Electronic Supplementary Material (ESI) for Chemical Communications.

Electronic Supplementary Information

Fluorescent chemodosimeter for quantification of Cystathionine-γ-synthase activity in plant extracts and imaging of endogenous biothiols

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Materials

Terephthaladehyde and ammonium acetate were purchased from Sigma Aldrich. Acetic anhydride, Acetonitrile (DMSO, HPLC grade), nitro methane and different amino acids were of reagent grade (S. D. Fine Chemical, India) and used as received without any further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 200/500 MHz FT NMR (Model: Avance-DPX 200/400/500) using trimethylsilane (TMS) as an internal standard. FTIR spectra were recorded as KBr pellets in a cell fitted with a KBr window, using a Perkin-Elmer Spectra GX 2000 spectrometer. ESI-Ms measurements were carried out on a Waters QTof-Micro instrument. Solution pH was evaluated using Mettler Toledo FEP20 pH meter. Absorption spectra were recorded using a Perkin Elmer Lambda 950 UV-Vis spectrophotometer equipped with cell holder having path length of 1cm. Fluorescence spectra were recorded on PTI QuantaMaster 400 spectrophotometer.

Synthesis

Synthesis of 2: The benzoxazinone intermediate **1** was prepared by following the previously reported method.[S1] 0.55 g (2.69 mmol) of the benzoxazinone intermediate **1**was suspended in 3.5 mL of acetic anhydride. To this suspension, 0.54 g (4.04 mmol) of terephthaldehydewas addedand temperature and the reaction mixture were maintained at 140°C for 4 h. The mixture was then cooled to room temperature to give a red precipitate, whichwas filtered and the residue was washed with cold diethyl ether to get the desired product in pure form. No further purification was required and an isolated yield of **2** was 360 mg (41%). ^{1}H NMR (500 MHz, $CDCl_3$, 25^{o}C, TMS as internal standard and values as provided in ppm): 10.04 (s, 1H (H_{CHO})), 7.96 (d, 1H,*J*=15 Hz (CH=CH)), 7.91 (d, 2H, *J*= 8 Hz (C_6H_4)), 7.78 (d, 2H, *J*=8 Hz (C_6H_4)), 7.59 (d, 1H,*J*= 15 Hz (CH=CH)),7.6 (d, 1H, *J* = 8.5 Hz (C_6H_3)), 6.75 (dd, 1H, *J*= 6.5 Hz, 2.5 Hz(C_6H_3)), 6.485 (s, 1H, (C_6H_3)), 3.14 (s,

6H ($N(CH_3)_2$)). ^{13}C NMR (100 MHz, $CDCl_3$, 25°C, TMS): 214.56, 167.61, 165.57, 10.84, 153.33, 151.92, 145.1, 143.17, 137.85, 134.96, 132.14, 129.95, 113.02, 96.5, 25.59. IR (KBr) ν_{max}/cm^{-1}: 1718, 1688, 1609, 1382. ESI-Ms (*m/z*): 318.80 [M - H^+]. Elemental analysis: $C_{19}H_{16}N_2O_3$ calculated C (71.24), H (5.03), N (8.74); found C (71.51), H (5.0), N (8.81).

Synthesis of L

Initially, 160 mg (0.5 mmol) of **2**was dissolved in 10 ml of nitromethane. To this, 385 mg (5 mmol) of ammonium acetate was added,and the resulting mixturewas maintained at 80 °C for 4 h. On cooling to room temperature a precipitate appeared, which was washed with water and then with 10 ml of diethyl ether. The residuewas further extracted from DCM/water, and the DCM layer was dried for isolating the desired pure product as brown solid. Yield; 130 mg (71%),^1H NMR (600 MHz, $CDCl_3$, δ (ppm)) δ: 7.98 (d, J = 13.6 Hz, 1H), 7.60 (s, 1H), 7.59 (d, J = 13.6 Hz, 1H), 7.56 (d, J = 8.3 Hz, 2H), 7.51 (dd, J = 12.3, 3.9 Hz, 3H), 7.29 (d, J = 8.8 Hz, 1H), 7.17 (d, J = 16.2 Hz, 1H), 6.59 (dd, J = 8.8, 2.5 Hz, 1H), 3.42 (s, 6H). ^{13}C NMR (150 MHz, CDCl3, δ (ppm)) δ:164.35, 152.20, 150.79, 142.30, 140.55, 135.09, 134.68, 134.45, 132.84, 129.21, 128.50, 128.21, 127.93, 122.03, 121.52, 119.48, 40.47. IR ν_{max}/cm^{-1}: 2914, 1728, 1616, 1467. HRMS (*m/z*): Calculated $[C_{20}H_{17}N_3O_4+H]^+$ 364.1292 and found 364.1285.

Preparation of solutions

1.0 mM stock solution of **L** in acetonitrile was prepared and used for further studies after appropriate dilution using 10 mM HEPES buffer to achieve the effective concentration of 1.0×10^{-5}M in 10 mM aq. HEPES buffer-CH_3CN (7:3, v/v; pH 7.2). Stock solutions of 100 mM of different amino acids were prepared in a 10 mM aq. HEPES buffer-CH_3CN (7:3, v/v; pH 7.2) medium. Stock solutions were further diluted with buffer solution as per requirement for a specific experiment.

^{1}H NMR of **2**

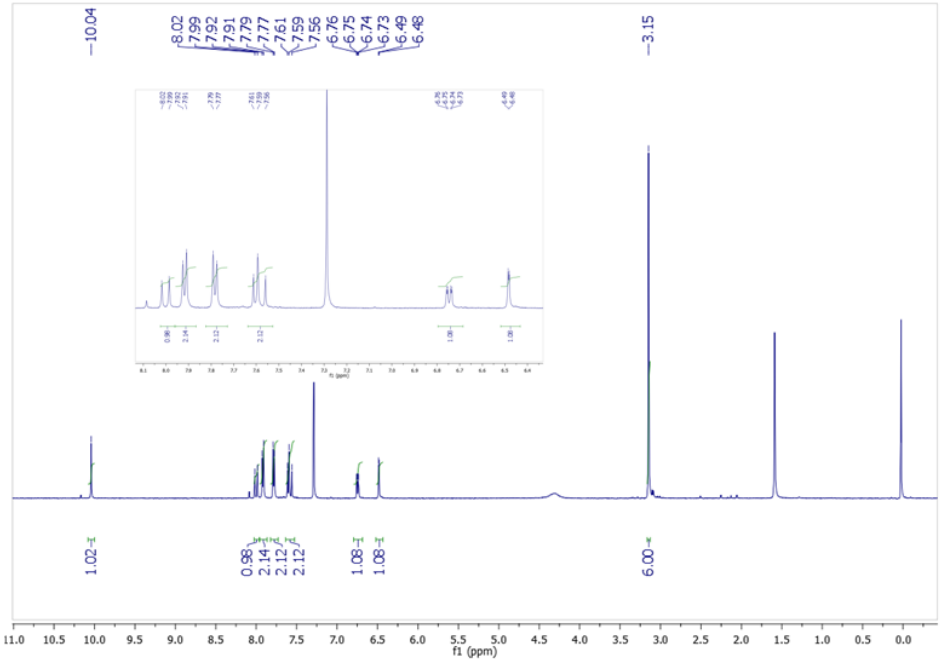


Fig. S1*^{1}H NMR spectra of **2** in $CDCl_3$.*

^{13}C NMR spectra of **2**

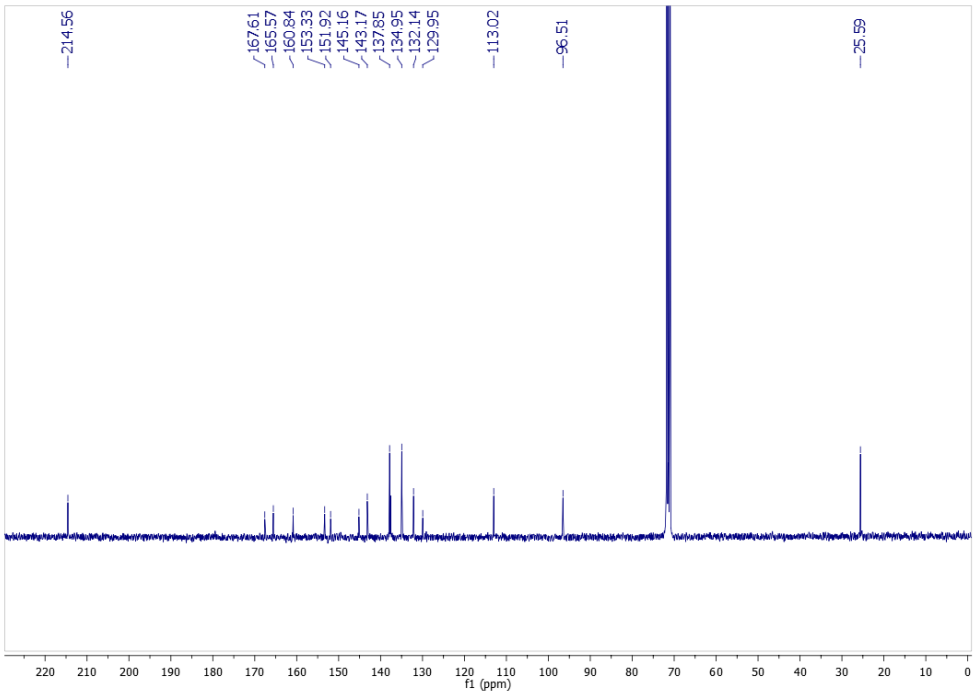


Fig. S2*^{13}C NMR spectra of **2** in $CDCl_3$.*

Mass spectra of **2**

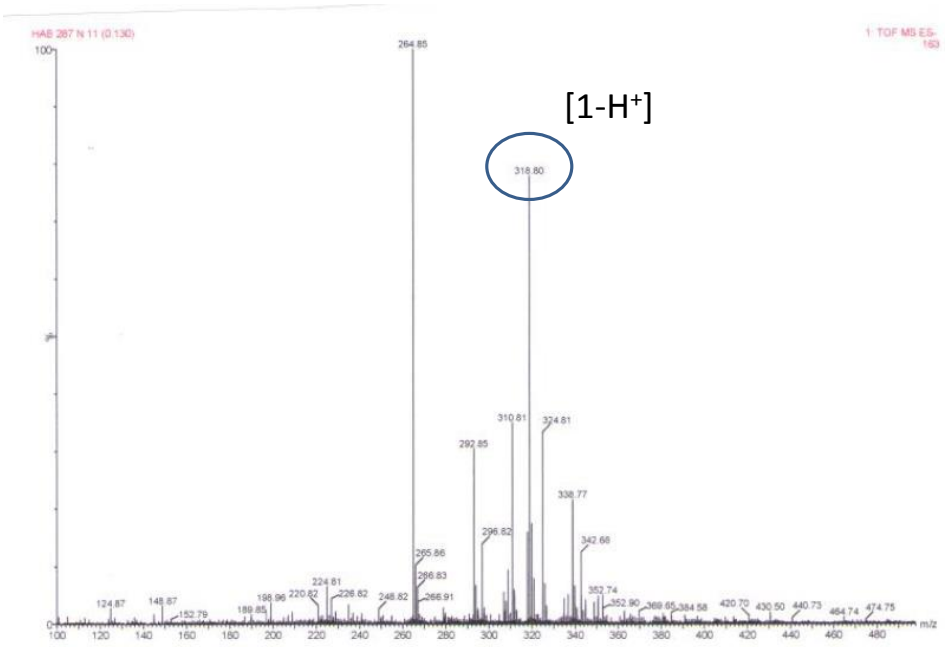


Fig. S3 *Mass spectra of **2**.*

^{1}H NMR of **L**

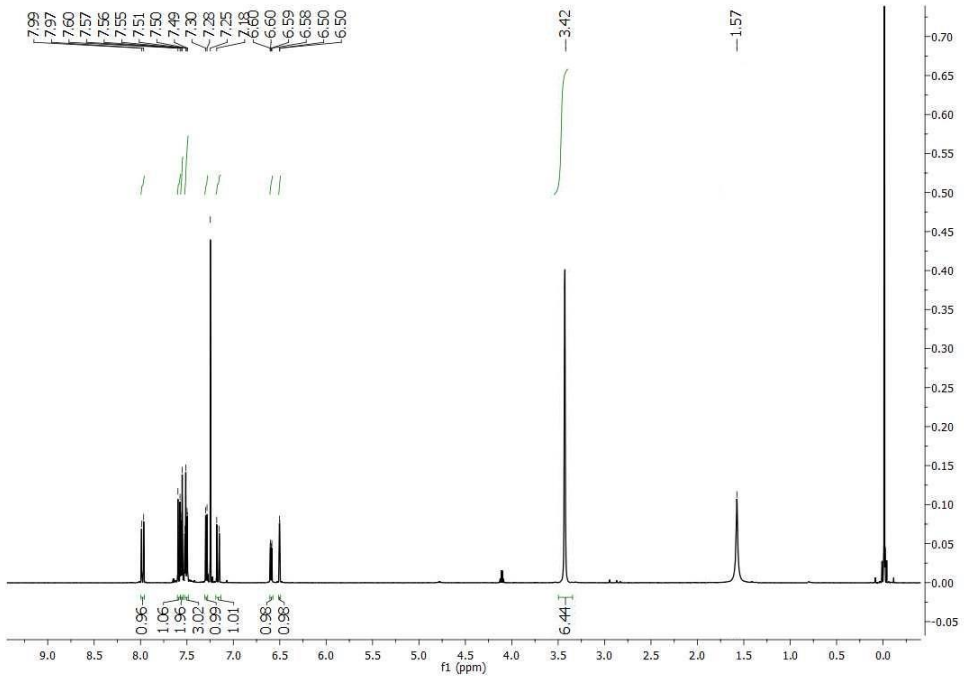


Fig. S4 *^{1}H NMR spectra of **L** in $CDCl_3$.*

^{13}C NMR of **L**

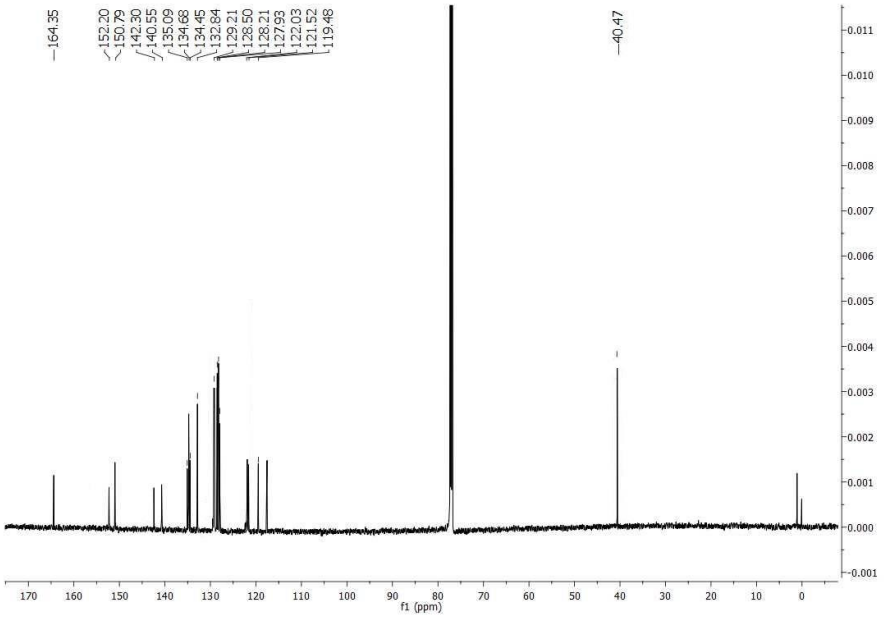


Fig. S5 *^{13}C NMR spectra of **L** in $CDCl_3$.*

FTIR spectra of **L**

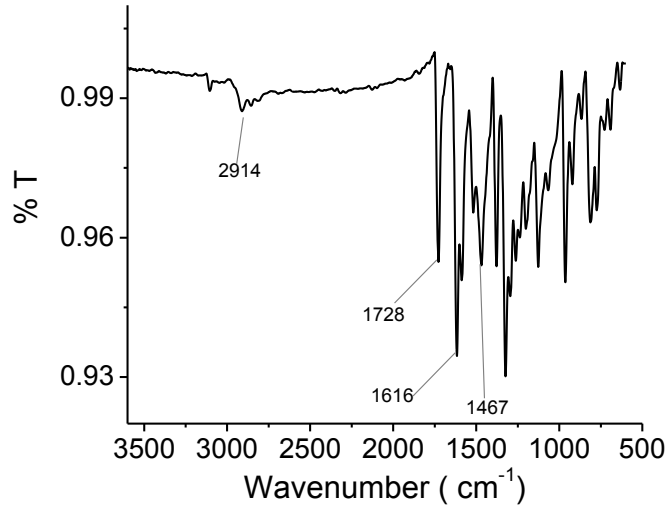


Fig. S6 *FTIR spectra of **L**.*

Mass spectra of **L**

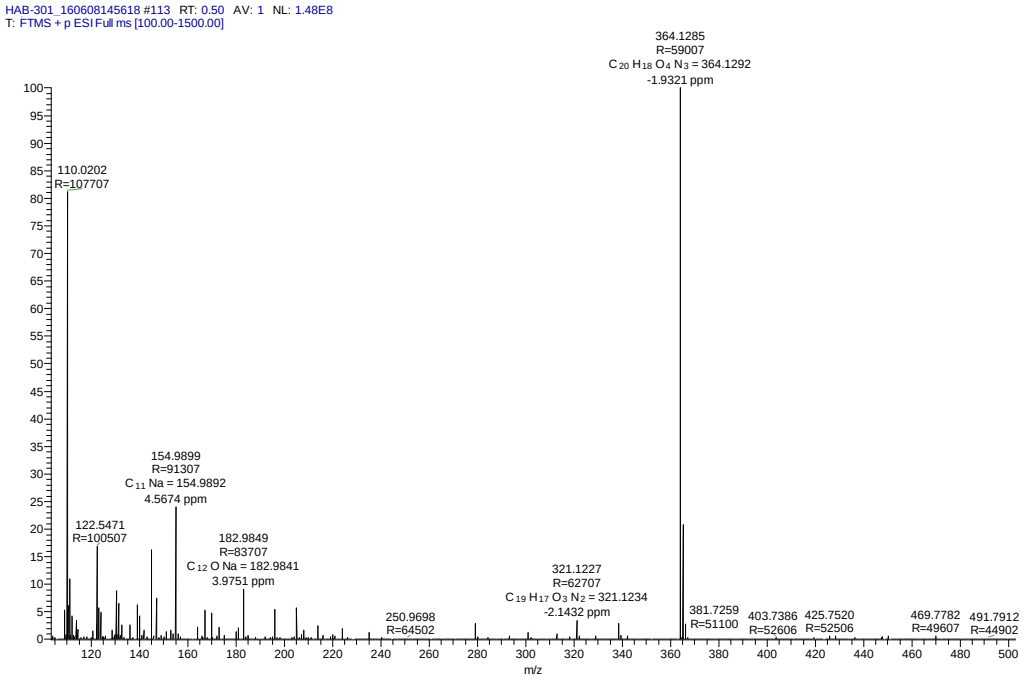
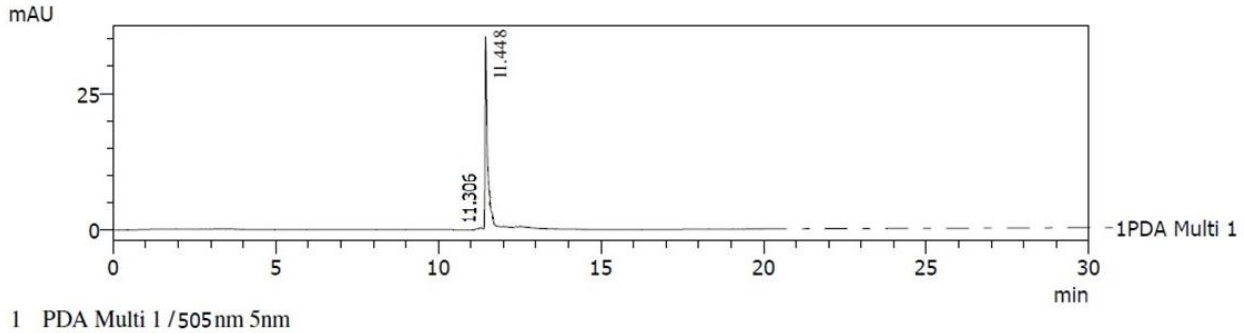


Fig. S7 *HRMS spectra of **L**.*

HPLC trace of L.



1 PDA Multi 1 / 505 nm 5nm

PeakTable

PDA Ch1 505nm 5nm

Peak#	Ret. Time	Area	Area %
1	11.306	1731	0.885
2	11.448	193870	99.115
Total		195601	100.000

Fig. S8 *HPLC trace of **L**.*

Elemental Analysis

Table ST2: Elemental Analysis of **L**.

CHN analysis of **L**	C	H	N
Chem Biodraw analysis data for **L**	66.11 %	4.72 %	11.56 %
Experimental analysis data for **L**	66.16%	4.78%	11.47%

Spectroscopic Study

Solvent dependent UV-Vis spectra of **L**

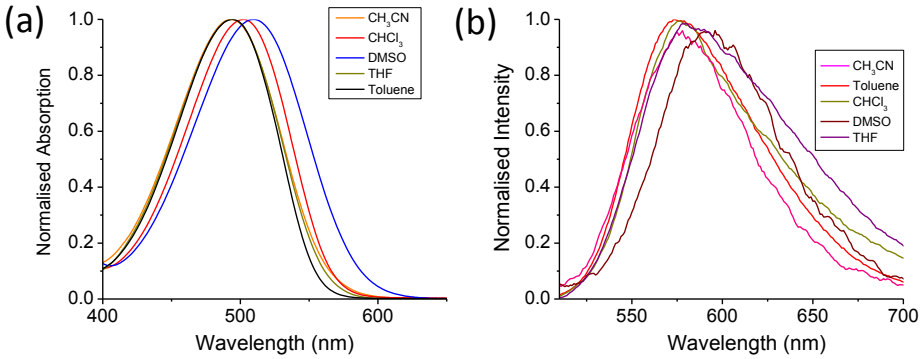


Fig. S9 *Normalized (a) Absorption spectra (b) emission spectra of* ***L*** *(10 μM) in solvents of different polarity.*

Absorption spectra of **L** in presence of different anions

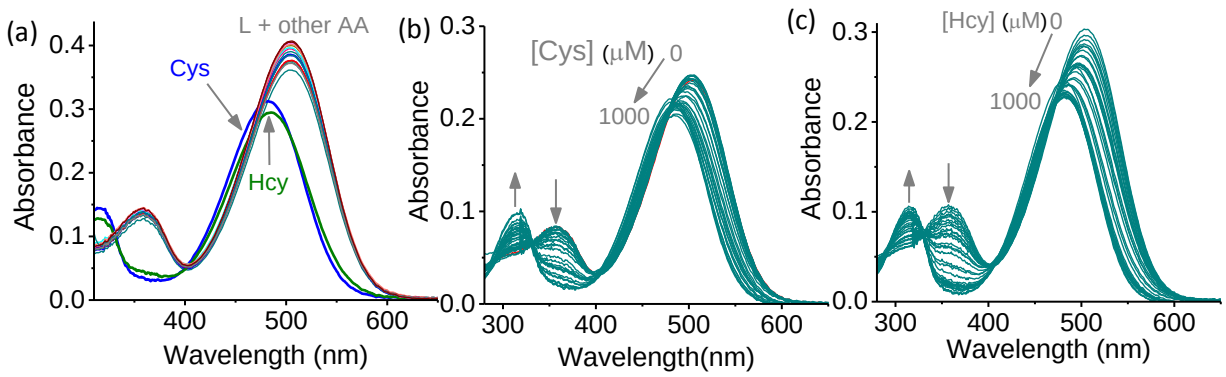


Fig.S10 *Absorption spectra of **L** (10 μM) (a) in absence and presence of 100 mole equivalent of different anions, cations and amino acids, (b) with varying [Cys] (0-100 mole equivalent), (c) with varying [Hcy] (0-100 mole equivalent) in aq. HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2).*

The absorption spectrum of **L** was recorded in absence and presence of 100 mole equivalent of different common anions (X: F^-, Cl^-, Br^-, $H_2PO_2^-$, OAc, HSO_4^-, CN^-, SO_3^{2-} and HSO_3^-), cations (Na^+, Ca^{2+}, Mg^{2+},Fe^{2+}, Fe^{3+}, Cu^{2+}, Cr^{3+}, Ni^{2+}, Zn^{2+}) and natural amino acids (AAs: histidine (His), leucine (Leu), methionine (Met), isoleucine(Ile), phenylalanine (Phe), tryptophon (Trp), tyrosine (Tyr), valine (Val), serine (Ser), alanine (Ala), arginine (Arg), glycine (Gly), glutamine (Gln), proline (Pro), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), lysine (Lys), methionine (Met)), and biothiols like glutathione (GSH), Homocysteine (Hcy) and Cysteine (Cys) (Figure 2a). A hypsochromic shift of ~ 25nm was observed for the 505 nm band only when spectra were recordedin the presence of Cys and Hcy and this attributed to a visually detectable change in solution colour from red to orange. Other analytes failed to induce any detectable change in absorption spectra. The observed hypsochromic shift in absorption spectra supports the formation of Michael adducts with Cys and Hcy which interrupted the extended conjugation as well as disfavor the push-pull effect and the ICT process.

Emission spectra of probe L different equivalent of Cys.

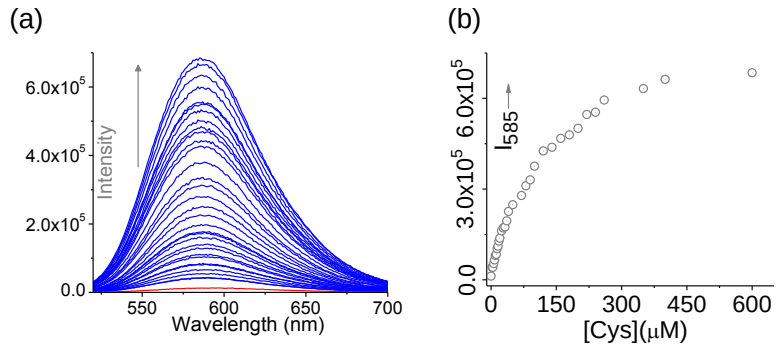


Fig. S11 *Emission spectra of probe L (10 μM) (a) with 0 - 100 mole equivalent of Cys. (b) show a change in intensity at 585 nm as a function of [Cys] in aq. HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2). For all studies, λ_{Ext} = 505 nm.*

Emission spectra of **L** in presence of various concentration of GSH

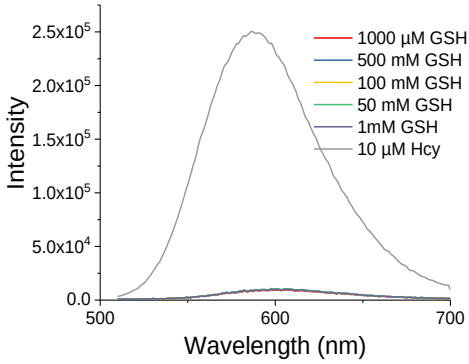


Fig. S12 *Emission spectra of probe L (10 μM) (a) with 0 - 1000 mole equivalent of GSH in aq. HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2). λ_{Ext} = 505 nm.*

Calculation of Quantum yield

The quantum yields are calculated based on following equation when both sample and reference were excited at a point where they have same absorbance. Here Φ stands for quantum yield, η for refractive index, I for integrated fluorescence intensity for sample and reference.

$$\Phi_s = \Phi_{ref} \times \left(\frac{\eta_s^2}{\eta_{ref}^2}\right) \times \left(\frac{I_s}{I_{ref}}\right)$$

Coumarin 343 in ethanol (Φ=0.63 in ethanol) is used as reference. η for sample and reference are 1.33 and 1.36 respectively.

For L $\Phi_L = 0.63 \times [(1.33)^2/ (1.36)^2] \times [602330/30649462] = 0.01$

For L $\Phi_L = 0.63 \times [(1.33)^2/ (1.36)^2] \times [11417051/30649462] = 0.22$

Calculation of Lowest Detection Limit

The detection limit of Cys/Hcy was calculated by following equation 1

DL= K. σ /s …..Equation 1

Where K= 3, σ is the standard deviation of blank measurement, s is the slope of intensity *vs.* [Cys/Hcy] plot.

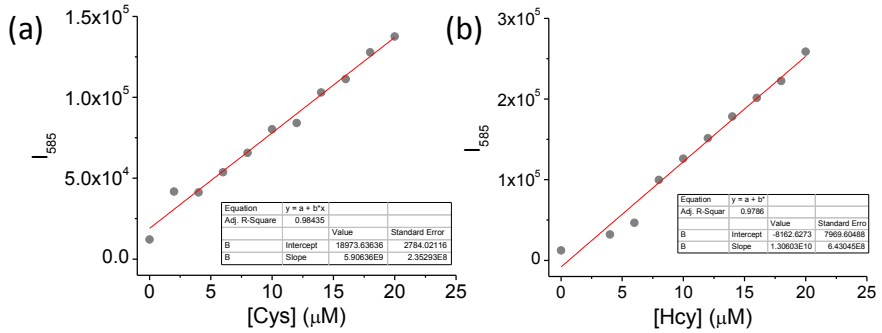


Fig. S13 *Calibration curve for determining lowest detection limit for (a) Cys (b) Hcy. Measurements were performed in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using* $\lambda_{Ext}/\lambda_{Em}$*: 480/585nm.*

Here σ is found to be 40.42

DL calculated for Cys is 2.05×10^{-8} M.

DL calculated for Hcy is 0.93×10^{-8} M.

Interference Study

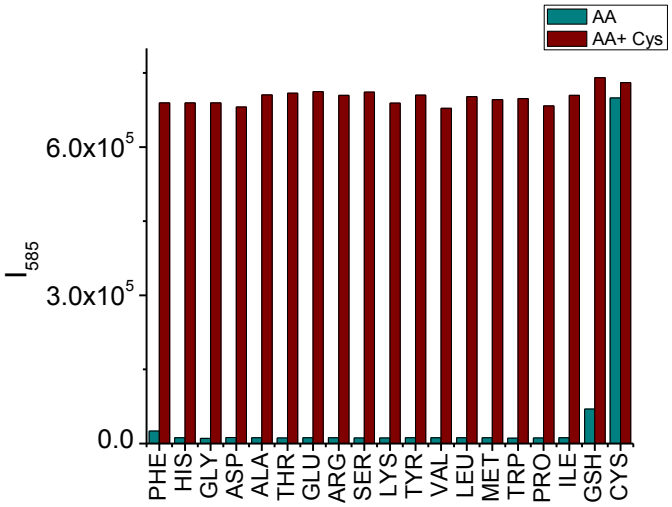


Fig. S14 *Interference study in presence of* ***L*** *(10 mM) in presence of 100 mole equivalent of Cys and 200 mole equivalent of other aminoacids in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using* $\lambda_{Ext}/\lambda_{Em}$*: 480/585nm.*

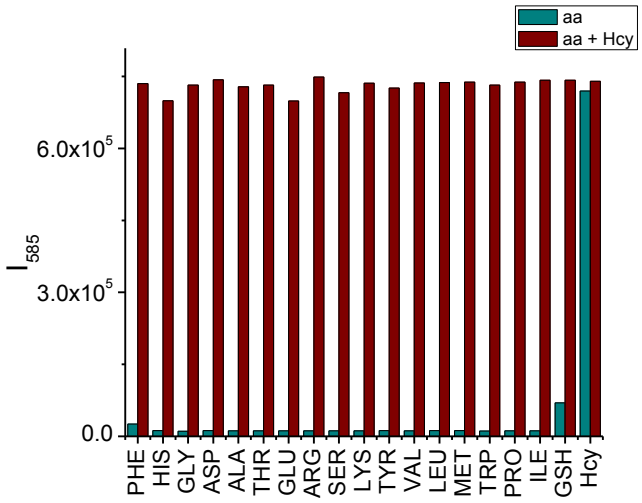


Fig. S15 *Interference study in presence of* ***L*** *(10 mM) in presence of 100 mole equivalent of Hcy and 200 mole equivalent of other aminoacids in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using* $\lambda_{Ext}/\lambda_{Em}$*: 480/585nm.*

Time dependent Study

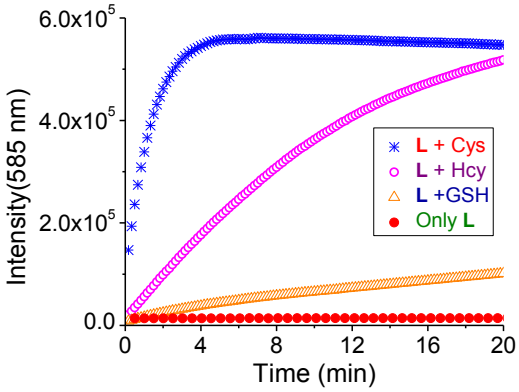


Fig. S16 *Time dependent emission change (585 nm) in absence and presence of 20 equivalents of Cys, Hcy and GSH of probe* ***L*** *(10 μM) in HEPES buffer: CH_3CN medium (7 :3, v/v; pH 7.2) excited at 480 nm.*

Kinetic Studies

Time dependent studies of (10 μM) of **L** with different concentrations of Cys/Hcy were carried out by mixing, and monitored by fluorescence measurements in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using $\lambda_{Ext}/\lambda_{Em}$: 480/585nm. 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:

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

Where, I_t 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.

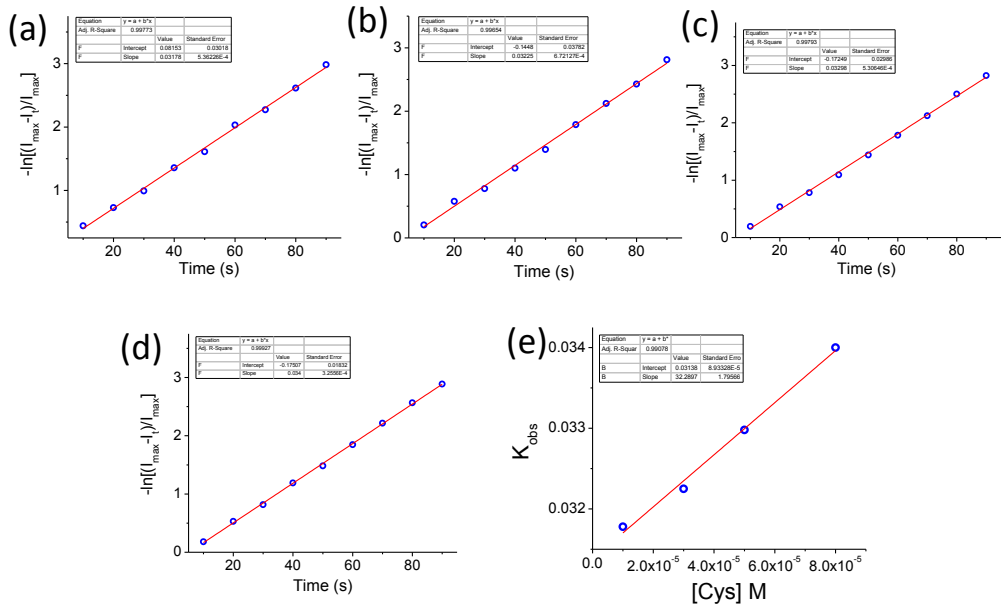


Fig. S17 *Plot of –ln[(*I_{max}*-*I_t*)/*I_{max}*)] vs time with 10 μM of* ***L*** *in presence of different concentration of Cys (a)* 1×10^{-5} *M (b)* 3×10^{-5} *M (c)* 5×10^{-5} *M (d)* 8×10^{-5}*M and (e)* K_{obs} *vs [Cys] plot in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using* $\lambda_{Ext}/\lambda_{Em}$*: 480/585nm.*

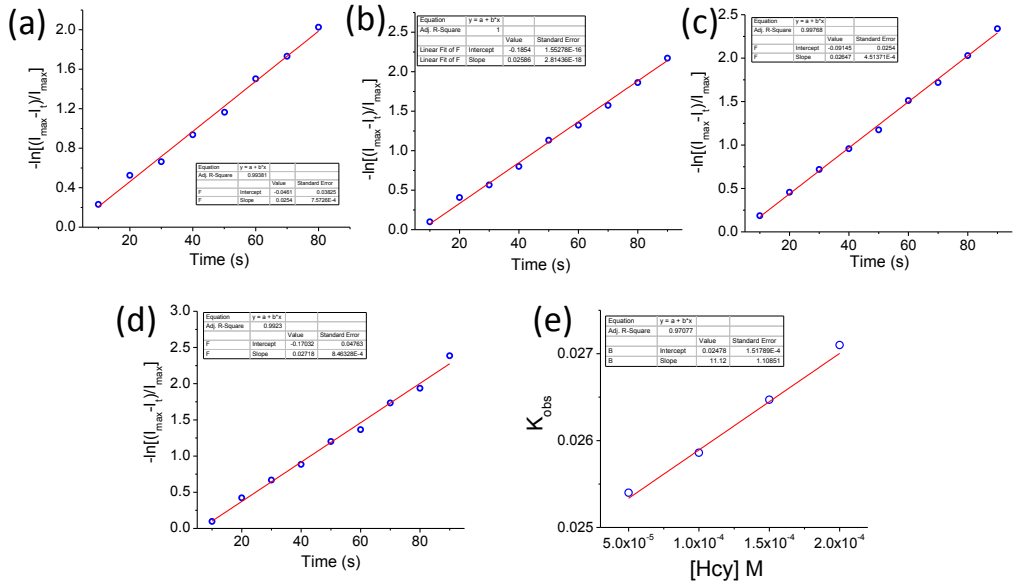


Fig. S18 *Plot of –ln[(*I_{max}*-*I_t*)/*I_{max}*)] vs time with 10 μM of* ***L*** *in presence of different concentration of Hcy (a)* 5×10^{-5} *M (b)* 10×10^{-5} *M (c)* 15×10^{-5} *M (d)* 20×10^{-5} *M and (e)* K_{obs} *vs [Hcy] plot in 10 mM HEPES aq. Buffer: acetonitrile (7:3, v/v) pH 7.2 using* $\lambda_{Ext}/\lambda_{Em}$*: 480/585nm.*

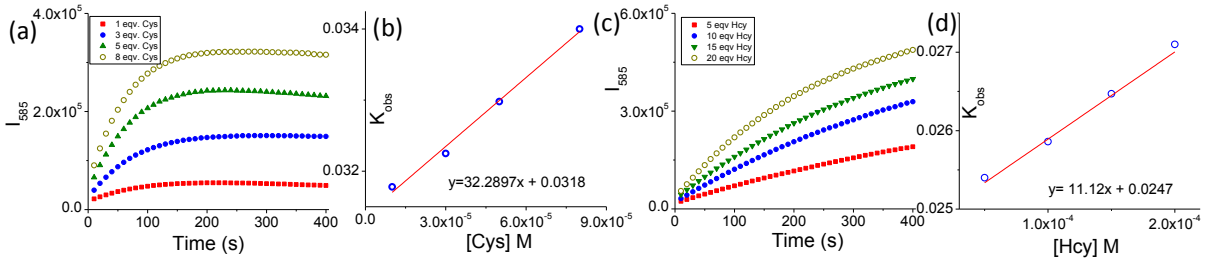


Fig. S19 *Time dependent spectra of* ***L*** *(10 μM) in presence of different concentration of (a) Cys (c) Hcy; and* K_{obs} *vs Concentration plot of (b) Cys and (d) Hcy, in aq. HEPESbuffer:*CH_3CN *medium (7:3, v/v; pH 7.2),* λ_{exc}/ λ_{mon} *=480/585 nm*

pH dependent Study

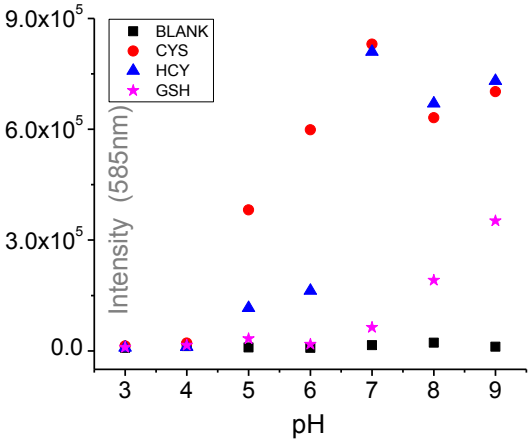


Fig. S20 *pH dependent emission change (585 nm) in absence and presence of 20 equivalents of Cys, Hcy and GSH of probe* ***L*** *(10 μM) in HEPES buffer:* CH_3CN *medium (7 :3, v/v; pH 7.2) excited at 480 nm.*

Mass Spectra of L with Cys

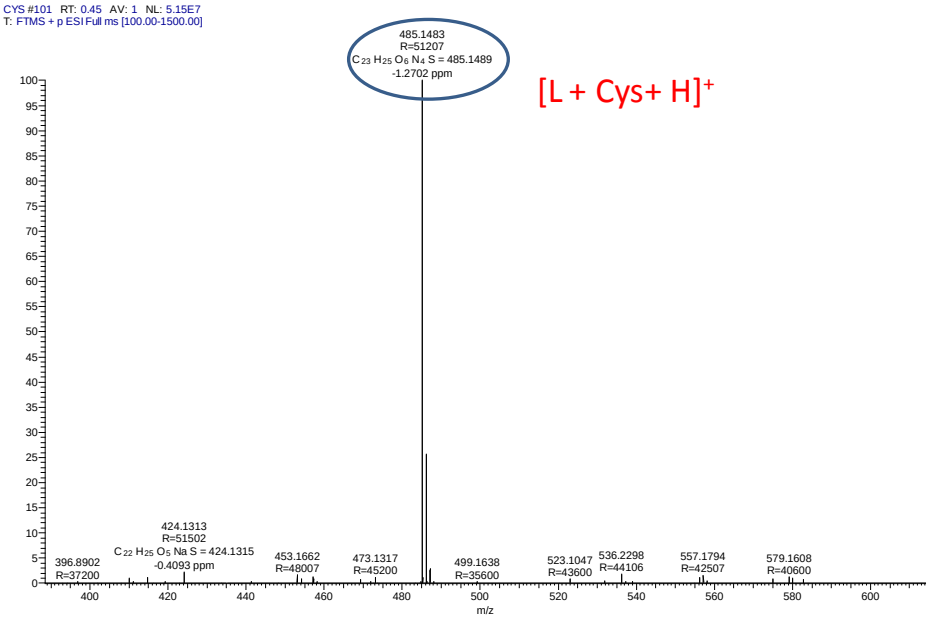


Fig. S21 *HRMS spectra of* ***L*** *in presence of Cys.*

Mass Spectra of L with Hcy

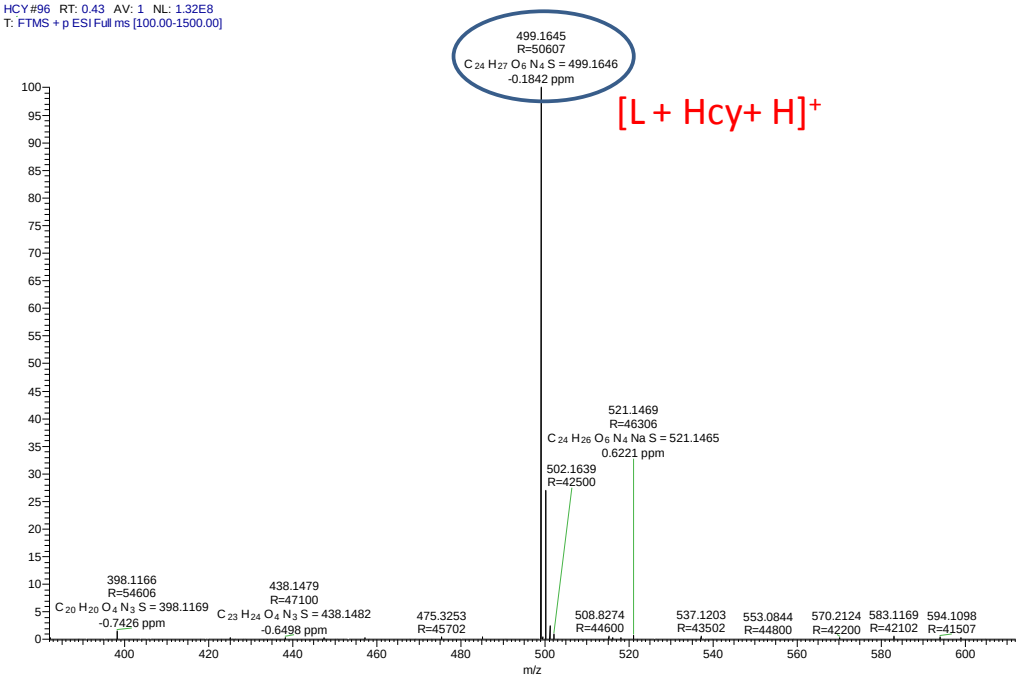


Fig. S22 HRMS *spectra of* ***L*** *in presence of Hcy.*

^{1}H NMR Spectra of L with Cys and Hcy

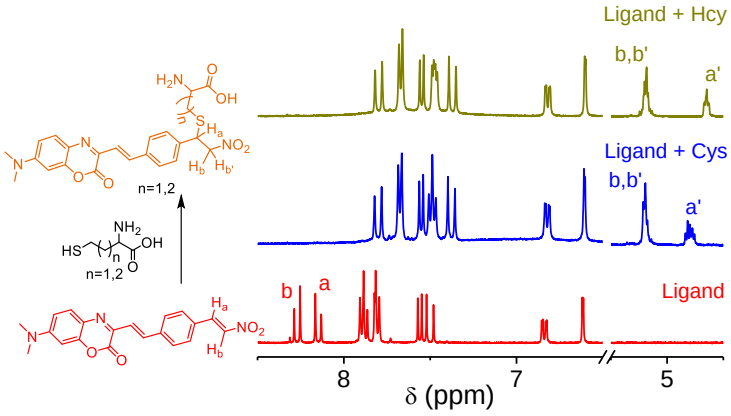


Fig. S23 *Partial ^{1}H NMR spectra of probe **L** in absence and presence of 100-mole equivalents of Cys and Hcy in DMSO-d_6. Adduct formation between **L** and Cys/Hcy is also shown.*

Proposed Michael addition reaction between **L** and the Cys and Hcy was also established based on the results of the ^{1}H NMR spectroscopy. Spectra for **L**were recorded in the absence and presence of 100 mole-equivalents of Cys and Hcy, respectively, in DMSO-d_6. Spectrum for **L** showed signals at 8.14 and 8.27 ppm due to the protons associated with the nitroolefin group (H_a, H_b). On addition of Cys, this signals disappeared, and a new set of peak appeared at 4.79 and 5.19 ppm. Similarly, for Hcy new set of signals appeared at 4.64 and 5.18 ppm. These confirmed the 1,4-adduct formation between the corresponding thiolate moiety of Cys/Hcy and the nitroolefincenter of the molecular probe, **L**. As anticipated, other aromatic protons showed an upfield shift and these could be rationalized based on the loss of extended conjugation.

Michaelis constant for CgS using probe L.

Michaelis constant was evaluated by using series of enzyme assay by varying OPH and sodium sulfide concentration from (0.1 – 0.5 mM) with fixed amount of CgS. Initial rates were evaluated from the plot of Log [F_t -F_0] vs time (in sec), where F_t is the luminescence intensity for L at 600 nm (λ_{Ext} = 485 nm) at time t and F_0 is the initial luminescence intensity. Linear plots were obtained for OPH (0.1 – 0.5mM). A plot of 1/V vs. 1/[S] would give intercept of $1/k_2[E]_0$ and slope of $Km/k_2[E]_0$. Thus, {slope/intercept} would result K_m.

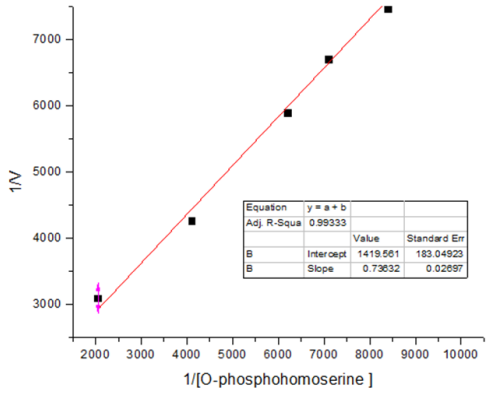


Fig. S24 *plot of 1/v vs 1/[o-phosphohomoserine]*

Michaelis constant for CgS using probe L_1.

Michaelis constant was evaluated by using series of enzyme assay by varying OPH and Na_2S concentration from (0.1 – 0.5 mM) with fixed amount of CgS. Initial rates were evaluated from the plot of Log [F_t -F_0] vs time (in sec), where F_t is the luminescence intensity for L_1 at 678 nm (λ_{Ext} = 620 nm) at time t and F_0 is the initial luminescence intensity. Linear plots were obtained for OPH (0.1 – 0.5mM). A plot of 1/V vs. 1/[S] would give intercept of $1/k_2[E]_0$ and slope of $Km/k_2[E]_0$. Thus, {slope/intercept} would result K_m.

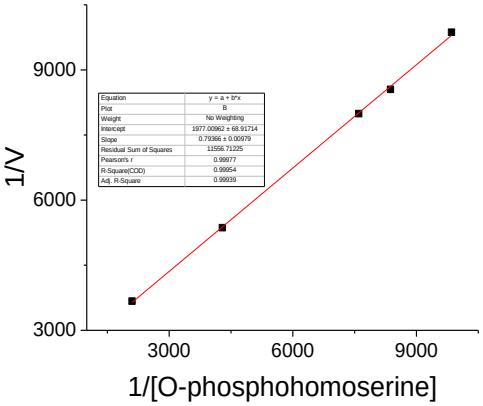


Fig. S25 *plot of 1/v vs 1/[o-phosphohomoserine]*

Plot of $\log(F_t - F_0)$ vs. time for evaluating k_{obs}^{CgS} (s^{-1}) by using **L**

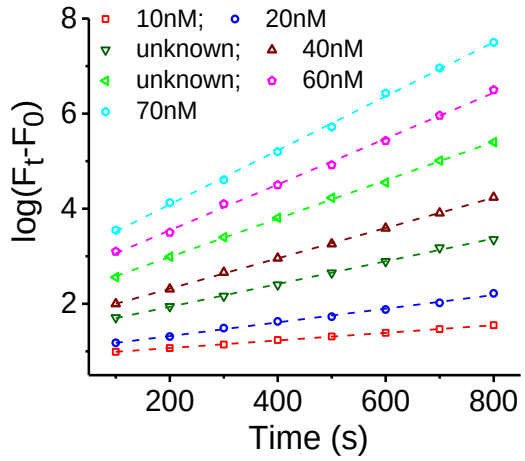


Fig. 26 *Plot of $\log(F_t - F_0)$ vs. time for evaluating k_{obs}^{CgS} (s^{-1}) for known, but different [CgS] (10 nM, 20 nM, 40 nM, 60 nM and 70 nM) and unknown [CgS] added to TLE.*

Comparison studies with fluorescamine assay method

In order to corroborate our method, we have compared our results with existing fluorescamine based assay method.[S2] Fluorescamine assay is a well-known technique used for the measurement of enzyme. The free amine which is produced in the enzymatic reaction reacts further with fluorescamine reagent and gives turn-on fluorescence signal. Fluorescamine assay was performed

for three different unknown samples in aq. HEPES buffer:CH_3CN medium (7:3, v/v; pH 7.2). Here fluorescamine was excited at 390 nm and emission was recorded at 475nm. Concentration of the unknown sample was determined by using the calibration curve of the standard protocols.

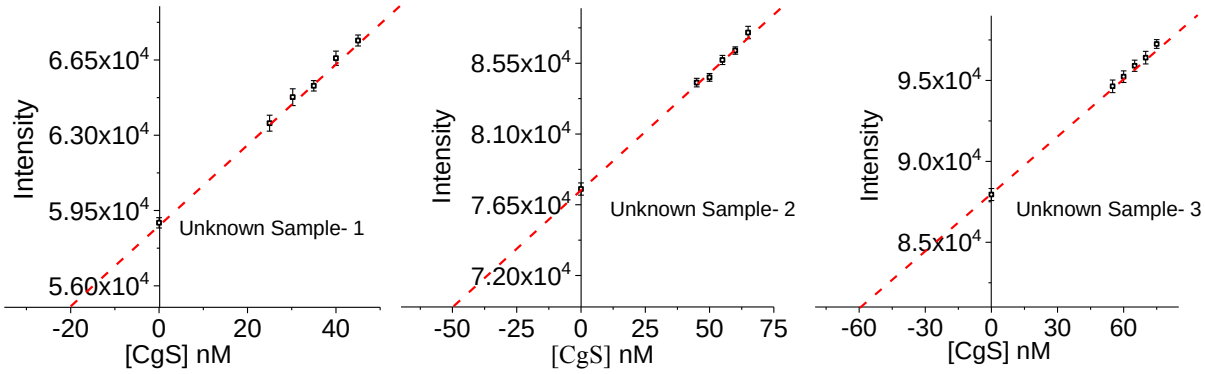


Fig. S27 *Determination of unknown concentration of CgS in different samples (Unknown 1, 2 & 3) by fluorescamine assay method. (Unknown concentration of CgS determined from the graph were given in the below table).*

Table ST2: *Unknown [CgS] samples (1, 2 &3) quantified by reagent L and fluorescamine assay method.*

Unknown CgS camples	Cgs (in nM) quantified by reagent L using addition method	CgS (in nM) quantified by fluorescamine method
Unknown sample 1	20.45	18.50
Unknown sample 2	49.88	50.10
Unknown sample 3	60.38	59.78

Cell culture

HeLa cells were incubated in DMEM supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) and 1% (*w*/*v*) penicillin-streptomycin (PS) at 37 ˚C in a humidified atmosphere of 5% of CO_2 in the air. Cellswere passagedwhen they reached approximately 80% confluence. Cells were seeded onto a cell culture dish at a density of 1.0×10^5 cells, which was incubated at 37 ˚Covernight under 5%

CO_2 in the air.For the negative control experiment, the cells were pre-incubated in NEM (1 mM) for 30 min, washed with PBS (phosphate buffered saline) buffer for three times, and then incubated with the probe (10 μM) for 30 min. For the positive control experiment, the cells were incubated in N-acetyl Cystiene(NAC, 0.7 mM) for 15 min, washed with PBS buffer solution for three times, and then incubated with the probe (10 μM) for 30 min. Those cells incubated finally with theprobe were washed with PBS buffer three times and then fixed with 4% formaldehyde solution for the microscopic imaging.

In-Vitro imaging study

HeLa cells were incubated with 10 μM of this molecular probe for 30 min and then were thoroughly washed with PBS buffer. Confocal images (Fig. 6) with probe **L** showed bright fluorescence from cells due to the reaction of biothiols with probe within the cells. To ascertain that emission originated due to the presence of biothiols, a control experiment was carried out in the presence a thiol-blocking agent, namely N-ethylmaleimide (NEM). Cells pre-incubated with NEM (1 mM) showed week fluorescence compared to the only probes. This confirmed that the observed emission was due to the reaction of L with intracellularbiothiols. Further, an additional experimentwas also carried out in the presence of N-acetyl cysteine (NAC). NAC is known to participate inenzymatic hydrolysis induced by aminoacylase-1 that is commonly present in Hela cells. Cys is produced as the hydrolysis product, and this further reacted with **L** to cause an enhanced intracellular fluorescence. This is in agreement with our previous report and the solution study.

Cell viability assay

As a proof-of-concept, the probe **L** was tested for cytotoxicity. HeLa cells were plated into 96 well plated at a density of 1.0 x 10^5 cells/well. The cells were left to incubate at 37 °C in a 5 % CO_2 incubator for 24 h after which they were washed with 1xPBS. A total of six wells per condition were taken and 100 µL of the different required conditions were added to the respective wells: blank (supplemented IMDM medium), cell death, 100 µM, 50 µM, 20 µM and 10 µM of probe **L** was in supplemented IMDM medium. The cells were left to incubate for 24 h in the incubator, after which they were washed 3 times with 1xPBS. Then 100 µL of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution, 10 % MTT solution in supplemented IMDM medium, was added to each well and left to incubate for another 4 h. The solution was then transferred to a dark plate and the fluorescence measured at 570 nm. The experiment was done in triplicate.

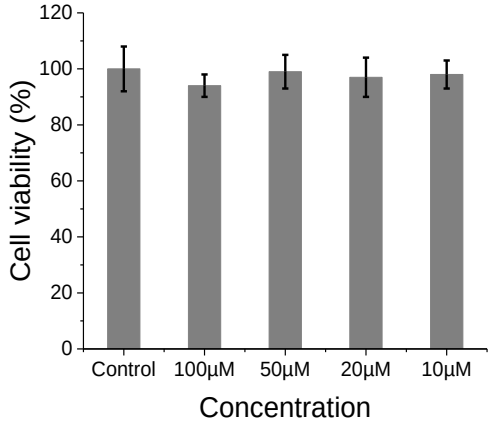


Fig. S28 *Cell viability of HeLa cells after 24 h exposure to a concentration range of probe **L**, determined using the MTT assay. Data represent mean ± standard deviation of three replicates.*

Fig S26 shows that cell viability levels remained stable as compared to a control group, no decrease below 97 % was observed after exposure (24 h) to different concentrations of probe **L**. This confirms that the synthesized probe **L** is biocompatible. The concentrations used are notably higher

than the range used in medical applications such as bio-imaging, usable for biomedical applications. One-way Anova statistical analysis was used to determine if a concentration effect was present for the sample. At $p < 0.01$ no statistically significant difference between the concentrations used was present, thereby indicating no dose-dependent effect for the used concentration range.

Zebrafish Experiment

Zebrafish Maintenance and Embryo Harvesting

Zebrafish culture and handing procedures were in agreement with the guidelines evaluated and approved by the ethics committee of CSIR-CCMB, Hyderabad. Zebrafish (strain Danio rerio) were purchased from the local market and maintained them at a constant temperature of 28 °C and pH 7.2 with a 12/12 h dark/light cycle. For spawning, one adult male and two female fish were chosen and placed at opposite sides of a small breeding tank separated by a tank divider, at 18.00 of the previous day of the experiment. On the next day, the tank divider was removed at 10:30 and allowed the fish to breed for 10min and checked for embryos. Embryos were collected immediately, and we transferred them to embryo media E3 (50 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), and we used these for further experimentations.

Fluorescence imaging of Zebra Fish Embryos

Method

10 μM probe prepared by dissolving appropriate amounts of **L** in the E3 medium. In a petri dish, 20 embryos (0.5 hpf and 96 hpf (hours post fertilization)) were soaked in the E3 medium containing 10 μM probe. The zebrafish embryos (0.5 hpf) were used at the one-cell stage to ensure

that the probe is permeated into the embryos and dispersed throughout the zebrafish cytoplasm. After 1.5 h, the zebrafish embryos were washed three times with the E3 medium and then placed on a glass slide with a small amount of the same medium. This glass slide was viewed through a fluorescent stereomicroscope (Leica M165 FC, Leica Microsystems, Heerbrugg, Switzerland). For the negative control experiment, the zebrafish were pre-incubated in NEM (1 mM) for 30 min, washed with E3 medium for three times, and then incubated with the probe (10 μM) for 1.5 h. For the positive control experiment, the cells were incubated in N-acetyl Cysteine (NAC, 0.7 mM) for 30 min, washed with E3 medium for three times, and then incubated with the probe (10 μM) for 1.5 h.

Results

Zebrafish embryos of two developmental stages, 0.5 and 96 hpf (hours post fertilization) were considered, and divided them in each developmental stage into four groups. The control groups of both developmental stages were treated with the regular E3 medium. It was observed that for the live zebrafish embryos (of both the 0.5 and 96 hpf) that were soaked in 10 μM of the solution of **L**. Studies revealed that the probe **L** could easily permeate the embryos across the chorion and the germ ring around the yolk sac. Fluorescence microscopic images with probe **L** showed bright fluorescence from zebrafish embryos due to the reaction of endogenous biothiols (within the embryos) with **L** (Fig. 7). These studies confirmed that **L** could easily permeate the embryos across the chorion and the germ ring around the yolk sac. Further control experiments in the presence of NEM (1 mM) confirmed that that the observed emission of embryos was solely due to the presence of endogenous biothiols. Thus these results revealed that this molecular probe is tissue permeable and efficient for mapping Cys/Hcy present in tissues.

Biological Toxicity and Biocompatibility of probe L.

Viabilities of more than 85% were observed for the **Zebra Fish** embryo and larvae incubated with buffer solution of 10 mg mL^{-1}, and it was more than 95% for the concentrations lower than 5.0 mg mL^{-1}. Most interestingly, no malformation was observed in the **Zebra Fish** embryo and larvae incubated with the probe solutions at various concentrations (0.50, 1.5, 3, and 5 mg mL^{-1}), showing very low toxicity to the living fish. These results also confirm the good biocompatibility of the probe after soaking.

Reference

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S2. Anila, H. A.; Ali, F.; Kushwaha, S.; Taye, N.; Chattopadhyay S.; Das, A. *Anal. Chem.*, 2016, **88**, 12161-12168.