

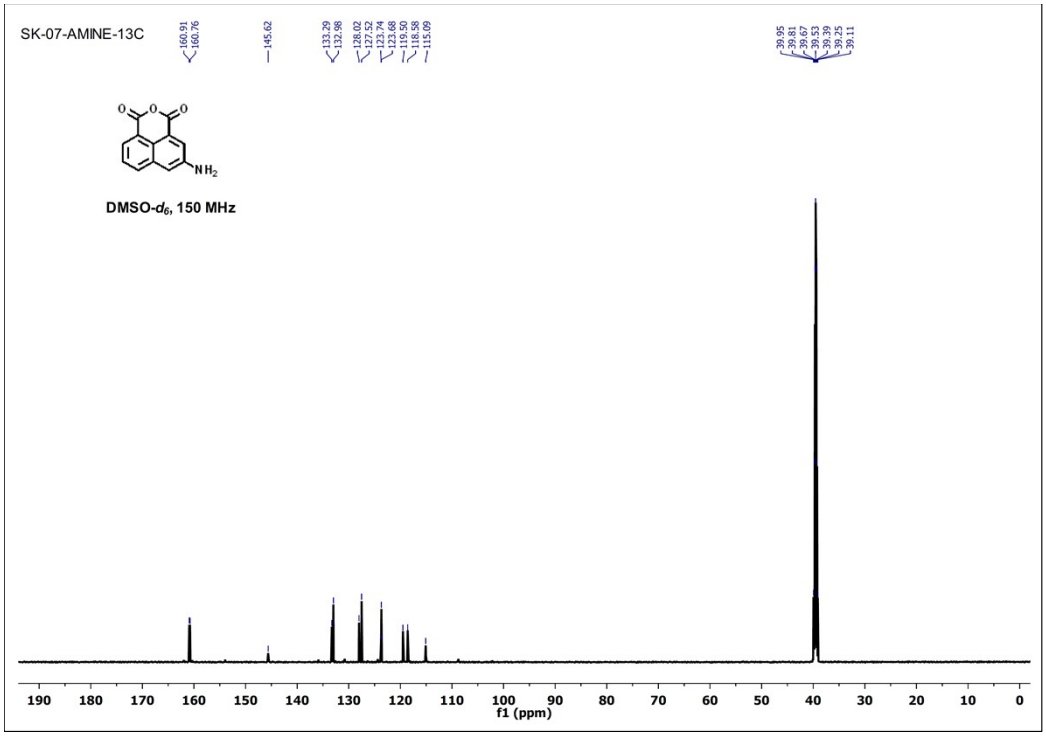


Figure S13. ^{13}C NMR spectrum (DMSO-d_6,150 MHz) spectrum of compound **2**.

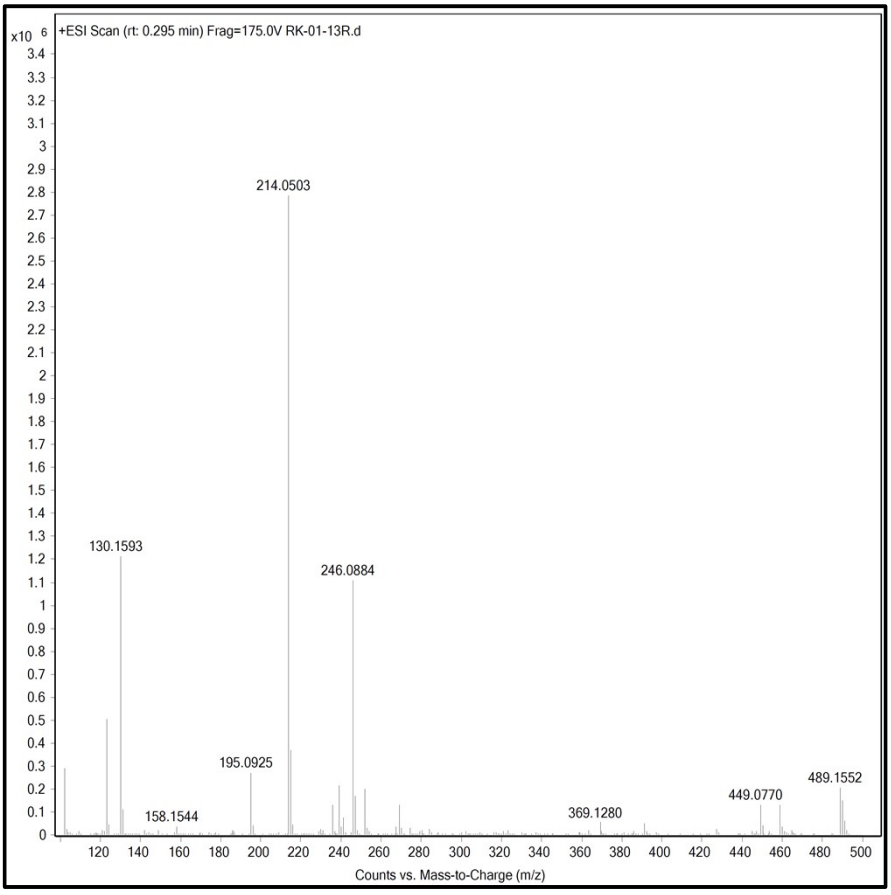


Figure S14. ESI-MS spectrum of compound **2**. ESI-MS *m/z* calcd. for $C_{16}H_8NO_3$ $[M + H]^+$ = 214.0504; obs. 214.0503.

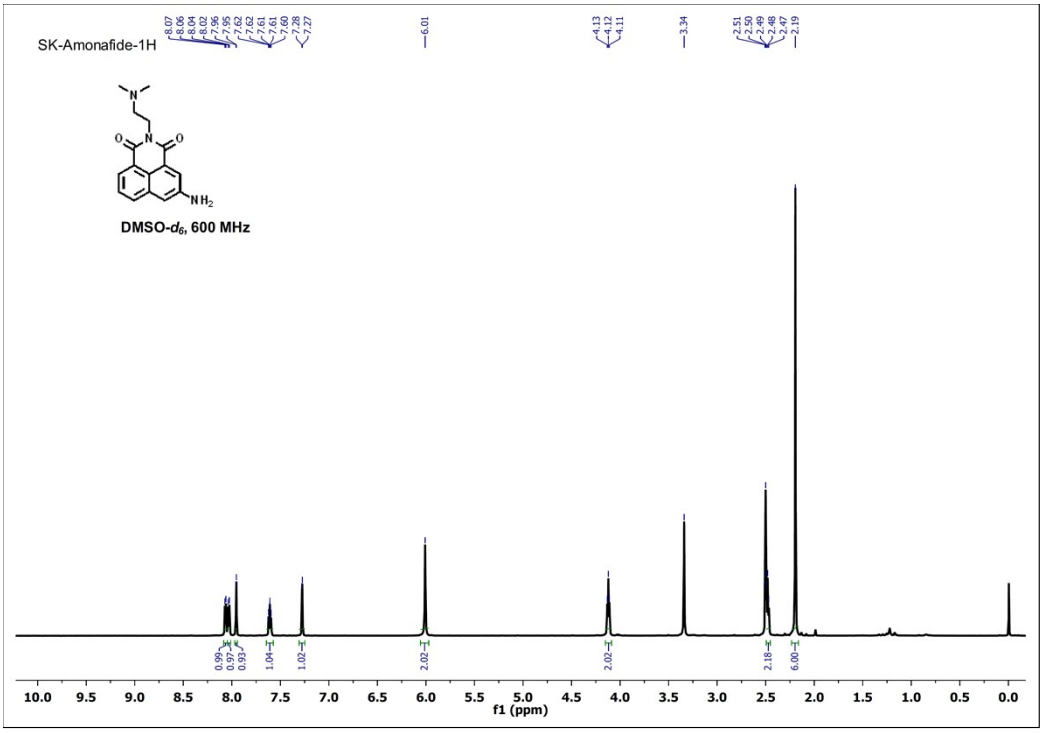


Figure S15. ^{1}H NMR spectrum (DMSO-d_6,600 MHz) spectrum of Amonafide.

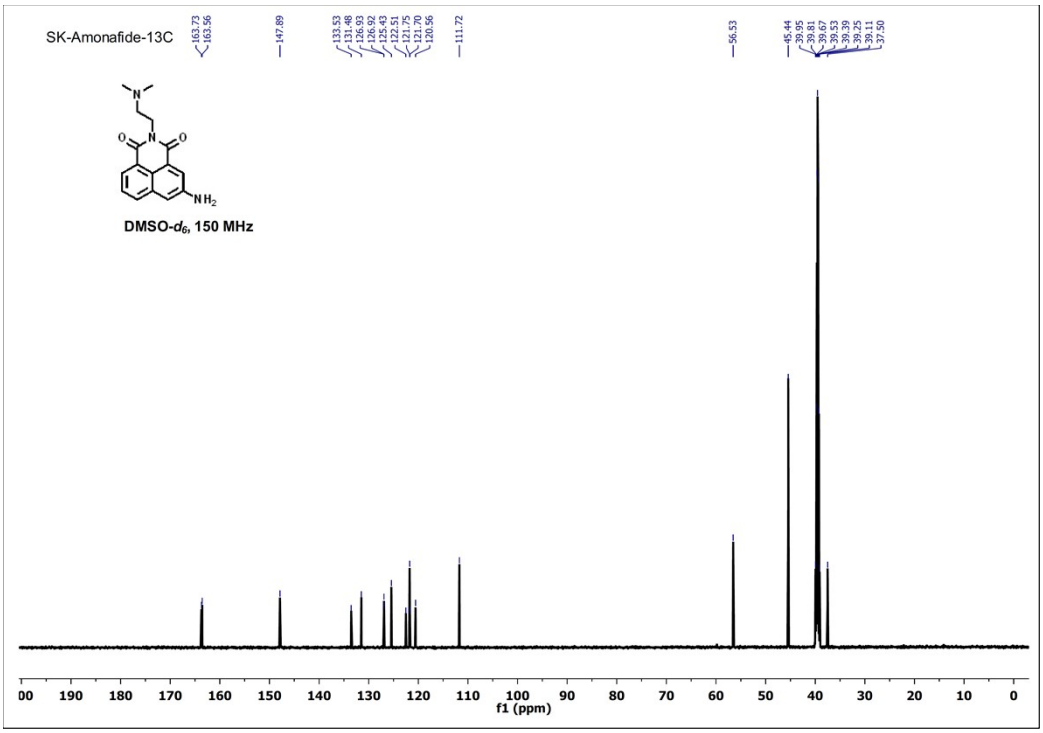


Figure S16. ^{13}C NMR spectrum (DMSO-d_6, 150 MHz) spectrum of Amonafide.

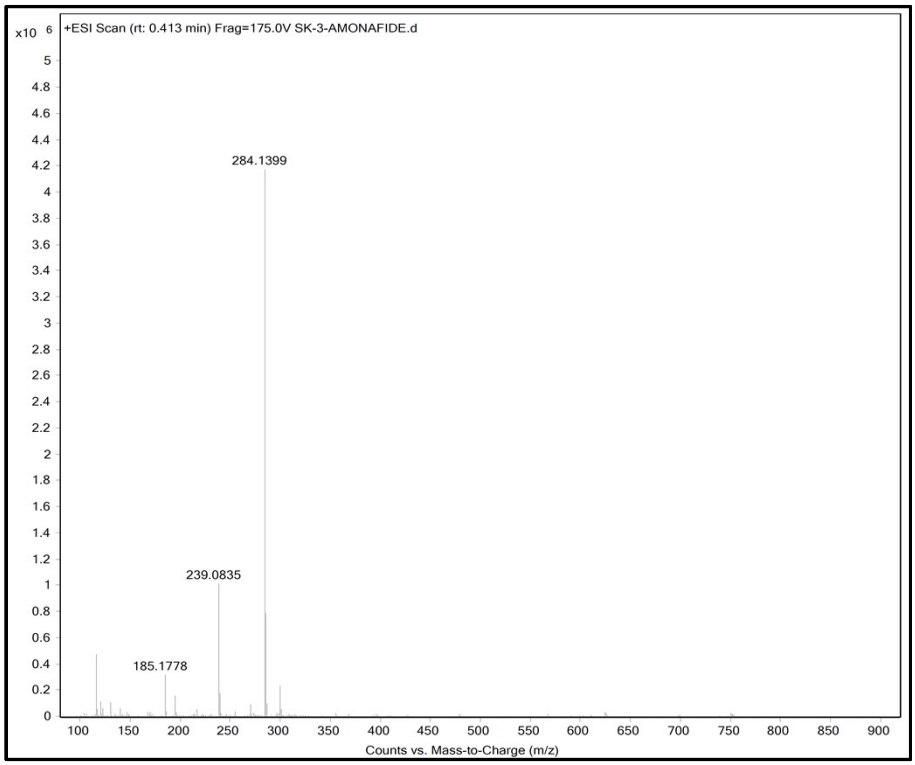


Figure S17. ESI-MS spectrum of Amonafide. ESI-MS *m/z* calcd. for $C_{16}H_{18}N_3O_2$ $[M + H]^+$: 284.1399, obs. 284.1399.

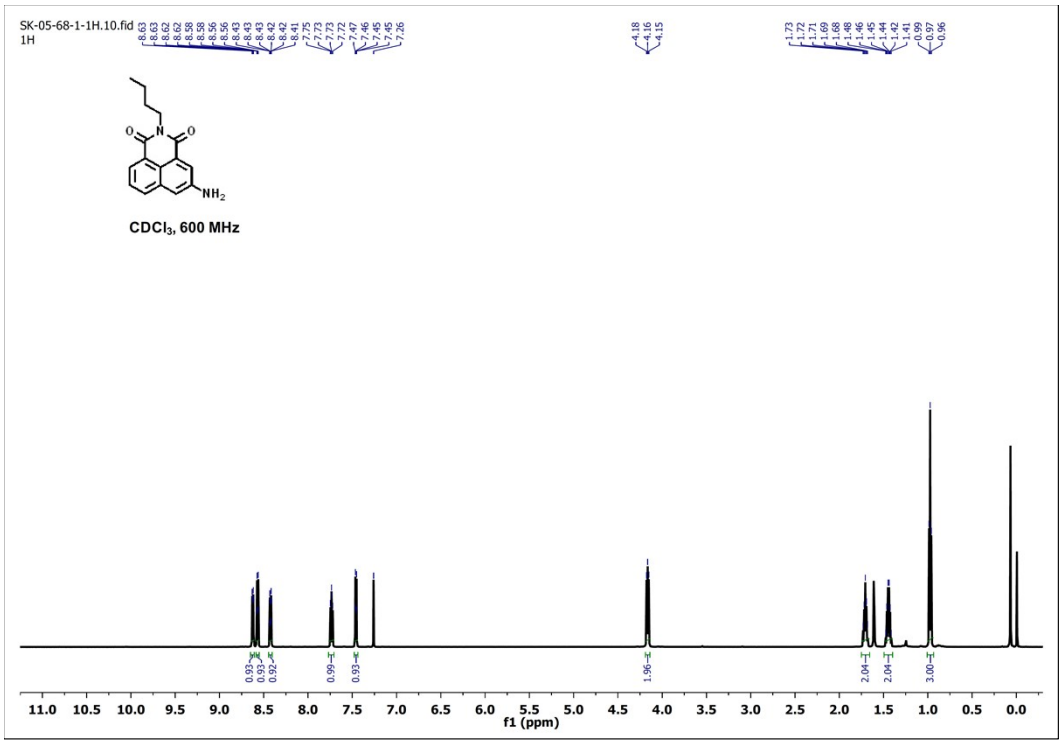


Figure S18. ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) spectrum of NAB.

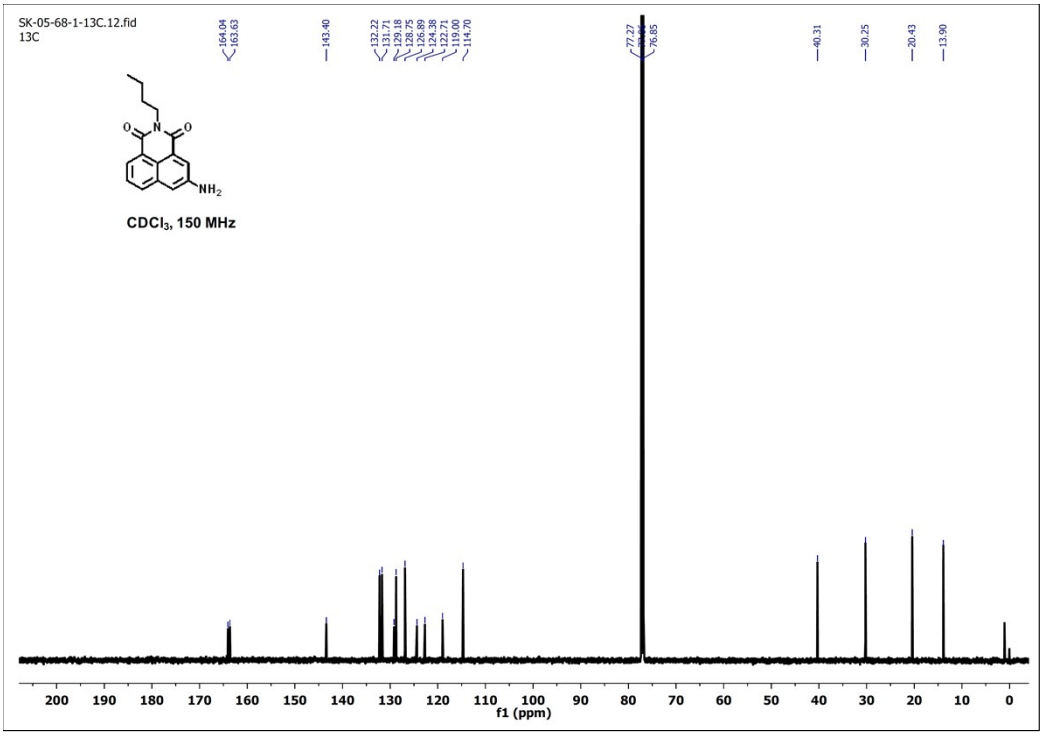


Figure S19. ^{13}C NMR spectrum ($CDCl_3$, 150 MHz) spectrum of NAB.

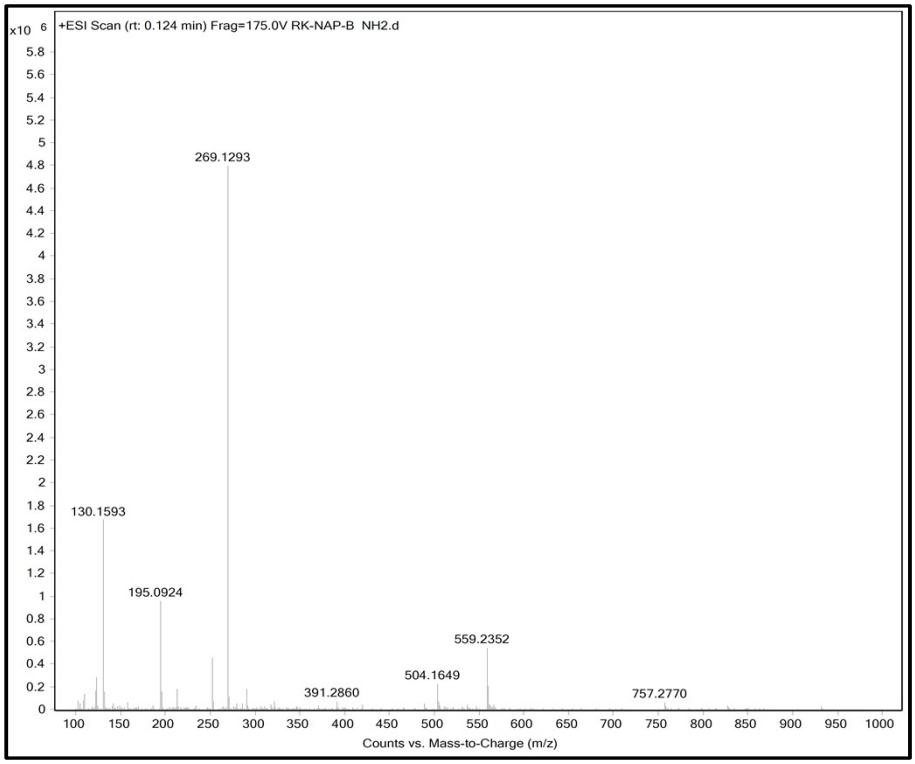


Figure S20. ESI-MS spectrum of NAB**.** ESI-MS *m/z* calcd. for $C_{16}H_{17}N_2O_2$ $[M + H]^+$: 269.1290, obs. 269.1293.

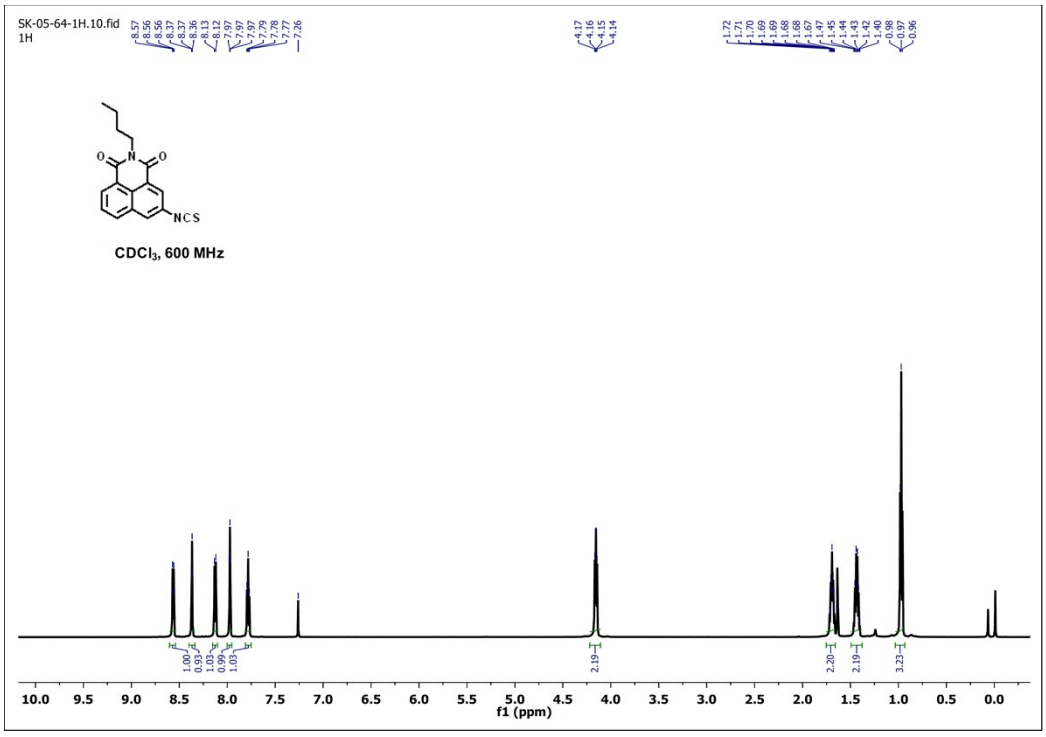


Figure S21. ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) spectrum of **NB-ITC**.

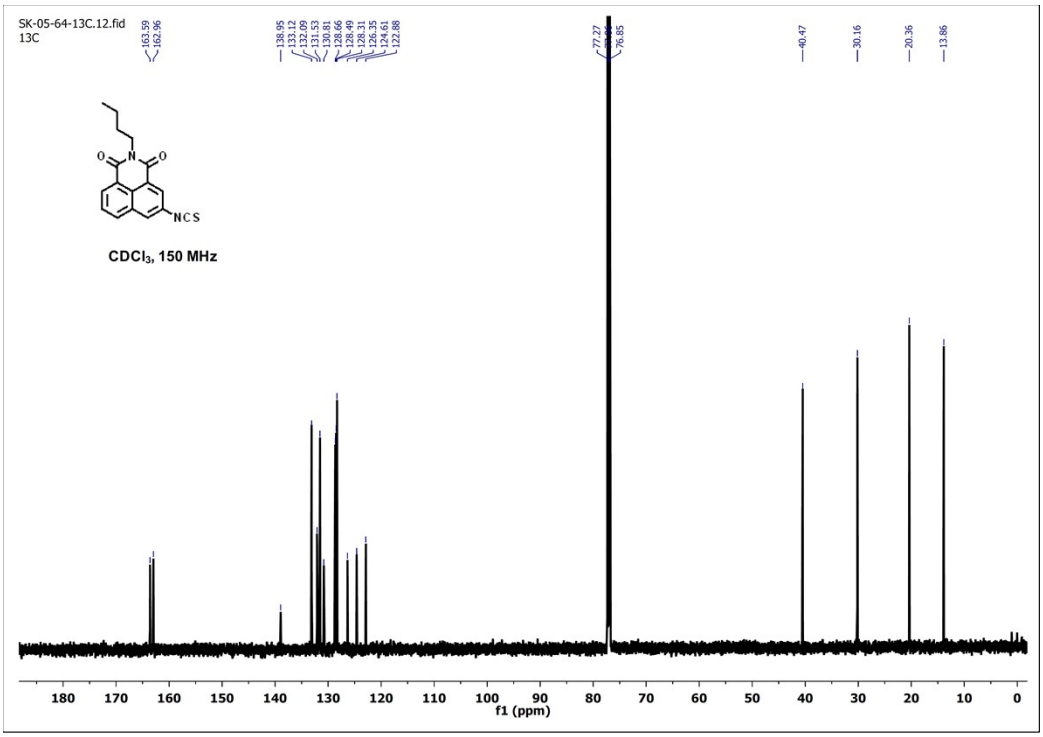


Figure S22. ^{13}C NMR spectrum ($CDCl_3$, 150 MHz) spectrum of **NB-ITC**.

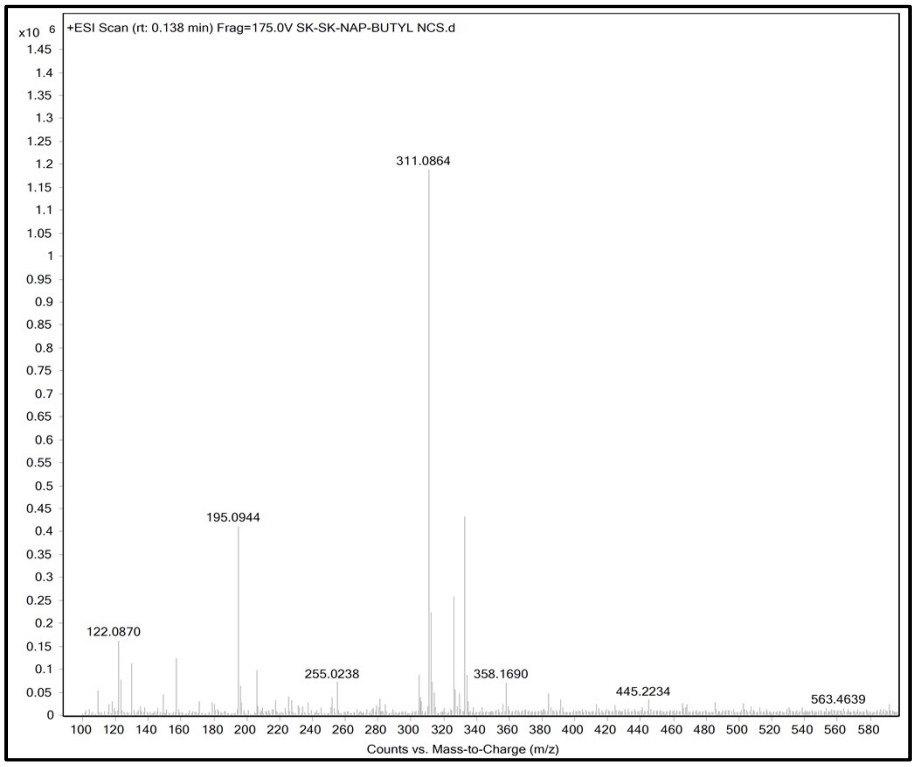


Figure S23. ESI-MS spectrum of **NB-ITC**. ESI-MS *m/z* calcd. for $C_{17}H_{15}N_2O_2S$ $[M + H]^+$ = 311.0854, obs. 311.0864.

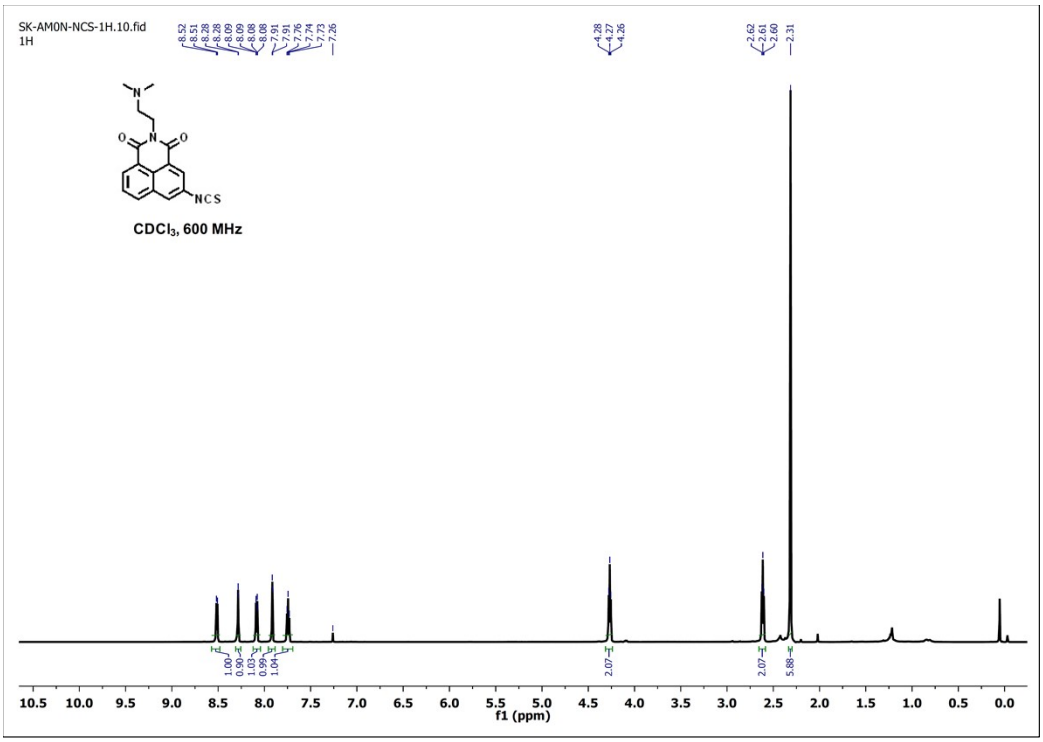


Figure S24. ^{1}H NMR spectrum ($CDCl_3$, 600 MHz) spectrum of **AM-ITC**.

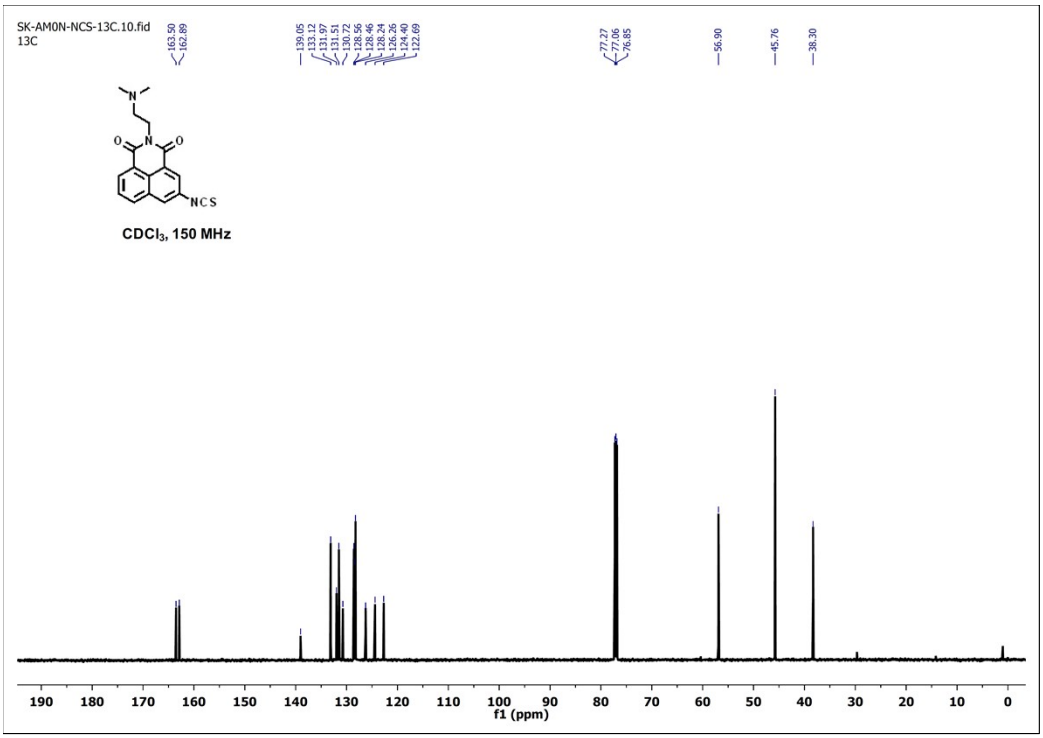


Figure S25. ^{13}C NMR spectrum ($CDCl_3$, 150 MHz) spectrum of **AM-ITC**.

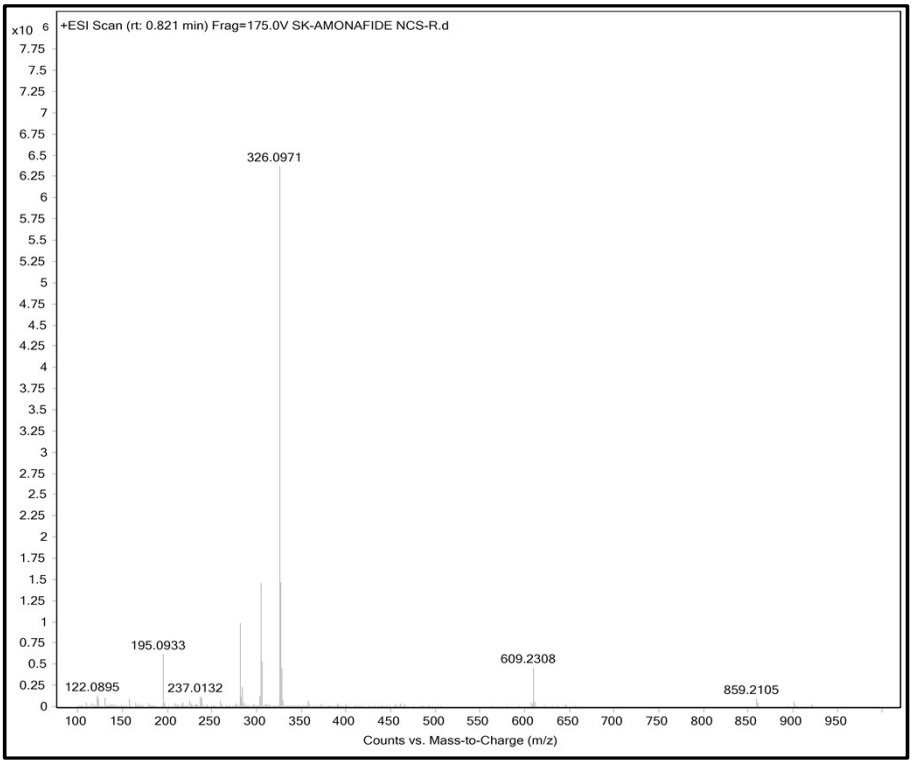


Figure S26. ESI-MS spectrum of **AM-ITC**. ESI-MS *m/z* calcd. for $C_{17}H_{16}N_3O_2S$ $[M + H]^+$ = 326.0963, obs. 326.0971.

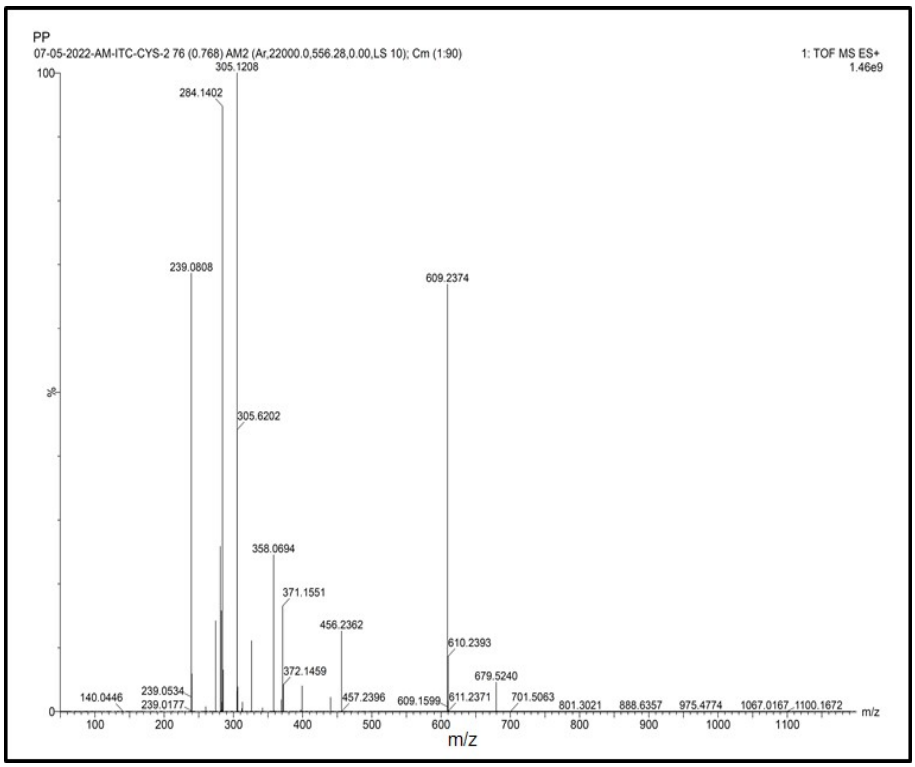


Figure S27. ESI-MS (+ve) spectrum for the reaction of **AM-ITC** with Cys in acetonitrile. The mass of the released compound amonafide was observed under mass spectrometric conditions. ESI-MS (+ve) calculated for $C_{16}H_{18}N_3O_2$ $[M+H]^+$ = 284.1399, obs. 284.1402.

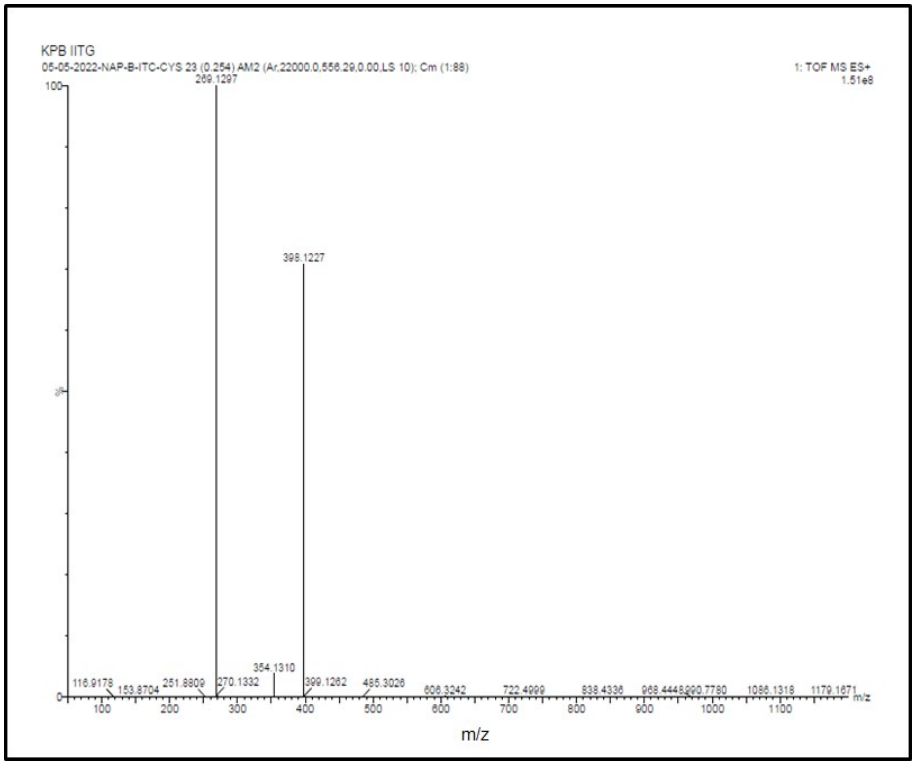


Figure S28. ESI-MS (+ve) spectrum for the reaction of **NB-ITC** with Cys in acetonitrile. The mass of the released compound NAB and the intermediate **6b** were observed under mass spectrometric conditions. ESI-MS (+ve) calculated for NAB $C_{16}H_{17}N_2O_2$ $[M+H]^+$ = 269.1290, obs. 269.1297. ESI-MS (+ve) calculated for **6b** $C_{20}H_{20}N_3O_4S$ $[M+H]^+$ = 398.1175, obs. 398.1227.

References

1. J. S. Sidhu, A. Singh, N. Garg and N. Singh, *ACS Appl. Mater. Interfaces*, 2017, **9,** 25847-25856.

2. E. V. Quaquebeke, T. Mahieu, P. Dumont, J. Dewelle, F. Ribaucour, G. Simon, S. Sauvage, J-F, Gaussin, J. Tuti, M. E. Yazidi, F. V. Vynckt, T. Mijatovik, F. Lefranc, F. Darro and R. Kiss, *J. Med. Chem.*, 2007, ***50***, 4122-4134.

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

4. B. Peng, W. Chen, C. Liu, E. W. Rosser, A. Pacheco, Y. Zhao, H. C. Aguilar and M. Xian, *Chem Eur J*, 2014, **20**, 1010-1016.
