Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

A novel fluorescence probe for estimation of Cysteine/Histidine in human blood plasma and recognition of endogenous Cysteine in live Hct116 cells

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Materials.

Dansyl chloride, Hydrazine Monohydrate (98%), Phthalic anhydride, 2-Pyridinecarboxaldehyde, Sodium triacetoxyborohydride, Copper (II)perchlorate hexahydrate, were obtained from Sigma Aldrich and were used as received. Cysteine, Histidine, Glutathione, Arginine, iso- Leucine, Proline, Methionine, Glycine, Alanine, Serine, Threonine, Tryptophan, Tyrosine, Valine, Leucine were purchased from SD Fine Chemicals in India. Solvents used for synthesis of various intermediates and final compounds were of AR grade (S.D. Fine Chemicals) and were used as received without further purification. HPLC grade (S.D. Fine Chemicals) solvents were used for various spectroscopic studies.

Analytical Methods:

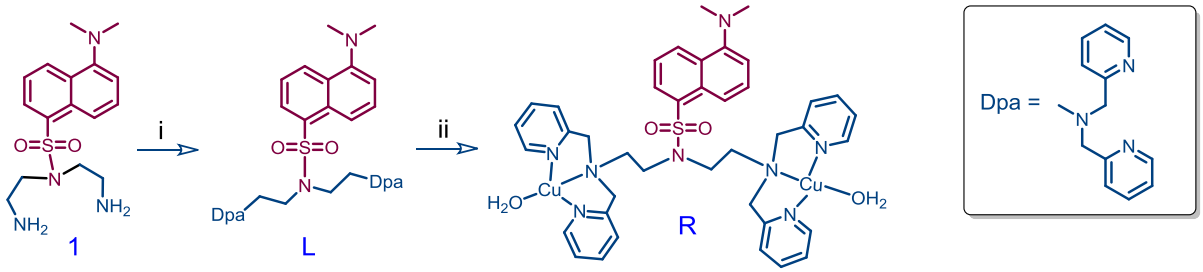
^{1}H NMR spectra was recorded on a Bruker 500 MHz FT NMR (Model: Avance-DPX 500) using $CDCl_3$ as the solvent and tetra methyl silane (TMS) as an internal standard. ESI-Ms measurements were carried out on a Waters QT of-Micro instrument. Uv spectra's was recorded with a Shimadzu UV-3101 PC spectrophotometer; while fluorescence spectra were recorded using an Edinburgh instrument Xe 900 spectrofluorometer.

General experimental procedure for UV-Vis and Fluorescence studies:

1.0×10^{-4} M, solution of the **L** in aq. HEPES buffer: acetonitrile (96: 4 (v/v)) and **R** in pure aq.-HEPES buffer medium was prepared and stored in dark. This solution was used for all spectroscopic studies after appropriate dilution. 10 mM and pH 7.4 solution of aq. HEPES buffer was used for all spectroscopic studies unless mentioned otherwise. 1.0×10^{-2} M of amino acid solutions were prepared in 10 mM HEPES buffer (pH 7.4). Solution of the compound **R** was further diluted for spectroscopic titrations, and the effective final concentration of the solution of compound **R** used for the fluorescence study was 2.0×10^{-5}

M, while the final analyte concentration during emission spectral scanning was 4.0 x 10^{-3} M. For all luminescence measurements, λ_{Ext} = 350 nm with an emission slit width of 3 nm. The relative fluorescence quantum yields (ϕ_f) were estimated using equation 1 in acetonitrile medium by using the integrated emission intensity of dansyl amide (ϕ_f = 0.37) for **L** and **R** as a reference.[1]

Synthesis:



i. 2-Pyridine carboxyldehyde, $NaBH(OAC)_3$, 1,2 Di chloroethane, RT, 48 h.
ii.$Cu(ClO_4)_2.H_2O$, MeOH, RT, 8h.

Scheme 1: Methodologies that were adopted for synthesis of **1**, **L** and **R.**

Synthesis of L: Synthetic procedure that was adopted for synthesis of **1** from our previous literature.[1]

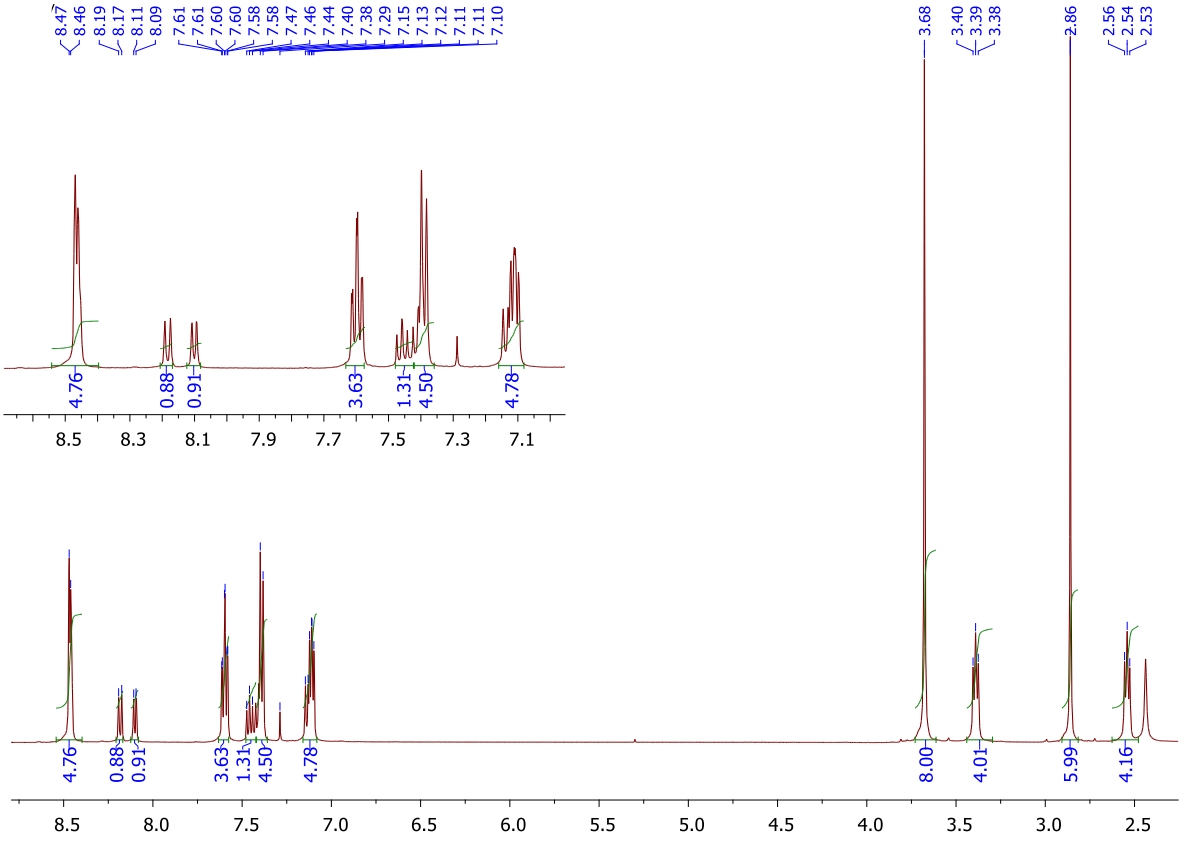
Synthesis of L[2]**:** Compound 1 (210 mg, 0.625 mmol) and 2-Pyridinecarboxaldehyde (401 mg, 3.75mmol) were dissolved in 7 ml of 1, 2 dichloromethane and refluxed it for 1 h. To this reaction mixture, Sodium triacetoxyborohydride (791 mg, 3.75 mmol) in 1,2 dichloromethane (10mL) was added. The reaction mixture was allowed to stirring at room temperature for 48 h. Progress of the reaction was monitored by checking the TLC and stopped when no further change was observed. The reaction mixture was treated with saturated aqueous sodium bi carbonate solution and subsequent extraction using chloroform was performed. The organic layer was recovered, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was subjected to neutral alumina gel

chromatography using chloroform: methanol (99.5: 0.5, v/v) as eluent. Major fraction was collected and dried under vacuum, which afforded a sticky oil solid.

Yield: 270 mg, 61.78 %. ESI- Ms (m/z) calculated for $C_{40}H_{44}N_8O_2S$: 700, observed: 701 [M + H^+]. ^{1}H NMR [500 MHz, $CDCl_3$: δ (ppm)]: 8.46 (5H, d, *J* = 4.0 Hz, ArH); 8.18(1H, d, *J* = 8.5 Hz, ArH); 8.10 (1H, d, *J* = 6.5 Hz, ArH); 7.59 (4H, t, *J* = 8.0 Hz, ArH); 7.47-7.44 (1H, m , ArH);7.39 (5H, t, *J* = 12 Hz, ArH); 7.15-7.10 (5H, ArH); 3.67 (8H, s, CH_2); 3.39 (4H, t, *J* = 7.0 Hz, CH_2); 2.77 (6H, s, CH_3); 2.54 (4H, t, *J* = 7.0 Hz, CH_2). ^{13}C NMR (500 MHz, $CDCl_3$, δ (ppm)): 173.60, 164.71, 161.18, 145.80, 138, 137, 135.17, 129, 127.74, 124.24, 119, 50.24, 40.17, 32 and 31.60.

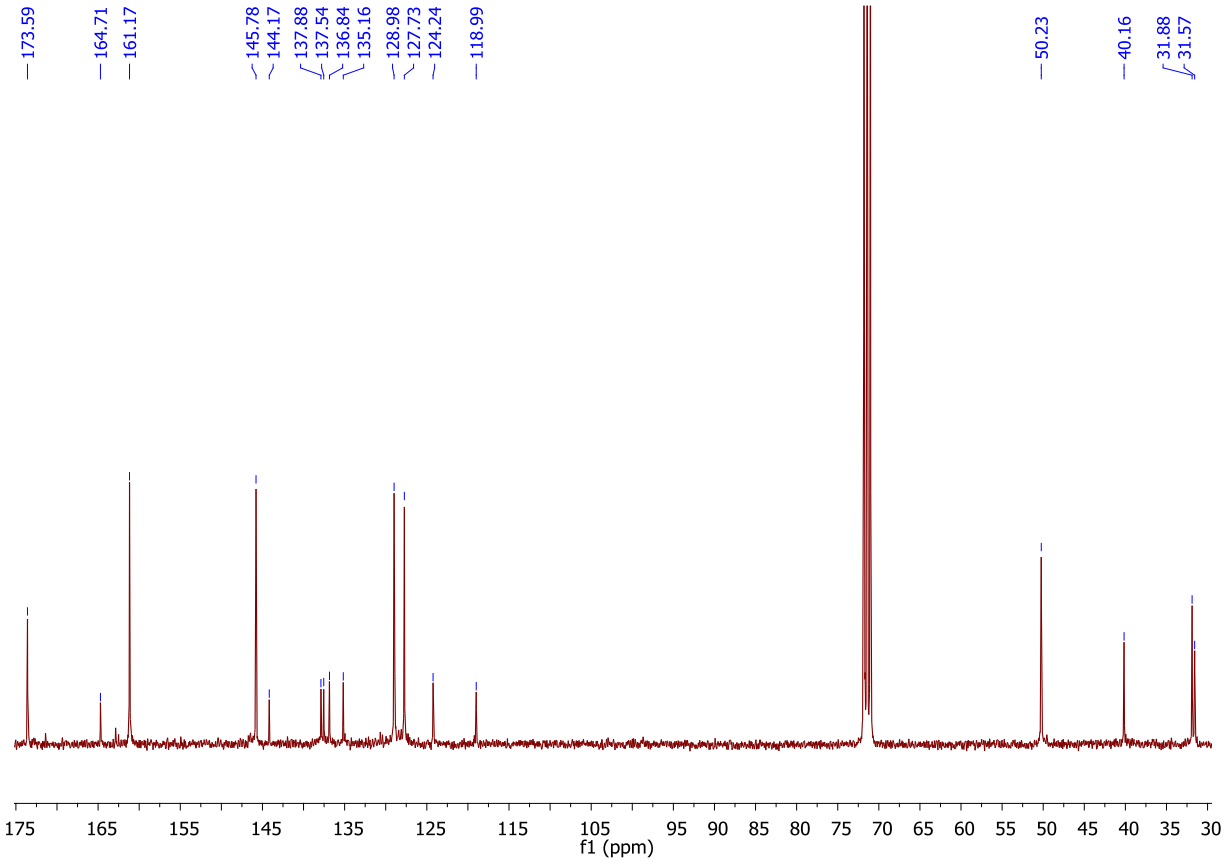
Synthesis of R^3: The compound L (120 mg, 0.17 mmol) was dissolved in 7.5 mL methanol, to this Cu $(ClO_4)_2.6H_2O$ (126 mg, 0.342 mml) was added. Solution colour was changed immediately. The reaction mixture was stirred for 8 h and then transferred into a beaker and allowed to evaporation at room temperature to precipitate the desired compound. Light bluish white solid compound was isolated through filtration and was further carefully washed with cold Dichloromethane. Yield 120 mg, 42.25%. ESI-Ms (m/z) calculated for $C_{40}H_{44}Cu_2N_8$ $.2H_2O$: 862, observed: 863 [M+H^+].

^{1}H NMR spectra of L



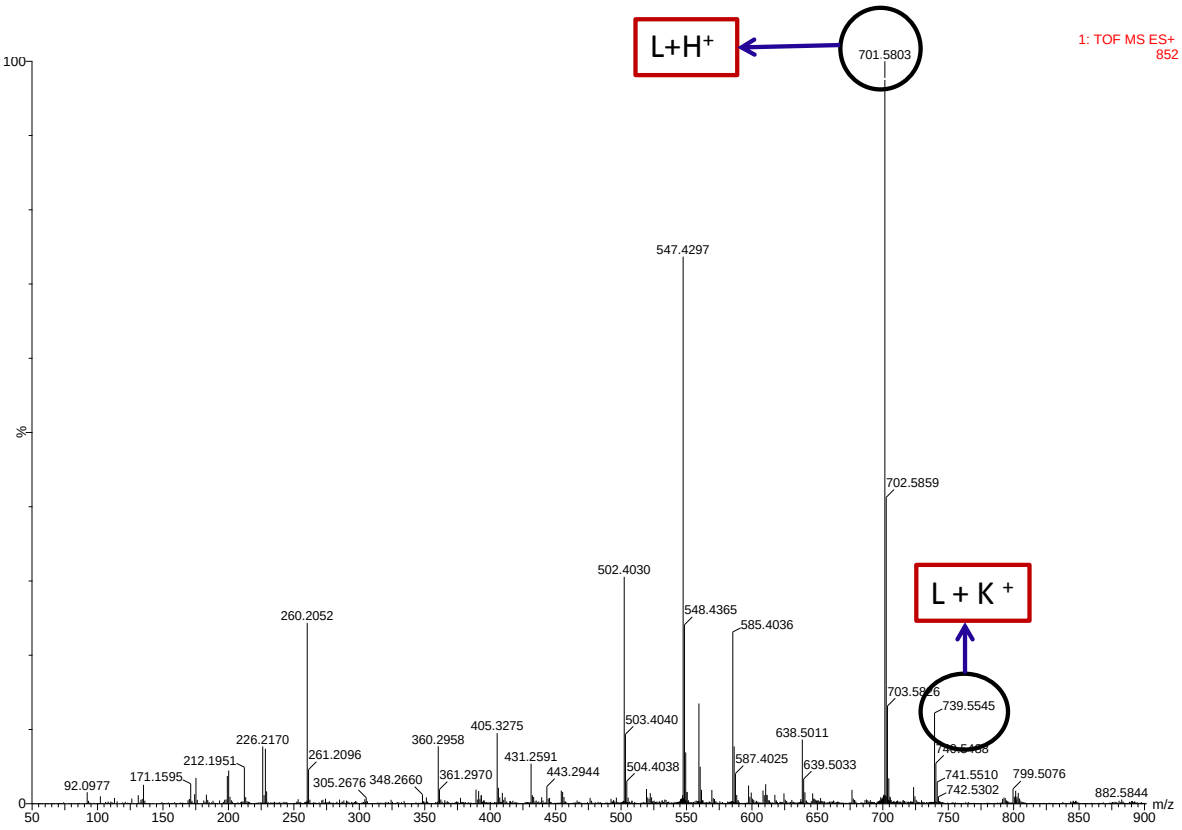
SI Figure 1: ^{1}H NMR spectra of **L** in $CDCl_3$ medium.

^{13}C NMR Spectra of L



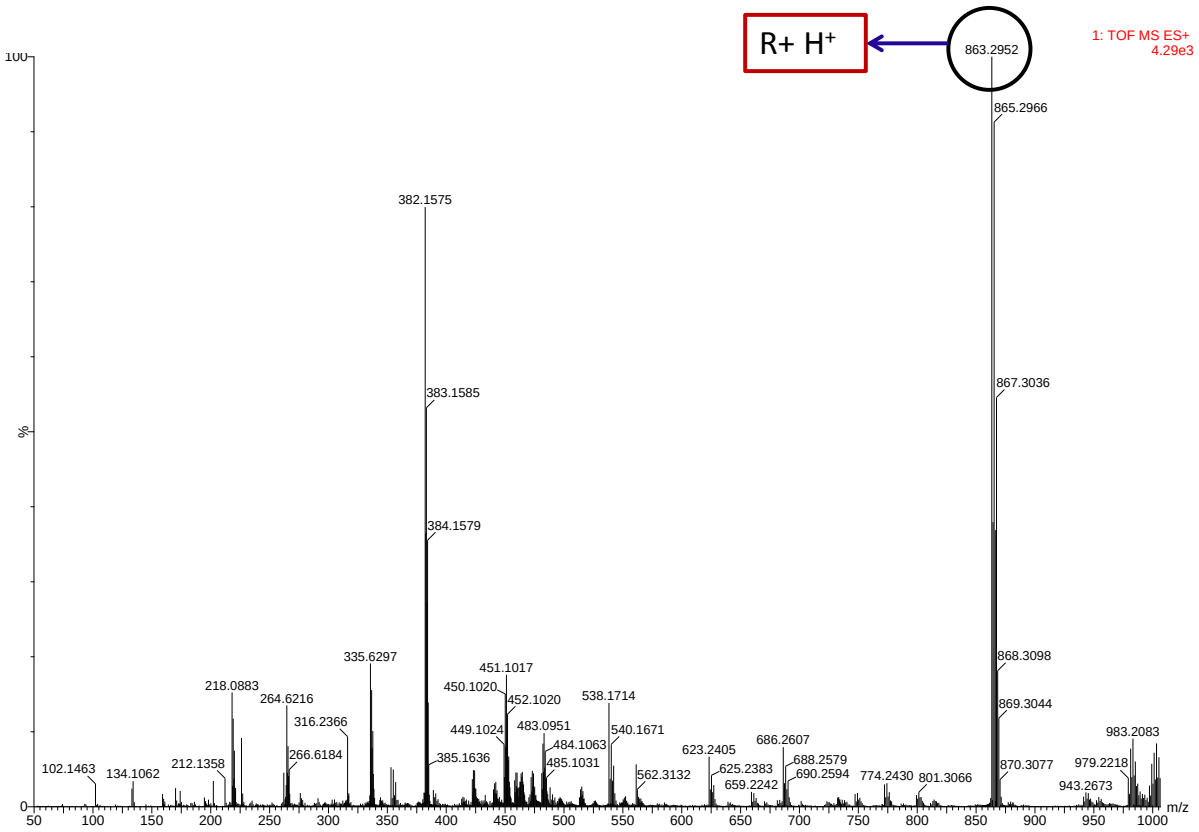
SI Figure 2: ^{13}C NMR spectra of L in $CDCl_3$ medium.

Mass spectra of L



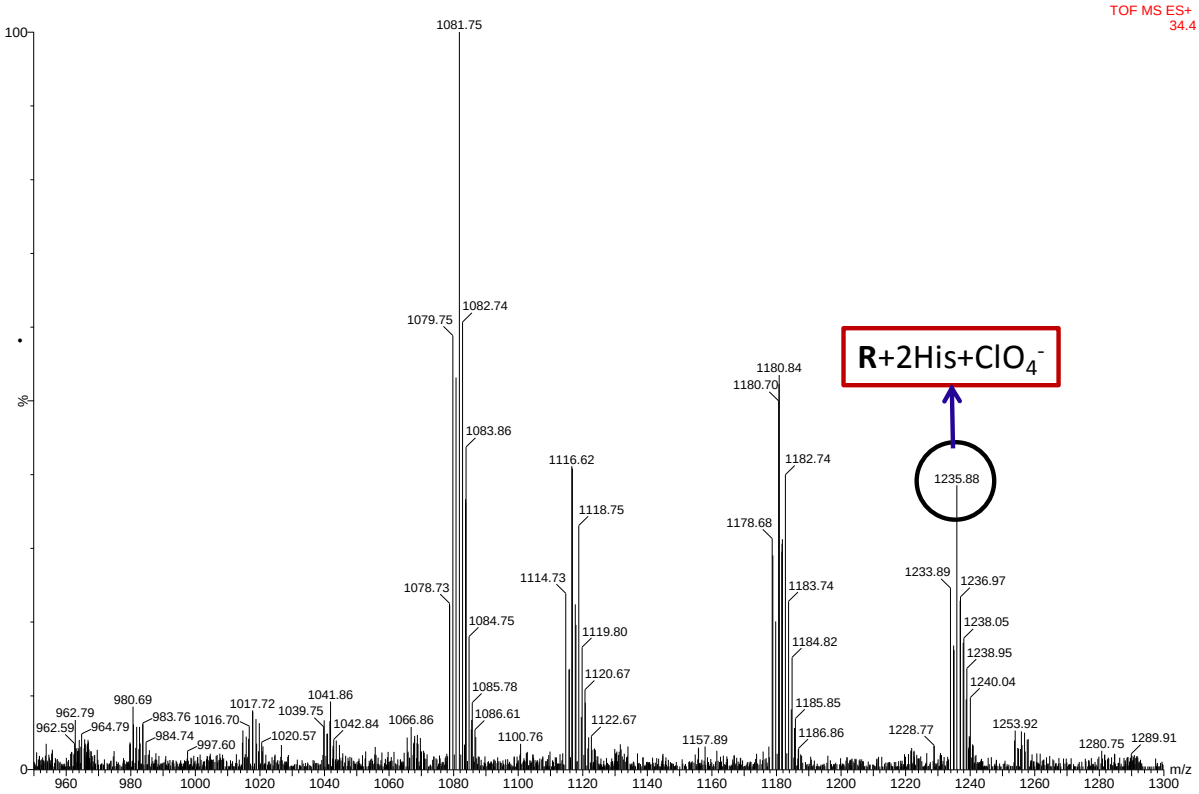
SI Figure 3: ESI- Ms spectrum of **L** in CH_3OH.

Mass spectra of R



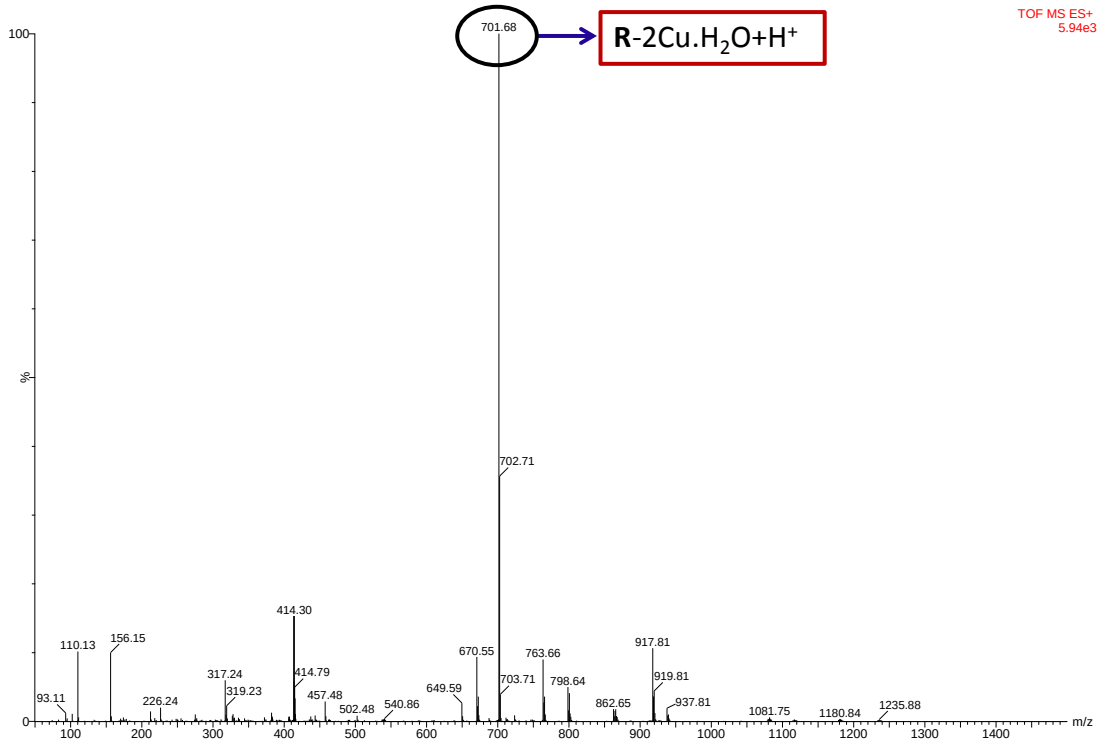
SI Figure 4: ESI- Ms spectrum of **R** in H_2O**.**

Mass spectra of R with His



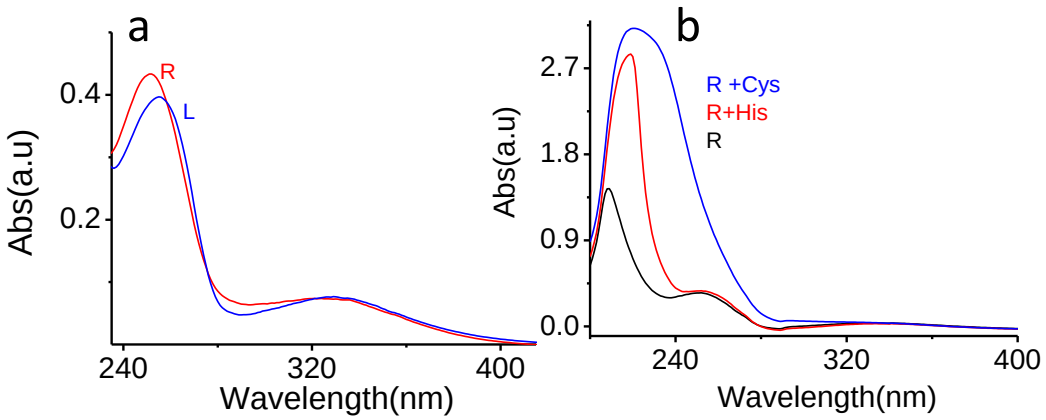
SI Figure 5: ESI- Ms spectrum of **R** with His in H_2O.

Mass spectra of R with Cys



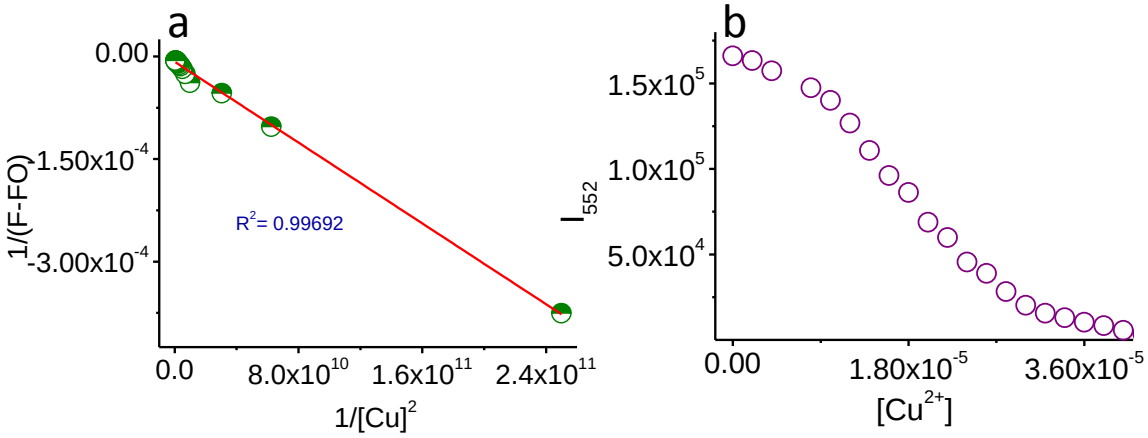
SI Figure 6: ESI- Ms spectrum of **R** with Cys in H_2O.

Absorbance spectra of L, R and R in absence and presence of Cys and His



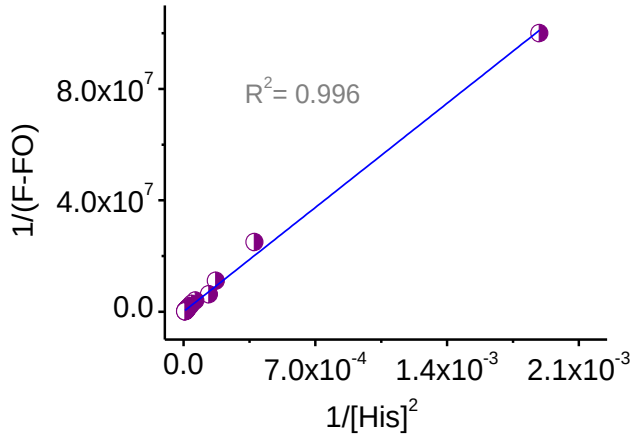
SI Figure 7 (a) Absorbance spectra of **L** (2.0 x 10^{-5} M) and **R** (2 x 10^{-5} M) (b) Absorbance spectra of **R** (2.0 x 10^{-5} M) in presence of His and Cys were performed in aq HEPES buffer medium(10 mM, pH 7.4).

Benesi-Hildebrand plot for binding studies of Cu^{2+} towards L



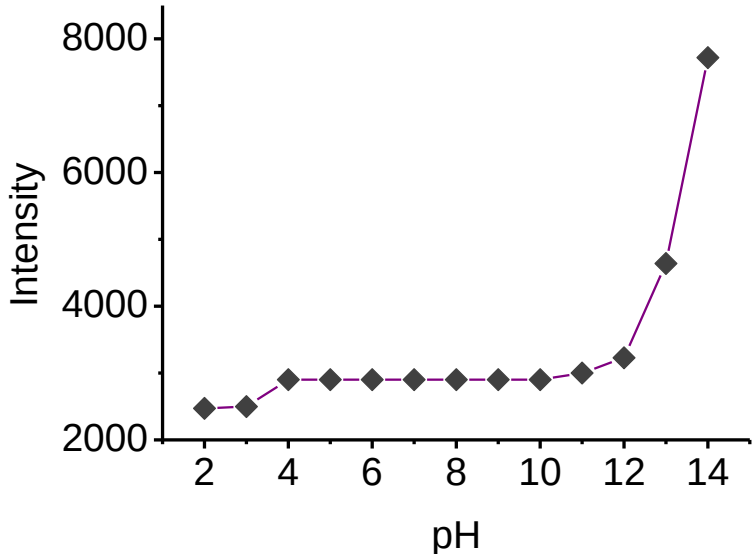
SI Figure 8 (a) BH plot of L (2 x 10^{-5} M) for varying [Cu^{2+}] (0 to 4.6 x 10^{-5} M) λ_{ext} = 350 and λ_{Mon} = 552 nm. Good linear fit confirms the 1: 2 binding stoichiometry in aq.-HEPES buffer-CH_3CN (96: 4(v/v); 10mM, pH 7.4) medium. (b) Luminescence titration profile.

Benesi-Hildebrand plot for binding studies of [His] towards R



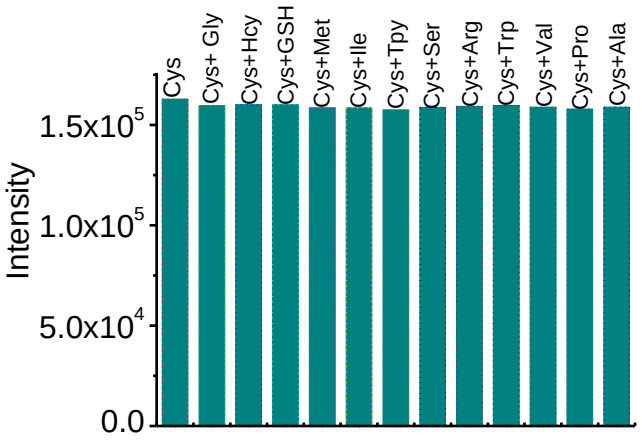
SI Figure 9. Benesi-Hildebrand plot of R (2 x 10-5 M) for varying [His] (0 to 3.2x 10^{-3} M) λ_{Ext} = 350 and λ_{Mon} = 552 nm. Good linear fit confirms the 1: 2 binding stoichiometry in aq.-HEPES (10 Mm, pH 7.4) medium.

Change of Emission intensity of R at 500 nm as a function of the solution pH



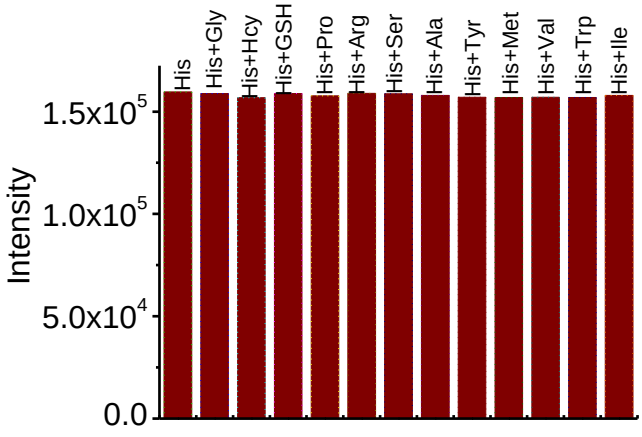
SI Figure 10. Change in emission intensity at 500 nm with variation in pH of the solution for **R** (20 μM) by using 10 mM HEPES buffer λ_{Ex}= 350 nm.

Interference study for interaction of Cys with R in presence of various Amino Acids (AAs)



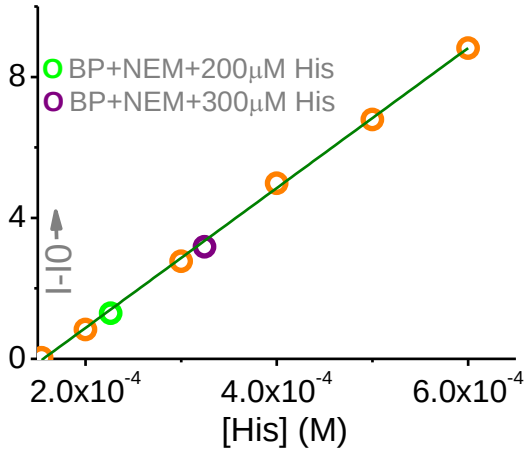
SI Figure 11. Spectrophotometric interference study of **R**(2 x 10^{-5}M) with Cys (2.8 x 10^{-3}M) in presence of various metal ions (2.8 x 10^{-3}M) in HEPES buffer by using l_{Ext} = 350 and l_{Mon} = 552 nm.

Interference study for interaction of His with R in presence of various AAs



SI Figure 12. Spectrophotometric interference study of R(2 x 10^{-5}M) with His (3.2 x 10^{-3}M) in presence of various metal ions (3.2 x 10^{-3}M) in HEPES buffer by using l_{Ext} = 350 and l_{Mon} = 552 nm.

Detection of His in human blood plasma



SI Figure 13. Plot of DI = (I_0- I) vs. [His], where I_0 and I are emission intensities of receptor **R** at 552 nm (λ_{Ext} = 350 nm) in presence of known [His] and blood plasma samples spiked with a known [His] + 10 mM NEM.

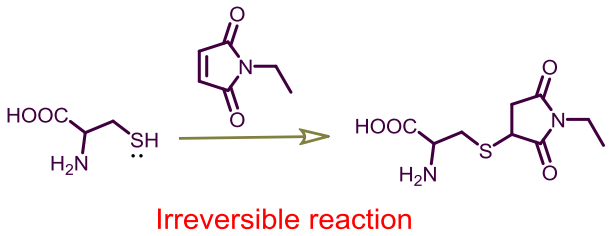
Pretreatment of the healthy human blood plasma for estimation of C_{Cys} & C_{His}[4]

Fresh and human blood samples (5 mL) with added Lithium anticoagulant were centrifuged in a vacutainer tube at 3000 rpm for 15 min. The supernatant solution (plasma), which contains proteins and amino acids, was collected. 2 ml of collected plasma was vigorously mixed with appropriate amount of $NaBH_4$ and incubated for 5 minutes at room temperature in order to hydrolyse the disulphide bond. Proteins present in the sample after reduction were precipitated by the addition of methanol, followed by centrifugation (18 500g) of the sample for 15 minutes. The supernatant liquid, which contained Cys and His in blood plasma, was used for the spectroscopic studies.

Evaluation of C_{Cys} and C_{His}, in human blood plasma sample

Methodology 1:

Calculation of the C_{His} in Human Blood Plasma (HBP) sample that contains Cys and His. To the 20μM HBP sample 10 mM NEM was added. NEM is known to react irreversibly with Cys and yield a non fluorescent compound (Scheme 2 shown below). Then this resultant solution was used for quantitative analysis of His.



Scheme 2

Concentration of His in HBP is low and thus to avoid any error in its detection, HBP samples (pre-treated with excess NEM) were spiked with two known concentration of His (100μM and 200μM, respectively). Thus fluorescence intensity of such HBP samples spiked with 200 and 300 μM of His were $I_{HBP+200}$ and $I_{HBP+300}$, respectively. Fluorescence intensities for pure aqueous HEPES buffer solution having pH of 7.4 was evaluated for [His] of 200 and 300 μM and these values were I_{200} and I_{300}, respectively.

Thus, the difference between $I_{HBP+200}$ and I_{200} should lead to the actual concentration of His ($[His]_1$) in HBP sample.

Similarly, the difference between $I_{HBP+300}$ and I_{300} should lead to the actual concentration of His ($[His]_2$) in HBP sample.

Arithmetic mean of $[His]_1$ and $[His]_2$ led us to the value ((23 ± 2.1) μM) for C_{His} in HBP sample. Please note that the data reported for C_{His} is an average of three independent evaluations for $[His]_1$ and $[His]_2$.

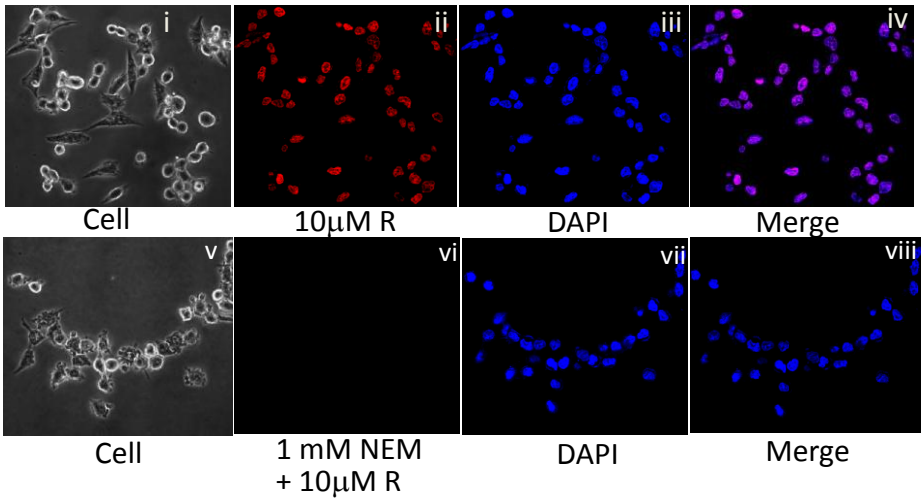
Emission intensity measurements for HBP samples treated with the reagent R directly gave the summation of concentration of C_{Cys} and C_{His} ($C_T = C_{Cys} + C_{His}$).

Thus, $C_T - C_{His} = C_{Cys}$.

Methodology 2:

C_{Cys} was also evaluated from the experiments with NEM, that reacted specifically with Cys. Emission intensity for HPB samples treated with R was evaluated (I_T), which reflects the total concentration of Cys and His present in HBP sample. Then this solution was treated with NEM. NEM reacted with Cys present in HBP sample and the product was non emissive. The emission intensity (I_{NEM}) for the resulting solution was evaluated and the difference in intensities ($DI = I_T - I_{NEM}$) was used for evaluation of [Cys] in HBP sample. Value evaluated for [Cys] in HBP sample, following this methodology, agreed well with the value that was evaluated by adopting the methodology 1.

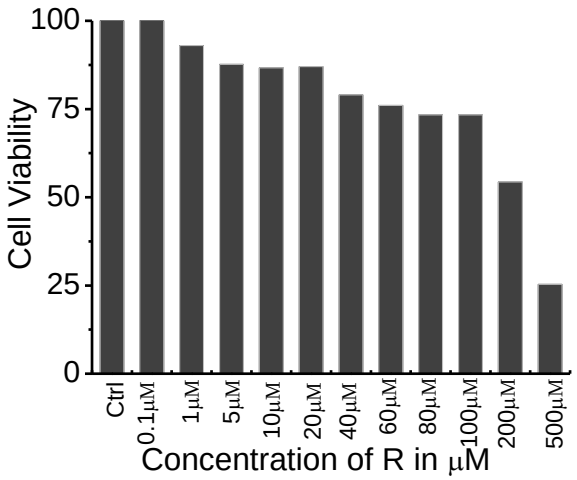
<u>Cell culture and fluorescence imaging</u>



SI Figure 14.Confocal laser fluorescence microscopic images of Hct116 cells treated with 10 μM of **R** in HEPES buffer and various reagents mentioned in the Figs. iv & viii are overlay of the merged images and confirmed the intracellular fluorescence.

Hct116 cells (3 x 10^5) were seeded on coverslips placed in 6 well plates. After 24 hours cells were treated with **R** (10μM) for 30 minutes or pre-treated with N-Ethyl Maleimide (NEM, a thiol specific blocking reagent (1mM) for 30 minutes before adding **R** (10μM) for 30 minutes. Cells were then washed thrice with Phosphate Buffer Saline (1X PBS) and fixed with 4% PFA for 20 minutes and washed again with 1X PBS. Permeabilization of the cells was done using 0.2% Triton X 100 for 5 minutes. Again three washes were given and then coverslips mounted using Fluor shield with DAPI (Sigma) mounting medium. Nail paints was used to seal the coverslips mounted on the glass slides. Images were acquired in Olympus Fluoview Microscope.

MTT assay for evolution of cytotoxicity of the reagent R towards Hct116 cells



SI Figure 15. MTT assay to determine the cell viability percentage in Hct116 colon cancer cells. The concentration of the **R** ranges from 0.1- 500μM and treated for 12 hours. IC_{50} has been calculated to be 200 μM.

The *in vitro* cytotoxicity of **R** on Hct116 cells (Colon cancer cell) were determined by conventional MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow

tetrazole) assay. Hct116 colon cancer cells (7 x 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% Fetal Bovine Serum and 100 Units of Penicillin Streptomycin antibiotics. After 24 hours the cells were treated with different concentrations of the **R** in triplicates for 12 hours. After treatment cells were added with 0.5µg/ml of MTT reagent. The plate was then incubated for 4 hours at 37°C and then later added to each well with 100 µl of Isopropyl Alcohol. The optical density was measured at 570nm using Multiskan Go (Thermo Scientific) to find the concentration of the cell inhibition. IC_{50} value has been calculated to be 200µM.

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

Cell viability (%) = (means of Absorbance value of treated group/ means of Absorbance value of untreated control) X 100.

Determination of detection limit:[5]

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

DL = K * Sb1/S

Where K = 2 or 3 (we take 2 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 = 4.85x 10^4, and Sb1 value is 0.0004

Thus using the formula we get the Detection Limit = 1.64x 10^{-8} M.

i.e. **R** can detect Cys in this minimum concentration by fluorescence techniques.

References

(1) U. Reddy G, R. Lo, S. Roy, T. Banerjee, B. Ganguly and A. Das, *Chem. Commun*., 2013, **49**, 9818. (2) U. Reddy G, P. Das, S. Saha, M. Baidya, S. K. Ghosh and A. Das, *Chem. Commun*., 2013, **49**, 255-257. (3) M. Ikeda, A. Matsu-ura, S. Kuwahara, S.S. Lee and Y. Habata, *Org. Lett*., 2012, **14**, 1564-1567. (4) P. Das, A. K. Mandal, U. Reddy G., M. Baidya, S. K. Ghosh, A. Das, *Org. Biomol. Chem*., 2013, **11**, 6604-6614. (5) S.Goswami, S.Das, K. Aich, B. Pakhira, S. Panja, S. Kanti Mukherjee and S. Sarkar, *Org. Lett*., 2013, **15**, 5412.