Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

A Three-Photon Probe with Duel Emission Colors for Imaging of Zn(II) Ion in Living Cell

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1. General Experimental Section:

AIBN, 1-bromo-6-chlorohexane, 1-bromo-2-(2-(2-methoxyethoxy)ethoxy)ethane, 3-bromoprop-1-yne, 18-crown-6, *N*-bromosuccinimide, 5,5'-dimethyl-2,2'-bipyridine, phosphorus oxychloride, potassium tertiary butoxied, 1H-pyrrole-2-carbaldehyde, sodium azide, triethyl phosphite and all other chemicals used were purchased from Sigma-Aldrich Chem. Co. and were used as received without any further purification. All solvents were of reagent grade and were procured from local companies. All solvents were dried and distilled prior to use by following standard procedures. ^{1}H NMR spectra were recorded on a Bruker 300 MHz FT-NMR (model: Advance-DPX 300) spectrometer at 25°C. The chemical shift (δ) data and coupling constant (J) values were given in parts per million (ppm) and Hertz, respectively, throughout the Supporting Information unless otherwise mentioned. ESI MS measurements were carried out on a Waters QTof-Micro instrument. The high-resolution time-of-flight mass spectrometry (TOF MS) was performed on a Waters Q-tof Premier MS spectrometer. Elemental analysis was performed on a EuroVector Euro EA elemental analyzer. UV-vis spectra were obtained by using a Shimadzu UV-3600 UV-vis-NIR spectrometer. Steady-state emission spectra at room temperature were obtained using a Shimadzu RF-5301PC spectrofluorimeter. The measurements of lifetime were performed by using an Optronis Optoscope streak camera system, which has an ultimate temporal resolution of ~50 ps. The laser pulse from an OPA combined with TOPAS (1000 Hz, 100 fs, Spectra-Physics, Inc.) at the wavelength of 360 nm was used as the excitation source. Three photon microscopic image were acquired using a Multiphoton CLSM (LSM510 META NLO, PMT detector, Carl-Zeiss) with Ti:Sapphire laser system used as an excitation source, for which the excitation wavelength is tunable in the wavelength range from 260 to 2600 nm. Which produced 100 fs (HW1/e) pulse with a repetition of 1000 Hz respectively. All the acquired images were processed minimally, including adjustment of brightness and contrast.

2. Synthetic route of compound (**L1**):

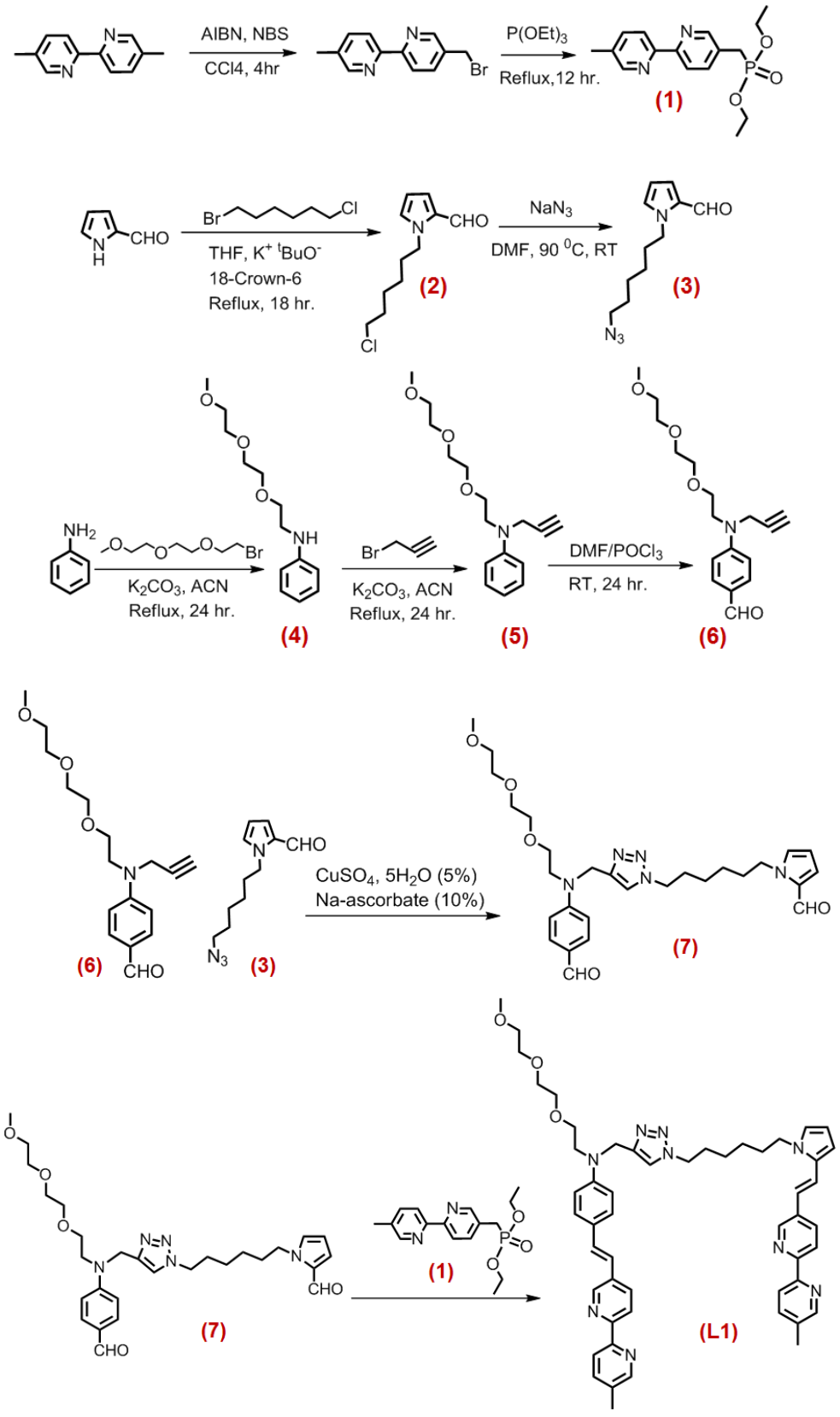
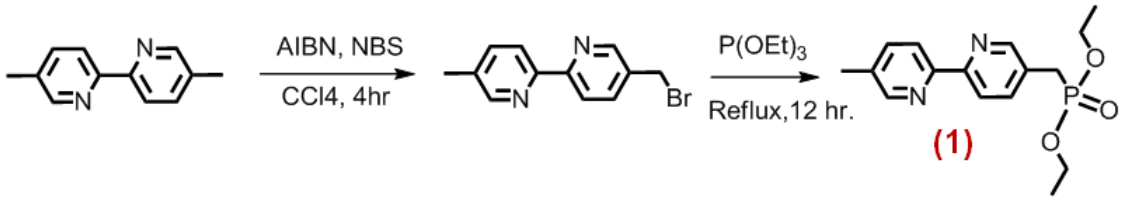


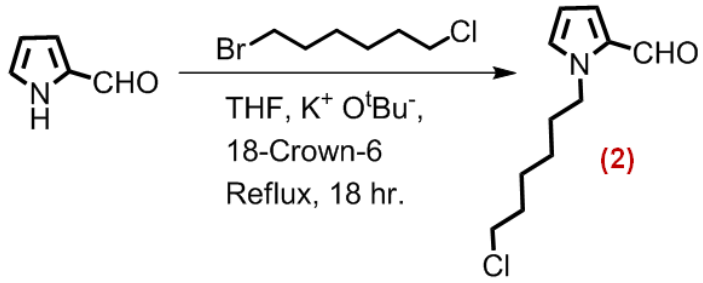
Figure S1. Methodologies adopted for the synthesis of (**L1**) (RT = room temperature).

3. Synthetic procedure of (**1**):



Synthesis of (**1**): 5,5'-Dimethyl-2,2'-bipyridine (1.00 g, 5.43 mmol) and *N*-bromosuccinimide (0.93 g, 5.21 mmol) were dissolved in anhydrous CCl_4 (40 mL), and the mixture solution was refluxed. To this solution catalytic amount of AIBN was added, and the reaction mixture was refluxed for 3 h. Then, the solvent was removed in reduced pressure at low temperature. The obtained crude residue was immediately dissolved in triethyl phosphite (5 mL), and the reaction mixture was refluxed for 12 h. Then, the excess triethyl phosphite was removed from the reaction mixture by using downward vacuum distillation setup. The crude product was purified on Si-gel column by using $CHCl_3$:MeOH (9.6:0.4 v/v) as an eluent to yield (**1**) (0.66 g, 40.0%) as a sticky light yellow mass. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.50 (1H, s), 8.27 (1H, d, J = 9.0 Hz), 8.21 (1H, d, J = 6.0 Hz), 7.74 – 7.70 (1H, m), 7.57 (1H, dd, J = 9.0 Hz), 4.06 – 3.96 (4H, m), 3.10 (2H, d, J = 21.0 Hz), 2.32 (3H, s), 1.21 (3H, t, J = 6.0 Hz). Elemental analysis: Calcd. for $C_{16}H_{21}N_2O_3P$: C, 59.99; H, 6.61; N, 8.75; P, 9.67. Found: C, 59.93; H, 6.59; N, 8.72; P, 9.64. ESI-MS: m/z calcd for $C_{16}H_{21}N_2O_3P$ 320.12, found 321.19 $[M + H]^+$.

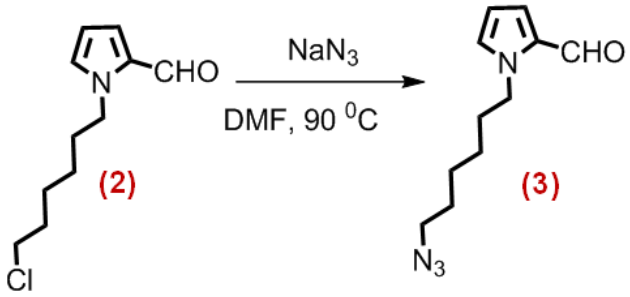
4. Synthetic procedure of (**2**):



Synthesis of (**2**): Potassium tertiary butoxied (5.29 g, 47.31 mmol) was dissolved in dry THF (20 mL) in a double neck round bottle under N_2 atmosphere. Then, 1H-pyrrole-2-carbaldehyde (3.00 g, 31.54 mmol) was dissolved in dry THF (10 mL), which was added dropwise to the above solution. The reaction mixture was stirred at room temperature for 3 h. 1-Bromo-6-chlorohexane (6.29 g, 31.54 mmol) and catalytic amount of 18-crown-6 were added and the reaction mixture was refluxed for 18 h. Then, the solvent was removed under reduced pressure. The crud product was extracted three times with $CHCl_3$. Organic layers were combined and dried over anhydrous sodium sulphate. Solvent was removed under reduced pressure and the crude product was purified on Silica-gel column using hexane/ethyl acetate (9:1, v/v) as an eluent to yield (**2**) (4.36 g, 65.0%) as a sticky light brown solid.

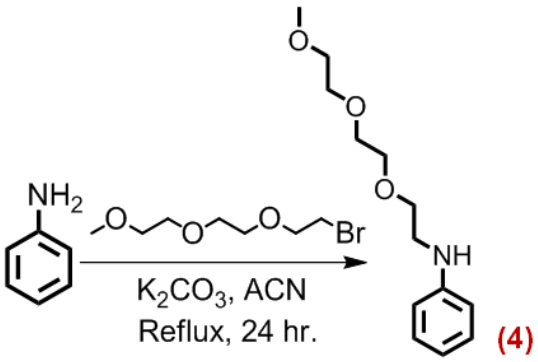
^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 9.53 (1H, s), 6.94 – 6.92 (2H, m), 6.22 (1H, t, J = 3.0 Hz), 4.31 (2H, t, J = 7.5 Hz), 3.51 (2H, t, J = 7.5 Hz), 1.82 – 1.71 (4H, m), 1.51 – 1.41 (2H, m), 1.35 – 1.26 (2H, m). Elemental analysis: Calcd. for $C_{11}H_{16}ClNO$: C, 61.82; H, 7.55; N, 6.55. Found: C, 61.79; H, 7.52; N, 6.53. ESI-MS: m/z Calcd. for $C_{11}H_{16}ClNO$ 213.09, found 236.11 $[M + Na]^+$.

5. Synthetic procedure of (**3**):



Synthesis of (**3**): Compound (**2**) (4.3 g, 20.17 mmol) was dissolved in dry DMF (40 ml). To this solution NaN_3 (3.9 g, 60.51 mmol) was added and the reaction mixture was stirred at 90 ^{0}C for overnight. Then, the solvent was removed in reduced pressure. The crude product was extracted three times with $CHCl_3$. After combining the organic layers, the solvent was evaporated in reduced pressure. The crude product was purified by passing through a Si-gel column using $CHCl_3$ as an eluent to yield (**3**) (3.33 g, 75.0%) as a sticky brown solid. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 9.50 (1H, s), 6.91 – 6.88 (2H, m), 6.19 (1H, t, J = 3.0 Hz), 4.27 (2H, t, J = 7.5 Hz), 3.21 (2H, t, J = 6.0 Hz), 1.78 – 1.69 (2H, m), 1.60 – 1.50 (2H, m), 1.35 – 1.23 (4H, m). Elemental analysis: Calcd. for $C_{11}H_{16}N_4O$: C, 59.98; H, 7.32; N, 25.44. Found: C, 59.95; H, 7.29; N, 25.41. ESI-MS: m/z calcd for $C_{11}H_{17}N_4O$ 221.13, found 222.08 $[M + H]^+$.

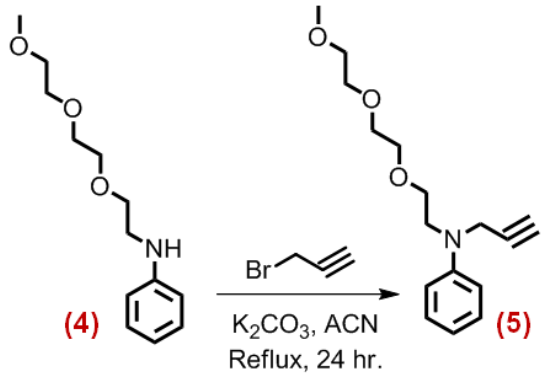
6. Synthetic procedure of (**4**):



Synthesis of (**4**): Aniline (1.08 g, 11.71 mmol) was dissolved in dry CH_3CN (20 mL) in a two-neck round-bottom flask. To this stirred solution 1-bromo-2-(2-(2-methoxyethoxy)ethoxy)ethane (2.66 g,

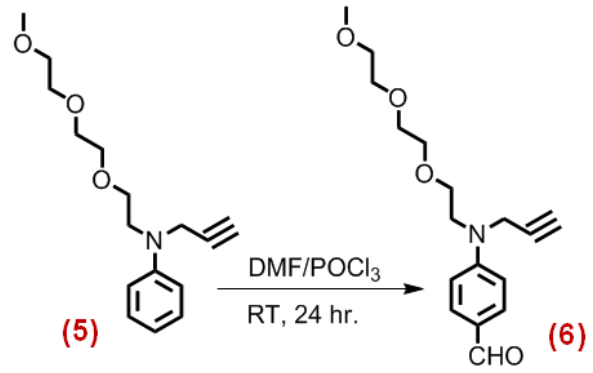
11.71 mmol) and K_2CO_3 (8.08 g, 58.59 mmol) were added. Then, the reaction mixture was refluxed for 24 h. Solvent was removed under reduced pressure and the residue was extracted three times with $CHCl_3$. The combined organic layers were dried over anhydrous sodium sulphate. After removing the solvent, the crude product was purified by passing through an silica column using ethyl acetate/hexane (3:7, v/v) as the eluent to yield (**7**) (1.68 g, 60.0%) as a sticky light yellow mass. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 7.15 (2H, t, J = 9.0 Hz), 6.69 (1H, t, J = 7.5 Hz), 6.63 (2H, d, J = 9.0 Hz), 3.68 (2H, t, J = 6.0 Hz), 3.64 (6H, bs), 3.54 (2H, t, J = 4.5 Hz), 3.37 (3H, s), 3.28 (2H, t, J = 4.5 Hz). Elemental analysis: Calcd. for $C_{13}H_{21}NO_3$: C, 65.25; H, 8.84; N, 5.85. Found: C, 65.21; H, 8.81; N, 5.82. ESI-MS: m/z calcd for $C_{13}H_{21}NO_3$ 239.15, found 240.09 $[M + H]^+$.

7. Synthetic procedure of (**5**):



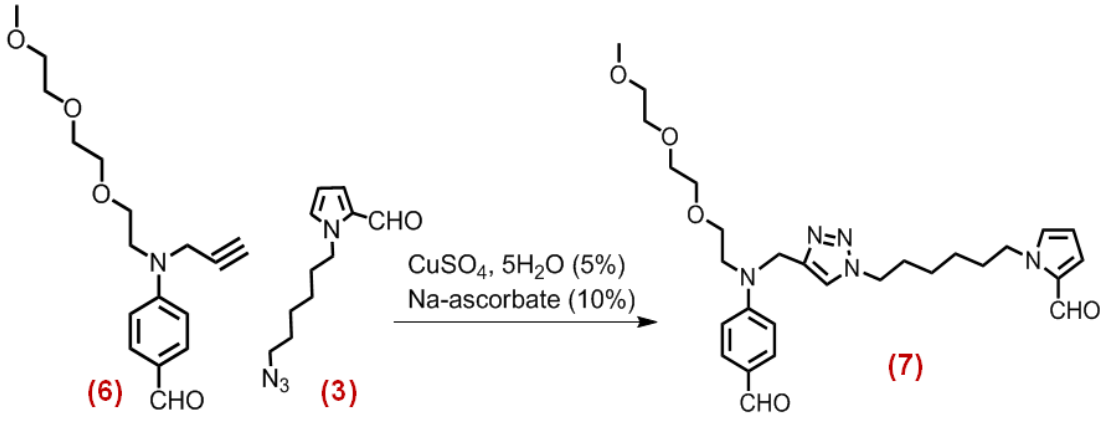
Synthesis of (**5**): Compound (**4**) (3.40 g, 14.22 mmol) was dissolved in dry CH_3CN (20 mL) in a two-neck round-bottom flask in N_2 atmosphere. To this stirred solution 3-bromoprop-1-yne (2.20 g, 18.49 mmol) and K_2CO_3 (5.88 g, 42.66 mmol) were added. Then, the reaction mixture was refluxed for 24 h. Solvent was removed under reduced pressure and the residue was extracted three times with $CHCl_3$. The combined organic layers were dried over anhydrous sodium sulphate. After removing the solvent, the crude product was purified by passing through an silica column using ethyl hexane/ethyl acetate (6:4, v/v) as the eluent to yield (**8**) (2.16 g, 55.0%) as a sticky light yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 7.26 (2H, t, J = 7.5 Hz), 6.86 (2H, d, J = 9.0 Hz), 6.76 (1H, t, J = 7.5 Hz), 4.08 (2H, d, J = 3.0 Hz), 3.70 – 3.53 (12 H, m), 3.37 (3H, s), 2.19 (1H, t, J = 3.0 Hz). Elemental analysis: Calcd. for $C_{16}H_{23}NO_3$: C, 69.29; H, 8.36; N, 5.05. Found: C, 69.27; H, 8.32; N, 5.03. ESI-MS: m/z calcd for $C_{16}H_{23}NO_3$ 277.16, found 278.15 $[M + H]^+$.

8. Synthetic procedure of (**6**):



Synthesis of (**6**): $POCl_3$ (5.19 g, 33.87 mmol) and DMF (2.47 g, 33.87 mmol) were stirred in a round bottle under N_2 atmosphere at room temperature for 30 min. Then, compound (**5**) (2.16 g, 7.79 mmol) was dissolved in DMF (7 mL), which was dropwise added to the above solution. The reaction mixture was stirred for 24 h. The solvent was removed under reduced pressure and the residue was poured into ice water (50 mL). The solution was neutralized by NaOH, and then was extracted three times with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulphate. After removing the solvent, the crude product was purified by passing through Si-gel column using hexane/ethyl acetate (7:3, v/v) as the eluent to yield (**9**) (1.78 g, 75.0%) as a sticky light brown solid. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 9.74 (1H, s), 7.75 (2H, d, J = 9.0 Hz), 6.86 (2H, d, J = 9.0 Hz), 4.18 (2H, d, J = 3.0 Hz), 3.72 – 3.59 (10H, m), 3.51 (2H, t, J = 4.5 Hz), 3.35 (3H, s), 2.25 (1H, t, J = 3.0 Hz). Elemental analysis: Calcd. For $C_{17}H_{23}NO_4$: C, 66.86; H, 7.59; N, 4.59. Found: C, 66.82; H, 7.56; N, 4.57. ESI-MS: m/z Calcd. For $C_{17}H_{23}NO_4$ 305.16, found 306.20 $[M + H]^+$.

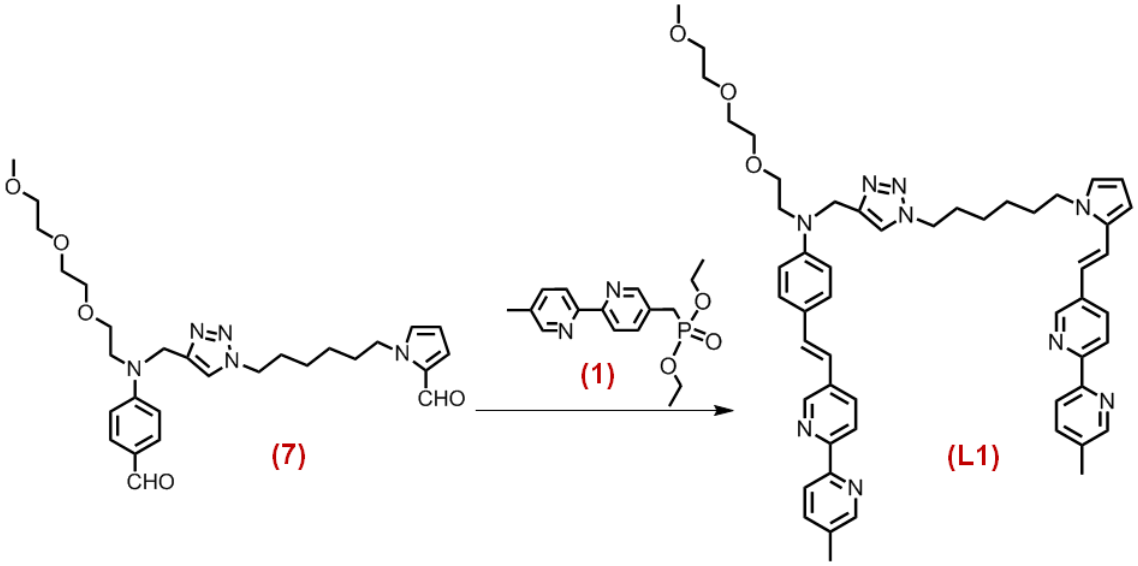
9. Synthetic procedure of (**7**):



Synthesis of (**7**): Compound (**6**) (0.74 g, 2.45 mmol) and compound (**3**) (0.53 g, 2.45 mmol) were suspended in a 1:1 mixture of water and tert-butyl alcohol (16 mL). Sodium ascorbate (0.24g, 0.22

mmol) was added, followed by the addition of copper(II) sulphate pentahydrate (0.03 g, 0.12 mmol). The heterogeneous mixture was stirred vigorously overnight until complete consumption of the reactants determined by the TLC analysis. The solvent was removed under reduced pressure. The crude reaction mixture was extracted three times with $CHCl_3$. The combined organic layers were dried over anhydrous sodium sulphate. After removing the solvent, the crude product was purified by passing through Si-gel column using hexane/ethyl acetate (5:5, v/v) as the eluent to yield (**11**) (0.99 g, 77.0%) as a sticky brown solid. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 9.68 (1H, s), 9.49 (1H, s), 7.66 (2H, d, J = 9.0 Hz), 7.51 (1H, s), 6.92 (2H, d, J = 6.0 Hz), 6.84 (2H, d, J = 6.0 Hz), 6.20 (1H, t, J = 3.0), 4.78 (2H, s), 4.30 – 4.21 (4H, m), 3.74 (4H, s), 3.62 – 3.57 (6H, m), 3.50 (2H, t, J = 6.0 Hz), 3.33 (3H, s), 1.85 (2H, t, J = 6.0 Hz), 1.70 (2H, t, J = 6.0 Hz), 1.31 – 1.26 (4H, m). Elemental analysis: Calcd. for $C_{28}H_{39}N_5O_5$: C, 63.98; H, 7.48; N, 13.32. Found: C, 63.94; H, 7.45; N, 13.29. ESI-MS: m/z calcd for $C_{28}H_{39}N_5O_5$ 525.29, found 526.38 $[M + H]^+$.

10. Synthetic procedure of (**L1**):



Synthesis of (**L1**): Compound (**7**) (0.61 g, 1.17 mmol) and compound (**1**) (0.75 g, 2.34 mmol) were dissolved in dry THF (20 mL) in a double neck round bottle. Sodium hydride (60%, 0.09 g, 2.34 mol) was dissolved in THF (15 mL), which was addeded dropwise to the reaction mixture at 0 °C. Then, the reaction mixture was stirred at room temperature for 30 min. After the reaction mixture was refluxed for 12 h, the solvent was removed under reduced pressure and the crude mass was extracted three times with $CHCl_3$. The combined organic layers were dried over anhydrous sodium sulphate. After removing the solvent, the crude product was purified by passing through Si-gel column using $CHCl_3$/MeOH (9.5:0.5, v/v) as the eluent to yield (**L1**) (0.52 g, 52.0%) as a yellow brown solid. ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 8.67 (2H, bs), 8.48 (2H, bs), 8.33 – 8.25 (4H, m), 7.88 – 7.81 (2H,

m), 7.60 (2H, d, J = 9.0 Hz), 7.36 (2H, d, J = 6.0 Hz), 7.33 (1H, s), 7.12 – 6.98 (2H, m), 6.89 - 6.84 (2H, m), 6.76 (2H, d, J = 9.0 Hz), 6.65 (1H, d, J = 6.0 Hz), 6.56 (1H, d, J = 6.0 Hz), 6.16 (1H, t, J = 3.0 Hz), 4.71 (2H, s), 4.24 (2H, t, J = 7.5 Hz), 3.92 (2H, t, J = 7.5 Hz), 3.69 – 3.57 (10H, m), 3.52 (2H, t, J = 4.5 Hz), 3.34 (3H, s), 2.37 (6H, s), 1.87 – 1.81 (2H, m), 1.71 – 1.63 (2H, m), 1.30 (4H, bs). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 154.4, 154.2, 153.5, 153.4, 149.8, 149.5, 147.8, 147.6, 147.2, 145.7, 145.6, 137.7, 137.4, 133.5, 133.2, 133.1, 132.7, 130.7, 130.5, 128.0, 125.6, 123.3, 121.7, 121.6, 120.5, 120.4, 118.7, 112.4, 108.7, 107.4, 71.8, 70.4, 69.0, 67.8, 59.1, 50.7, 50.1, 47.3, 46.7, 38.6, 31.1, 29.9, 25.8, 25.5, 18.3. Elemental analysis: Calcd. for $C_{52}H_{59}N_9O_3$: C, 72.79; H, 6.93; N, 14.69. Found: C, 72.73; H, 6.90; N, 14.65. HRMS Calcd. for $C_{52}H_{59}N_9O_3$ 857.4741, found 858.4810 $[M+H]^+$.

11. Solvatochromic behavior of (**L1**):

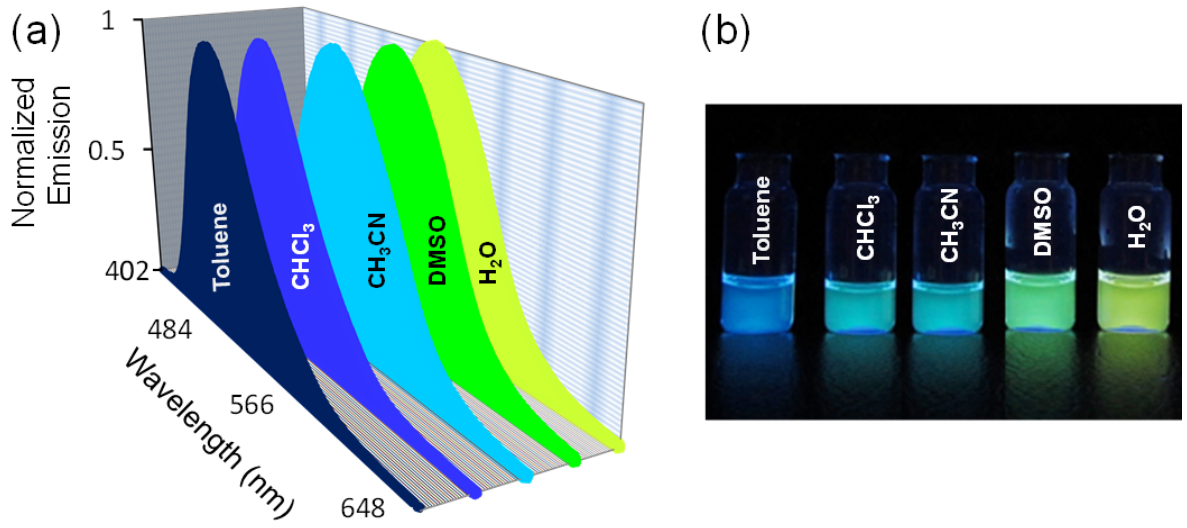


Figure S2. (a) Fluorescence emission spectra of the probe **L1** (5.98 x 10^{-6} M) in different solvents with increasing polarity under excitation at 380 nm. (b) Corresponding color change of the probe **L1** with increasing the solvent polarity under a hand-held UV light on excitation at 325 nm at room temperature.

12. UV-Vis spectral change of (**L1**) with different metal ions:

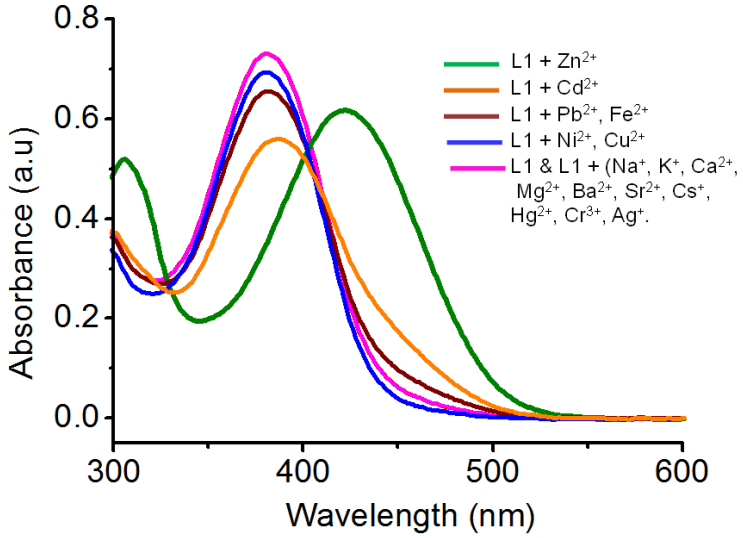


Figure S3. UV-vis spectra change of **L1** (1.18 x 10^{-5} M) in the presence of different metal ions (3.54 x 10^{-4} M) recorded in 50 mM of aqueous HEPES buffer of pH 7.2 at 25 ºC.

13. Fluorescence spectral change of (**L1**) with different metal ions:

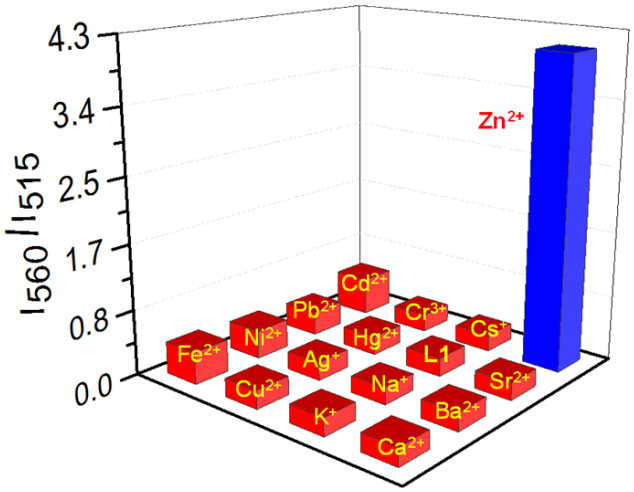


Figure S4. Fluorescence spectral change of **L1** (5.98 x 10^{-6} M) in the presence of different metal ions (1.79 x 10^{-4} M) recorded in 50 mM of aqueous HEPES buffer of pH 7.2 at 25 °C.

14. UV-Vis spectral response of (**L1**) with Zn^{2+}:

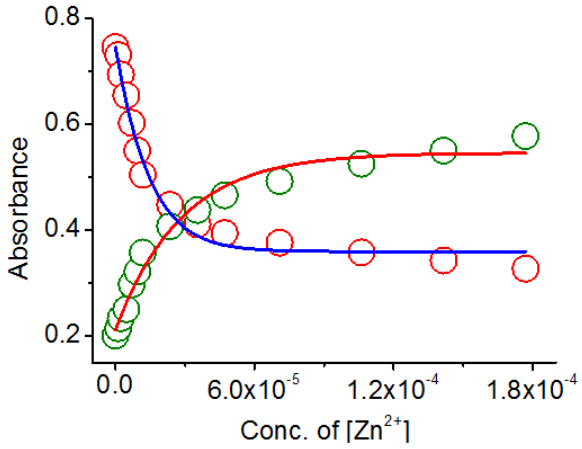


Figure S5. Changes of UV-vis spectral response of **L1** (1.18×10^{-5} M) upon increasing the concentration of Zn(II) ($0 - 1.77 \times 10^{-4}$ M) recorded in 50 mM of aqueous HEPES buffer of pH 7.2 and 25 °C. The absorption was monitored at 380 nm (red circle) and 424 nm (green circle), respectively.

15. UV-Vis spectral change of (**L1**) with TFA:

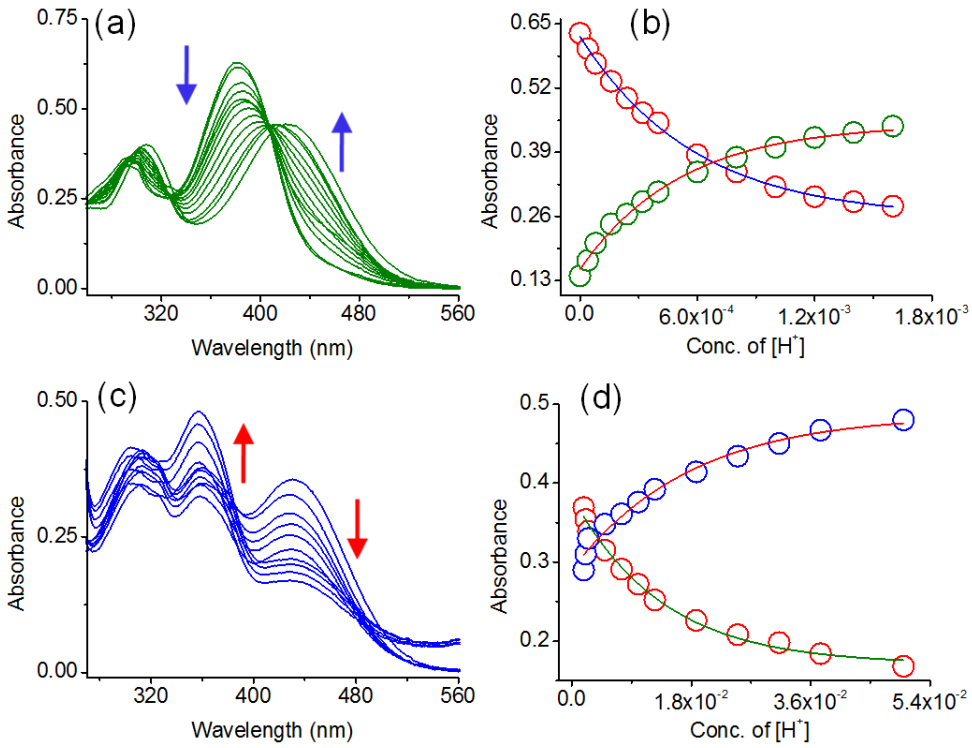


Figure S6. (a) UV-Vis spectral changes of the probe **L1** (1.05×10^{-5} M) with varying the concentrations of TFA ($0 - 1.60 \times 10^{-3}$ M) recorded in water (0.1 M KCl) at 25 °C. (b) Corresponding titration plot based on the absorbance changes of **L1** with different concentrations of TFA added.

Absorbance was monitored at 380 nm (red circle) and 433 nm (green circle). (c) UV-Vis spectral change of the probe **L1** (1.05 x 10^{-5} M) with varying the concentrations of TFA (1.80 x 10^{-3} – 5.00 x 10^{-2} M) recorded in water (0.1 M KCl) at 25 °C. (d) Corresponding titration plot based on the absorbance changes of **L1** with different concentrations of TFA added. Absorbance was monitored at 433 nm (red circle) and 357 nm (blue circle).

16. Fluorescence spectral response of (**L1**) with Zn^{2+} and TFA:

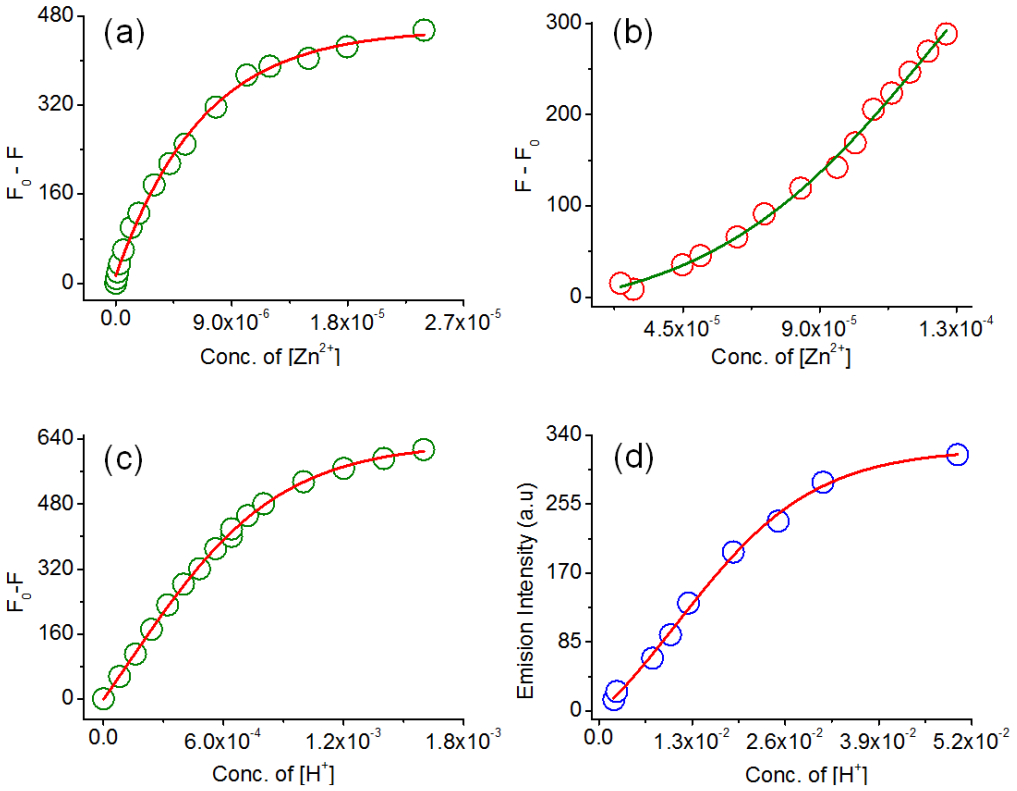


Figure S7. (a) Fluorescence titration plot for the changes of emission intensity of **L1** (5.98 x 10^{-6} M) monitored at 515 nm with different concentrations of Zn^{2+} added (0 – 2.39 x 10^{-5} M) (green spectra in the titration). (b) Fluorescence titration plot for the changes of emission intensity of **L1** (5.98 x 10^{-6} M) monitored at 566 nm with different concentrations of Zn^{2+} added (2.45 x 10^{-5} – 1.31 x 10^{-4} M) (red spectra in the titration). (c) Fluorescence titration plot for the changes of emission intensity of **L1** (1.05 x 10^{-5} M) monitored at 515 nm with varying the concentrations of TFA (0 - 1.6 x 10^{-3} M) (green spectra in the titration). (d) Fluorescence titration plot for the changes of emission intensity of **L1** (1.05 x 10^{-5} M) monitored at 465 nm with varying the concentrations of TFA (1.8 x 10^{-3} - 5.0 x 10^{-2} M) (blue spectra in the titration).

17. Summary of the one-photon photo-physical data:

Table S1. Summary for the photo-physical parameters of the probe **L1** in the presence of Zn(II) and TFA at room temperature: absorption maximum (λ_{abs}), emission maximum (λ_{emi}), extinction co-efficient (ε) at the maximum absorption, emission quantum yield (Φ), and excited state life time (τ).

Compounds	λ_{abs} (nm)	λ_{emi} (nm)	$\varepsilon \times 10^4$ ($M^{-1}cm^{-1}$)	Φ[c] (%)	τ[d] (ns)
L1[a]	380	515	6.33	27.3	$\tau_1 = 1.44$ $\tau_2 = 1.17$
L1+Zn^{2+}[a]	425	566	5.26	18.2	$\tau_1 = 1.27$ $\tau_2 = 0.70$
L1+$H^+_{(high)}$[b]	355	465	4.57	26.1	$\tau_1 = 1.31$ $\tau_2 = 0.72$
L1+$H^+_{(low)}$[b]	425	515	4.35	14.3	$\tau_1 = 0.88$ $\tau_2 = 0.71$

[a] Spectroscopic measurements were performed in50 mM of aqueous HEPES buffer of pH 7.2. [b] Spectroscopic measurements were performed in water (0.1 M KCl). [c] Luminescence quantum yields (±5% error) were determined using quinine sulphate as the standard (Φ_f = 0.54 in 0.1 M H_2SO_4). [d] Excited state lifetimes were measured through bi-exponential convolution fit of the fluorescence decay profiles. Under the excitation at 380 nm, the emission was monitored at 515 nm for **L1**, 570 nm for (**L1**+Zn^{2+}), 465 nm for (**L1**+H^+_{high}), and 515 nm for (**L1**+H^+_{low}). The fitting decays were judged through the reduced χ^2 values. The fits were accepted for χ^2 = 0.8 -1.2.

18. Determination of binding constant:

The binding constants were determined by using Benesi-Hildebrand equation based on the UV-Vis and fluorescence spectral changes of the probe (**L1**) in the presence of Zn^{2+}.

$$\frac{1}{A - A_0} = \frac{1}{K(A_{max} - A_0)[Zn^{2+}]^n} + \frac{1}{A_{max} - A_0}$$

where A_0 is the absorbance of the probe (**L1**), A is the absorbance in the presence of Zn^{2+}, A_{max} is the absorbance obtained with excess amount of Zn^{2+}, K is the association constant, and [Zn^{2+}] is the concentration of Zn^{2+}added.

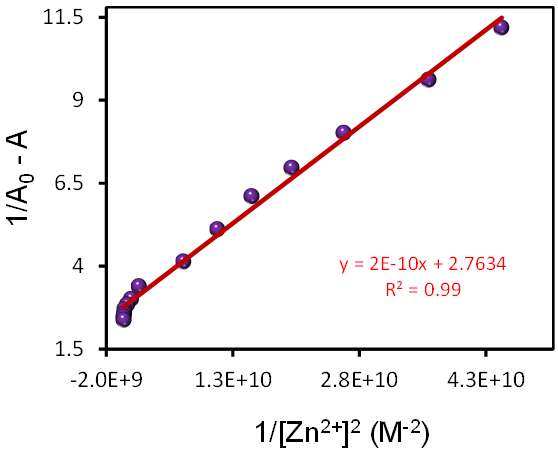


Figure S8: Benesi-Hildebrand plot obtained from UV-Vis spectral change for evaluation of the binding constant of **L1** with Zn^{2+}, recorded in 50 mM of aqueous HEPES buffer of pH 7.2 and 25 ºC. The linearity of this plot with $R^2 = 0.99$ confirms the 1:2 binding stoichiometry.

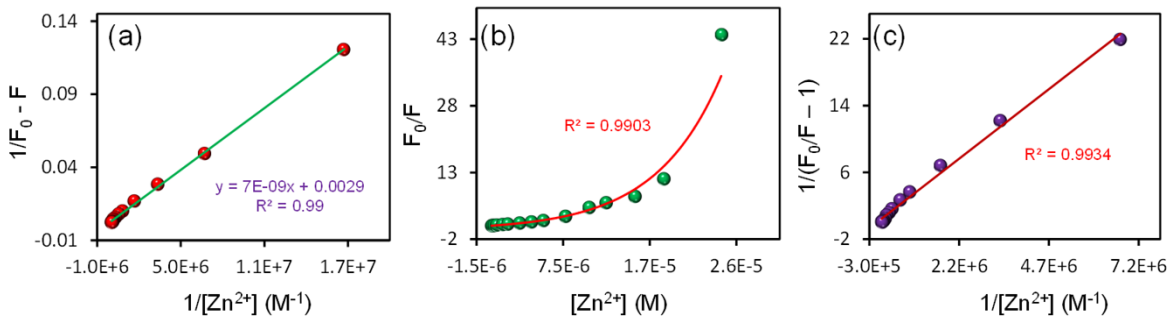


Figure S9: (a) Benesi-Hildebrand plot obtained from fluorescence spectral change for the evaluation of first binding constant (K_1) of **L1** with Zn^{2+}, recorded in 50 mM of aqueous HEPES buffer of pH 7.2 and 25ºC. (b) Non-linear nature of the plot for the change of fluorescence ratio (F_0/F) as a function of varying [Zn^{2+}]. (c) Linear double reciprocal plot suggesting the 1:1 complex formation.

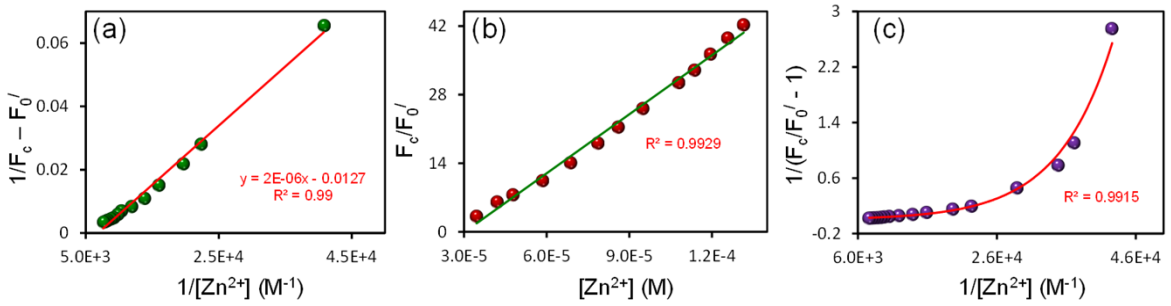


Figure S10: (a) Benesi-Hildebrand plot obtained from fluorescence spectral change for the evaluation of second binding constant (K_2) of **L1** with Zn^{2+}, recorded in 50 mM of aqueous HEPES buffer of pH 7.2 and 25ºC. (b) The linear nature of the plot for the change of fluorescence ratio (F_C/F'_0) as a function of varying [Zn^{2+}], (c) The non-linear nature of double reciprocal plot suggesting the formation of higher-order complex.

19. Schematic representation of the photo-physical response of the probe (L1) in presence of Zn(II) and TFA:

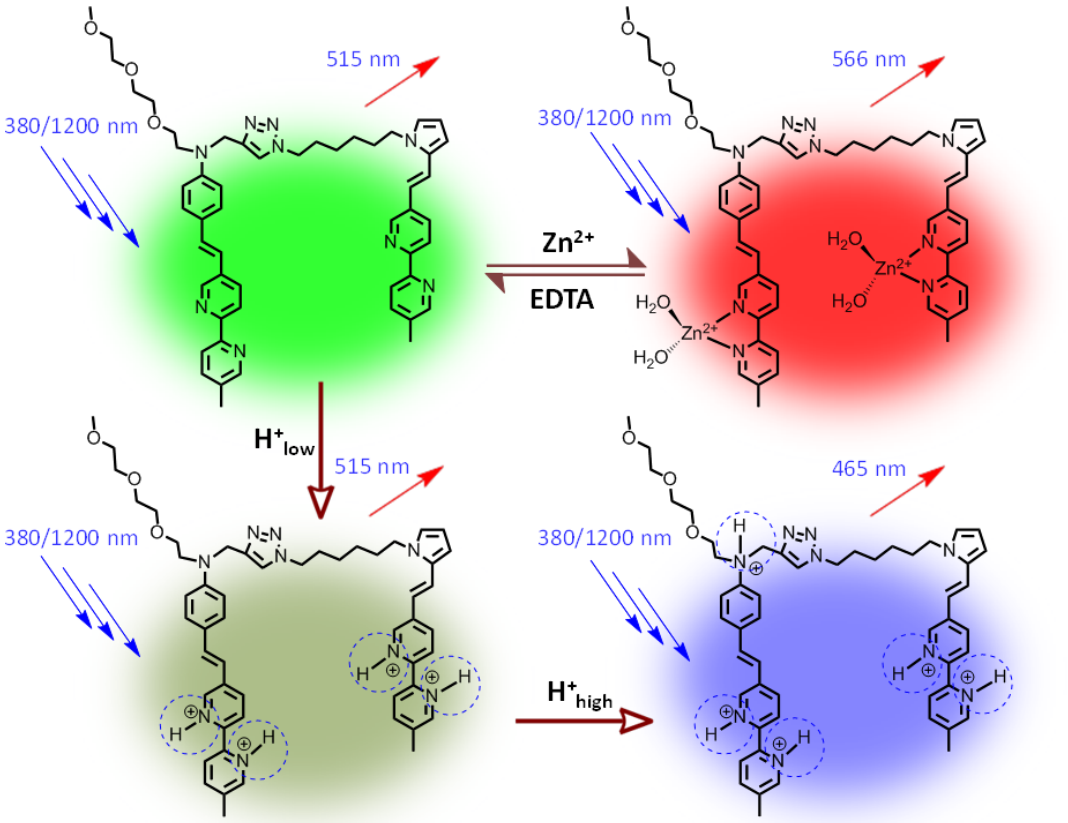


Figure S11. Schematic representation of the complexes formed from the probe **L1** in the presence of Zn(II) or TFA, and their corresponding photo-physical response.

20. Molar ratio plot for the complexation of (**L1**) with Zn^{2+}:

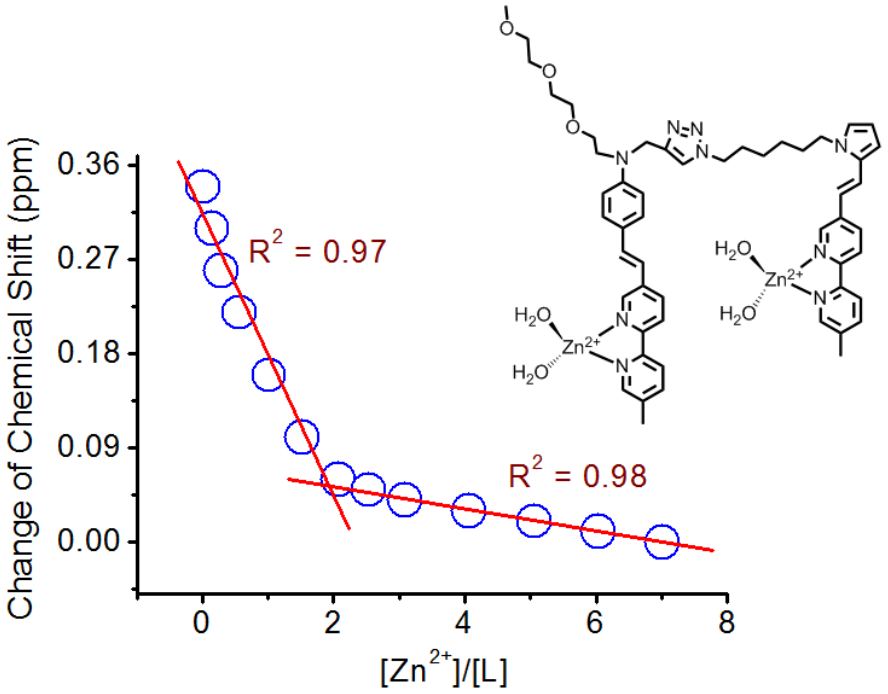


Figure S12: Molar ratio plot derived from the ^{1}H NMR titration for the complexation of **L1** with $Zn(ClO_4)_2$ recorded in CD_3CN at 25 °C. The inflection point at ~2 in the X-axis suggests the 1:2 stoichiometry of the complex {**L1**$(Zn^{2+})_2$}.

21. HRMS spectrum for the complexation of (**L1**) with Zn^{2+}:

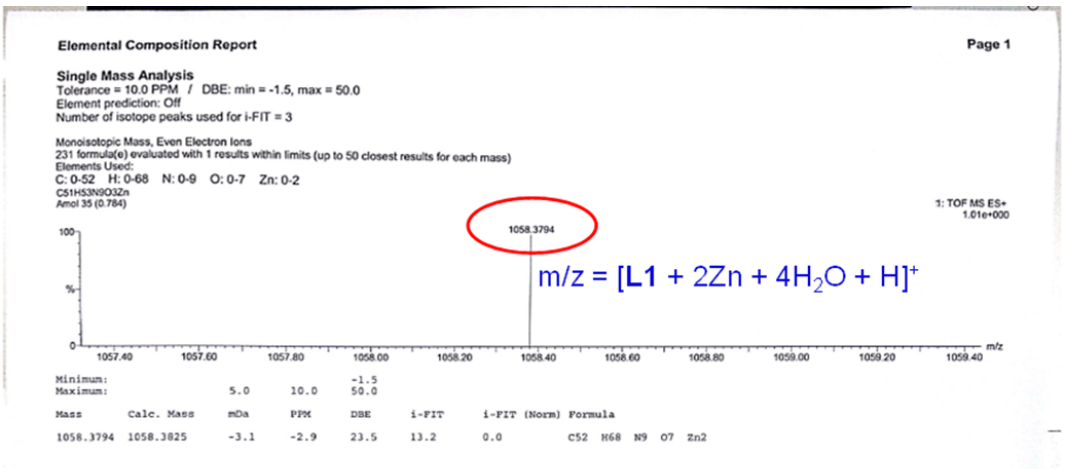


Figure S13: HRMS for the complex {**L1**$(Zn^{2+})_2$•$4H_2O$} suggesting the 1:2 stoichiometry of the complex.

22. Quantum yield measurements:

Fluorescence quantum yields were measured by the relative comparison procedure by using quinine sulphate in 0.1 M H_2SO_4 as the reference compound with quantum yield of 0.54. The quantum yield was calculated from Equation (1) shown below:

$$\phi_f = \phi_f' \, (I_{sample}/I_{std})(A_{std}/A_{sample})(\eta^2_{sample}/\eta^2_{std}) \quad \ldots\ldots\ldots\ldots\ldots \quad (1)$$

where ϕ_f' is the absolute quantum yield of the reference compound, I_{sample} and I_{std} are the integrated emission intensities, A_{sample} and A_{std} are the absorbance at the excitation wavelength, and η^2_{sample} and η^2_{std} are the respective refractive indices.

23. Fluorescence decay profiles for (**L1**) in presence of Zn^{2+} and TFA:

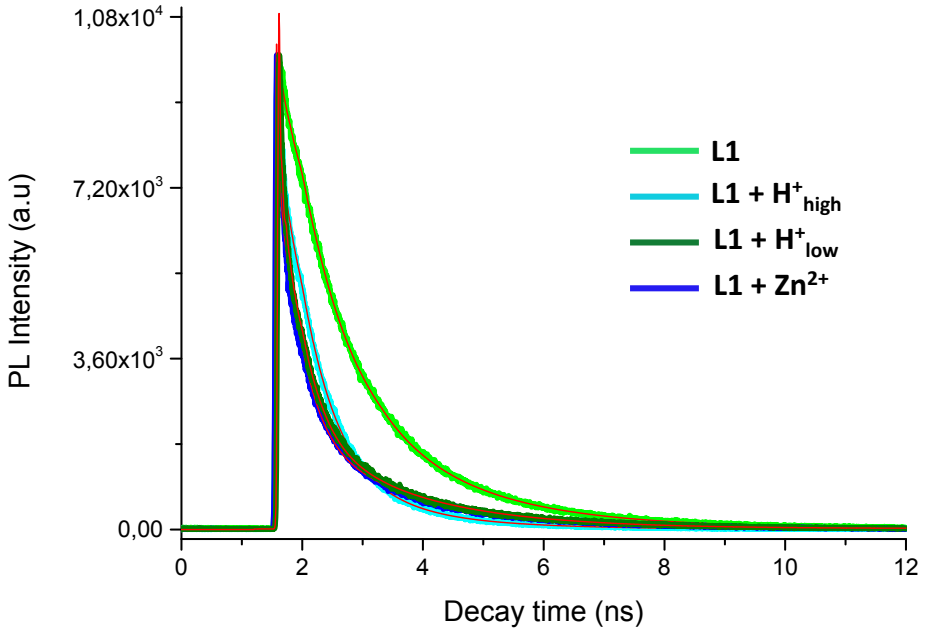


Figure S14: Fluorescence decay profiles measured for **L1** (5.98 x 10^{-6} M) in the presence of Zn^{2+} (1.31 x 10^{-4} M) as well as low (9.10 x 10^{-4}M, H^+_{low}) and high (2.84 x 10^{-2} M, H^+_{high}) concentrations of TFA. Decay profiles were measured with excitation at 380 nm and the emission at 515 nm for **L1**, 570 nm for **L1**+Zn^{2+}, 465 nm for **L1**+H^+_{high} and 515 nm for **L1**+H^+_{low}. Decay profiles for **L1** and **L1** in the presence of Zn^{2+} were measured in 50 mM of aqueous HEPES buffer of pH 7.2 and the ones for **L1** in the presence of TFA were measured in water (0.1 M KCl) at 25 °C.

24. ^{1}H NMR spectral response of (**L1**) in presence of Zn^{2+}:

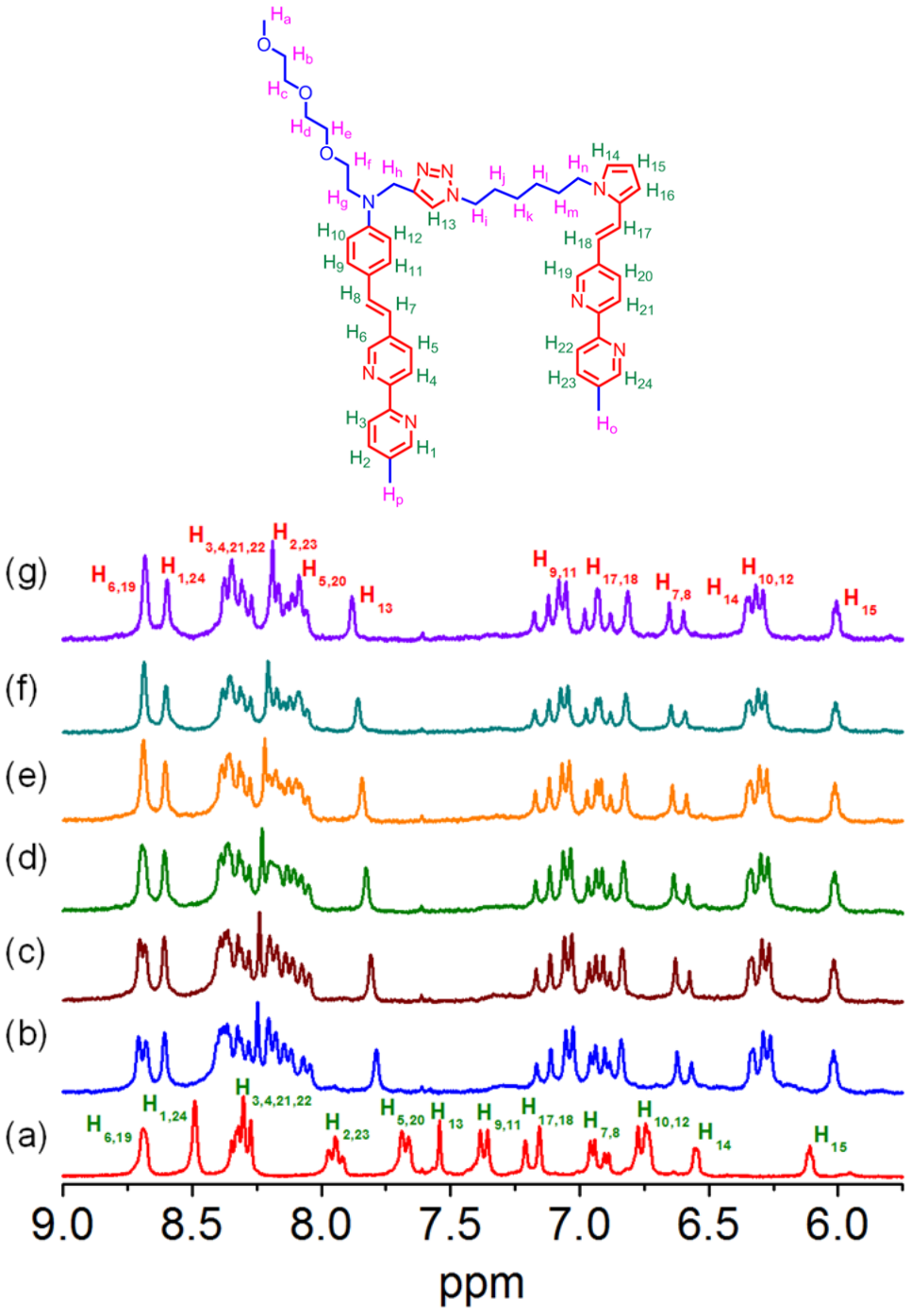


Figure S15: Partial ^{1}H NMR spectra (300 MHz, CD_3CN, δ ppm) recorded for (a) 8.2 mM of **L1**, and 8.2 mM of **L1** with (b) 8.2 mM of $Zn(ClO_4)_2$, (c) 16.4 mM of $Zn(ClO_4)_2$, (d) 24.6 mM of $Zn(ClO_4)_2$, (e) 32.8 mM of $Zn(ClO_4)_2$, (f) 41.0 mM of $Zn(ClO_4)_2$, and (g) 82.0 mM of $Zn(ClO_4)_2$.

25. ^{1}H NMR spectral response of (**L1**) in presence of TFA:

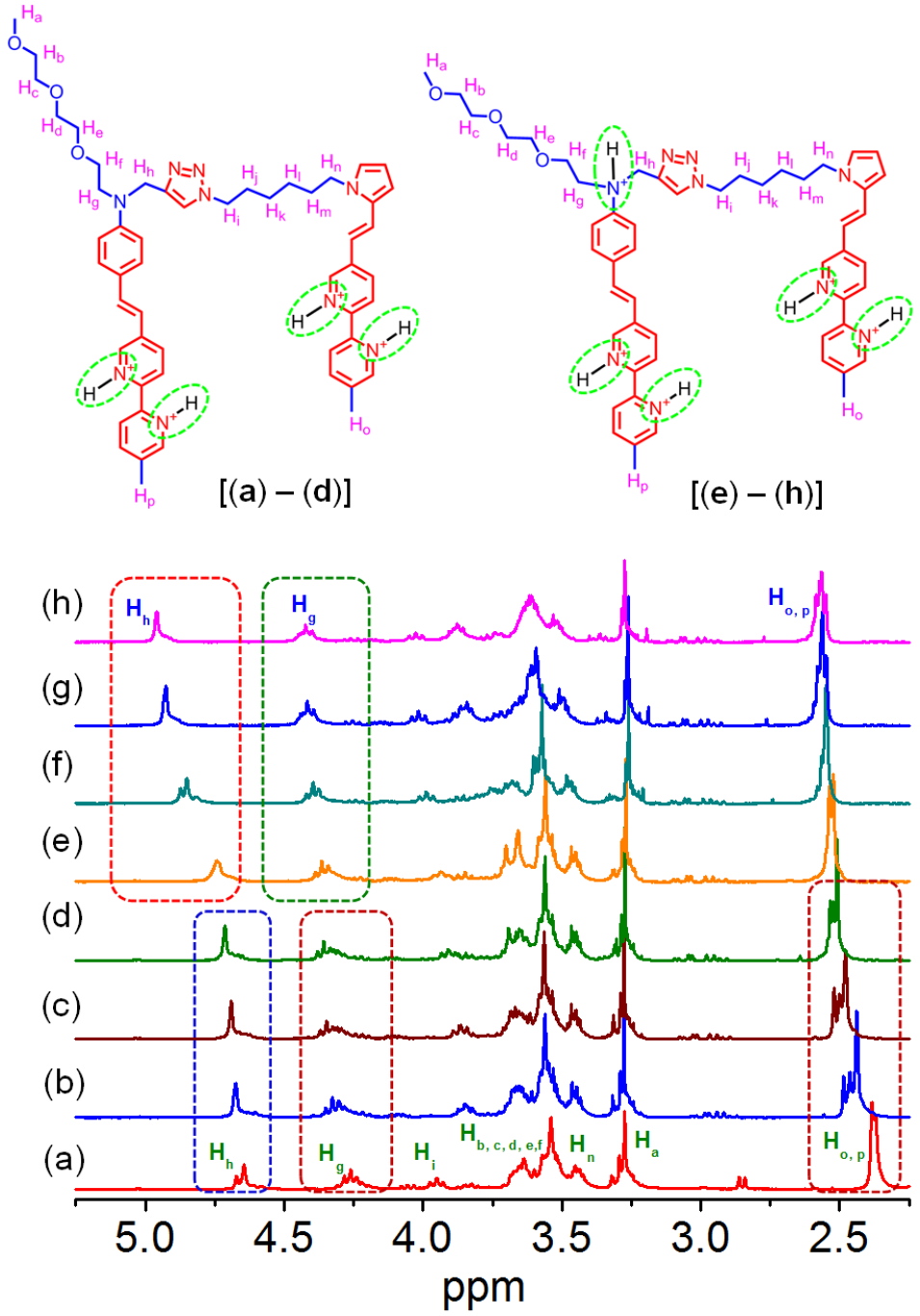


Figure S16: Partial ^{1}H NMR spectra (300 MHz, CD_3CN, δ ppm) recorded for (a) 8.2 mM of **L1**, and 8.2 mM of **L1** with (b) 8.2 mM of TFA, (c) 16.4 mM of TFA, (d) 32.8 mM of TFA, (e) 41.0 mM of TFA, (f) 49.2 mM of TFA, (g) 65.6 mM of TFA, and (h) 82.0 mM of TFA. The dotted boxes from (a) to (d) indicate the initial protonation of the bipyridine units. The dotted boxes from (e) to (h) indicate the protonation of the tertiary N-atom.

26. Measurements of three-photon absorption cross section:

Measurements of 3PA cross-section: 3PA spectra of samples were obtained by using multi-photon luminescence method with the (Rhodamine B) in water as reference. A Ti:sapphire system, which produced 100 fs (HW1/e) pulse in the wavelength range of 260–2600 nm with a repetition of 1000 Hz, was used as the excitation source. In the measurement process, the input laser beam was focused into the solutions. To avoid the self-reabsorption in the 3PA cross section measurements, the laser beam was focused as close as possible to the wall of the quartz cell and the PL signal was collected in back-scattering geometry, so that only the emission from the edge of the solution was collected. It is well known that the three-photon excited luminescence (F_3) is proportional to the cube of incident power intensity, while the two-photon excited luminescence (F_2) linearly depends on the square of incident power intensity:

$$F_3 \sim \eta\phi\sigma_3 N_3 I_0^3 \quad and \quad F_2 \sim \eta\phi\sigma_2 N_3 I_0^2 \ldots\ldots\ldots(1)$$

Where, F_3 and F_2 are the three- and two-photon excited luminescence intensity, respectively, η is the luminescence quantum efficiency, σ_3 and σ_2 are the 3PA cross section for samples and 2PA cross section for Rhodamine B, respectively, N_3 and N_2 are the numbers of luminescent molecules in the focal volume, and I_0 is the incident intensity at the focus. As we used the same solvent (water) for both the samples and reference, the refractive indexes were not included in equation (1). By comparing the three-photon excited luminescence intensity of samples with the two-photon excited luminescence intensity of Rhodamine B, the values of 3PA cross-section were achieved.

27. Summary of the three-photon photo-physical data:

Table S2. Summary of 3PA parameters for L1 in the presence of Zn(II) or TFA. 3PA cross-section (σ3) and action cross-section (η3σ3) along with the slope values of logarithmic plots of luminescence output vs. laser power at 1200 nm.

Compounds	σ_3 [$cm^6 s^2 photon^{-2}$]	$\eta_3\sigma_3$[c] [$cm^6 s^2 photon^{-2}$]	Slope[d]
L1[a]	1.38×10^{-78}	3.77×10^{-79}	2.90
L1 + Zn^{2+}[a]	3.03×10^{-78}	5.51×10^{-79}	3.00
L1 + H^+_{high}[b]	1.00×10^{-78}	2.61×10^{-79}	3.10
L1 + H^+_{low}[b]	2.18×10^{-78}	3.11×10^{-79}	3.00

[a] Spectroscopic measurements were performed in 50 mM of aqueous HEPES buffer of pH 7.2. [b] Spectroscopic measurements were performed in water (0.1 M KCl). [c] η_3 denotes luminescence quantum efficiency by assuming that one- and three-photon excited luminescence quantum efficiency remains the same. [d] Slopes were measured at laser power I_{peak} ~100 GW/cm^2.

28. Three-photon excited luminescence spectra of (**L1**) in presence of Zn^{2+} and TFA with varying laser power:

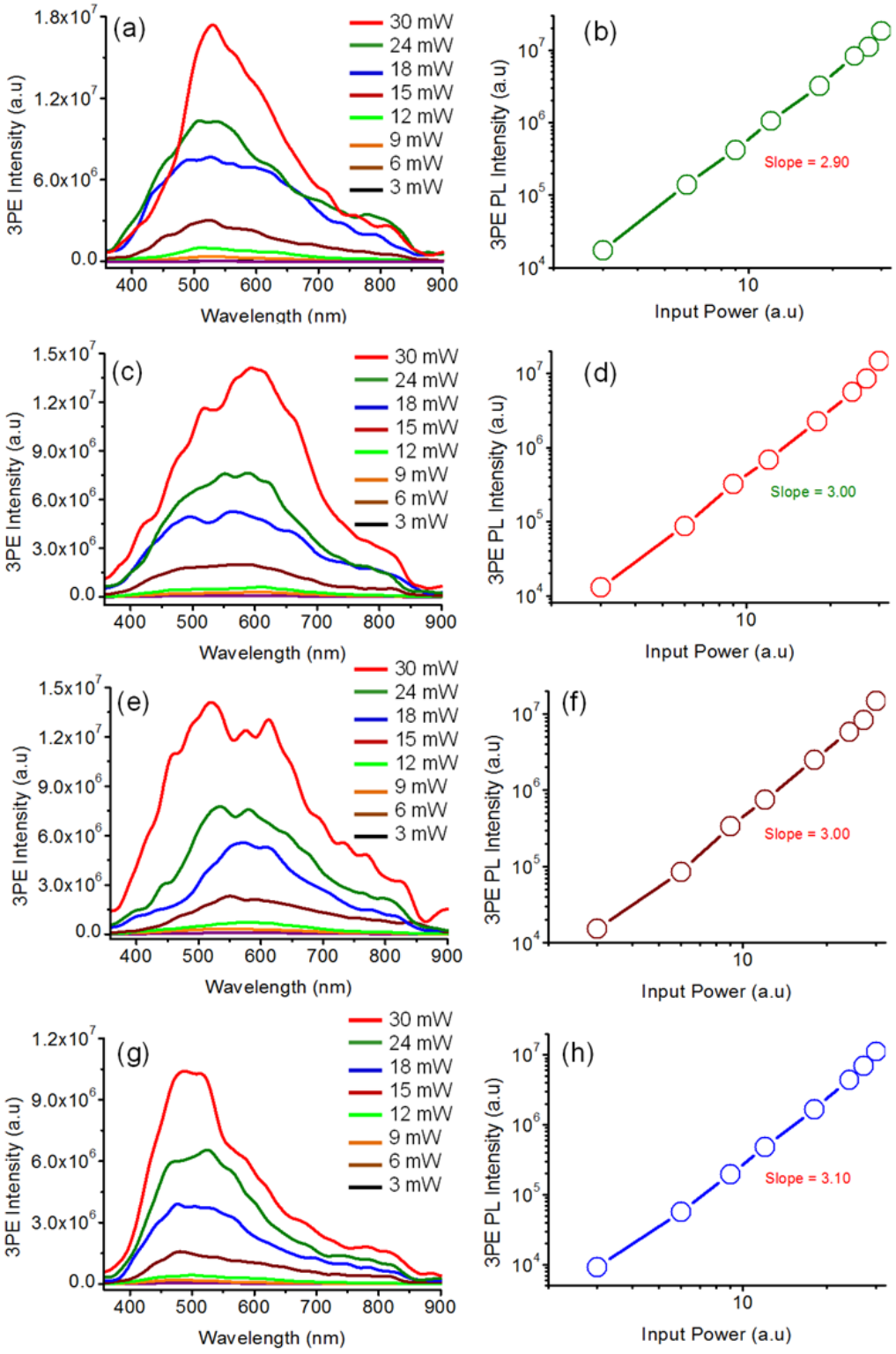


Figure S17: Three-photon excited luminescence spectral response with varying the laser power from 3 – 30 mW on excitation at 1200 nm. (a) 3PE luminescence spectra for **L1** (5.98 x 10^{-6} M) measured

in 50 mM of aqueous HEPES buffer of pH 7.2 . (b) Corresponding logarithmic plot of luminescence output vs. incident power intensity. (c) 3PE luminescence spectra for **L1** (5.98 x 10^{-6} M) in the presence of Zn^{2+} (1.31 x 10^{-4} M) measured in 50 mM of aqueous HEPES buffer of pH 7.2. (d) Corresponding logarithmic plot of luminescence output vs. incident power intensity. (e) 3PE luminescence spectra for **L1** (5.98 x 10^{-6} M) in the presence of TFA (9.10 x 10^{-4} M, H^{+}_{low}) measured in water (0.1 M KCl). (f) Corresponding logarithmic plot of luminescence output vs. incident power intensity. (g) 3PE luminescence spectra for **L1** (5.98 x 10^{-6} M) in the presence of TFA (2.84 x 10^{-2} M, H^{+}_{high}) measured in water. (h) Corresponding logarithmic plot of luminescence output vs. incident power intensity. Inside color lines in the spectra (a, c, e, g) indicate corresponding input laser power.

29. Three-photon excited luminescence spectra of (**L1**) with varying the concentration of Zn^{2+}:

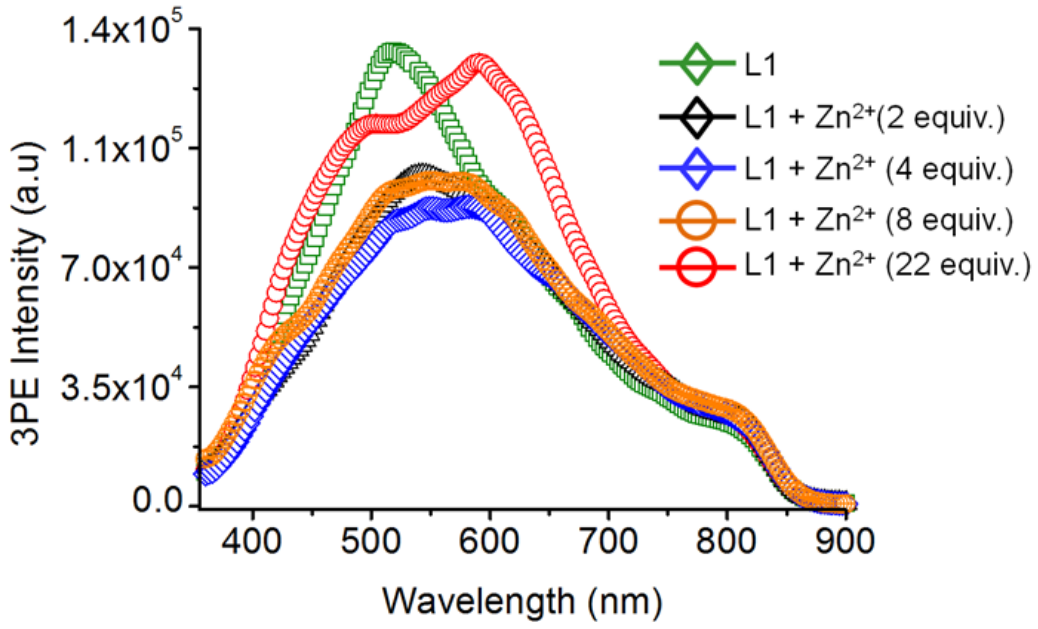


Figure S18: Three-photon excited luminescence spectra of **L1** (5.98 x 10^{-6} M) with increasing the concentrations of Zn^{2+} measured in 50 mM of aqueous HEPES buffer of pH 7.2 and 25 °C on excitation at 1200 nm.

30. Reversible binding of (**L1**) towards Zn^{2+}:

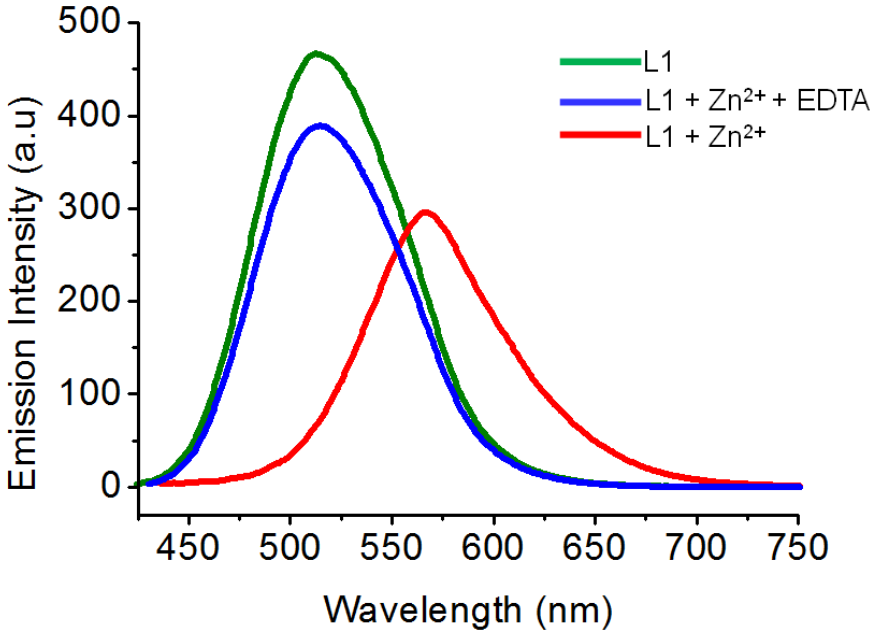


Figure S19: Fluorescence spectral changes recorded in 50 mM aqueous HEPES buffer (pH 7.2) at 25 °C for **L1** (5.98 x 10^{-6} M, green line), **L1** (5.98 x 10^{-6} M) in the presence of $Zn(ClO_4)_2$ (1.31 x 10^{-5} M, red line), and **L1** (5.98 x 10^{-6} M) in the presence of $Zn(ClO_4)_2$ (1.31 x 10^{-5} M) and excess EDTA (blue line).

31. Cell culture:

HeLa cells were seeded and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum at 37 °C. These samples were trypsinized and about 1 × 10^4 cells were added in each well in a 6-well culture plate. After 24 h of growth, all the cell lines were incubated with solutions (10 μM each) of the probe (**L1**) at 37°C in the culture medium for 4 h. Then, to these solutions, $ZnCl_2$ solutions (90 μM each) were added and the cells were further incubated for 2 h. After washing with PBS three times to remove remaining probe and $ZnCl_2$, cells were viewed under fluorescence microscope (IX7, inverted fluorescent microscope, Olympus) at an excitation of 1200 nm.

32. MTT assay for cytotoxicity studies:

To investigate the cytotoxicity of the probe **L1** on HeLa cell line, conventional MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used.[1] All the cells were seeded

into a 96-well plate at a density of 1 × 10^4 cells/well in DMEM medium. After 24 h exposure, the medium in the wells were replaced with fresh medium (100 µL) containing the probe **L1**, with gradually diluted concentrations according to standard procedure.[1,2] After incubation for another 24 h, the medium was removed and a medium (100 µL) containing MTT (0.5 mg mL^{-1}) was added. After further incubation for 4 h, the medium was replaced with DMSO (100 µL). The plate was gently shaken for 30 min. Then, the absorbance at 560 nm was recorded using a microplate reader (infinite 200 PRO, Tecan). The cell viability related to the control wells that only contain cell culture medium was calculated by $[A]_{test} / [A]_{control}$, where $[A]_{test}$ and $[A]_{control}$ are the average absorption intensities of the test and control samples, respectively.

[1] X. Ma, K. T. Nguyen, P. Borah, C. Y. Ang, Y. Zhao. *Adv. Healthcare Mater.* **2012**, *1*, 690-697.

[2] Q. Hang, F. Liu, K. T. Nguyen, X. Ma, X. Wang, B. Xing, Y. Zhao. *Adv. Funct. Mater.* **2012**, *22*, 5144–5156.

33. Cytotoxicity study for the probe (**L1**) in HeLa cells:

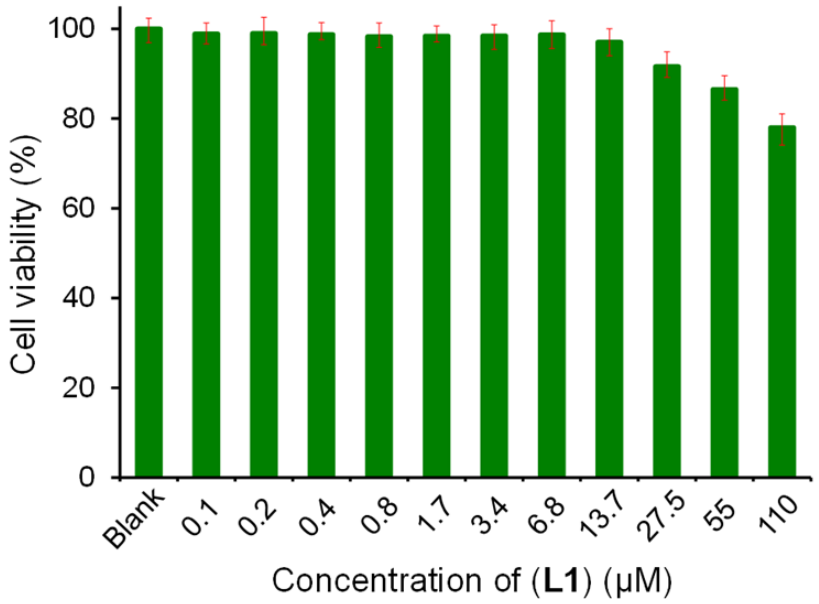


Figure S20. Cell viability (%) estimated by MTT proliferation test versus incubation concentration of **L1**. HeLa cells were cultured in the presence of 0-110 µM of **L1** at 37 °C for 24 h. (%) of viability was calculated by considering 100% cell growth in the absence of **L1**.

34. Determination of the water solubility of the probe (L1):

To determine the solubility of the probe (L1) in water we have followed a previously reported procedure.[3] First a small amount of the probe was dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to ($9.0 \times 10^{-3} \sim 1.0 \times 10^{-4}$) M and added to a cuvette containing 3.0 mL of buffer (50 mM HEPES, pH 7.2) by using a micro syringe. In all cases, the concentration of DMSO in H_2O was maintained to be 0.2 %.[4] The plots of fluorescence intensity against the dye concentration were linear at low concentration and showed downward curvature at higher concentration. The maximum concentration in the linear region was taken as the solubility. The solubility of the probe in HEPES buffer is ~ 13.0 μM.

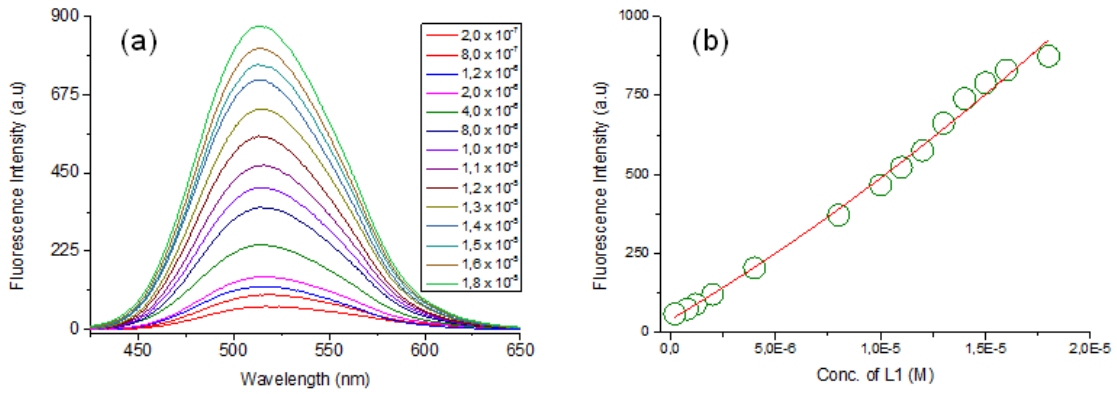


Figure S21. (a) One-photon excited fluorescence spectra and (b) plot of fluorescence intensity against the concentration of the probe (L1) in aqueous HEPES buffer (50 mM HEPES, pH 7.2) recorded at 25 °C. The excitation wavelength was 380 nm.

3. C. S. Lim, G. M. H. J. Kim, J. H. Han, H. M. Kim, B. R. Cho, *J. Am. Chem. Soc.* **2011**, *133*, 11132–11135.

4. H. M. Kim, H. J. Choo, S. Y. Jung, Y. G. Ko, W.-H. Park, S. J. Jeon, C. H. Kim, T. Joo, B. R. Cho, *Chem. Bio. Chem.* **2007**, *8*, 553–559.

35. ^{1}H-NMR & ESI-Mass spectra of (**1**):

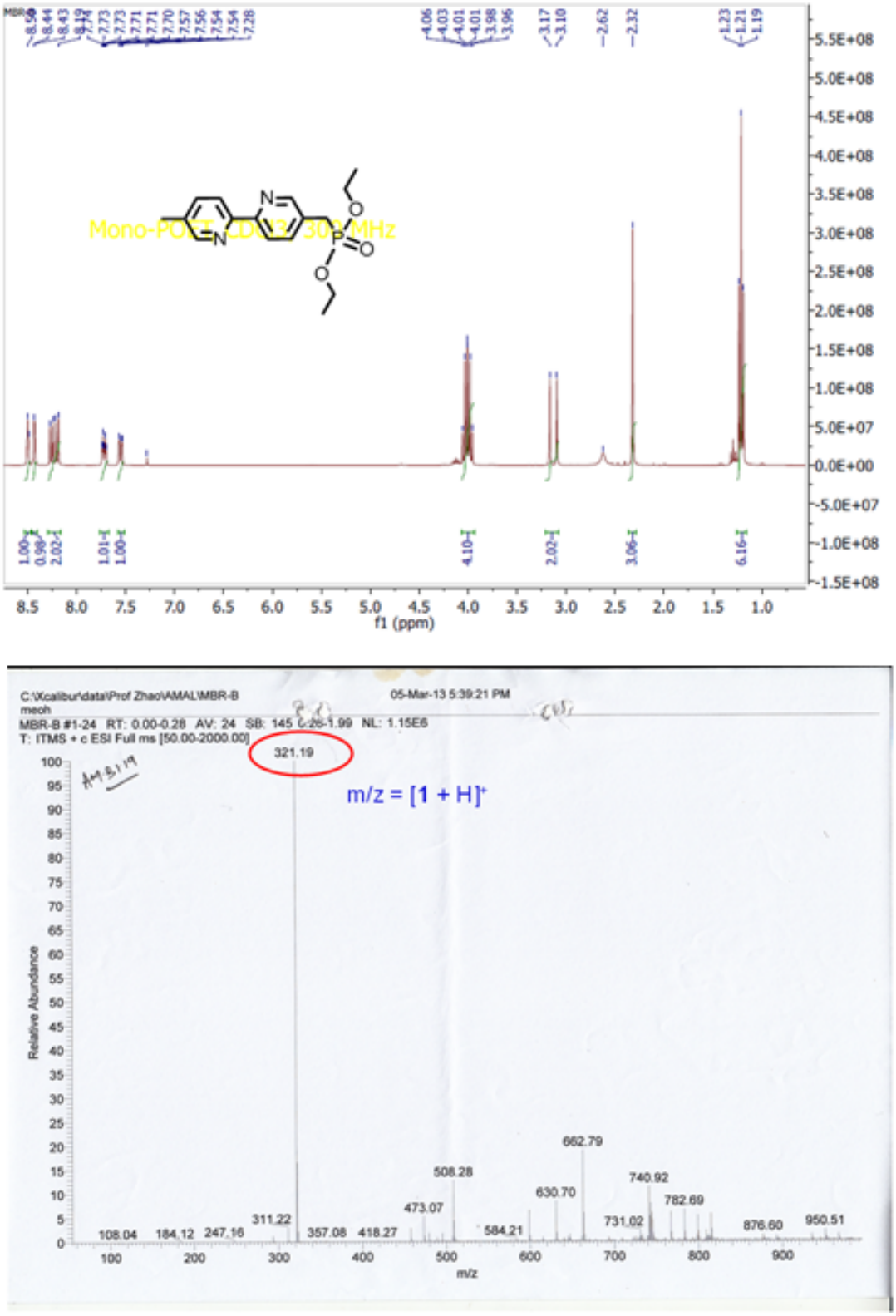


Figure S22. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and the ESI-Mass spectra for the compound (**1**) recorded in room temperature.

36. ^{1}H-NMR & ESI-Mass spectra of (**2**):

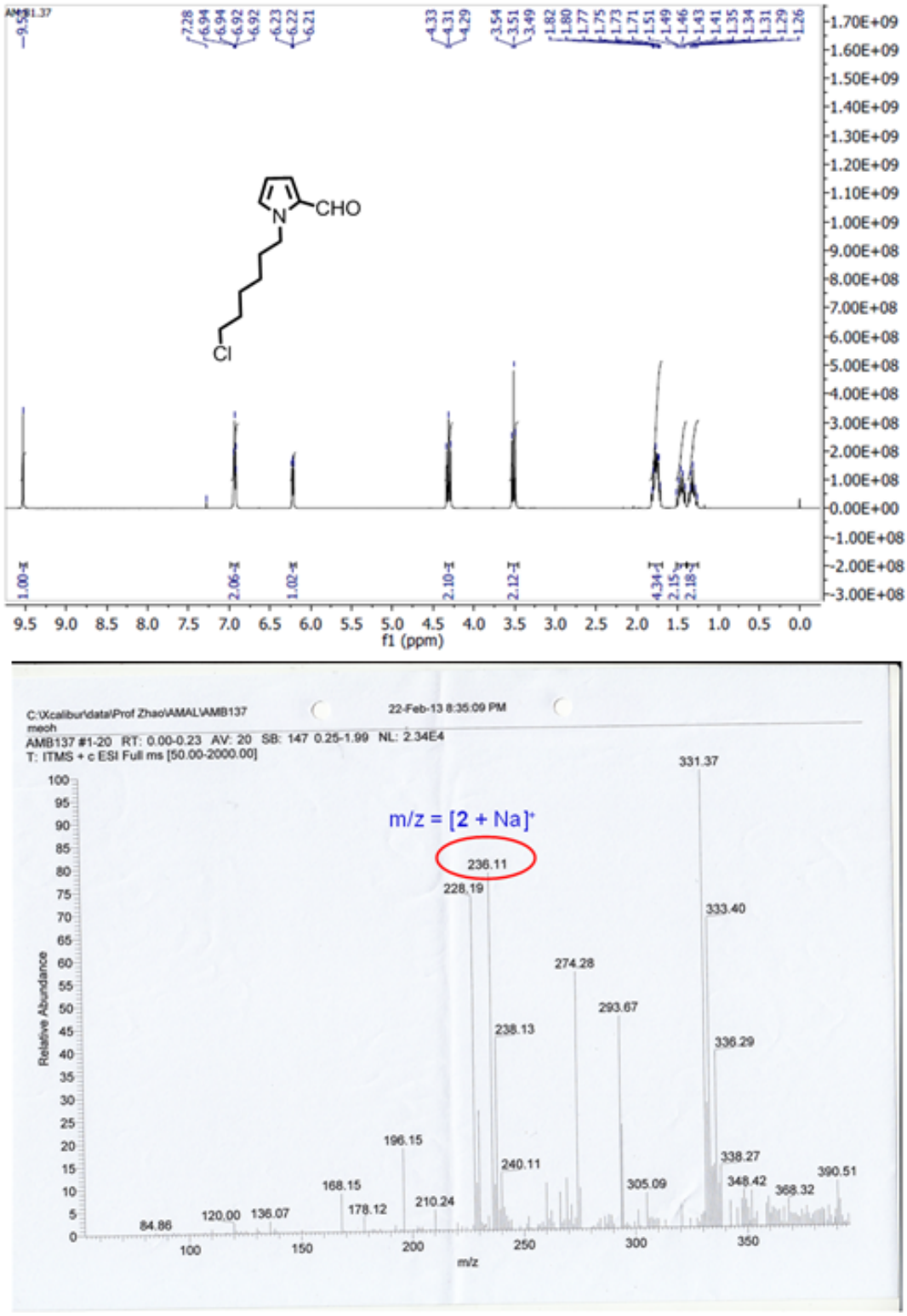


Figure S23. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and the ESI-Mass spectra for the compound (**2**) recorded in room temperature.

37. ^{1}H-NMR & ESI-Mass spectra of (**3**):

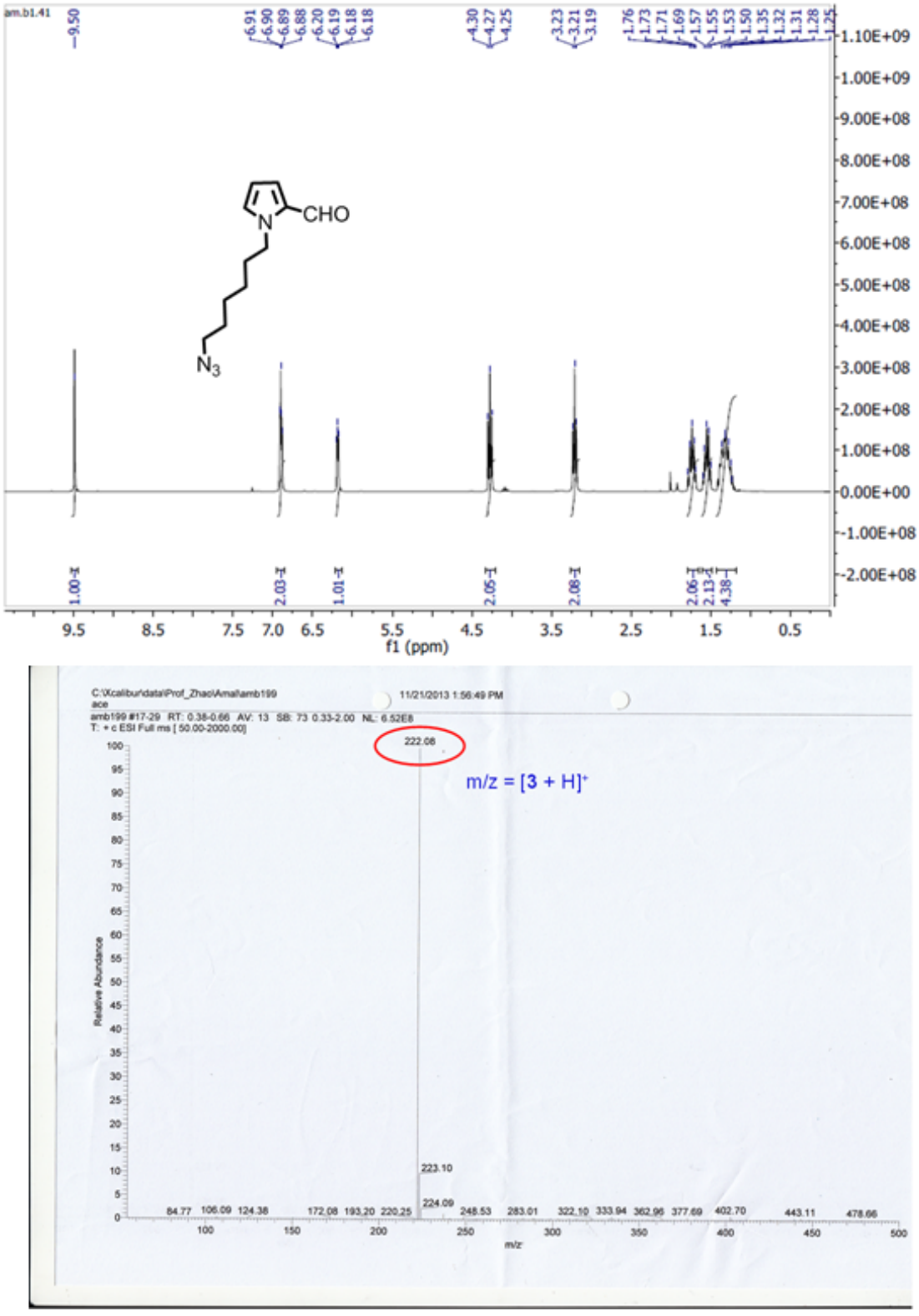


Figure S24. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and the ESI-Mass spectra for the compound (**3**) recorded in room temperature.

38. ^{1}H-NMR & ESI-Mass spectra of (**4**):

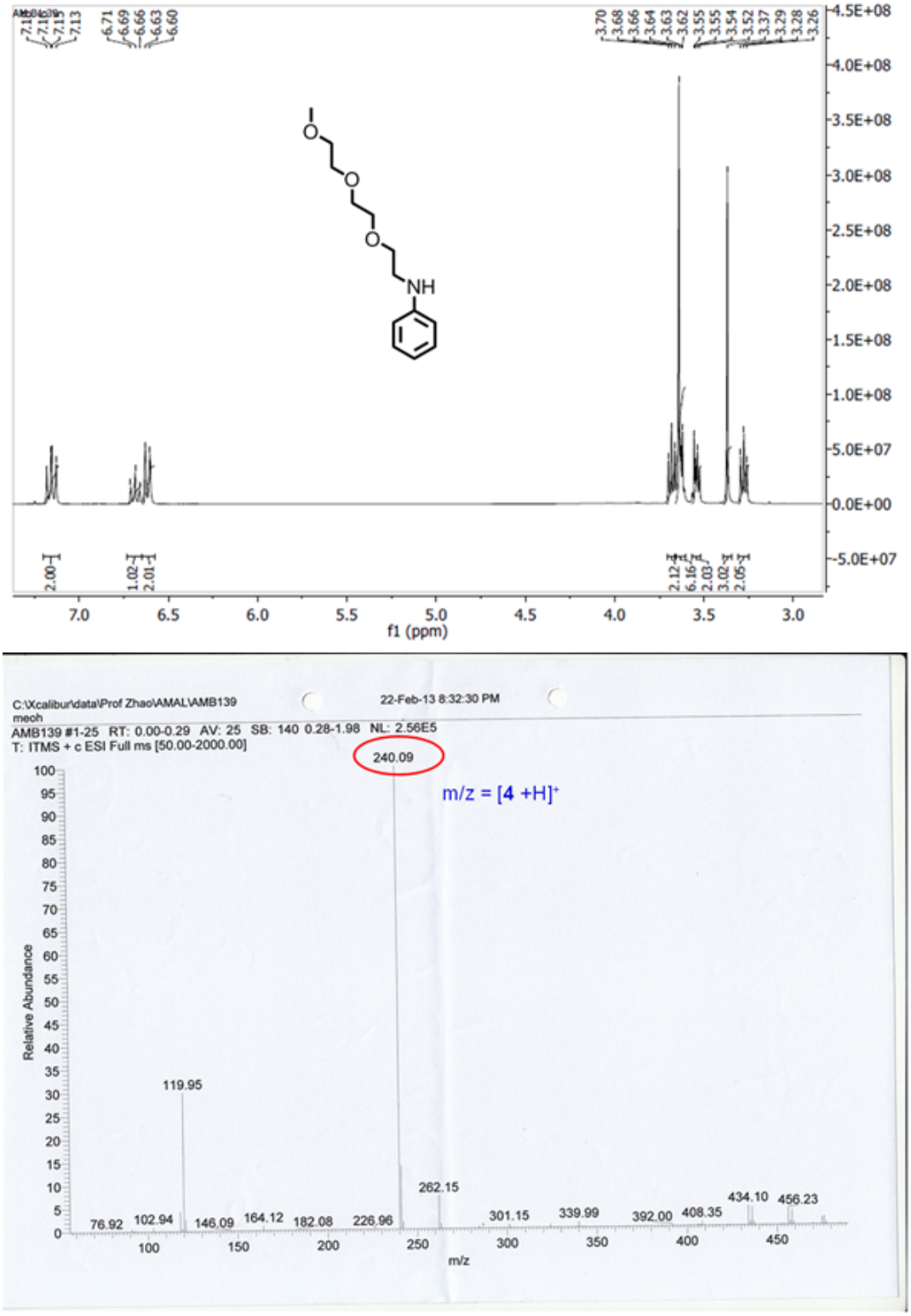


Figure S25. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and the ESI-Mass spectra for the compound (**4**) recorded in room temperature.

39. ^{1}H-NMR & ESI-Mass spectra of (**5**):

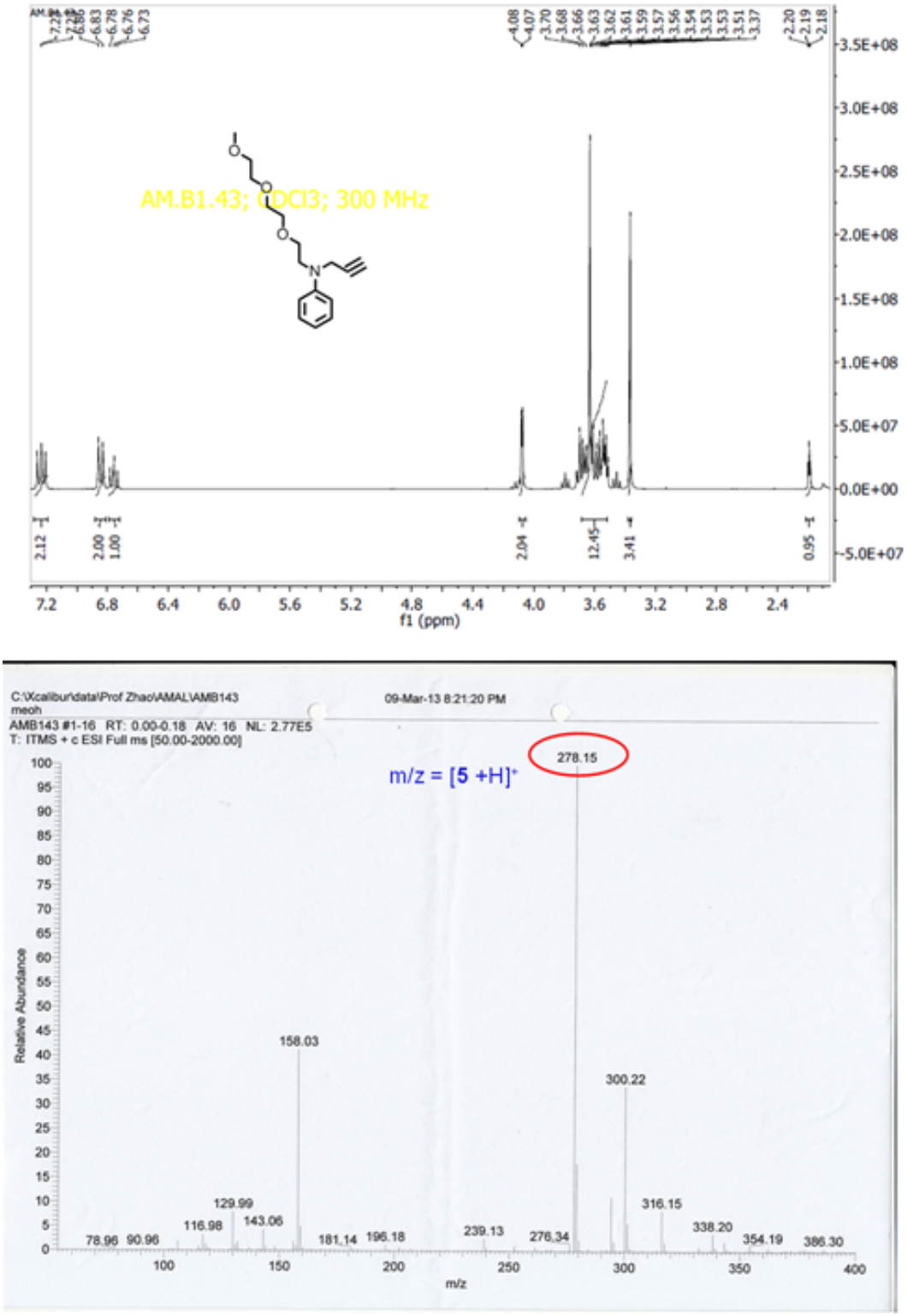


Figure S26. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and the ESI-Mass spectra for the compound (**5**) recorded in room temperature.

40. ^{1}H-NMR & ESI-Mass spectra of (**6**):

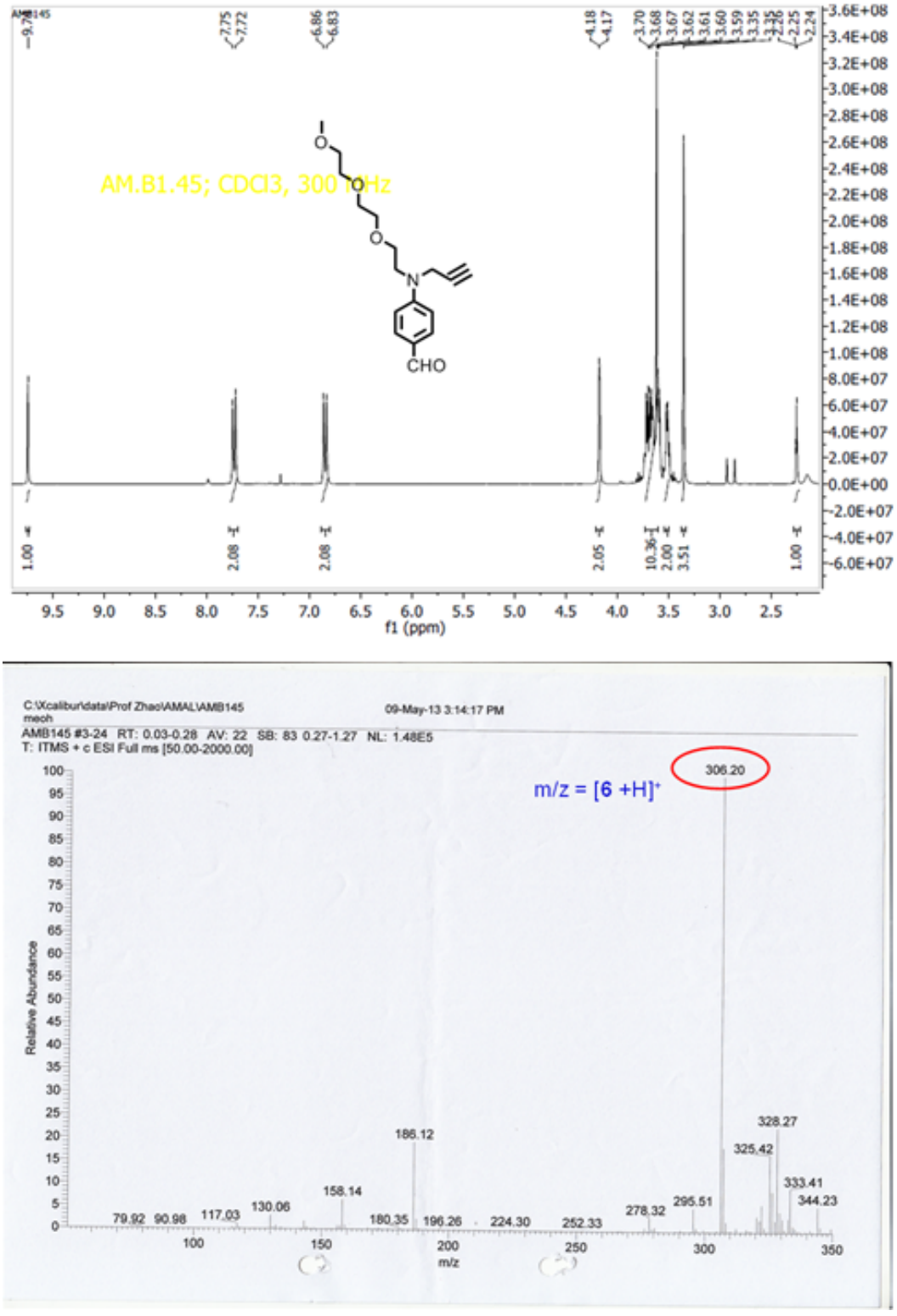


Figure S27. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and the ESI-Mass spectra for the compound (**6**) recorded in room temperature.

41. ^{1}H-NMR & ESI-Mass spectra of (**7**):

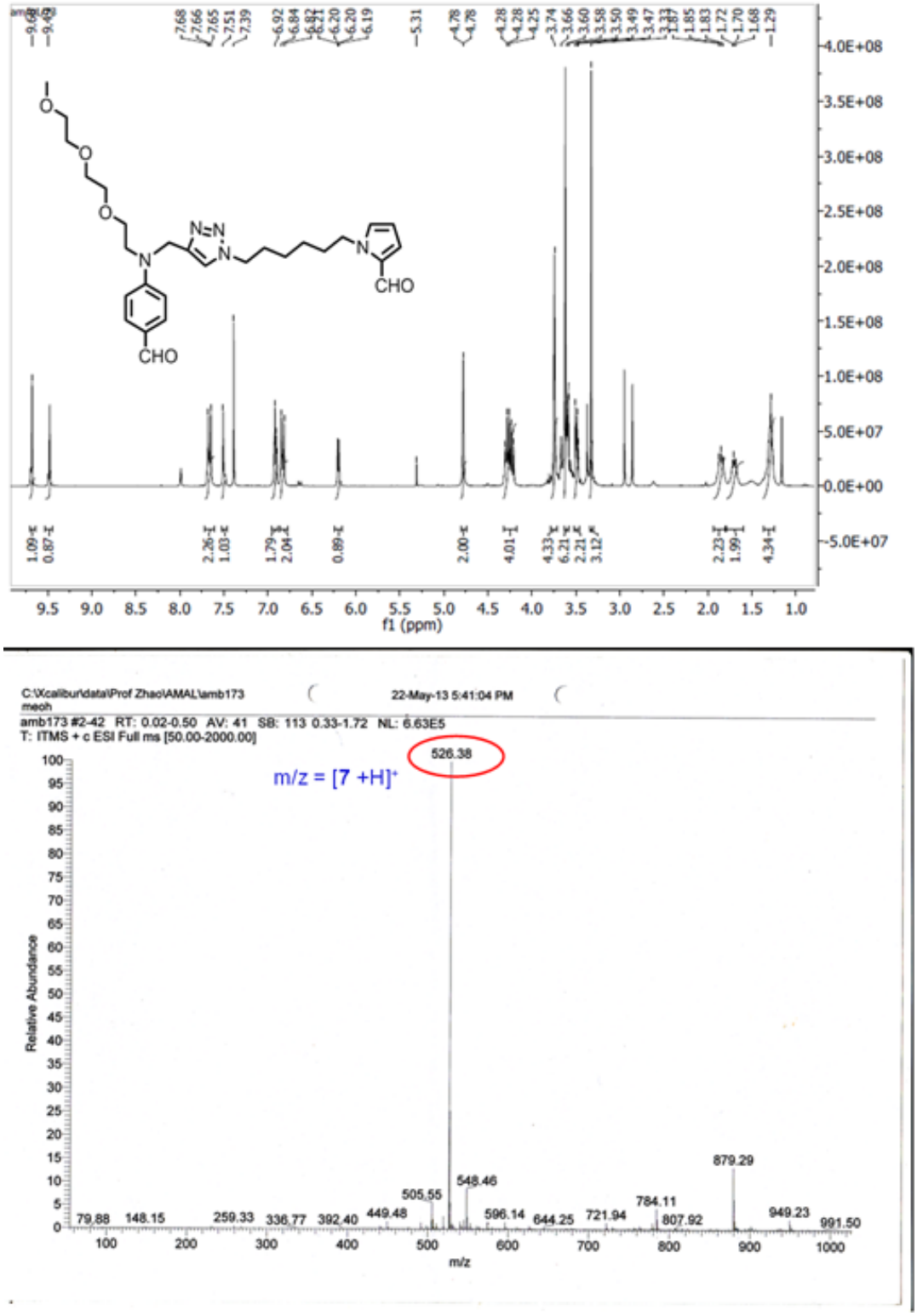


Figure S28. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and the ESI-Mass spectra for the compound (**7**) recorded in room temperature.

42. ^{1}H-NMR & ^{13}C-NMR spectra of (**L1**):

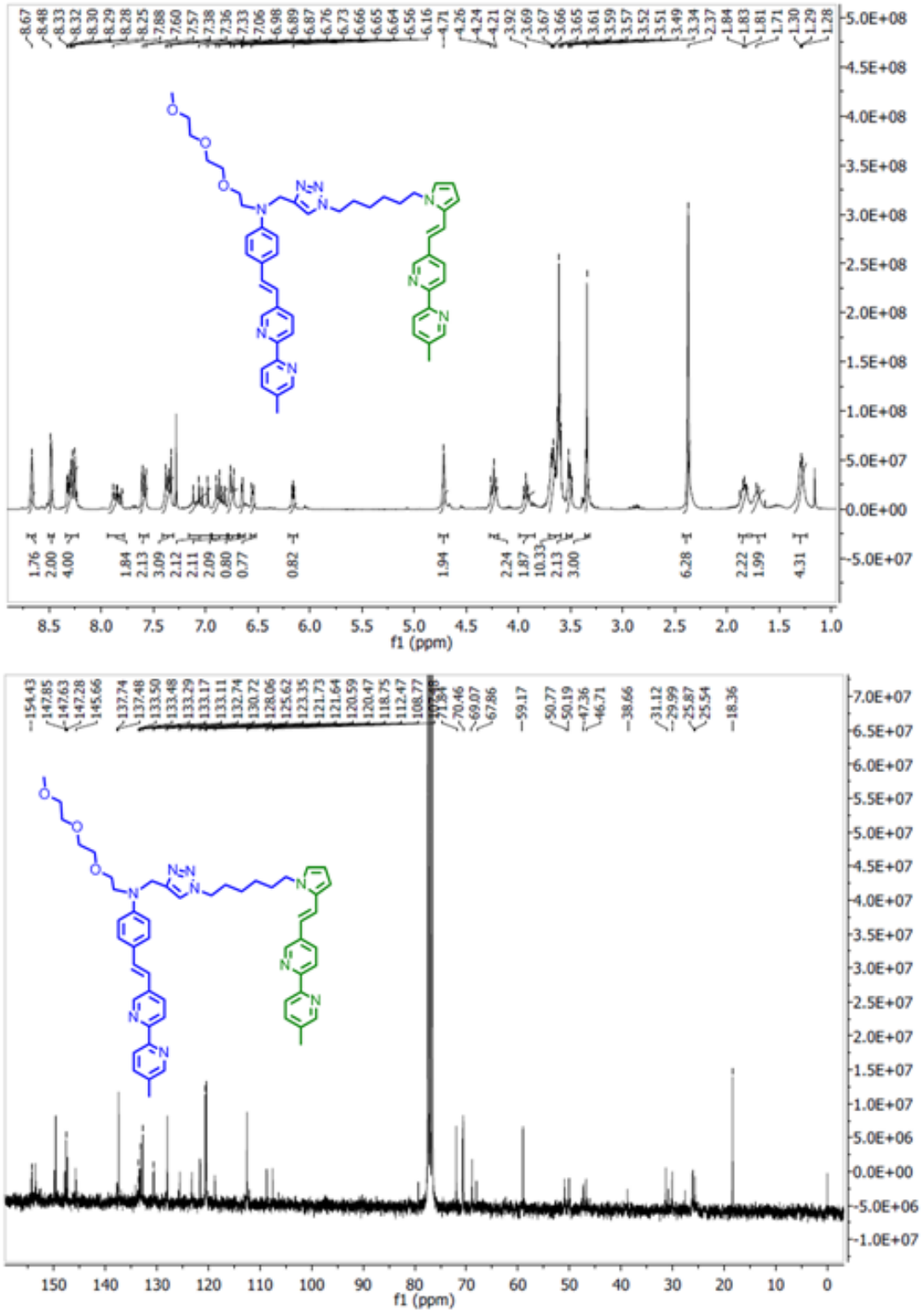


Figure S29. Partial ^{1}H NMR (300 MHz, $CDCl_3$, 298K) and ^{13}C-NMR (300 MHz, $CDCl_3$, 298K) spectra for the compound (**L1**) recorded in room temperature.

43. ESI & HRMS mass spectra of (**L1**):

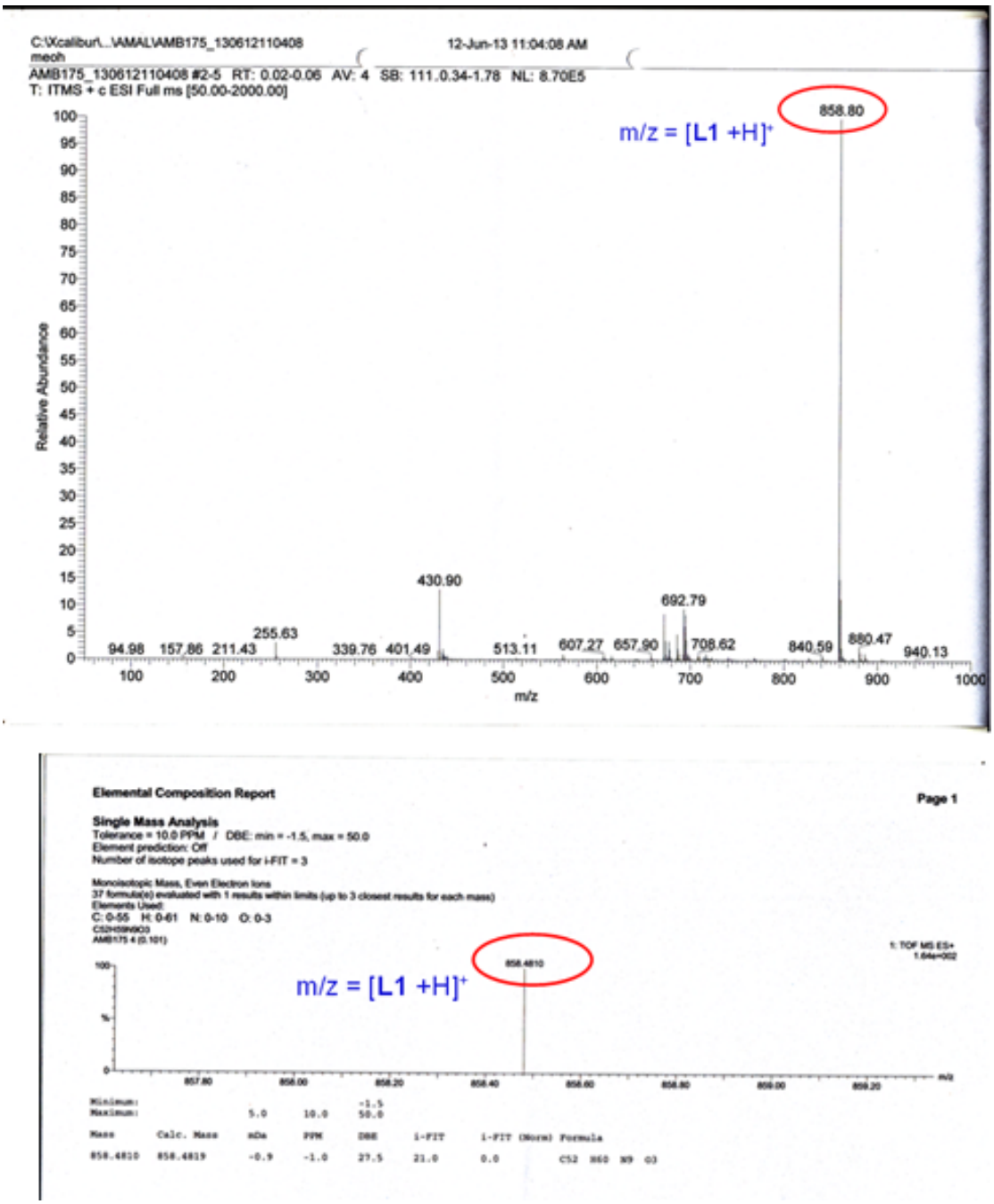


Figure S30. ESI and HRMS spectra for the compound (**L1**) recorded in room temperature.