Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

An activity-based fluorescent sensor with a penta-coordinate N-donor binding site detects Cu ions in living systems

Kunika Gupta and Ankona Datta*

* Corresponding Author: Ankona Datta, Department of Chemical Sciences, Tata Institute of Fundamental Research, Mumbai-400005, India, *Email*: ankona@tifr.res.in

TABLE OF CONTENTS

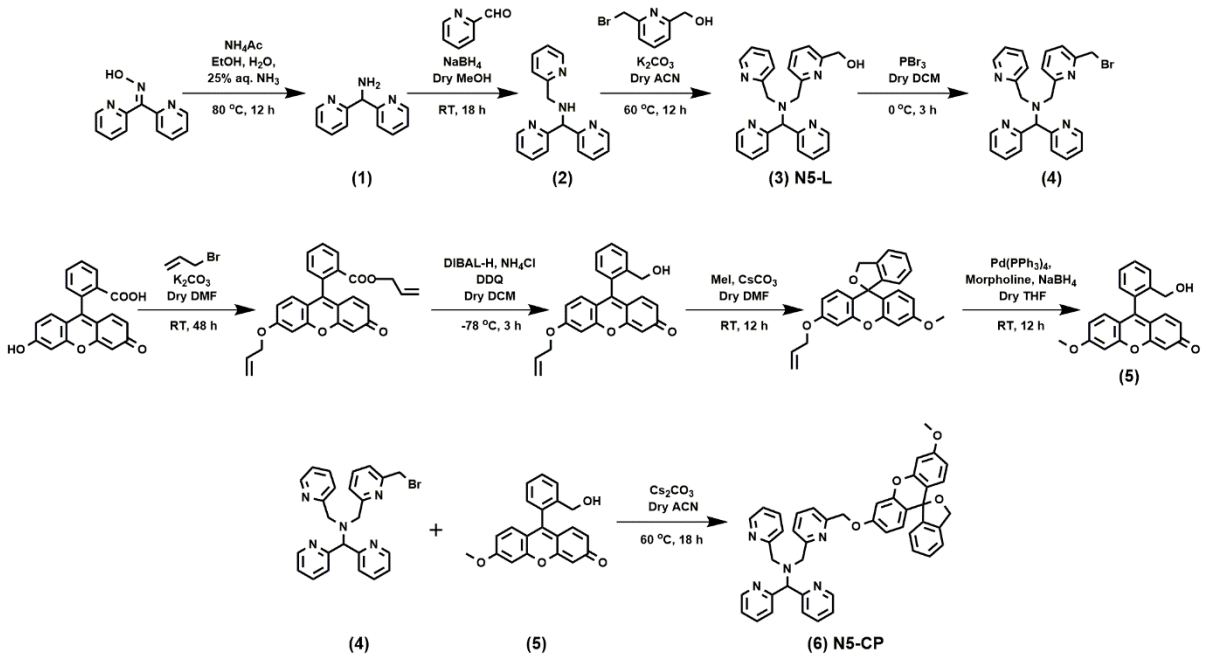
General materials and methods

Chemicals were purchased from either Sigma–Aldrich®, or SD Fine-Chem Ltd., or TCI India Research Chemicals, or Alfa Aesar, and used without further purification unless otherwise noted. All cell culture reagents were purchased from either SigmaAldrich® or Gibco®. Acetonitrile, dichloromethane, N,N-dimethylformamide, and tetrahydrofuran were dried using activated molecular sieves. HEK 293T cells were purchased from American Type Culture Collection (ATCC®). All experiments were performed in deionized water taken from a Milli-Q ® Integral 3 water purification unit (Millipore Corp. Billerica, MA, USA). Silica gel (230-400 mesh size, Loba Chemie Pvt. Ltd.) and Alumina (Brockmann grade, SD Fine-Chem Ltd.) were used for column chromatography. Eluting systems for column chromatography purifications were determined by thin layer chromatography (TLC) analysis. TLC analyses were performed on silica gel 60 F_{254} (Merck & Co., Inc.) and aluminium oxide 60 F_{254} (Merck & Co., Inc.) TLC plates and the plates were visualized under UV light, 254 nm and 365 nm. Analytical grade solvents were used for separation and used without distillation. Solvents were evaporated under reduced pressure using a rotary evaporator (BÜCHI Labortechnik AG).

^{1}H NMR and ^{13}C NMR spectra were collected in either $CDCl_3$ or $(CD_3)_2CO$ as a solvent (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C on either a Varian 600 MHz spectrometer or Bruker 800 MHz at the National NMR facility, Tata Institute of Fundamental Research, Mumbai, India. All chemical shifts are reported in the standard notation of parts per million (ppm) using the peaks of proton signals of residual solvents for calibration. The abbreviations used for the proton spectra multiplicities are: s, singlet; br, broad singlet; d, doublet; t, triplet; m, multiplet. UV-Visible spectrophotometric experiments were performed on a Thermo Scientific Multiskan Go spectrophotometer in a quartz cuvette having a path length of 1 cm with 10 mm x 4 mm (Hellma® Analytics) inner dimensions. Liquid chromatography mass spectrometry (LCMS) analyses were carried out on a Shimadzu LCMS 2020 with an ESI probe (positive and negative ion modes). High-resolution mass spectrometry analyses were carried out at the Chemistry Department, Indian Institute of Technology, Bombay, India on a maXisTM impact ESI-qTOF mass spectrometer (Bruker Corp.). Electron paramagnetic resonance (EPR) was carried out in a Bruker EMX-Micro X-band EPR spectrophotometer.

For cell viability experiments, a Biotech Cytation 5 Analyzer microplate reader was used for measuring absorbance at 570 nm in a 96 well plate. For all measurements, stock solutions of **N5-CP** were prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C. Stock solutions were then diluted in appropriate aqueous buffer and care was taken such that the percentages of DMSO never exceeded 5% in each experiment. Zebrafish were bred and maintained and experiments were performed in accordance with guidelines and protocols approved by the institutional animal ethics committee of the Tata Institute of Fundamental Research.

Synthetic procedures for N5-CP



Scheme S1: Synthetic Scheme for **N5-CP**.

Di(pyridin-2-yl)methanamine (1): Compound **1** was synthesized according to a previously reported procedure.[1] (92 %)

1,1-Di(pyridin-2-yl)-N-(pyridin-2-ylmethyl)methanamine (2): Compound **2** was synthesized according to a previously reported procedure.[1] (67%)

(6-(((di(pyridin-2-yl)methyl)(pyridin-2-ylmethyl)amino)methyl)pyridin-2-yl)methanol (3): A solution of 6-(Bromomethyl)-2-pyridinemethanol (146.25 mg, 0.724 mmol) in 5 mL degassed acetonitrile was added to 25 mL solution of compound **2** (100 mg, 0.362 mmol) and potassium carbonate (269.1 mg, 1.947 mmol) in degassed acetonitrile. The reaction mixture was heated at 60 °C for 12 h under argon atmosphere. After completion, the reaction mixture was filtered through a Whatman no.1 filter paper to remove the undissolved potassium carbonate and the solvent was evaporated under reduced pressure. The desired compound was obtained after column chromatography on alumina (5% methanol in ethyl acetate) as a yellow sticky solid (114.9 mg, 80%).

^{1}H NMR (800 MHz, $CDCl_3$, 298 K) δ (ppm): 8.57 (1H, d, 3.2 Hz), 8.50 (1H, d, 3.2 Hz), 7.72 (1H, d, 8 Hz), 7.66 (1H, t, 7.2 Hz), 7.63 (1H, t, 15.2 Hz), 7.58 (1H, t, 7.2 Hz), 7.32 (1H, d, 7.2 Hz), 7.14 (1H, t, 5.6 Hz), 7.11 (1H, t, 5.6 Hz), 7.01 (1H, d, 8 Hz), 5.35 (1H, s), 4.71 (2H, s), 3.96 (2H, s), 3.92 (2H, s).

^{13}C NMR (200 MHz, $CDCl_3$, 298 K) δ (ppm): 160.1, 160.0, 159.7, 158.4, 157.7, 149.2, 148.8, 136.9, 136.5, 136.43, 123.9, 123.1, 122.3, 121.9, 118.7, 73.0, 64.0, 57.1, 56.3.

HR-MS (ESI): m/z observed 398.2001 $[M+H]^+$, calculated 398.1981 for $C_{24}H_{24}N_5O$.

9-(2-(hydroxymethyl)phenyl)-6-methoxy-3H-xanthen-3-one (5): The reduced fluorescein dye (**5**) was synthesized in four steps following a reported protocol.[2,3] (57.2%)

Synthesis of 6 (N5-CP): Phosphorus tribromide (23.92 µL, 0.251 mmol) was added dropwise to a solution of **3** (20 mg, 0.025 mmol) in 20 mL degassed dichloromethane kept in ice bath at 0 °C. The

reaction mixture was left for 3 h. Phosphorus tribromide was quenched by saturated sodium carbonate solution. The product was extracted with dichloromethane. The organic layer was washed with water, dried over sodium sulphate and then reduced under rotary evaporator. Within one hour of synthesis, the brominated product **(4)** was utilised in the synthesis of the final molecule (**N5-CP**) without further purification as we found that the molecule was not stable.

ESI-MS m/z observed as bromine isotopic pattern 460, 462 $[M]^+$ and 482, 484 $[M+Na]^+$ calculated 460.38 for $C_{24}H_{22}N_5Br$

To solution of **5** (16.72 mg, 0.05 mmol), in 20 mL dry acetonitrile, cesium carbonate (32.82 mg, 0.1 mmol) was added. To this reaction mixture, a solution of **4** in dry acetonitrile was added dropwise. The reaction mixture was heated at 60 ^oC for 18 h under argon atmosphere. After completion, the reaction mixture was filtered through a Whatman filter paper to remove the undissolved cesium carbonate and the solvent was evaporated under reduced pressure. The desired compound was obtained after column chromatography on alumina (5% methanol in dichloromethane) as a brownish yellow sticky solid. (7.7 mg, 21%)

^1H NMR (800 MHz, $CDCl_3$, 298 K) δ (ppm): 8.57 (2H, d, 4.8 Hz), 8.51 (1H, br),7.71 (2H, d, 9.6 Hz), 7.66 (2H, t, 10.4 Hz), 7.64-7.56 (3H, m), 7.48 (1H, d, 9.6 Hz), 7.36-7.34 (2H, br), 7.29 (1H, d, 9.6 Hz), 7.25 (1H, t, 10.4 Hz), 7.15 (2H, t, 8.8 Hz), 7.10 (1H, br), 6.89-6.84 (3H, m) 6.79-6.77 (1H, br), 6.69-6.67 (2H,m), 6.60 (1H, dd, 11.2 Hz), 5.41 (1H, s), 5.27 (2H, s), 5.16 (2H, s), 4.02 (2H, s), 4.01 (2H, s), 3.80 (3H, s).

^{13}C NMR (200 MHz, $CDCl_3$, 298 K) δ(ppm): 162.5, 160.3, 159.1, 156.1, 151.5, 149.2, 144.8, 139.2, 137.2, 136.4, 129.9, 129.8, 128.4, 128.1, 124.1, 123.9, 122.2, 121.9, 120.7, 111.6, 111.1, 101.7, 100.4, 83.5, 72.3, 71.9, 70.8, 57.6, 57.2, 55.5, 53.5

HR-MS (ESI): m/z observed 712.2936 $[M+H]^+$ calculated 712.2924 for $C_{45}H_{38}N_5O_4$.

Thin Layer Chromatography Confirming Product Formation as Newly Developed Non-Fluorescent Spot

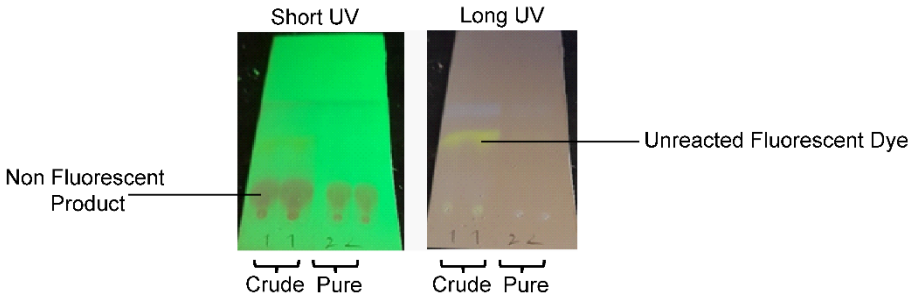


Figure S1: Images of silica gel TLC of **N5-CP** (reaction mixture before and after purification). Unreacted fluorescent dye and non-fluorescent product **N5-CP** indicated by arrows. TLC mobile phase, 5% methanol in dichloromethane.

Purity and Stability Analysis of N5-CP

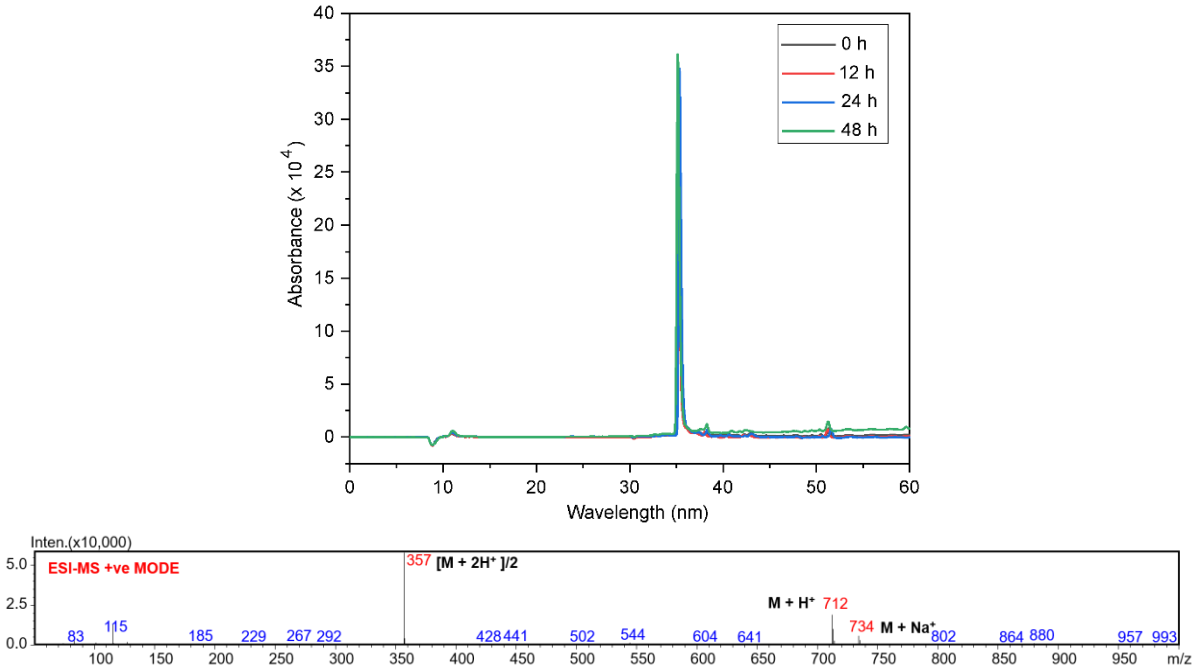


Figure S2: LC-ESI-MS of **N5-CP** confirming sample purity and stability (LC depicting absorption intensity at 301 nm). **N5-CP** (stock solution in DMSO) was diluted in 5% acetonitrile in (50% methanol in water) (final volume 400 µL), injected into the column and the stability of the ligand was monitored over time. A linear gradient from 5 % solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in 1 : 1 water : methanol) to 100 % solvent B in A was run at 0.2 mL/min for 60 min on a Kromasil® C8(2) (5 µm, 4.6 mm × 150 mm) column.

Fluorescence Measurements

All spectroscopic measurements were performed at room temperature. Samples were prepared in Millipore water. Fluorescence spectra were recorded on a FluoroLog®-3 (Horiba Jobin Yvon Inc.) spectrofluorometer using quartz cuvettes with 10 mm x 2 mm (Hellma® Analytics) inner dimensions. Fluorescence spectra were obtained by excitation at 470 nm with slit width 2 nm for both excitation and emission. $CuCl_2$ was used as the source of Cu^{2+} ions.

All spectroscopic measurements were performed in 20 mM HEPES buffer with 100 mM KNO_3, at pH 7.1. For a typical florescence measurement experiment: **N5-CP** (1 µM) was taken with 20 eq. of Cu^{2+} ions. 1 µL **N5-CP** was taken (from 200 µM stock solution in DMSO) and was added to buffer, followed by 100 µL of 10 mM GSH. The final volume was made 200 µL by adding buffer to it to make the final concentration of **N5-CP** and GSH to be 1 µM and 5 mM, respectively. To this, 1 µL of $CuCl_2$ solution (4 mM stock prepared in buffer) was added to make the final concentration of Cu^{2+} ions to 20 µM in the cuvette. The final solution was mixed well with a pipette every time before recording the fluorescence. Fluorescence was recorded at intervals of 30 min for four hours. Other metal ions were used to test the metal ion selectivity of the probe. Fe^{2+}, Ni^{2+}, Mn^{2+}, Zn^{2+}, and Hg^{2+} ions were delivered in the form of their chlorides as, $FeCl_2$, $NiCl_2$, $MnCl_2.4H_2O$, $ZnCl_2$, $HgCl_2$ from 4 mM stock solutions prepared in buffer. Na^+, K^+, Ca^{2+} and Mg^{2+} were delivered in the form of their chlorides as NaCl, KCl, $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ from 200 mM stock solutions prepared in HEPES. Cu^+ was supplied as $[Cu(CH_3CN)_4]PF_6$ and Fe^{3+} was supplied as $Fe(NO_3)_2.9H_2O$ from a 4 mM stock solution in acetonitrile. 20 µM d-block

metal ions and 1 mM of s-block metal ions were used as the final concentrations for the in vitro fluorescence experiments. For experiments under deoxygenated conditions, the buffer was bubbled with argon for 2 h prior to the experiments.

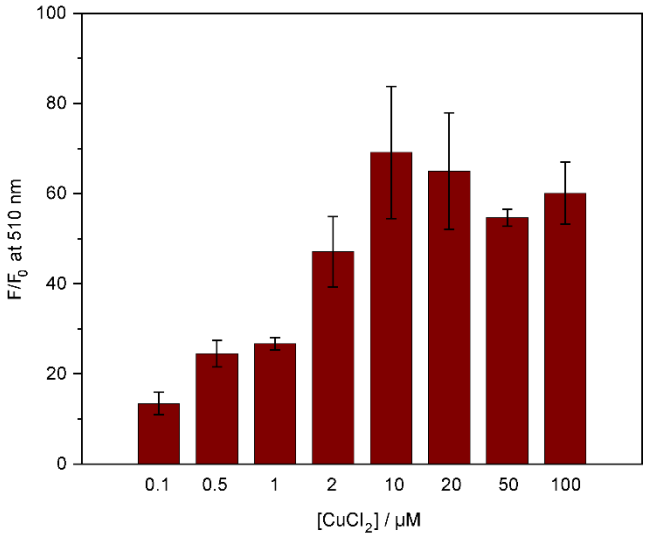


Figure S3: Fluorescence response of **N5-CP** (1 µM) with increasing concentrations of Cu^{2+} ions in 20 mM HEPES, pH 7.1, 100 mM KNO_3, 5 mM GSH under ambient oxygen after 4 hours. λ_{ex} = 470 nm.

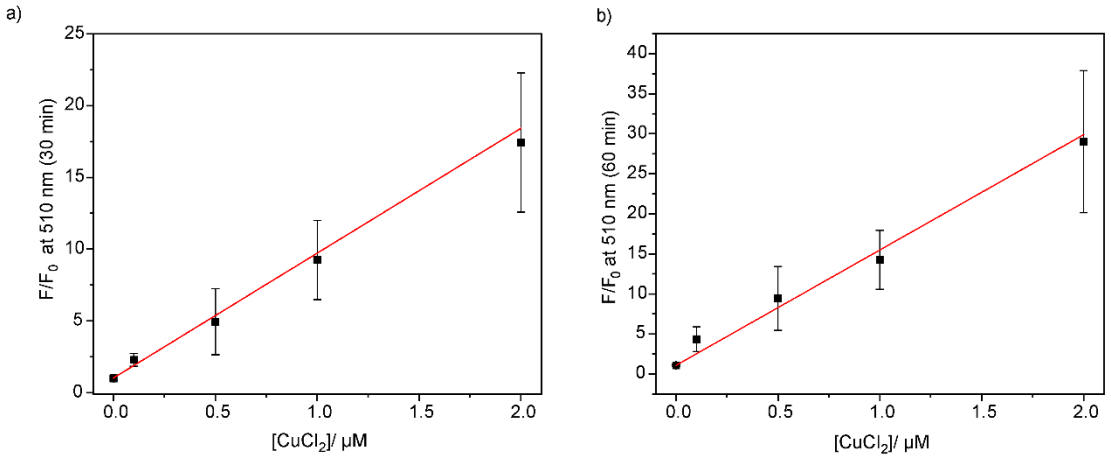


Figure S4: Fluorescence response of **a**) **N5-CP** (1 µM) with increasing concentrations of Cu^{2+} ions in 20 mM HEPES, pH 7.1, 100 mM KNO_3, 5 mM GSH under ambient oxygen at 30 min following Cu ion addition; λ_{ex} = 470 nm. The limit of detection was calculated by using the formula 3σ/k where σ was the standard deviation and k was the slope obtained from the F/F_0 versus $CuCl_2$ concentration plot. The LOD was 95 nM; **b**) **N5-CP** (1 µM) with increasing concentrations of Cu^{2+} ions in 20 mM HEPES, pH 7.1, 100 mM KNO_3, 5 mM GSH under ambient oxygen at 60 min following Cu ion addition; λ_{ex} = 470 nm.

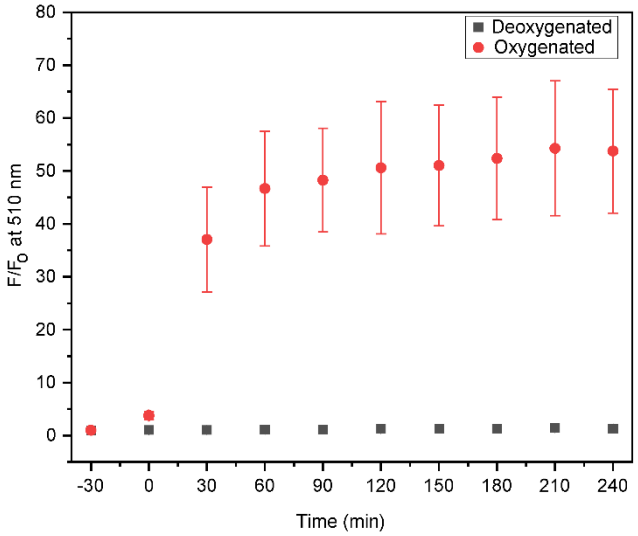


Figure S5: Time dependent fluorescence response of **N5-CP** (1 μM) with $[Cu(CH_3CN)_4]PF_6$ (20 μM) under deoxygenated (gray squares) and ambient (red circles) conditions in 20 mM HEPES (pH 7.1) containing 100 mM KNO_3 and 5 mM GSH; λ_{ex} = 470 nm.

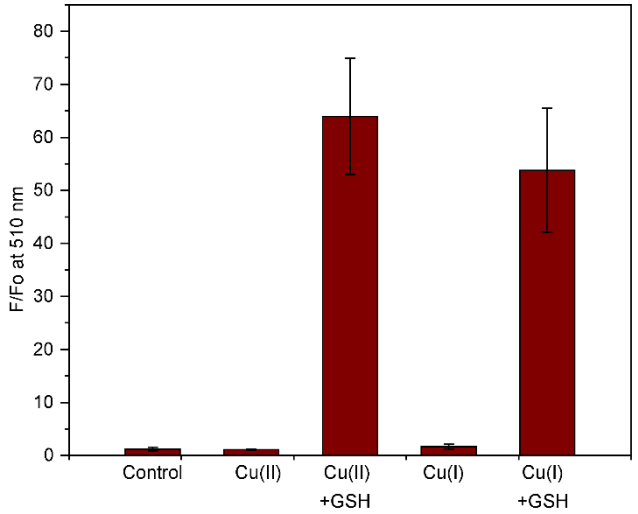
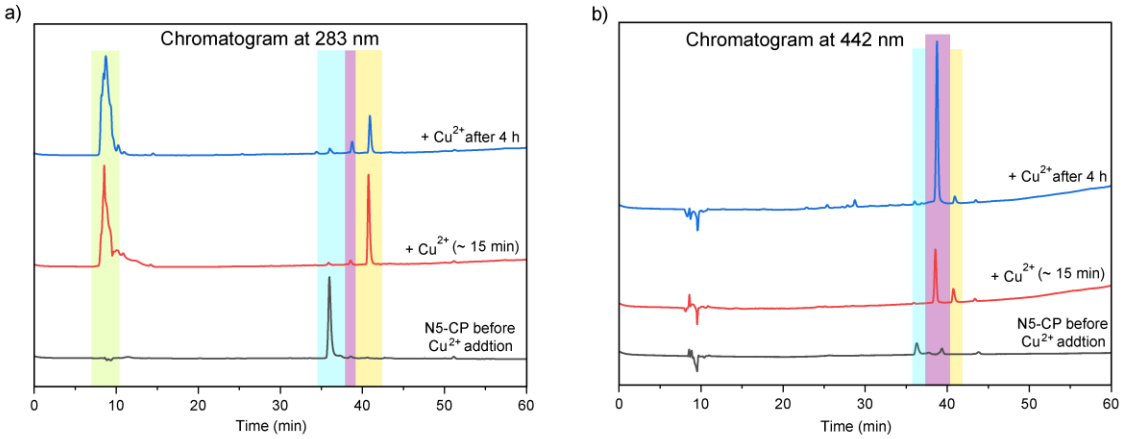
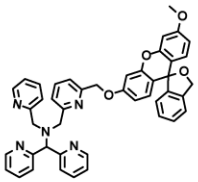


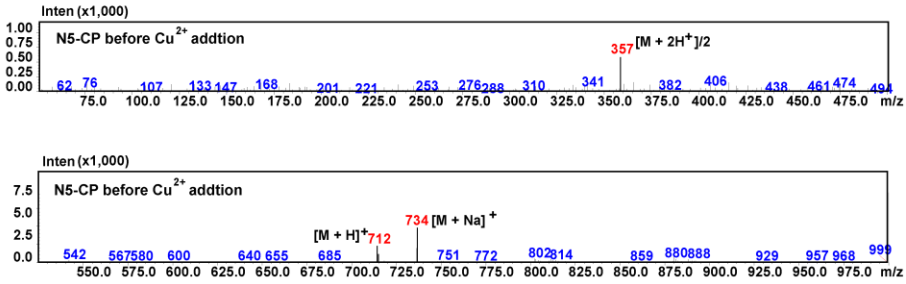
Figure S6: Fluorescence response of **N5-CP** (1 μM) in 20 mM HEPES, pH 7.1, 100 mM KNO_3 with either Cu^{2+} or Cu^{+} ions (20 μM) after 4 hours in absence and presence of 5 mM GSH under ambient oxygen. λ_{ex} = 470 nm.

LC-ESI-MS Traces of N5-CP Before and After Reaction with Cu ions

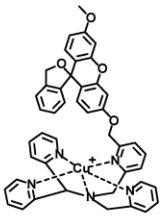


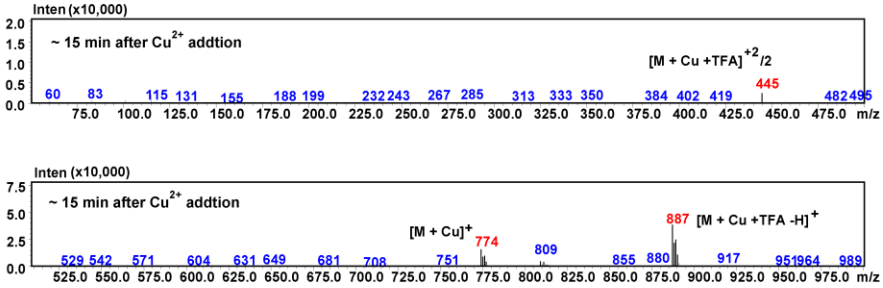
c) Retention time: 35.9 min (cyan)



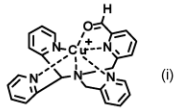


d) Retention time 40.9 min (yellow)

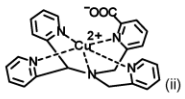




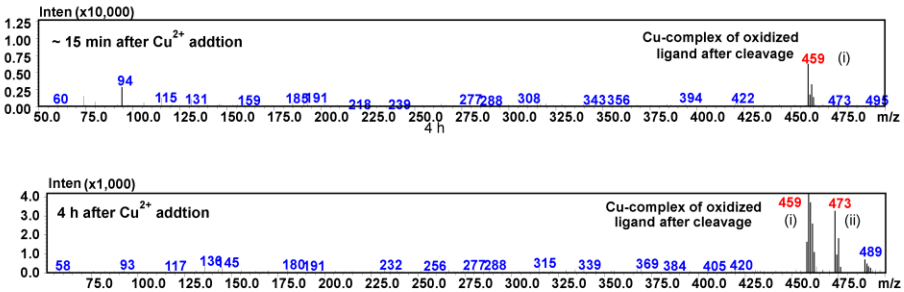
e) Retention time 8.4 min (green)



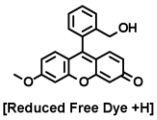
(i)



(ii)



f) Retention time: 38.7 min (pink)



[Reduced Free Dye +H]

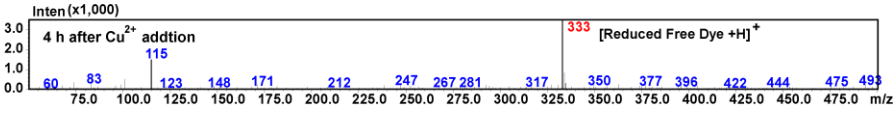


Figure S7: LC Chromatograms depicting reaction of **N5-CP** (50 µM) with $CuCl_2$ (1 mM) in presence of GSH (5 mM) and ambient oxygen. LC traces at 283 nm (**a**) and 442 nm (**b**) depicting reaction mixtures before Cu addition (lowest), after ~ 15 min of Cu addition (middle), and after 4 hours of Cu addition (top). ESI MS Data corresponding to peaks shown in **a** and **b**. Mass spectrum of peak at (**c**) retention time 35.9 min (cyan), before addition of Cu; (**d**) retention time 40.9 min (yellow), ~ 15 min after addition of Cu; (**e**) retention time 8.4 min (green), ~ 15 min and 4 h after addition of Cu; (**f**) retention time 38.7 min (pink), 4 h after addition of Cu. A linear gradient from 5 % to 100 % solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in 1 : 1 water : methanol) was run at 0.2 mL/min for 60 min on a Kromasil® C8(2) (5 µm, 4.6 mm × 150 mm) column. The mass peaks in the +ve mode are highlighted in red. **N5-CP** (50 µM) was prepared in 5% acetonitrile in (1: 1 water: methanol).

<u>Thin Layer Chromatography Confirming the Dye Cleavage upon Reaction with Cu^{2+} ions</u>

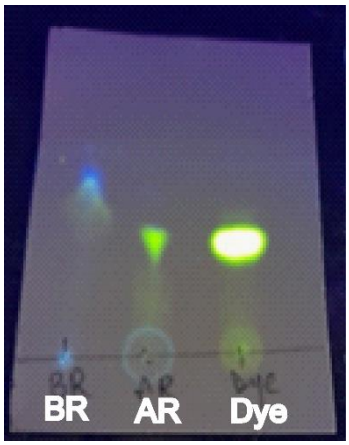


Figure S8: TLC of **N5-CP** before reaction (BR), after reaction (AR), and compound 5 (reduced fluorescein dye). TLC done on silica gel with 30 % ethyl acetate in hexane as mobile phase.

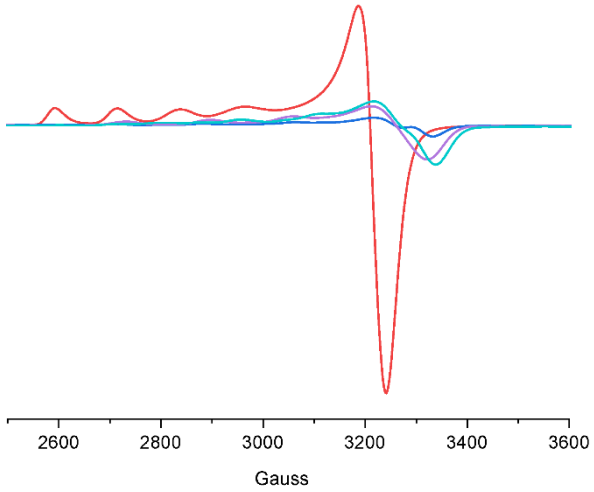


Figure S9: X-band EPR spectrum of: Cu^{2+} ions, red; Cu^{2+} ions in presence of GSH, blue; **N5-L** and Cu^{2+} ions, purple; **N5-L**, Cu^{2+} ions, and GSH (5 mM), cyan. All measurements were performed in presence of ambient oxygen. Concentration of ligand was 2.2 mM. Cu^{2+} ions were added as $CuCl_2$, concentration was 1 mM in 20 mM HEPES, 100 mM KNO_3 with 20% glycerol at pH 7.1. Spectra were collected on a Bruker EMX-Micro X-band EPR spectrophotometer at 106 K; microwave power, 2.37 mW; modulation amplitude, 1 G; time constant, 83.92 ms; conversion time, 100 ms; microwave frequency 9.3208 GHz.

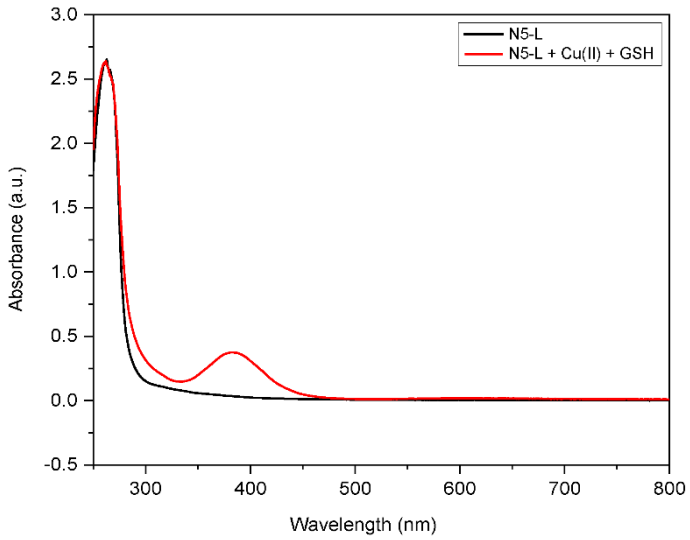


Figure S10: Absorption spectra of black: control molecule **N5-L** (20 µM) in presence of ambient oxygen; red: **N5-L** with $CuCl_2$ (20 µM) in presence of ambient oxygen and 5 mM GSH in 20 mM HEPES, 100 mM KNO_3 at pH 7.1.

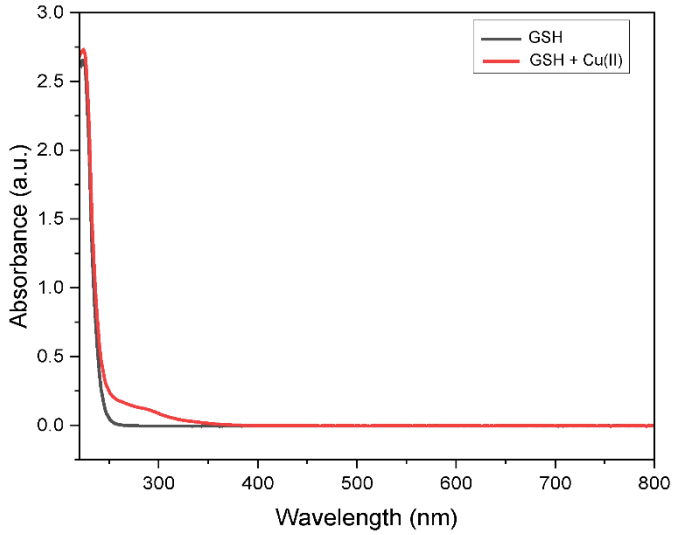


Figure S11: Absorption spectra of black: GSH (5 mM) in presence of ambient oxygen; red: GSH (5 mM) with $CuCl_2$ (20 µM) in ambient oxygen in 20 mM HEPES, 100 mM KNO_3 at pH 7.1.

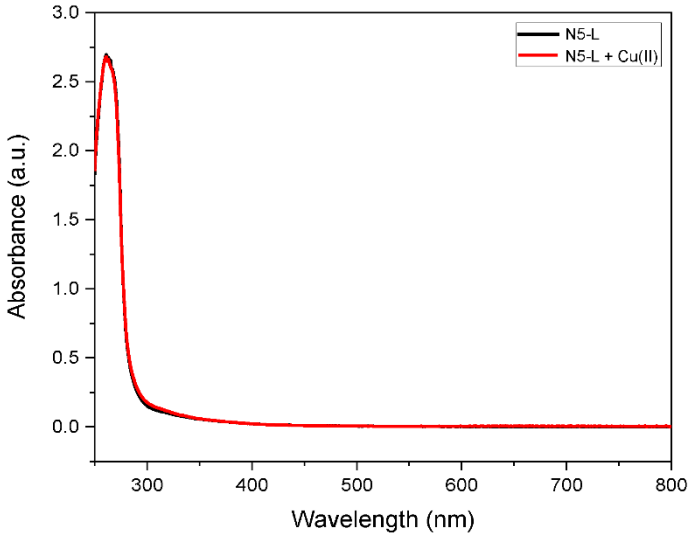


Figure S12: Absorption spectra of black: control molecule **N5-L** (20 µM) in presence of ambient oxygen; red: **N5-L** (20 µM) with $CuCl_2$ (20 µM) in presence of ambient oxygen without GSH in 20 mM HEPES, 100 mM KNO_3 at pH 7.1.

Cell Studies and Confocal Imaging

HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, SigmaAldrich®) supplemented with Fetal Bovine Serum (10%, Gibco®), Penicillin (50 units/mL, Gibco®) and Streptomycin (50 µg/mL, Gibco®) in T25 culture plates at 37 °C under humidified air containing 5% CO_2. Cells were plated on flame sterilised glass coverslips kept on petriplates (35 mm diameter, Tarsons) precoated with polylysine (0.2 mg/mL), a day before the experiment. Fluorescence images of the cells were recorded on a confocal microscope (LSM 880, Carl Zeiss, Germany) using 40x oil immersion objectives. 488 nm laser (Argon source) was used for probe excitation and emission was collected between 492-605 nm. DMEM-F12 without phenol red (imaging media, IM) was used during

the confocal studies. A stock solution of **N5-CP** (2 mM) was prepared in DMSO. The cells were washed with IM and incubated with **N5-CP** (5 μM in IM) for 1 h. After staining, the cells were washed three times with IM and imaged. For Cu treatment, cells were incubated with $CuCl_2$ (100 μM in IM) for 1 h at 37 °C under humidified air containing 5% CO_2. Then the cells were washed with IM three times to remove excess $CuCl_2$, stained with **N5-CP** as mentioned above and imaged. For copper deficient experiments, the cells were incubated with $CuCl_2$ (100 μM) for 1 h. The cells were then washed twice with IM and incubated with chelator **2c** (40 μM) for 30 min. Following this, the cells were washed twice with IM and stained with **N5-CP** as mentioned above. The cells were washed twice before imaging. Each experiment was repeated thrice.

Analysis was done using two images from each experimental set i.e. 6 images for each analysis. The fluorescence intensity analysis was performed using ImageJ software. Error bars denote standard derivation (SD; $n = 6$). Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

Cell Viability Experiments:

For testing the toxicity of **N5-CP** on HEK 293T cells, cells were seeded and grown for at least 16 h in 96 well plates (cell density 10000 cells per well) in DMEM media with phenol red and supplemented with Fetal Bovine Serum. Different concentrations (1 μM, 3 μM, 5 μM, 10 μM) of the sensor were prepared from stock in DMEM, pH 7.4, with no phenol red and serum. Cells were incubated with **N5-CP** solutions (100 μL per well) for 1 h, 3 h and 5 h at 37 °C in an incubator. As control, cells were incubated with media containing no sensor for designated time durations. After incubation, the **N5-CP** solution was removed and the wells were filled with MTT solution (0.5 mg/mL, 200 μL per well) in DMEM, pH 7.4, with phenol red and serum. MTT treated cells were kept at 37 °C incubator for at least 3 h for the formation of formazan crystals. Then the media from each well was removed and the wells were refilled with DMSO (100 μL per well) and the plates were shaken for 5 min to dissolve the crystal. Absorption values at 570 nm of the resultant purple solutions were recorded for each well. As background control, absorbance of only DMSO was also recorded. Cell viability was calculated as follows: % Cell viability = [(Experimental value − Background control) / (Control − Background control)] x 100.

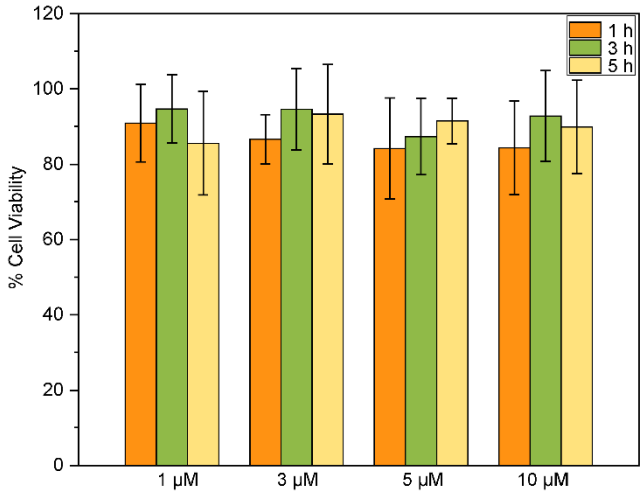


Figure S13: Cell viability in the presence of **N5-CP** at different concentrations (1, 3, 5, 10 μM) in HEK 293T cells after 1 h, 3 h, and 5 h of incubation at 37 °C in DMEM, pH 7.4, with no phenol red and serum. Percentage of viable cells was determined using MTT assay. Error bars represent the standard deviation of three replicate experiments.

Zebrafish Imaging Protocol:

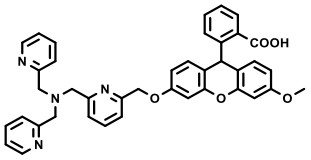
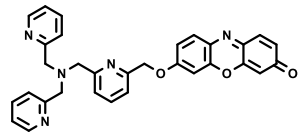
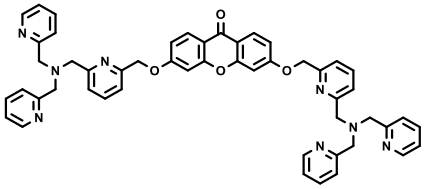
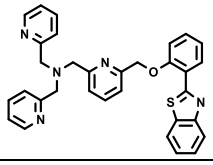
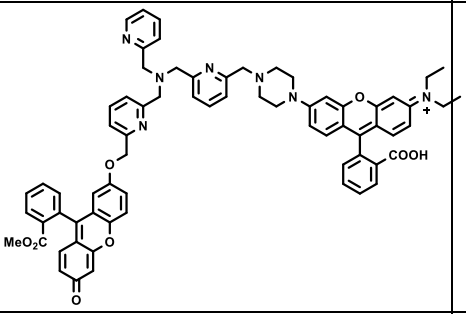
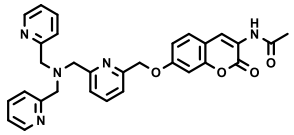
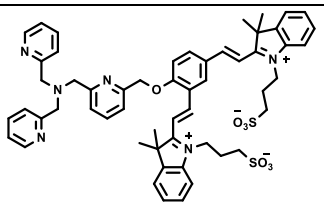
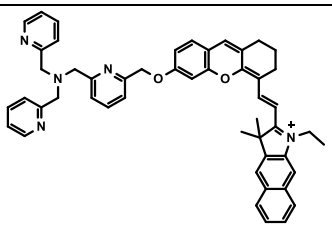
All experiments were performed using wildtype zebrafish larvae at 3 days post fertilization (dpf). Embryos were cultured at 28 °C in E3 medium containing NaCl (5 mM), KCl (170 μM), $CaCl_2$ (330 μM), and methylene blue (0.6 μM). Each treatment with chelators **2c** (60 μM), and **N5-CP** (10 μM) was carried out for 30 min. Following each treatment, the larvae were washed with fresh water. Confocal imaging was carried out with live anesthetized larvae using a 10x air objective on a confocal microscope (LSM 880, Carl Zeiss, Germany). 488 nm laser (Argon source) was used for probe excitation and emission was collected between 505-609 nm. Each experiment was repeated thrice with 3 larvae in each set. Fluorescence intensity analysis was done using Fiji ImageJ software utilising Stitching plugin. Two larvae from each experimental repeat were utilized for intensity analysis (total 6 larval images used per analysis). The yolk sac and eye regions were separately quantified. Error bars denote standard derivation (SD; n = 6). Statistical analyses were performed using an unpaired, two-tailed Student's t-test (***$p < 0.001$). Zebrafish were bred and maintained following protocols approved by institutional animal ethics committee (Tata Institute of Fundamental Research).

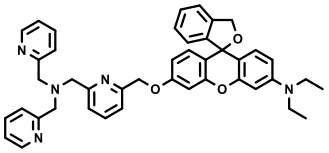
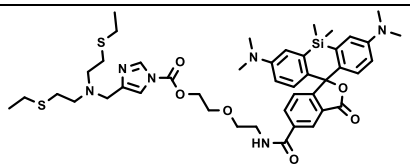
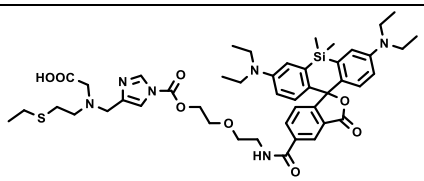
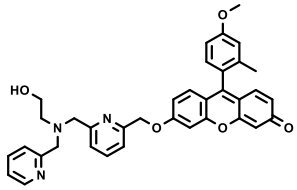
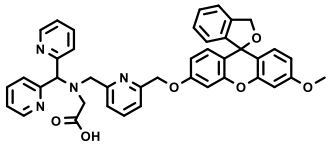
Imaging Data Analysis

ImageJ was utilised to analyse the images. As the fluorescence intensity varies within the cells, in order to have a uniform and unbiased selection of the region of interest (ROI), free hand ROIs were drawn around a group of cells. The proper boundary around each individual cell cannot to be discriminated from the other cell, hence, this method provides the unbiased quantification of the intensity. The intensity of each selection region was taken using the 'Measure' tool. For the background intensity, free hand ROIs were drawn at areas where there were no cells. The desired intensity was obtained by subtracting the background from the intensity obtained from cells.

Two images from each data set were taken for the analyses, i.e. data from 6 images were averaged to report average intensities.

<u>Table S1:</u> Previously reported activity-based sensors for first row transition metal ions.

Entry	Molecule Name	Structure	Detects	No. of N-Donors	Whether requires GSH and oxygen for sensing function
1.	FluTPA1[4]		Cu^+	4	Yes
2.	ResCu[5]		Cu^+	4	Yes
3.	XanCu[6]		Cu^+	4	Yes
4.	HBTCu[7]		Cu^+	4	Yes
5.	FCP1[8]		Cu^+	4	Yes
6.	Probe 1[9]		Cu^+	4	Yes
7.	TPACy[10]		Cu^+	4	Yes
8.	NFL-TPA[11]		Cu^+	4	Yes

10.	RDITPA-Et_2[12]		Cu^{+}	4	Yes
11.	CD649[13]		Cu^{+}	2	GSH used (Oxygen not explicitly mentioned, but ambient conditions used)
12.	CD649.2[14]		Cu^{2+}	2	No (But ambient conditions used)
13.	CP1[15]		Co^{2+}	3	Yes
14.	IP1[3]		Fe^{2+}	4	Yes

References:

1 S. Megow, H. L. Fitschen, F. Tuczek and F. Temps, *J. Phys. Chem. Lett.*, 2019, **10**, 6048–6054.

2 S. Ando and K. Koide, *J. Am. Chem. Soc.*, 2011, **133**, 2556–2566.

3 H. Y. Au-Yeung, J. Chan, T. Chantarojsiri and C. J. Chang, *J. Am. Chem. Soc.*, 2013, **135**, 15165–15173.

4 M. Taki, S. Iyoshi, A. Ojida, I. Hamachi and Y. Yamamoto, *J. Am. Chem. Soc.*, 2010, **132**, 5938–5939.

5 D. Maity, A. Raj, D. Karthigeyan, T. K. Kundu and T. Govindaraju, *RSC Adv.*, 2013, **3**, 16788–16794.

6 D. Maity, B. Sarkar, S. Maiti and T. Govindaraju, *Chempluschem*, 2013, **78**, 785–788.

7 D. Maity, V. Kumar and T. Govindaraju, *Org. Lett.*, 2012, **14**, 6008–6011.

8 C. Y. S. Chung, J. M. Posimo, S. Lee, T. Tsang, J. M. Davis, D. C. Brady and C. J. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 18285–18294.

9 K. K. Yu, K. Li, J. T. Hou and X. Q. Yu, *Tetrahedron Lett.*, 2013, **54**, 5771–5774.

10 D. Maity, A. Raj, D. Karthigeyan, T. K. Kundu and T. Govindaraju, *Supramol. Chem.*, 2015, **27**, 589–594.

11 X. Qian, W. Zhu, H. Yu, Y. Xu, W. Liu, H. Y. Wang and Y. Liu, *Dye. Pigment.*, 2021, **194**, 109561.

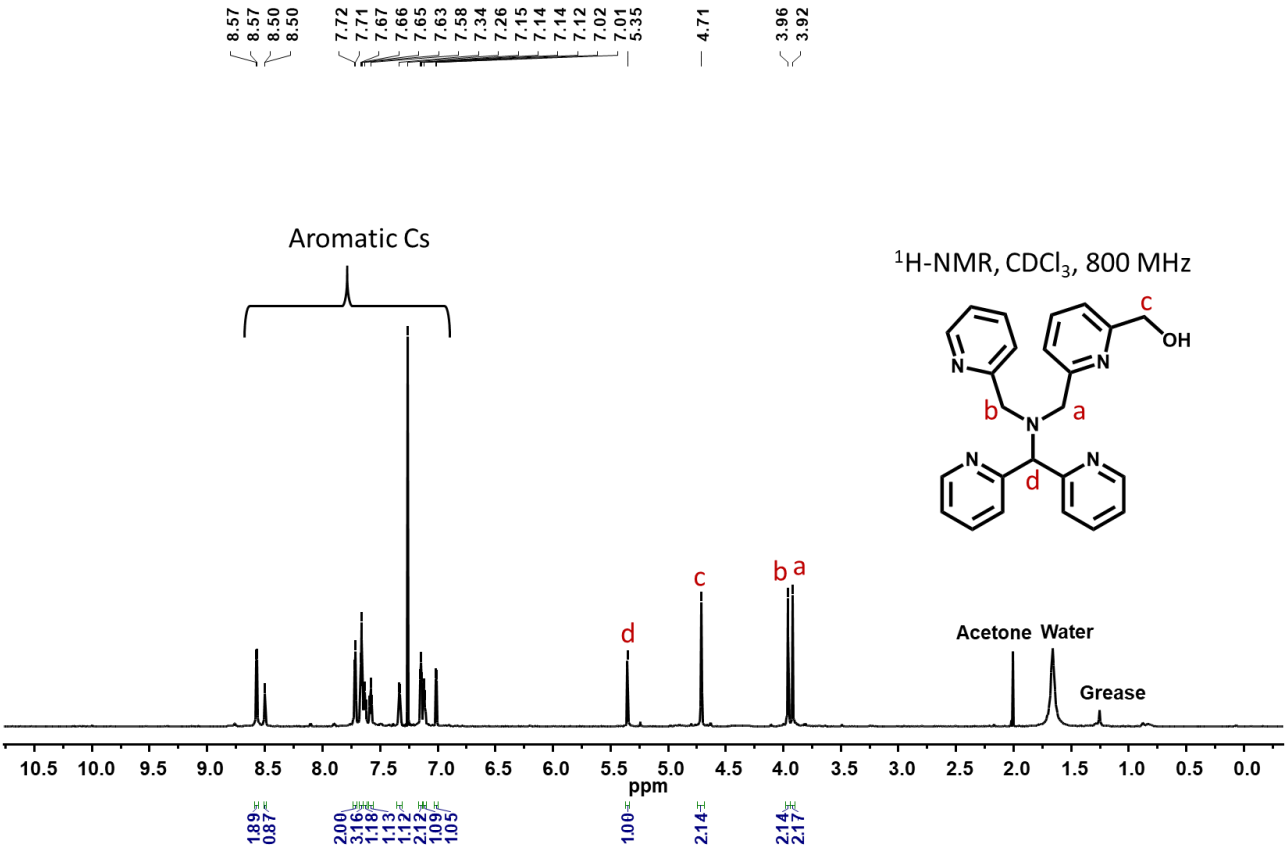
12 M. Taki, K. Akaoka, K. Mitsui and Y. Yamamoto, *Org. Biomol. Chem.*, 2014, **12**, 4999–5005.

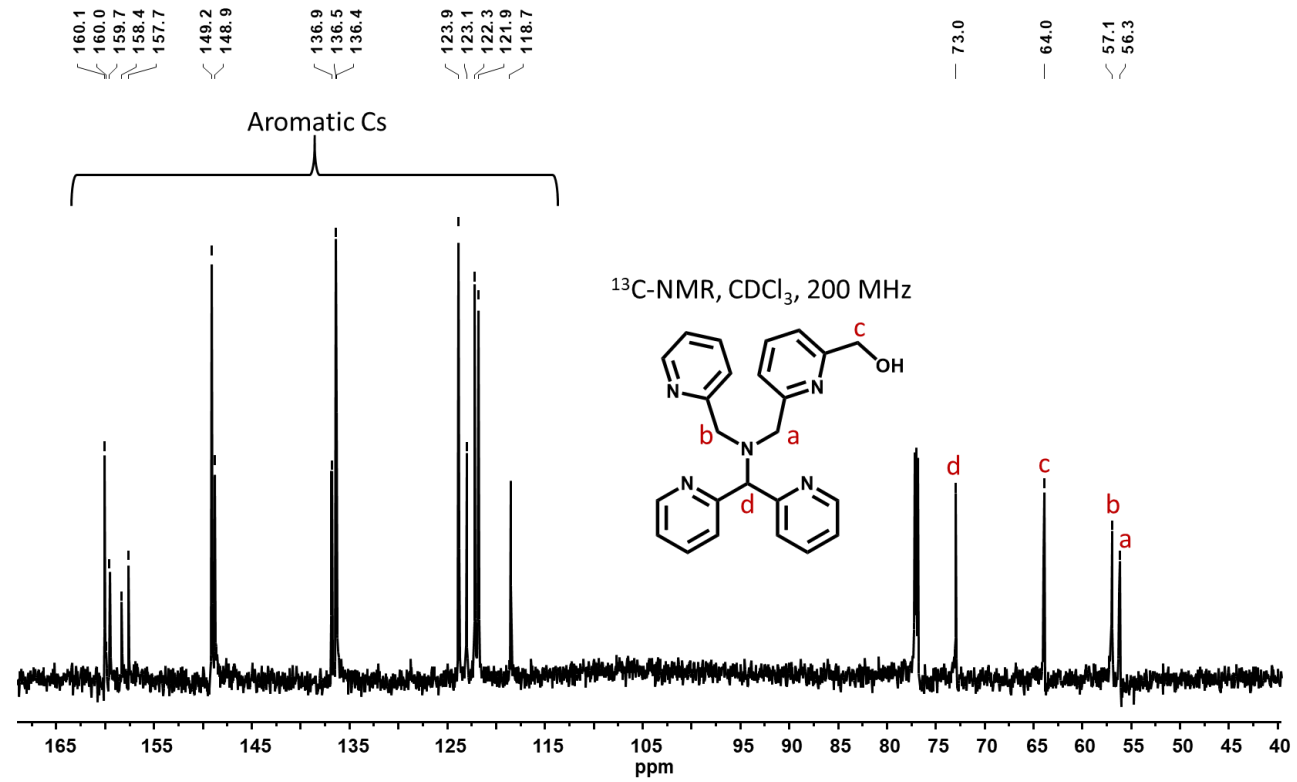
13 S. Lee, C. Y. S. Chung, P. Liu, L. Craciun, Y. Nishikawa, K. J. Bruemmer, I. Hamachi, K. Saijo, E. W. Miller and C. J. Chang, *J. Am. Chem. Soc.*, 2020, **142**, 14993–15003.

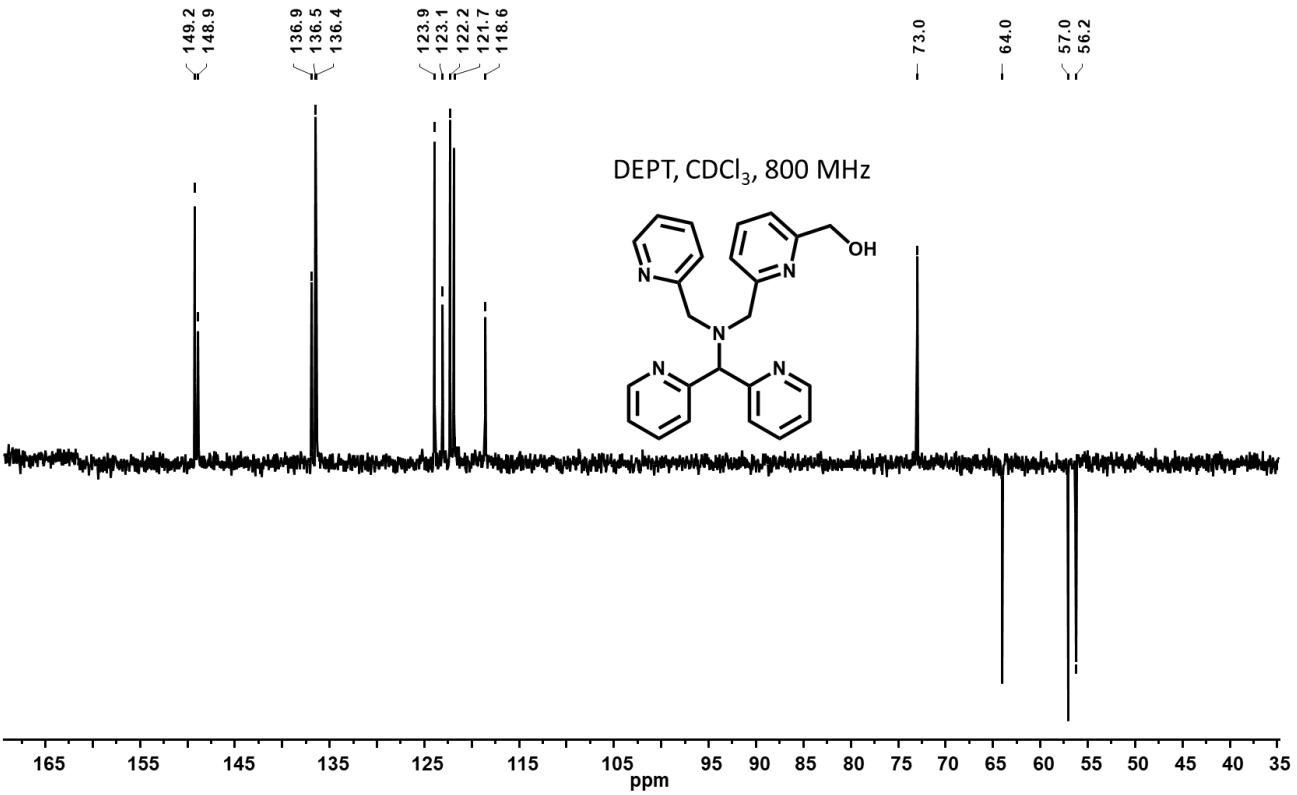
14 A. T. Pezacki, C. D. Matier, X. Gu, E. Kummelstedt, S. E. Bond, L. Torrente, K. L. Jordan-Sciutto, G. M. DeNicola, T. A. Su, D. C. Brady and C. J. Chang, *Proc. Natl. Acad. Sci. U. S. A.*, 2022, **119**, 1–9.

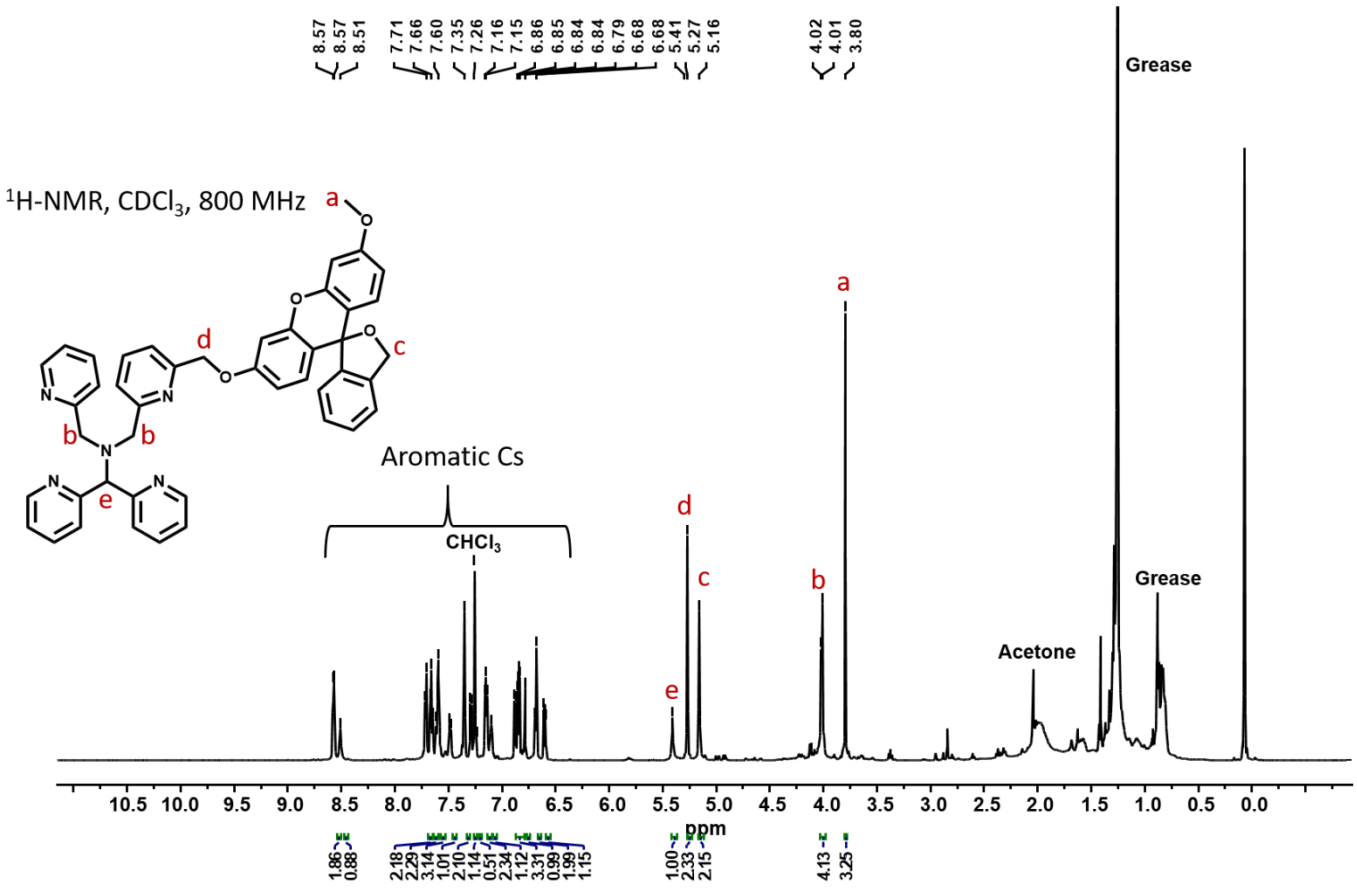
15 H. Y. Au-Yeung, E. J. New and C. J. Chang, *Chem. Commun.*, 2012, **48**, 5268–5270.

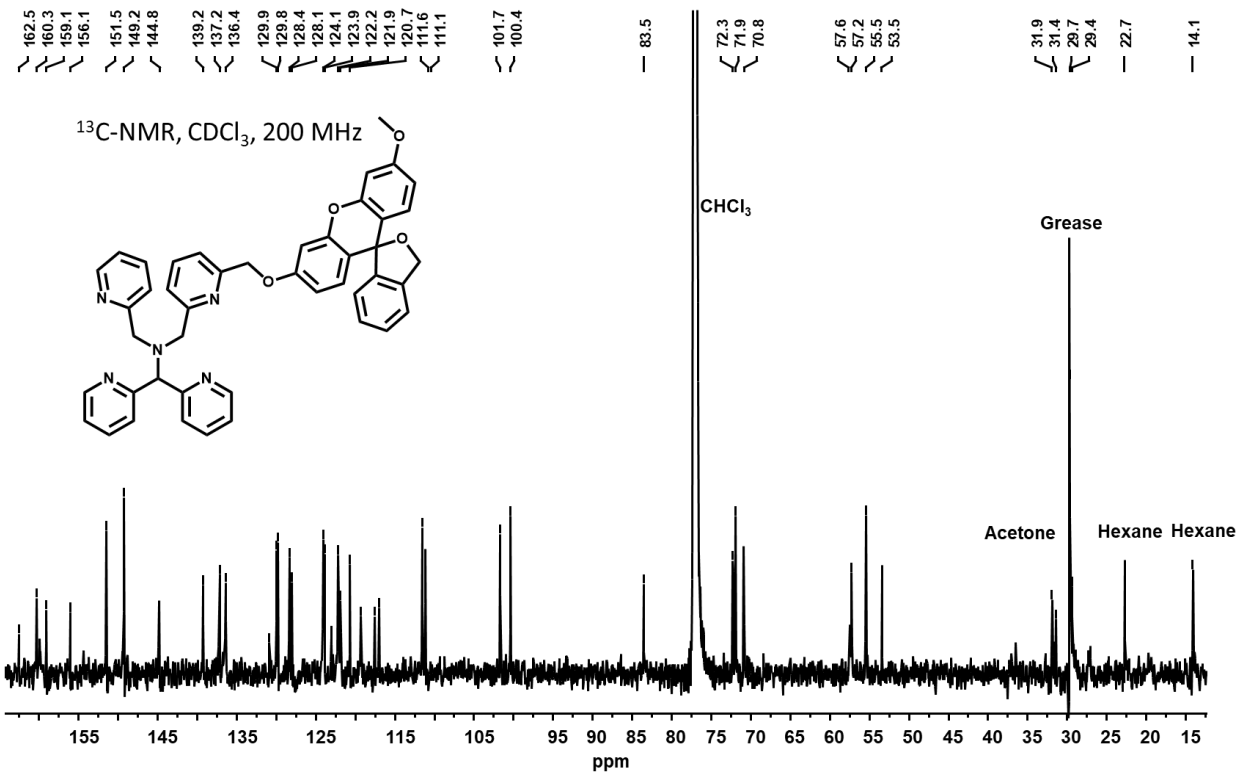
^{1}H NMR and ^{13}C Spectra




160.1
160.0
159.7
158.4
157.7
149.2
148.9
136.9
136.5
136.4
123.9
123.1
122.3
121.9
118.7
73.0
64.0
57.1
56.3
Aromatic Cs
13C-NMR, CDCl3, 200 MHz
c
OH
N
b
a
N
d
N
N
d
c
b
a
165
155
145
135
125
115
105
95
90
85
80
75
70
65
60
55
50
45
40
ppm


149.2
148.9
136.9
136.5
136.4
123.9
123.1
122.2
121.7
118.6
73.0
64.0
57.0
56.2
DEPT, CDCl3, 800 MHz
OH
N
N
N
N
N
165
155
145
135
125
115
105
95
90
85
80
75
70
65
60
55
50
45
40
35
ppm


^{1}H-NMR, $CDCl_3$, 800 MHz
Aromatic Cs
$CHCl_3$
Grease
Grease
Acetone
ppm


^{13}C-NMR, $CDCl_3$, 200 MHz
$CHCl_3$
Grease
Acetone
Hexane
Hexane
ppm