Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Fluorescent Probe for Specific Detection of Cysteine in Lipid Dense Region of Cells

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Experimental details:

Materials & Methods:

All reagents were procured from commercial suppliers and used without further purification. All aqueous solutions and buffers were prepared by using Millipore-Q water. TLC analysis were performed using pre-coated silica plates. ^{1}H spectra were recorded on Bruker 400/500 MHz FT NMR instrument; while ^{13}C NMR spectra were recorded in 125 MHz FT NMR instrument using Advance-DPX 500. High-resolution mass spectra were recorded on JEOL JM AX 505 HA mass spectrometer. FT-IR spectra were recorded using a Bruker Alpha FT IR spectrometer. UV-Vis spectra were recorded with a shimadzu 1800 spectrophotometer, while all emission spectra were performed using *PTI* Quanta Master™ Steady State Spectrofluorometer. Standard quartz cuvettes with a 2/2 nm slit width were used for all solution state studies while 1/1 nm slit width were used only for the solid state optical measurements. The 3D intensity plots were obtained by using ImageJ software. BODIPY core was synthesised by following literature procedures.[1] Quantum yield was recorded using standard methods and rhodamine B ($\bar{u}_f = 0.31$) as reference compound in aqueous medium.[2]

General experimental procedure for UV-Vis and Fluorescence studies:

Stock solution of probe **ER-F** (1×10^{-4} M) was prepared in HPLC grade acetonitrile. All the analytes stock solution (1×10^{-2} M) was prepared in aqueous HEPES buffer (10 mM) medium at pH 7.2. 500 µl of this stock solution of probe **ER-F** was added to 4.5 ml of HEPES (10 mM) aqueous buffer medium having solution pH 7.2 to make the effective **ER-F** concentration of (1×10^{-5}) M. This solution was used for all the photophysical studies. All the photophysical studies were performed in aq. HEPES: CH_3CN medium (9: 1, v/v) at pH 7.2. All emission studies were done using λ_{Ext} = 530 nm with an emission slit width of 2/2 nm, unless and otherwise mentioned.

Preparation of TLC test strips and solid state fluorescence studies:

TLC test strips were prepared by drop-casting of 20 εl of probe **ER- F** (0.1 mM) solution of CH_3CN on silica TLC plates. Then it was dried and different concentration of Cys solution in 10 mM aq. HEPES buffer (pH 7.2) was added on it by the same process. Again it was dried properly and after that the fluorescence colour changes were recorded. The same was repeated for Hcy, GSH and other analytes as well. Images were captured using Canon 630D camera. The silica coated TLC plates treated with different concentration of Cys were used for solid state fluorescence measurements using λ_{Ext} = 530 nm with an emission slit width of 1/1 nm.

General procedure for confocal imaging studies:

For confocal studies, Hct116 cells (3×10^5) were seeded on cover slips placed in 6 well plates. After 24 hours, Hct116 cells were treated with **ER-F** (1 εM) for 30 minutes at 37ęC in a 5% CO_2 air environment. Cells were then washed thrice with Phosphate Buffer (1X PBS) and fixed with 4% PFA for 20 minutes and washed again with 1X PBS. Nail paint was used to seal the cover slips mounted on the glass slides for each well plates. For control experiment Hct116 cells were pre-treated with 1 mM of N-Ethylmaleimide (NEM) for 30 minutes. Then cells were washed thrice with media and followed by incubation with **ER-F** (1 εM) for another 30 minutes under same conditions. Cells were again washed with 1XPBS buffer and fixed with 4% PFA for 20 minutes and washed again with 1X PBS. Confocal laser scanning microscopic (CLSM) images were acquired in Olympus Fluoview Microscope with $\lambda_{Ext}/\lambda_{Mon}$ = 530/573 nm.

For confocal studies with N-acetyl cysteine (NAC) drug we have used HepG2 liver cancer cells. HepG2 cells (3×10^5) were seeded on cover slips placed in 6 well plates. After 24 hours, cells were treated with 1 mM NEM for 30 minutes. Cells were then washed with media and treated with 25 εM of NAC and incubated for 1 hour. Further, cells were incubated with **ER-F** (1 εM) for 20 minutes. Cells were then washed thrice with Phosphate Buffer Saline (1X PBS) and fixed with 4% PFA for 20 minutes and again washed with 1X PBS. CLSM images were acquired in Olympus Fluoview Microscope with $\lambda_{Ext}/\lambda_{Mon}$ = 530/573 nm.

Synthesis of ER-S:

A mixture of BODIPY (400 mg, 1.23 mmol), 4-hydroxybenzaldehyde (165 mg, 1.35 mmol), 0.9 ml piperidine and 0.6 ml glacial acetic acid was refluxed in 30 ml toluene in a Dean-Stark apparatus for 3h. Then water was added into it and crude was extracted with dichloromethane. The organic layer was collected and dried over anhydrous sodium sulphate and solvent was removed under reduced pressure. It was then subjected to column chromatography using silica gel (100-200 mesh) as stationary phase and 10% EtOAc in hexane as mobile phase to get compound **ER-S** as red solid. Yield: 56%; ^{1}H NMR (500 MHz, $CDCl_3$, *J* in Hz, ŭ ppm): 7.52 (1H, d, *J* = 16.5), 7.47 (5H, m), 7.30 (2H, d, *J* = 8.0), 7.18 (1H, d, *J* = 16.0), 6.83 (2H, d, *J* = 8.5), 6.58 (1H, s), 5.99 (1H, s), 5.40 (1H, s), 2.59 (3H, s), 1.42 (3H, s), 1.38 (3H,s); ^{13}C NMR (125 MHz, $CDCl_3$, ŭ ppm): 156.80, 154.68, 153.47, 142.74, 142.40, 140.08, 136.24, 135.13, 132.84, 131.68, 129.38, 129.22, 129.09, 128.93, 128.25, 121.14, 117.55, 116.90, 115.82, 14.69, 14.59, 14.32. HRMS (ESI): m/z calcd for $C_{26}H_{23}BF_2N_2O$ [M+ H]: 429.1872, found 429.1943.

Synthesis probe ER-F:

In a 100 ml two neck r.b flask, **ER-S** (30 mg, 0.07 mmol) was dissolved in 10 ml of anhydrous dichloromethane. Then 100 ε l of Et_3N was added to the reaction mixture and allowed to stir for 10 minutes at room temperature under N_2 atmosphere. Then 20 ε l of acryloyl chloride was added to this and resulting mixture was stirred at room temperature until all the starting material was consumed, monitored by TLC. Then water was added to it and organic layer was extracted using dichloromethane. The organic layer was collected and dried over anhydrous sodium sulphate and solvent was removed under reduced pressure. It was then subjected to column chromatography using silica gel (100-200 mesh) as stationary phase and 5% EtOAc in hexane as mobile phase to get probe **ER-F** as solid. Yield: 70%. ^{1}H NMR (500 MHz, $CDCl_3$, *J* in Hz, ŭ ppm) : 7.55 (1H, d, *J*= 15.5), 7.53 (3H, d, *J* = 8.2), 7.41 (3H, d, *J* = 5.8), 7.25 ï 7.21 (2H, m), 7.13 (1H, d, *J* = 16.3), 7.07 (2H, d, *J* = 8.4), 6.56 (1H, s), 6.52 (1H, s), 6.25 (1H, dd, *J* = 17.3, 10.5), 5.95 (2H, d, *J* = 11.1), 2.52 (3H, s), 1.35 (3H, s), 1.31 (3H, s); ^{13}C NMR (125 MHz, $CDCl_3$, ŭ ppm): 164.35, 155.92, 152.21, 150.90, 143.27, 142.34, 140.65, 135.03, 134.69, 134.39, 132.78, 132.05, 129.15, 129.01, 128.44, 128.14, 127.86, 121.88, 121.55, 119.41, 117.48, 14.79, 14.57, 14.42. HRMS (ESI): m/z calcd for $C_{29}H_{26}BF_2N_2O_2$ [M + H]: 483.1977 found 483.2057.

^{1}H NMR spectrum of ER-S:

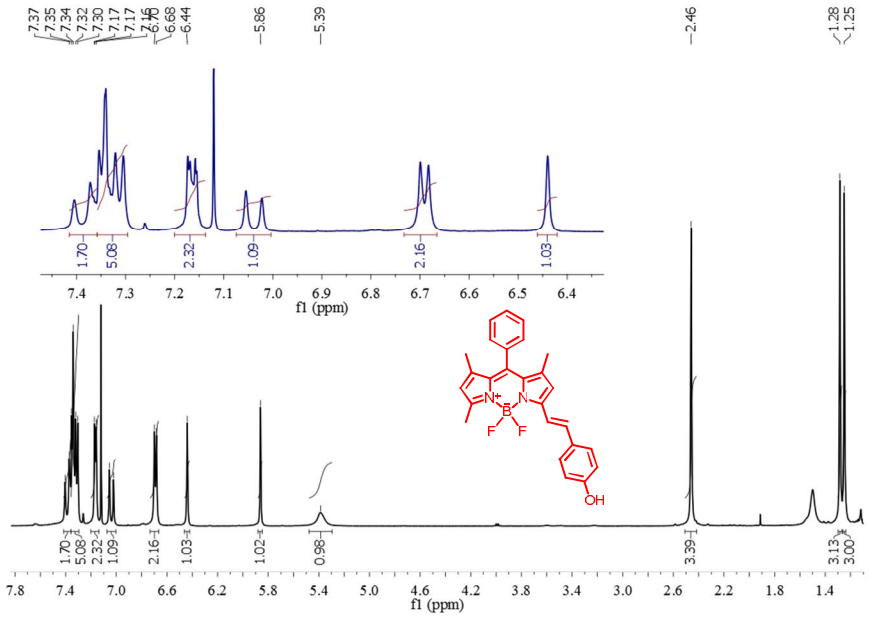

Fig. S 1: ^{1}H NMR of **ER-S** in $CDCl_3$.

^{13}C NMR spectrum of ER-S:

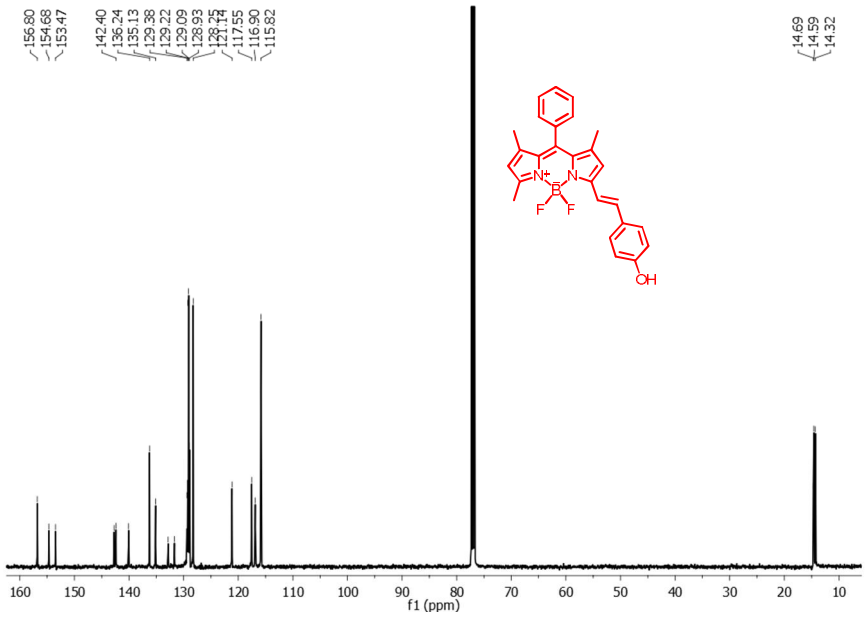

Fig. S 2: ^{13}C NMR of **ER-S** in $CDCl_3$ recorded in 125 MHz machine.

^{1}H NMR spectrum of probe ER-F:

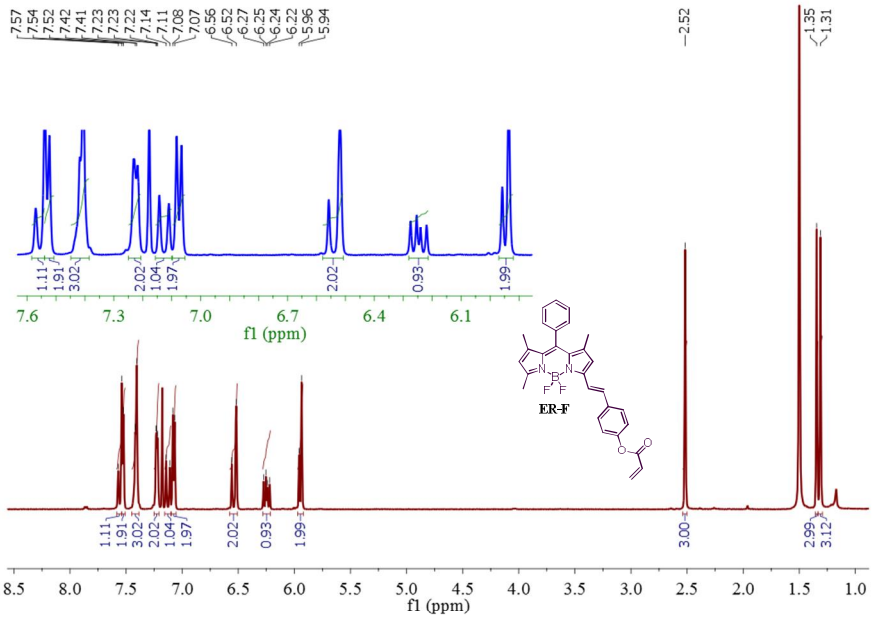

Fig. S 3: ^{1}H NMR of probe **ER-F** in $CDCl_3$.

^{13}C NMR spectrum of probe ER-F:

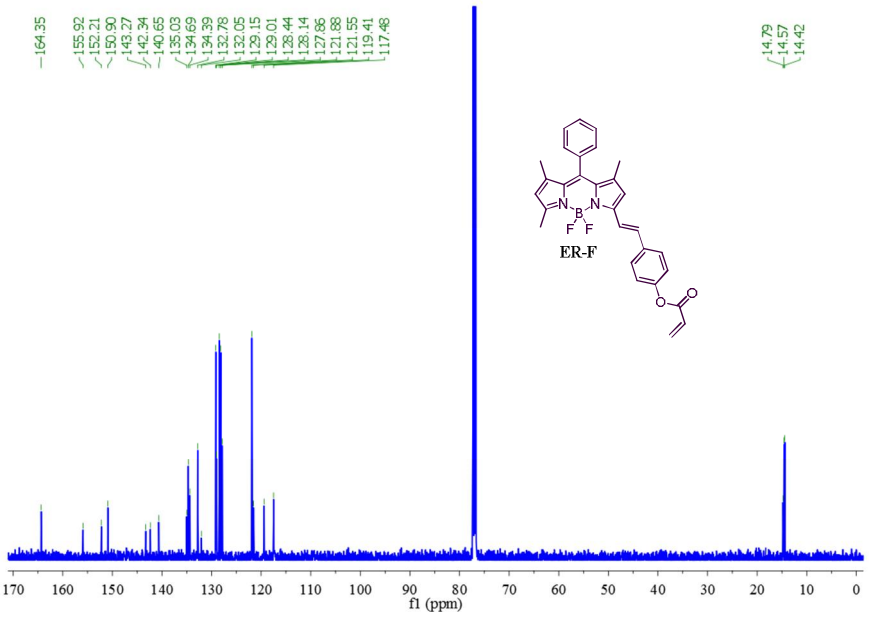

Fig. S 4: ^{13}C NMR of probe **ER-F** in $CDCl_3$ recorded in 125 MHz machine.

HRMS spectrum of ER-S:

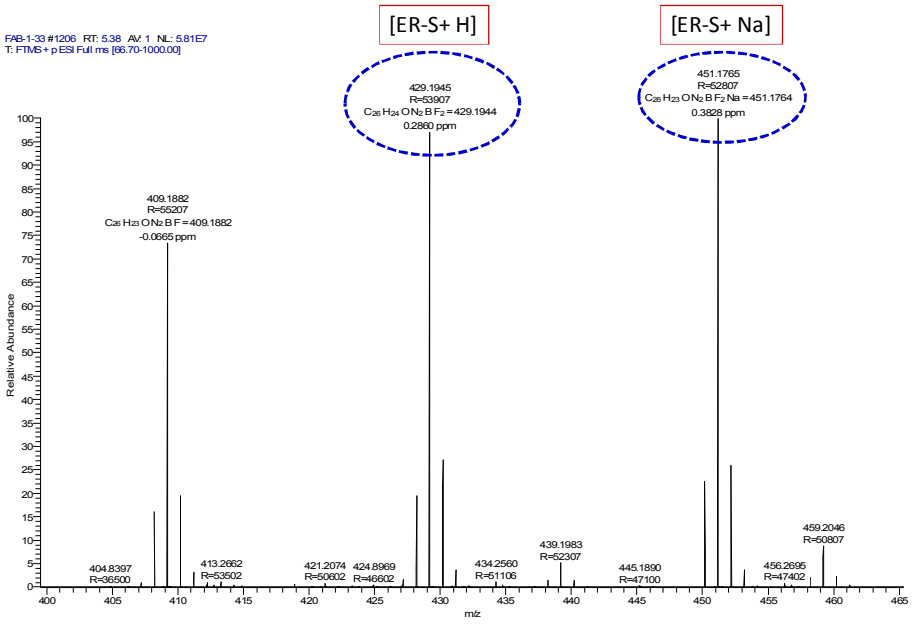

Fig. S 5: HRMS spectrum of **ER-S** in Methanol.

HRMS spectrum of ER-F:

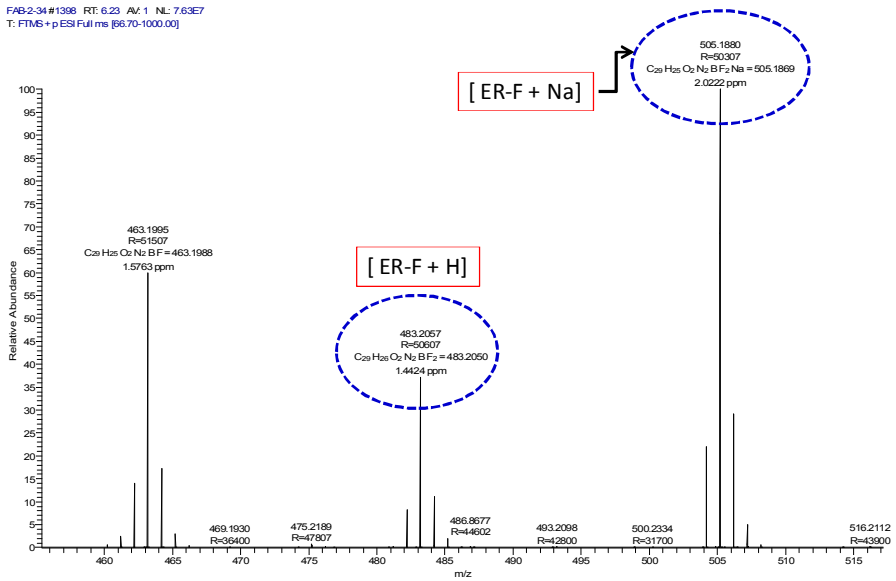

Fig. S 6: HRMS spectrum of probe **ER-F** in Methanol**.**

IR spectrum of ER-F:

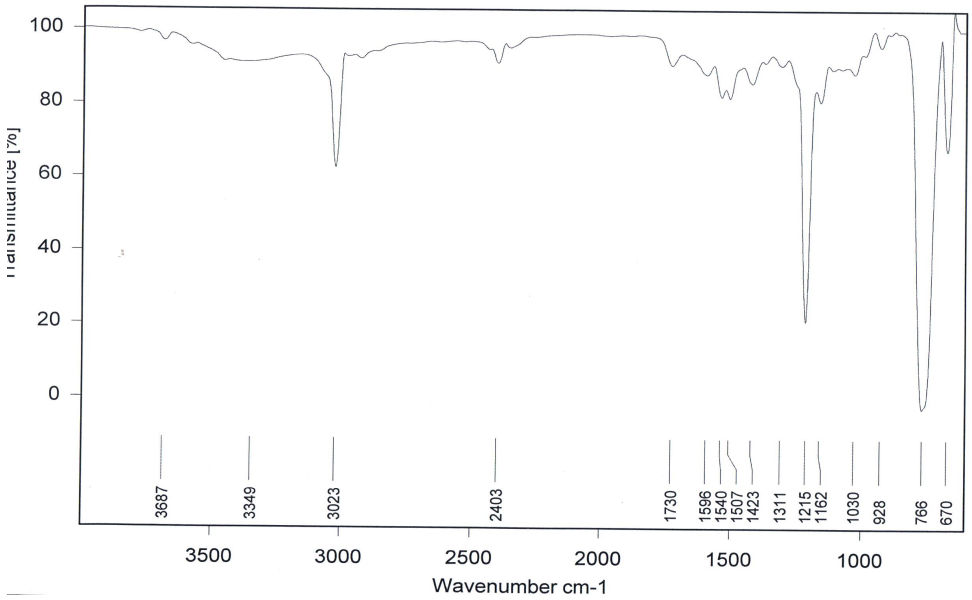

Fig. S 7: IR spectrum of probe **ER-F**.

IR spectrum of ER-S:

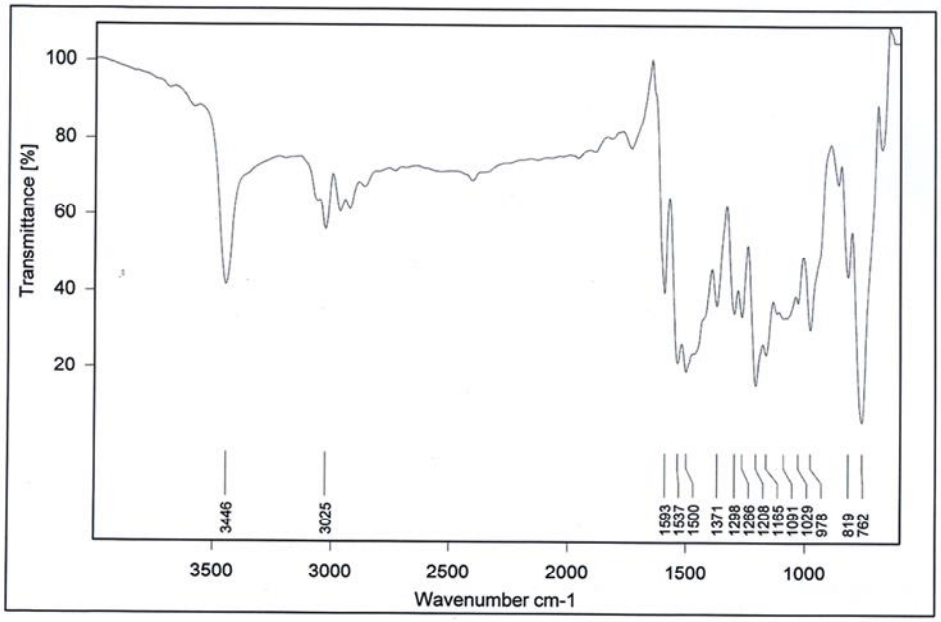

Fig. S 8: IR spectrum of probe **ER-S**.

X-ray crystallography

Single crystal of suitable dimension was chosen under an optical microscope and mounted on a glass fiber for data collection on a Bruker SMART APEX diffractometer equipped with CCD area detector at 150K. Intensity data for crystal was collected using graphite-mono chromated MoK_α (λ=0.71073 Å) radiation. The data integration and reduction were performed with SAINT software.[3] Data were subjected to empirical absorption correction using SADABS.[4] The structure was solved by direct methods using SHELXTL[5] and was reýned by full matrix least square procedures based on F^2 using the program SHELXL-97.[6] All non-hydrogen atoms were reýned anisotropically till convergence was reached. Hydrogen atoms, attached to the organic moieties, were either located from the difference of Fourier map or stereochemically ýxed in the compound. Details of crystallographic data for compound **ER-F** are provided **SI table 1.**

SI Table1: Crystal data and structure refinement for ER-F.

Identification code	EF-F	
CCDC	1420800	
Empirical formula	C_{29} H_{25} B F_2 N_2 O_2	
Formula weight	482.32	
Temperature	296(2) K	
Wavelength	0.71073 Å	
Crystal system	Triclinic	
Space group	P-1	
Unit cell dimensions	a = 10.4699(7) Å	α= 108.143(3)°
	b = 10.9521(7) Å	β= 95.509(4)°
	c = 12.2593(7) Å	γ = 106.047(4)°
Volume	1258.03(14) $Å^3$	
Z	2	
Density (calculated)	1.273 Mg/m^3	
Absorption coefficient	0.089 mm^{-1}	
F(000)	504	
Crystal size	0.45 x 0.38 x 0.32 mm^3	
Theta range for data collection	1.78 to 27.22°.	
Index ranges	-13<=h<=13, -14<=k<=14, -15<=l<=15	
Reflections collected	24454	
Independent reflections	5573 [R (int) = 0.0532]	
Completeness to theta = 27.22°	99.00%	
Absorption correction	multi-scan	
Max. and min. transmission	0.9719 and 0.9608	
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	5573 / 0 / 329	
Goodness-of-fit on F2	1.031	
Final R indices [I>2sigma(I)]	R1 = 0.0516, wR2 = 0.1417	
R indices (all data)	R1 = 0.0758, wR2 = 0.1606	
Extinction coefficient	0.014(3)	
Largest diff. peak and hole	0.521 and -0.325 e.$Å^{-3}$	

UV-Vis spectra of ER-S and ER-F in presence and absence of Cys:

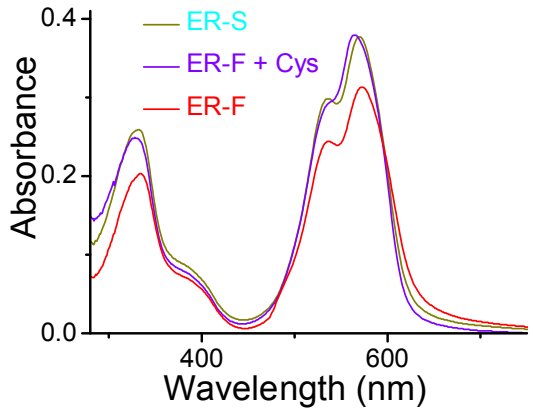

Fig. S 9: Absorbance spectra of **ER-F** (10 ε M), **ER-S** (10 ε M) and **ER-F** (10 ε M) in presence of Cys (100 ε M) in aqueous HEPES: CH_3CN (9:1, v/v) medium at pH 7.2.

TLC plate images of ER-F in presence and absence of different analytes:

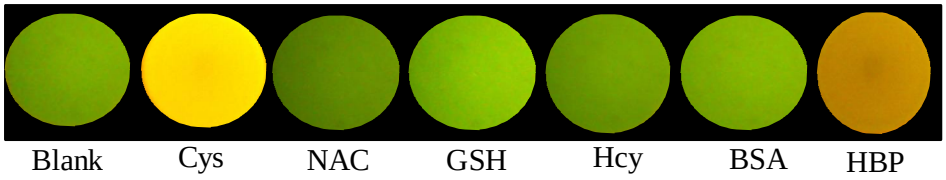

Fig. S 10: Snap shot of the visually detectable changes in fluorescence of silica surface, modified with the probe **ER-F** in absence and presence of (100 ε M) different analytes (Cys, NAC, GSH, Hcy, BSA (Bovine serum albumin) and diluted HBP sample). Fluorescence colour changes were observed using 365 nm UV lamp; [**ER-F**] used for the study was 0.1 mM.

3D intensity plot of ER-F in presence and absence of Cys:

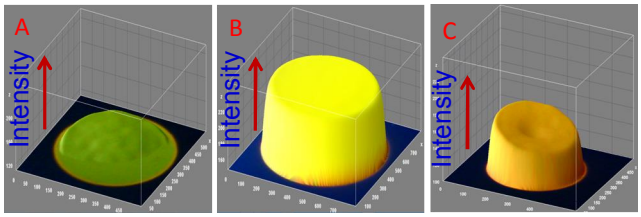

Fig. S 11: Interactive 3D surface plot of **ER-F** coated silica TLC plates in absence (A) and presence (B) of Cys (100 ε M) and (C) diluted HBP sample using ImageJ software.

Emission response of ER-F with Cys and different concentrations of GSH:

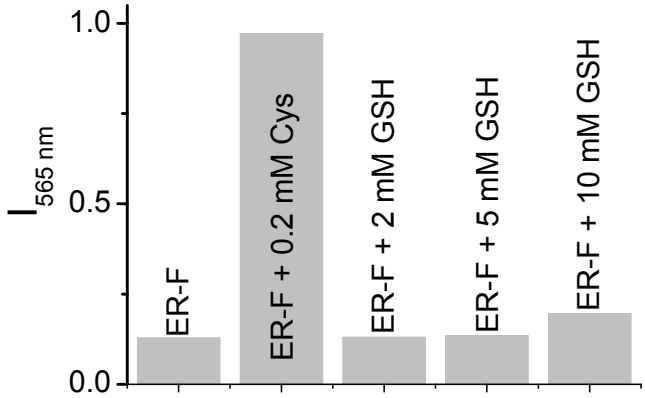

Fig. S 12: Relative emission response of **ER-F** (10 ε M) with Cys (0.2 mM) as well as with different concentrations of GSH in aqueous HEPES: CH_3CN (9:1, v/v) medium at pH 7.2. ∂_{Ext} = 530 nm, ∂_{Mon} = 565 nm.

Selectivity studies: Scanning with different amino acids.

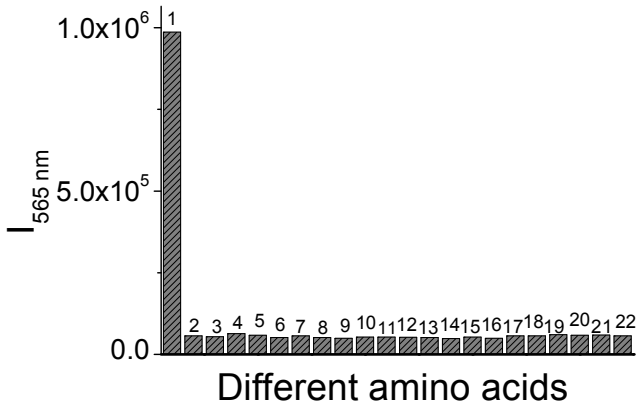

Fig. S 13: Emission response of **ER-F** (10 ε M) in the absence and presence of various amino acids (20 mole equiv. each). (From 1-22, Cysteine, **ER-F** only, Homocysteine, Glutathione, Histidine, Leucine, Methionine, Phenylalanine, Tryptophon, Tyrosine, Valine , Alanine, Arginine, Glycine, Glutamine, Proline, Serine, Aspartic acid, Glutamic acid, Threonine, Isoleucine, Lysine) in 10 mM HEPES: CH_3CN (9:1, v/v) at pH 7.2, ∂_{Ext} = 530 nm, ∂_{Mon} = 565 nm.

Interference studies with various cations and anions:

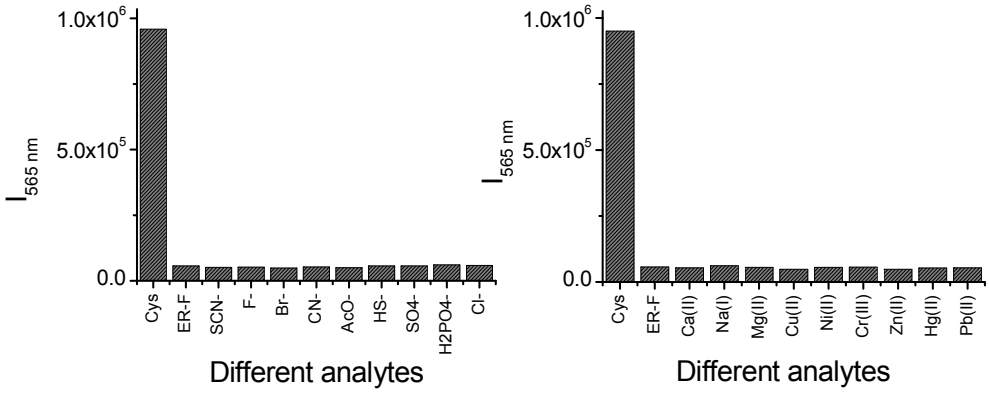

Fig. S 14: Emission response of **ER-F** (10 εM) upon addition of common cations, anions, BSA and Cys (20 equiv) in 10 mM HEPES: CH_3CN (9:1, v/v) at pH 7.2 using λ_{Ext} = 530 nm, λ_{Mon} = 565 nm.

Intensity v/s Concentration plot

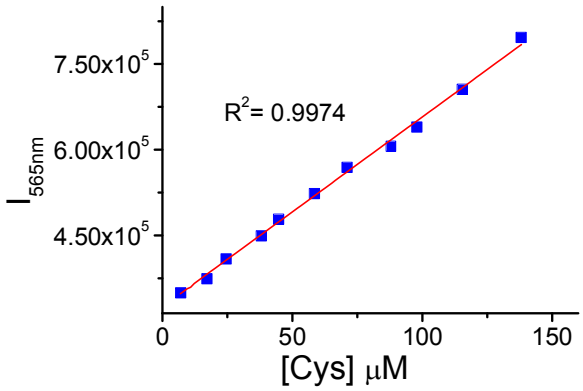

Fig. S 15: Fluorescence intensity of **ER-F** at 565 nm upon addition of Cys (0-150 εM) in 10 mM HEPES: CH_3CN (9:1, v/v) at pH 7.2, λ_{Ext} = 530 nm, λ_{Mon} = 565 nm.

Solvent dependent studies:

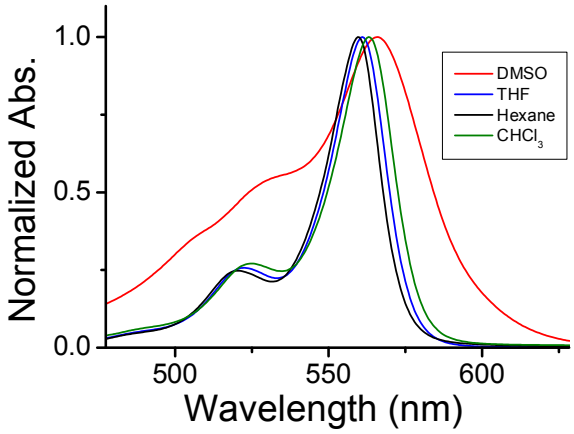

Fig. S 16: Normalised absorbance spectra of **ER-F** with different solvent.

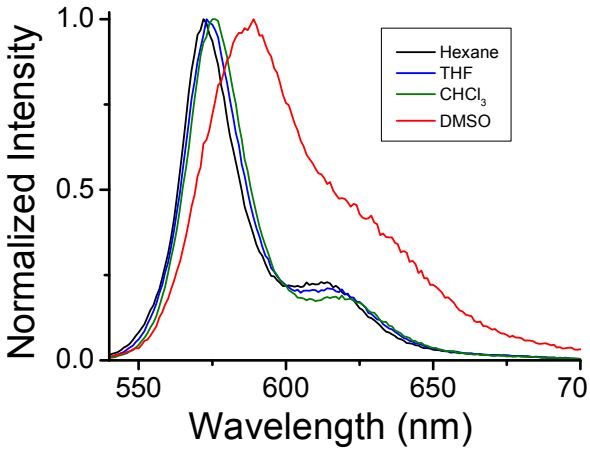

Fig. S 17: Emission spectra of **ER-F** with different solvent, λ_{Ext} = 530 nm.

Change in emission intensity of probe ER-F with pH:

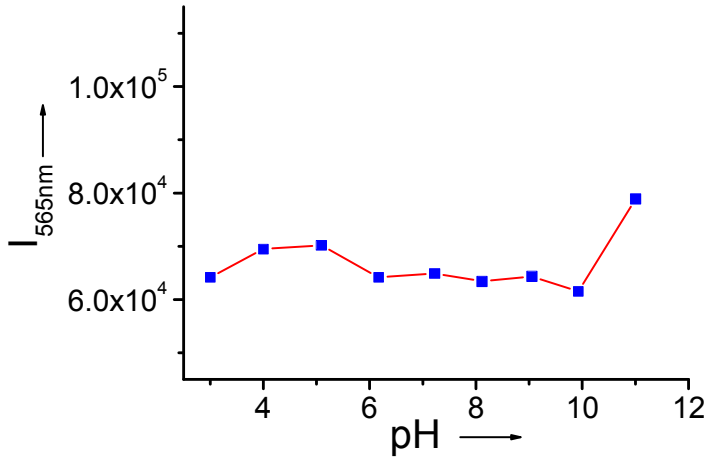

Fig. S 18: Emission spectrum of **ER-F** at different pH, λ_{Ext} = 530 nm, λ_{Mon} = 565 nm.

Methodology for the estimation of Cys in Human blood plasma (HBP) sample[7]:

Fresh human blood samples (6 ml) with added lithium anticoagulant were centrifuged in a vacationer tube at 3000 rpm for 15 min. The supernatant solution (blood plasma) was collected. 2 ml of collected blood plasma was vigorously mixed with appropriate amount of $NaBH_4$ in order to hydrolyse the disulphide bond and incubated for 5 minutes at room temperature. Proteins present in the sample after reduction were precipitated by the addition of methanol, followed by centrifugation of the sample for 10 minutes. The supernatant liquid, which contained Cys was used for the spectroscopic studies.

Calculation of detection limit[8]:

The detection limit was calculated based on the fluorescence titration. To determine the S/N ratio, the emission intensity of **ER-F** without Cys was measured 10 times and the standard deviation of blank measurements was determined. The detection limit (DL) of **ER-F** for Cys was determined from the following equation:

DL = K * Sb1/S

Where K = 2 or 3 (we took 3 in this case);

Sb1 is the standard deviation of the blank solution;

S is the slope of the calibration curve.

From the graph we get slope = 4.99×10^8, and Sb1 value is 2.49

Thus using the formula we get the Detection Limit = 15×10^{-9} M.

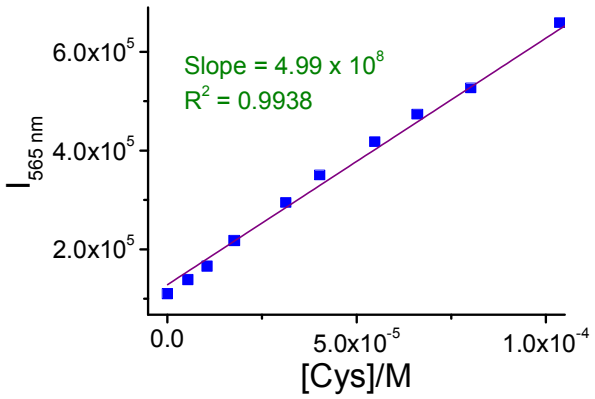

Fig. S 19: Fluorescence intensity at 565 nm upon addition of Cys (0 - 0.1 mM) in 10 mM HEPES: CH_3CN (9:1, v/v) at pH 7.2, λ_{Ext} = 530 nm, λ_{Ext} = 565 nm.

B-H plot obtained from the emission titration of ER-F (10 µM) with Cysteine:

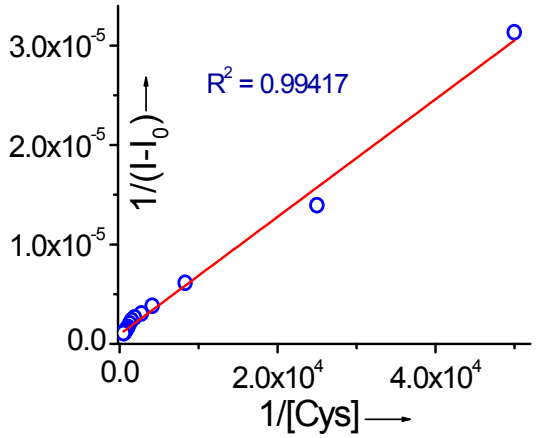

Fig. S 20: Benesi-Hildebrand (B-H) plot obtained from the emission titration of **ER-F** (10 εM) with Cysteine supported 1:1 reaction stoichiometry.

Kinetic studies[9]:

Time dependent studies of (10 ε M) **ER-F** with 2 mM of different analytes like Cys, Hcy, GSH and CN^- were carried out by mixing the reactants and monitored by fluorescence measurements in aq-HEPES-CH_3CN (9:1, v/v) medium of pH 7.2 at 15^0C. ∂_{Ext} = 530 nm. ∂_{Mon} = 565 nm. Data were collected under pseudo-first-order conditions. The pseudo-first order rate constant for the reaction was determined by fitting the fluorescence intensity changes of the samples to the pseudo first-order equation:

$$\ln[(I_{max}-I)/I_{max}] = -k_{obs}\, t$$

Where, I and I_{max} represent the fluorescence intensities at times t and the maximum value obtained after the reaction was complete. k_{obs} is the observed rate constant of the reaction.

From the slope we get k_{obs} value for each reaction.

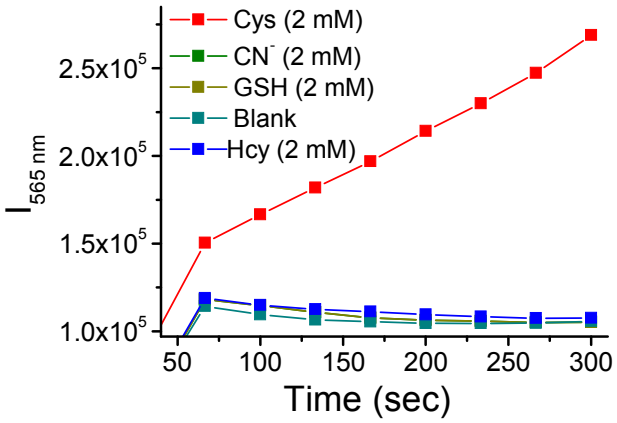

Fig. S 21: Time dependent emission studies of **ER-F** (10 ε M) in absence and presence 2 mM of Cys, Hcy, GSH and CN^- in aq-HEPES-CH_3CN (9:1, v/v) medium of pH 7.2 at 15^0C. ∂_{Ext} = 530 nm. ∂_{Mon} = 565 nm.

MTT assay:

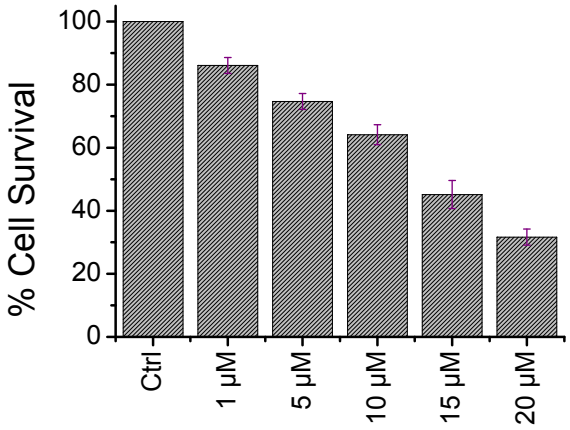

Fig. S 22: MTT assay to determine the cell viability percentage in presence of **ER-F** in Hct116 colon cancer cells.

The *in vitro* cytotoxicity of **ER-F** on Hct116 cells (Colon cancer cell) were determined by conventional MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Hct116 colon cancer cells (7×10^3) were seeded in each well of a 96 well plate and cultured in a 37°C incubator supplied with 5% CO_2. Cells were maintained in DMEM medium, supplemented with 10% Foetal Bovine Serum and 100 Units of Penicillin Streptomycin antibiotics. After 24 hours the cells were treated with different concentrations of the **ER-F** in triplicates for 12 hours. After the treatment, cells were added with 0.5 εg/ml of MTT reagent. The plate was then incubated for 4 hours at 37°C. 100 εl of Isopropyl Alcohol was added to each well. Optical density was measured at 570 nm using Multiskan Go (Thermo Scientific) to find the concentration of the cell inhibition. IC_{50} value has been calculated to be 14 εM.

The formula used for the calculation of the MTT assay for evaluation of the cell viability was as follows:

Cell viability (%) = (Means of absorbance value of treated group/ Means of absorbance value of untreated control) × 100.

CLSM images of HepG2 cells with N-acetyl cysteine (NAC) drug:

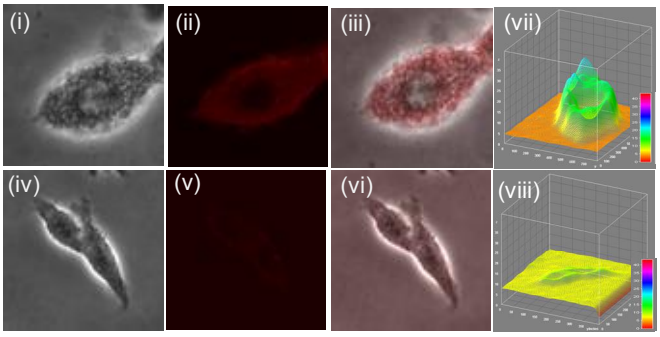

Fig. S 23: CLSM images of live HepG2 cells: (i) - (iii): bright field, dark field and overlay images of cells treated first with 1 mM NEM and then treated with 25 εM NAC for 1h, followed by incubation of 1 εM of **ER-F** for 20 min, respectively; (vii): 3D intensity profile plot of image (ii) ; (iv) - (vi): Cells treated with 1 mM NEM, and followed by incubation of 1 εM of **ER-F** for 20 min; (viii): 3D intensity profile plot of image (v).

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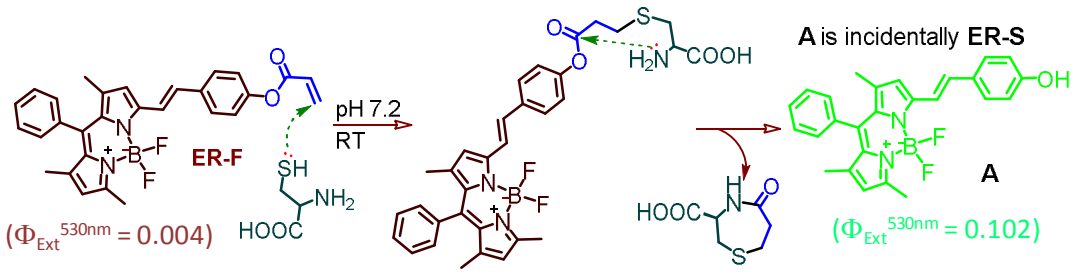
Sensing mechanism of probe ER-F for Cys:



SI scheme 1: Proposed mode of sensing mechanism.

To a 100 ml r.b flask **ER-F** (70 mg, 0.14 mmol) and cysteine (1.21 eqv.) were added in methanol-water mixture (7:3, v/v). Then Et_3N (40 εl) was added to it and allowed to stir at RT for 1h. After that solvent was removed under vacuum and the crude was subjected to column chromatography using 10% ethyl acetate in hexane mixture to get product **A** (yield: 42 %) and 8 mg cyclic bi-product. All the products were analysed by HRMS and ^{1}H NMR spectra. ^{1}H NMR spectrum of **A** was found to be identical with **ER-S**.

HRMS spectrum of Bi-Product:

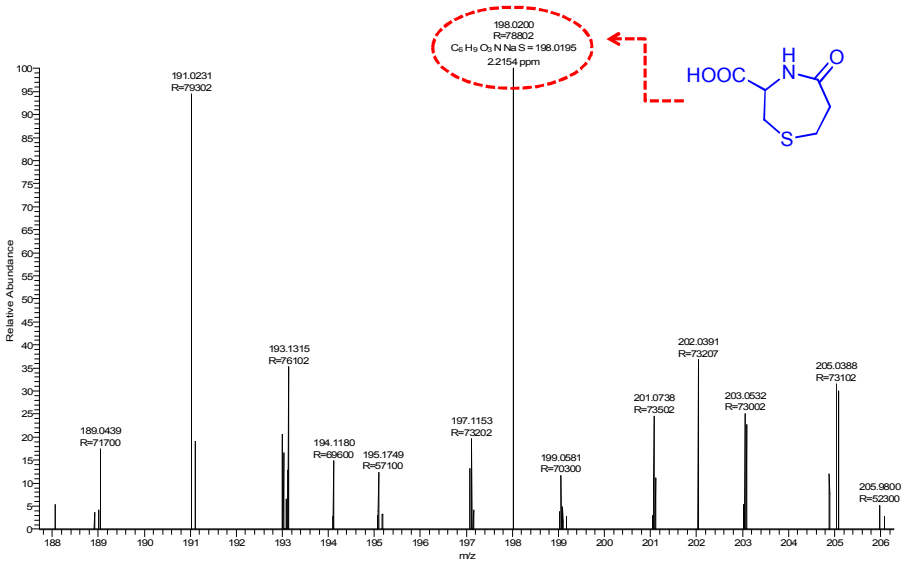


Fig. S 24: HRMS spectrum of Bi-product.

^{1}H NMR spectrum of Bi-product:

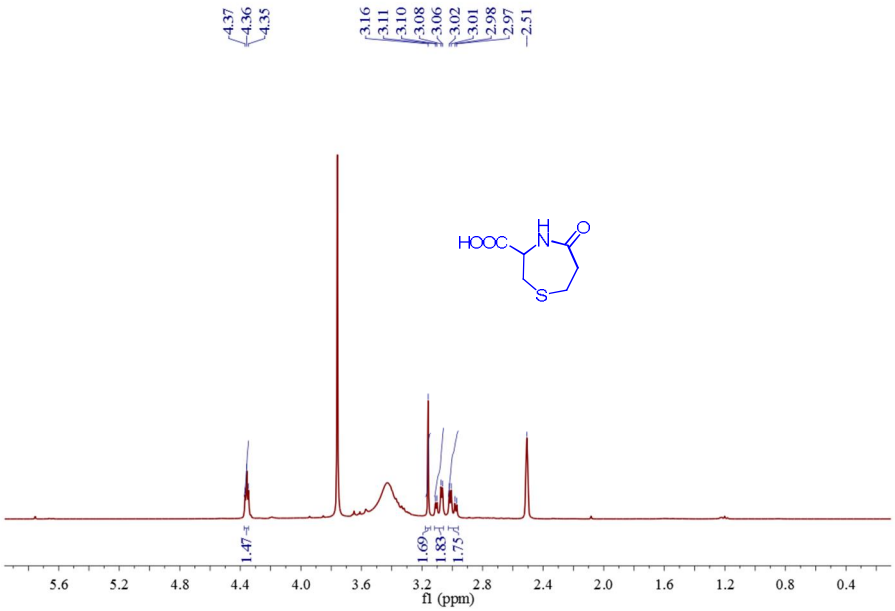


Fig. S 25: ^{1}HNMR spectrum of Bi-product in DMSO-d_6.

HRMS spectrum of ER-F with Cysteine:

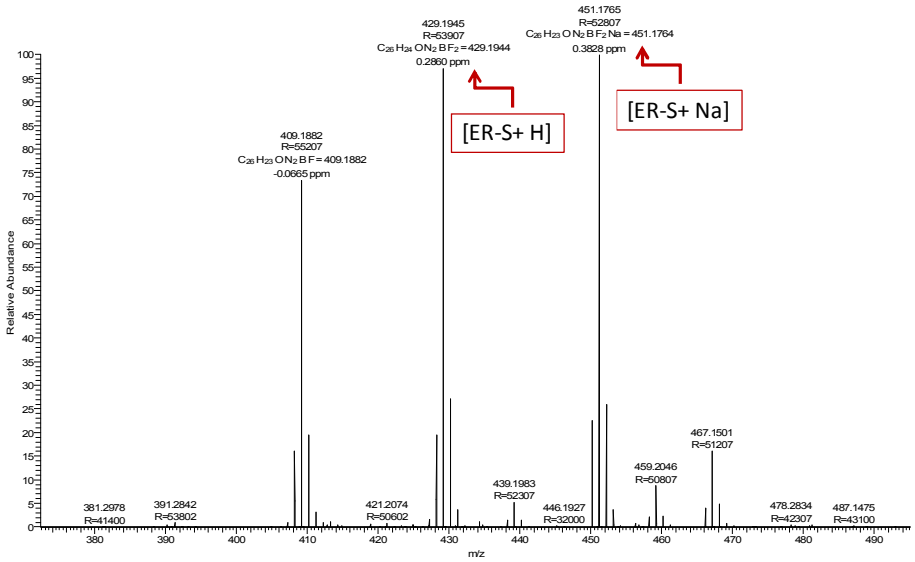


Fig. S 26: HRMS spectrum of **ER-F** with Cysteine. HRMS spectrum showing **ER-S** was produced by the reaction of **ER-F** with Cys.

^{1}H NMR spectrum of 'A' isolated from reaction of ER-F with Cys:

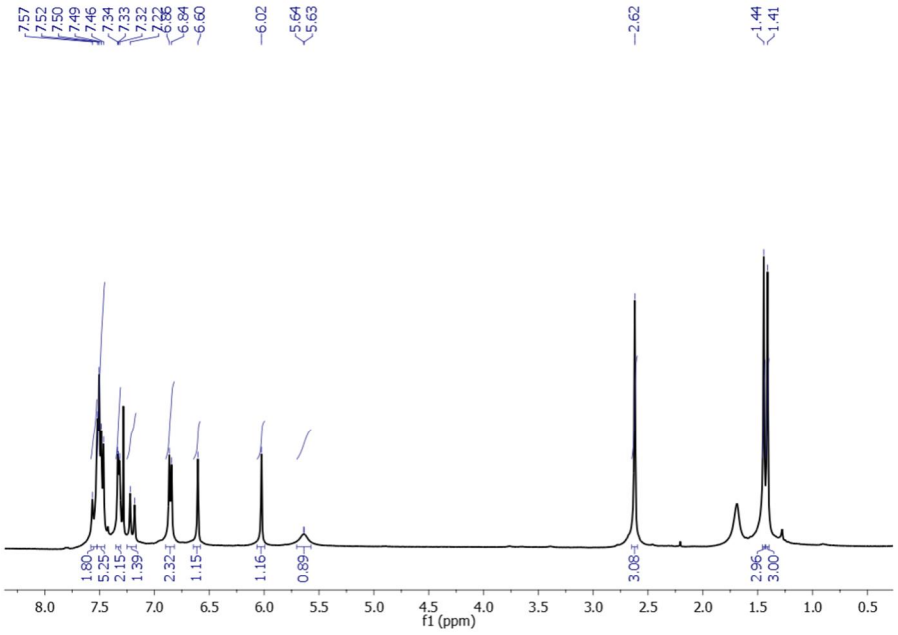


Fig. S 27: ^{1}H NMR spectrum of ó**A'** isolated from reaction of **ER-F** with **Cys** in $CDCl_3$, which is found to be identical with compound **ER-S**.

References:

1. S. Saha, H. Agarwalla, H. Gupta, M. Baidya, E. Suresh, S. K. Ghosh and A. Das, *Dalton Trans*., 2013, **42**, 15097ï 15105.
2. Q. Zhang, D. Yu, S. Ding and G. Feng, *Chem. Commun*., 2014, **50**, 14002-14005.
3. G. M. Sheldrick, SAINT 5.1 ed., Siemens Industrial Automation Inc., Madison, WI, 1995.
4. SADABS, Empirical Absorption Correction Program, University of Göttingen, Göttingen, Germany, 1997.
5. G. M. Sheldrick, SHELXTL Reference Manual: Version 5.1, Bruker AXS, Madison, WI, 1997.
6. G. M. Sheldrick, SHELXL-97: Program for Crystal Structure Reýnement, University of Göttingen, Göttingen, Germany, 1997.
7. U. G. Reddy, H. Agarwalla, N. Taye, S. Ghorai, S. Chattopadhyay and A. Das, *Chem commu*., 2014, **50**, 9899-9902.
8. Goswami, K. Aich, S. Das, S. B. Roy, B. Pakhira and S. Sarkar, *RSC Adv*., 2014, **4**, 14210-14214.
9. F. Wang, L. Zhou, C. Zhao, R. Wang, Q. Fei, S. Luo, Z. Guo, H. Tiana and W.-H. Zhu, *Chem. Sci*., 2015, **6**, 2584ï 2589.