Supporting Information

Selective ratiometric red-emission detection of In³⁺ in aqueous solution as well as live cells using fluorescent peptidyl probe and metal chelating agent

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Contents

1. Synthesis and characterization of Cy and 1

A cyanostilbene ((E)-2-(2-(2-(benzo[d]thiazol-2-yl)-2-cyanovinyl)-5(diethylamino) phenoxy)acetic acid) fluorophore **(Cy)** was synthesized as a following procedure.[1] To a stirred solution of 4-(diethylamino)salicylaldehyde (5.17 mmol) and K_2CO_3(12.9 mmol) in 35mL of dry THF, a solution of tert-butyl bromoacetate (6.24 mmol) in 5mL of THF was added in drops. And the mixed solution was heated to reflux overnight at 70℃. After the reaction was complete, the reaction solution was evaporated under reduced pressure and extracted with ethyl acetate. The combined organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using hexane/ethyl acetate as the mobile phase to give first product as yellowish oil (89% yield). To a stirred solution of solution of product and 2-benzothiazoleacetonitrile (1.2 equiv.) in methanol (25 ml) 10equiv. of piperidine was added. The resulting mixture was stirred overnight at 25℃. The precipitates were collected by filter paper and washed with cold ethanol to give second product as a red solid. The product was dissolved in DCM/TFA (1:1, v/v) and the resulting solution was stirred at 25℃ for 4 h. After 4 hour, a gentle stream of nitrogen was used to remove the excess TFA. The crude solid was recrystallized in ethanol-dichloromethane solution to afford final product as a red solid (86%). The successful synthesis was characterized by ESI-MS, ^{1}H and ^{13}C NMR and its purity (>95%) was checked by analytical HPLC with a C_{18} column. (Figure S1-4).

Cy : red solid; ^{1}H-NMR (400 MHz, DMSO-D6) δ 8.54 (d, J = 6.9 Hz, 1H), 8.25-8.28 (m, 1H), 8.03-8.08 (m, 1H), 7.92-7.97 (m, 1H), 7.49-7.54 (m, 1H), 7.39-7.45 (m, 1H), 6.55 (dd, J = 9.1, 2.3 Hz, 1H), 6.16 (d, J = 2.3 Hz, 1H), 4.91 (d, J = 15.1 Hz, 2H), 3.47 (q, J = 7.0 Hz, 4H), 1.08-1.17 (m, 6H); 13C-NMR (101 MHz, DMSO-D6) δ 169.91 (s, 1C), 165.26 (s, 1C), 159.80 (s, 1C), 153.08 (d, J = 55.9 Hz, 2C), 140.48 (s, 1C), 133.63 (s, 1C), 129.45 (s, 1C), 126.77 (s, 1C), 125.14 (s, 1C), 122.15 (d, J = 11.6 Hz, 2C), 118.33 (s, 1C), 108.60 (s, 1C), 105.84 (s, 1C), 94.28 (d, J = 17.3 Hz, 2C), 65.15 (s, 1C), 44.29 (s, 2C), 12.51 (s, 2C); ESI-Mass (m/z): [**Cy** + H$^+$]$^+$ calcd: 408.14, obsd: 408.21.

A fluorescent peptidyl probe (**1**) was synthesized using a solid-phase synthesis with Fmoc chemistry.[2] (Scheme S2) Fmoc protected amino acid (0.5 mmol, 5 equiv) was assembled on Rink Amide MBHA resin (0.1 mmol) for the synthesis of **1**. DIC (0.5 mmol) and HOBt (0.5 mmol) for activation method were used as coupling reagents. The amino acid, Fmoc-Aad(OtBu)-OH with Fmoc as protecting group (0.5 mmol) was loaded to swollen wang resin (0.1 mmol). After deprotection of the Fmoc group of Aad with 25% piperidine in DMF after washing, activated Fmoc-Ser(tBu)-OH (0.4 mmol) was coupled. After deprotection of Fmoc group of Ser, to the resin bound dipeptide, the cyanostilbene fluorophore (0.4 mmol) in DMF was coupled with the resin in presence of DIC (0.4 mmol) and HOBt (0.4 mmol) for activation method. The cleavage of the peptide from the resin was treated by a solution of TFA/H_2O (95:2.5) at 25℃ for 5 h. After filtration of the resin by TFA, the excess TFA was removed by a mild stream of N_2 gas. The crude was precipitated in the presence of diethyl ether at -20℃ and centrifuged twice at 3000 rpm for 5 min at 0℃. The probe (**1**) was synthesized with a 70% yield using solid phase synthesis. The successful synthesis was characterized by ESI-MS, ^{1}H and ^{13}C NMR and its purity (>95%) was checked by analytical HPLC with a C_{18} column. (Figure S5-8).

1 : red solid; ^{1}H-NMR (400 MHz, DMSO-D6) δ 8.53 (d, J = 2.3 Hz, 1H), 8.23-8.28 (m, 2H), 8.18 (d, J = 8.2 Hz, 1H), 8.05-8.08 (m, 1H), 7.97 (d, J = 7.8 Hz, 1H), 7.49-7.54 (m, 1H), 7.41 (td, J = 7.6, 1.0 Hz, 1H), 6.56 (dd, J = 9.4, 2.1 Hz, 1H), 6.14 (d, J = 1.8 Hz, 1H), 4.76-4.82 (m, 2H), 4.50 (dd, J = 13.5, 5.7 Hz, 1H), 4.20 (td, J = 8.1, 5.2 Hz, 1H), 3.59-3.72 (m, 2H), 3.46 (q, J = 7.0 Hz, 4H), 2.19 (t, J = 7.1 Hz, 2H), 1.69-1.78 (m, 1H), 1.50-1.65 (m, 3H), 1.12-1.17 (m, 6H); 13C-NMR (101 MHz, DMSO-D6) δ 174.19 (s, 1C), 173.32 (s, 1C), 169.68 (s, 1C), 167.29 (s, 1C), 165.35 (s, 1C), 160.00 (s, 1C), 153.15 (d, J = 48.2 Hz, 2C), 140.62 (s, 1C), 133.73 (s, 1C), 129.48 (s, 1C), 126.79 (s, 1C), 125.17 (s, 1C), 122.19 (d, J = 19.3 Hz, 2C), 118.40 (s, 1C), 108.53 (s, 1C), 105.88 (s, 1C), 94.45 (s, 1C), 94.18 (s, 1C), 67.16 (s, 1C), 61.97 (s, 1C), 54.65 (s, 1C), 51.80 (s, 1C), 44.33 (s, 2C), 33.17 (s, 1C), 30.59 (d, J = 25.0 Hz, 1C), 20.99 (s, 1C), 12.56 (s, 2C); ESI-Mass (m/z): [**1** + H$^+$]$^+$ calcd: 637.2214, obsd: 637.97.

2. Preparation of aqueous buffered solutions with various pHs

Aqueous buffered solutions at different pH were prepared in distilled water using the following chemicals. Hexamethyltetramine (Hexamine) was used for the buffer solutions at pH ranging from 4.0 to 6.0. (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was used for the buffered solutions at pH ranging from 6.5 to 8.0. 2-(N-morpholino)ethanesulfonic acid (MES) was used for the buffered solutions at pH ranging from 1.5 to 3.5. N-cyclohexyl-2-aminoethanesulfonic acid (CHES) was used for the buffered solution at pH ranging from 8.5 to 10.5.

3. Determination of detection limit

The detection limits of **1** to Al^{3+} and In^{3+} were calculated based on a fluorescence titration. To determine the S/N ratio, the fluorescence emission intensity at 530 nm of 2 μM of **1** in aqueous solutions was measured 10 times, and the standard deviation of the blank measurements was determined. Three separate measurements of the emission intensity at 530 nm were measured in the presence of increasing Al^{3+} and In^{3+} concentrations, and the mean emission intensity at 530 nm was plotted as a function of the Al^{3+} and In^{3+} concentration to determine the slope. The detection limit was calculated using the following equation:

Detection limit = 3σ/m

where σ is the standard deviation of the intensity at 530 nm of **1** in the absence of Al^{3+} and In^{3+}, m is the slope of the emission intensity ratio (I_{600}/I_{568}) of 2 μM of **1** as a function of the Al^{3+} concentration, and m is the slope of the emission intensity ratio (I_{600}/I_{569}) of 2 μM of **1** as a function of the In^{3+} concentration.[3]

4. Determination of dissociation constant

The dissociation constant for tight 1:1 complex was calculated on the basis of the titration curve of the probe with metal ion. The fluorescence signal, Y, is related to the equilibrium concentration of the complex (ML) between metal ion (M) and Ligand (L) by the following equation:

$$Y = Y_0 + \Delta Y \times [ML]$$

$$[ML] = 0.5 \times [K_D + M_T + L_T - \{(-K_D - M_T - L_T)^2 - 4M_T L_T\}^{1/2}]$$

Where Y_0 is the emission intensity of the probe only and ΔY is the change in emission intensity due to the formation of ML. Dissociation constants were confirmed by a nonlinear least-squares fitting of the

data with the equation.[4]

5. Cell culture, fluorescence cell imaging analysis, and cell toxicity

MG63 cells were purchased from ATCC (Manassas, VA, U.S.A.) and maintained with Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories Inc., Logan, Utah, U.S.A.) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories Inc.) and 1% penicillin/streptomycin (P/S, Hyclone Laboratories Inc.) in at 37°C in a humidified incubator containing 5% CO_2 in air.

Cell imaging experiments were performed with an Olympus CKX53 fluorescent microscope (Olympus Inc., Center Valley, PA, U.S.A.) with 20 objective lens. Excitation at 460 nm was carried out. MG63 cells were attached to the plate 24 h before study. After cells were treated with 4 µM of 1 containing 1% DMSO for 30 min at 37°C and then washed twice with aqueous buffered solutions. The fluorescence of the cells was confirmed and then the cells were further incubated in 20 µM $In(NO_3)_3$ in aqueous buffered solutions for 30 min. Cells were washed three times with aqueous buffered solutions and fluorescent microscopy was recorded.

Cytotoxicity was assessed by WST-1 solution assay. MG63 cells (1×10^3) were seeded in each well of a 96-well plate and incubated for 18 h at 37°C in a humidified incubator containing 5% CO_2 in air. After incubation, cells were treated with DMSO (0.1%) as a control vehicle and the indicated concentration of the chemicals for 24 h. After incubation, 20 µl of WST-1 solution was added to each well for 4 h. Then, the visible absorbance at 460 nm of each well was quantified using a microplate reader. Non- treated cells were used as a control and incubated in the same conditions for the same time. The relative cell viability (%) was calculated by the following equation.

$$\%Cell\,Viability = \frac{(Optical\,density\,of\,sample)}{(Optical\,density\,of\,control)} \times 100\%$$
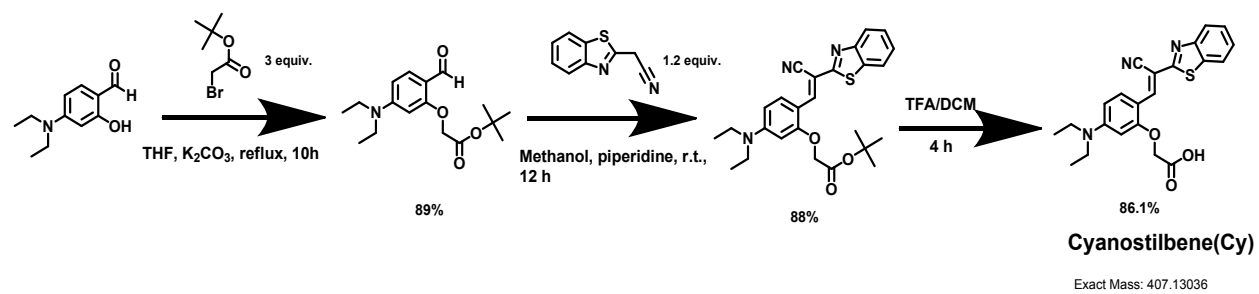
Scheme S1. Synthetic scheme of **Cy**
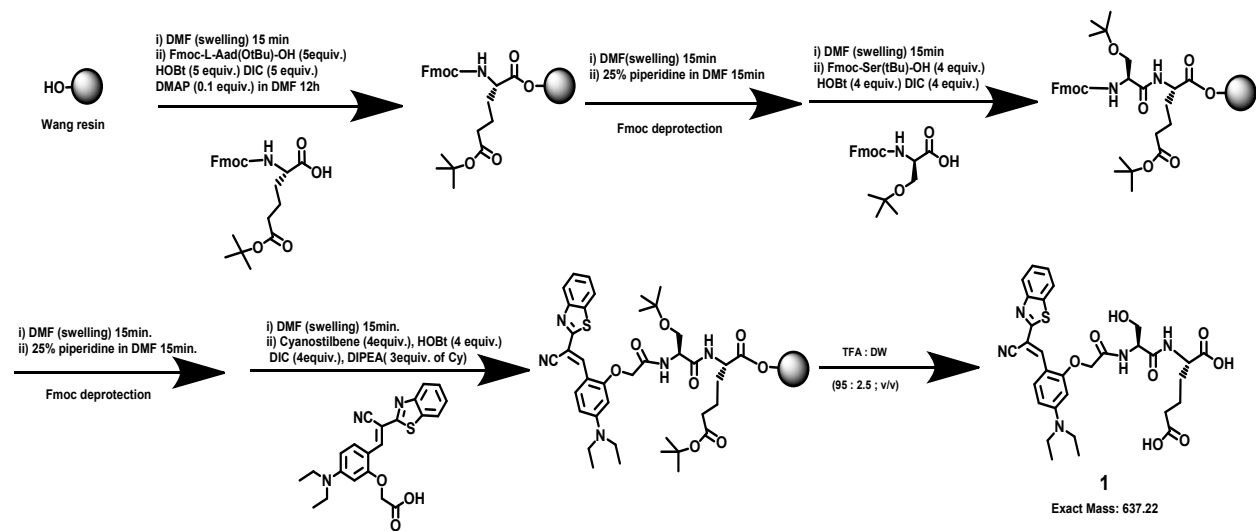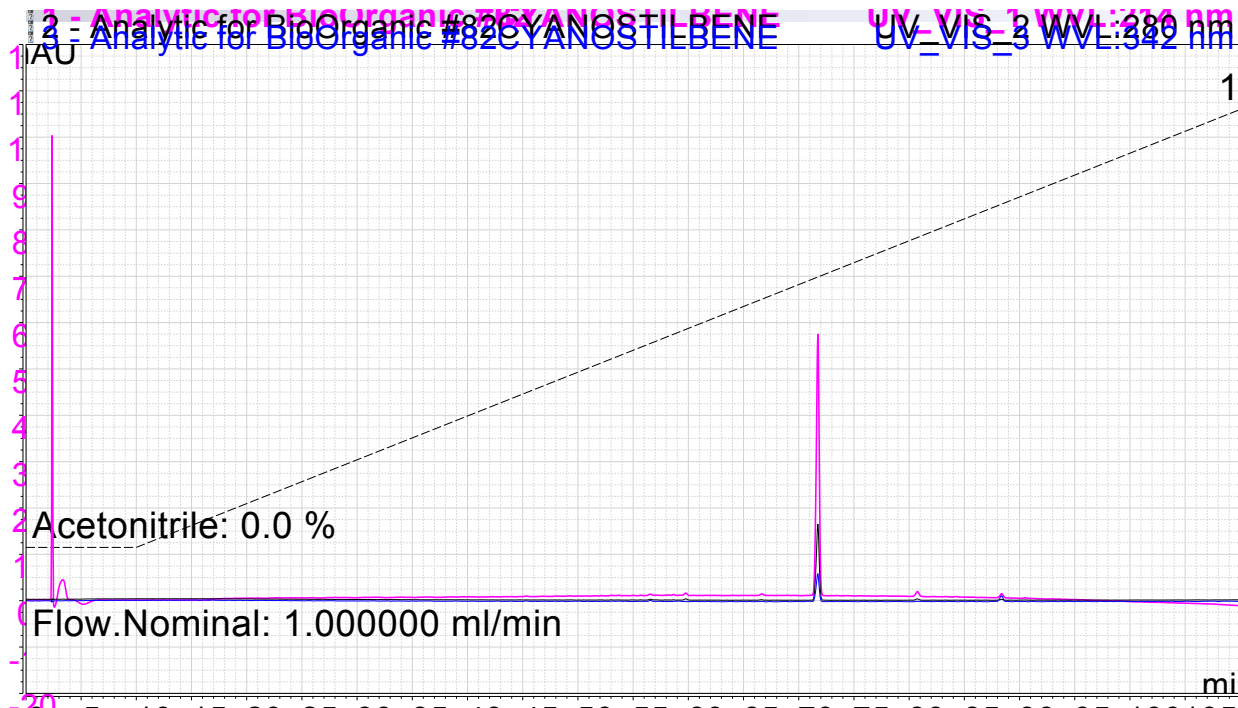
Scheme S2. Synthetic scheme of **1**

Figure S1. HPLC Chromatogram of **Cy**
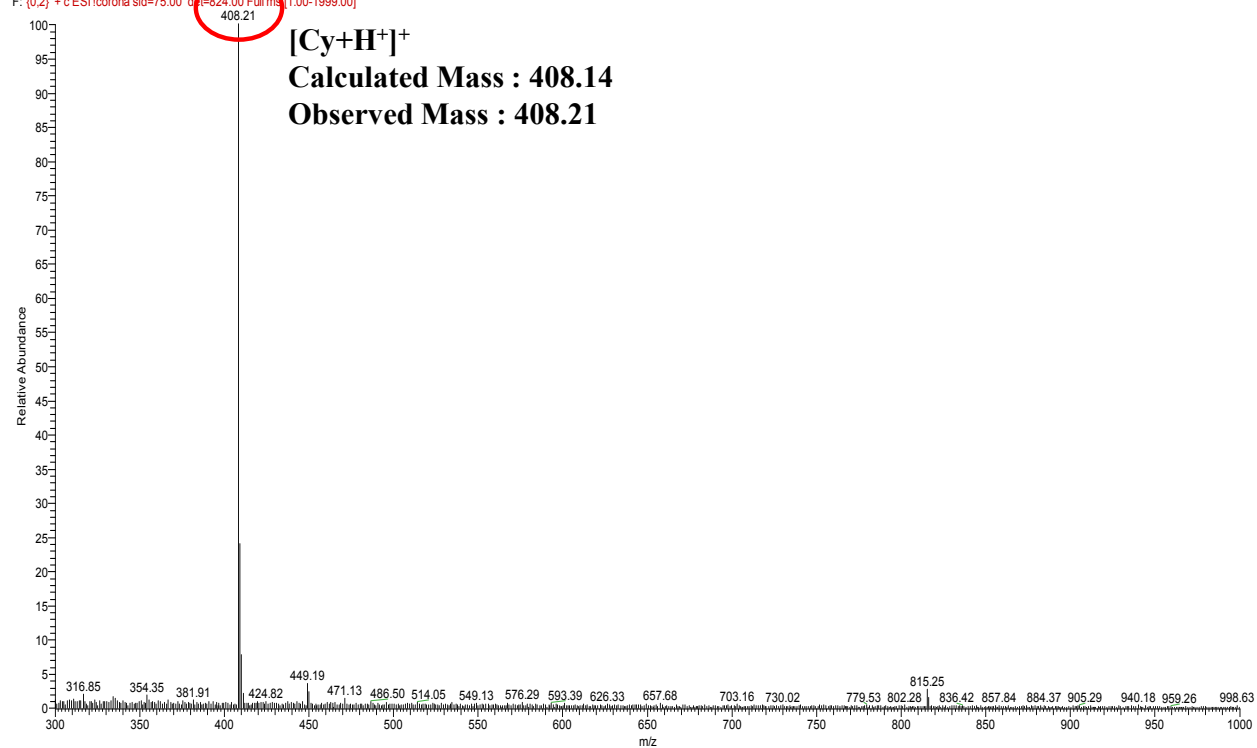
Figure S2. ESI-MS data of **Cy**
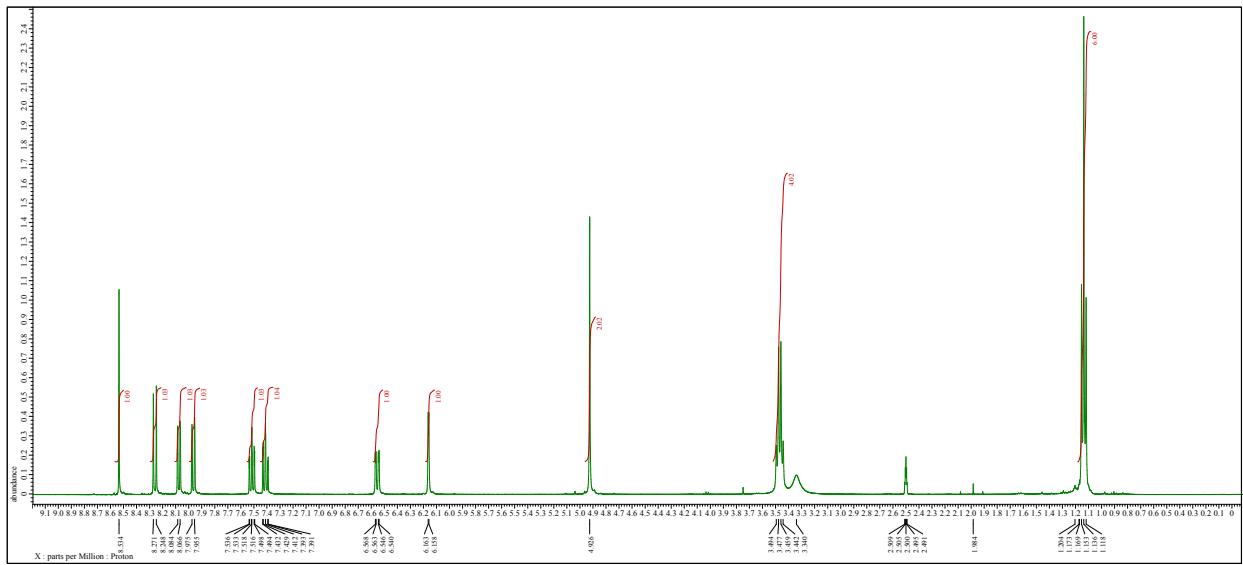
Figure S3. ^{1}H NMR of **Cy**
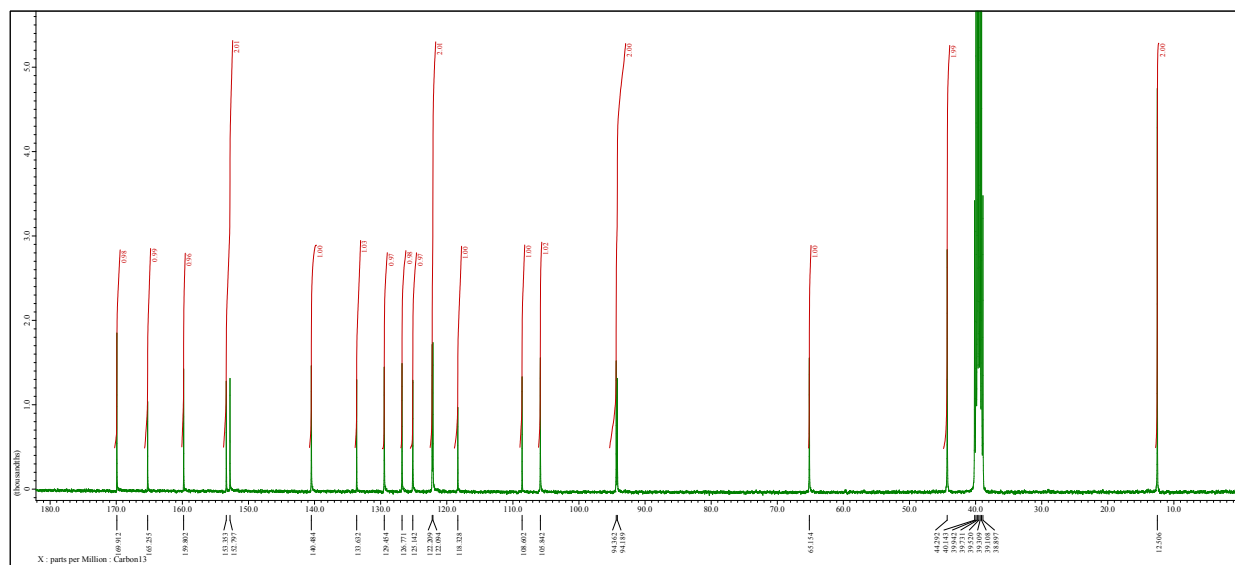
Figure S4. ^{13}C NMR of **Cy**

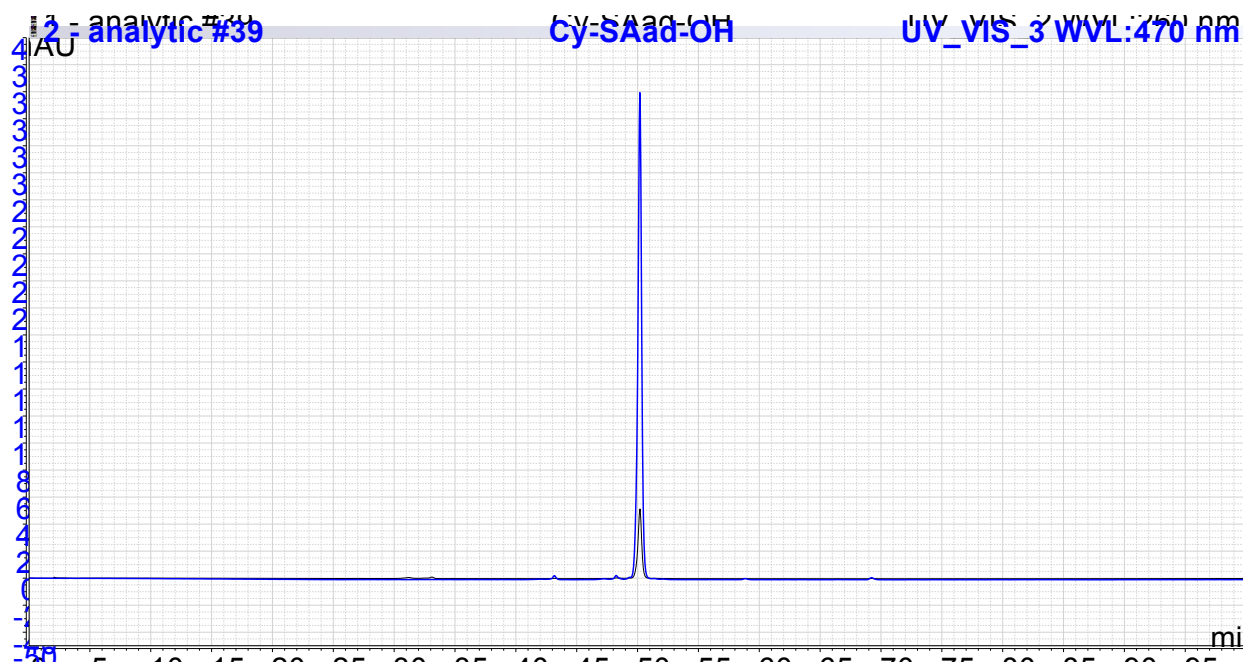**Figure S5.** HPLC Chromatogram of **1**
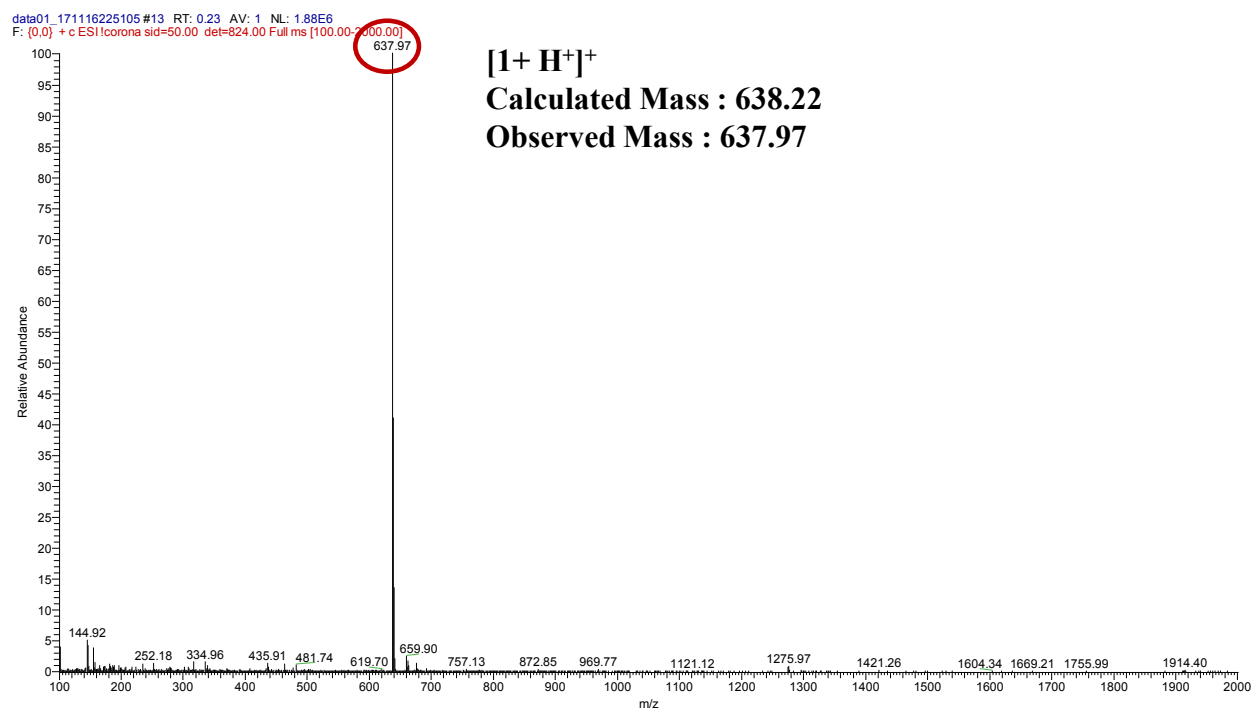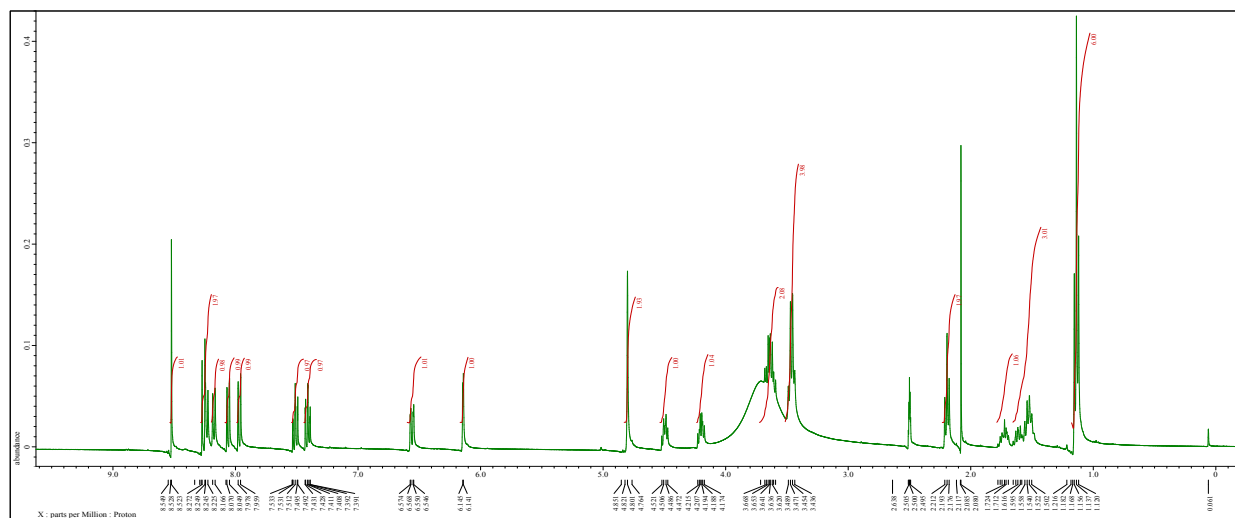
Figure S6. ESI-MS data of **1**

Figure S7. ^{1}H NMR of **1**
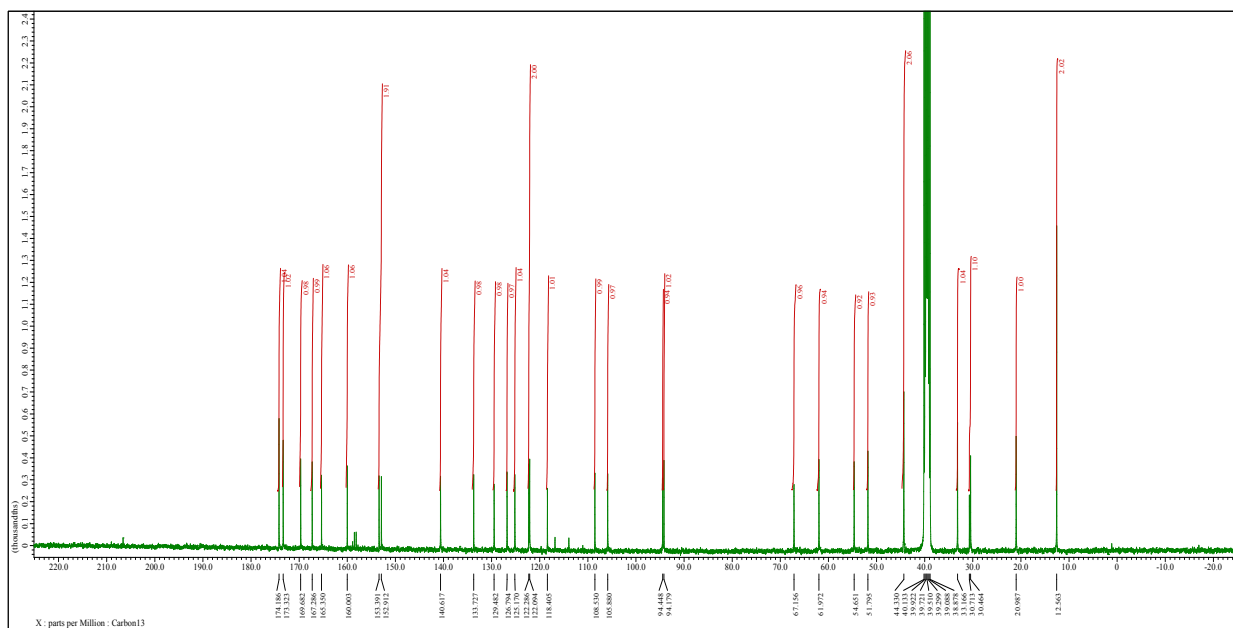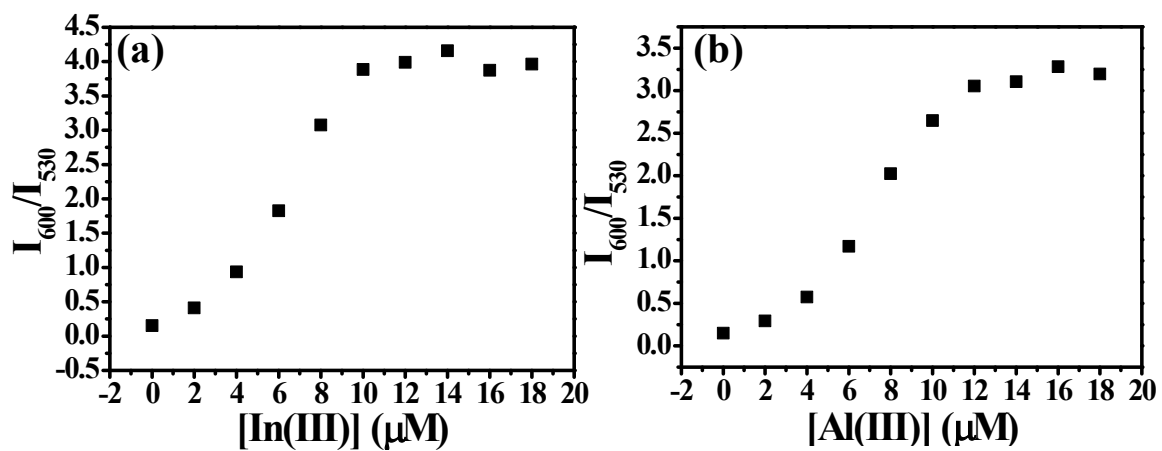
Figure S8. ^{13}C NMR of **1**

Figure S9. Intensity ratio change (I_{600}/I_{530}) of **1** (2 μM) as a function of (a) In^{3+} and (b) Al^{3+} in aqueous buffered solution (10 mM Hexamine, pH 5.0) containing 0.2% Acetonitrile (λ_{ex} = 450 nm).
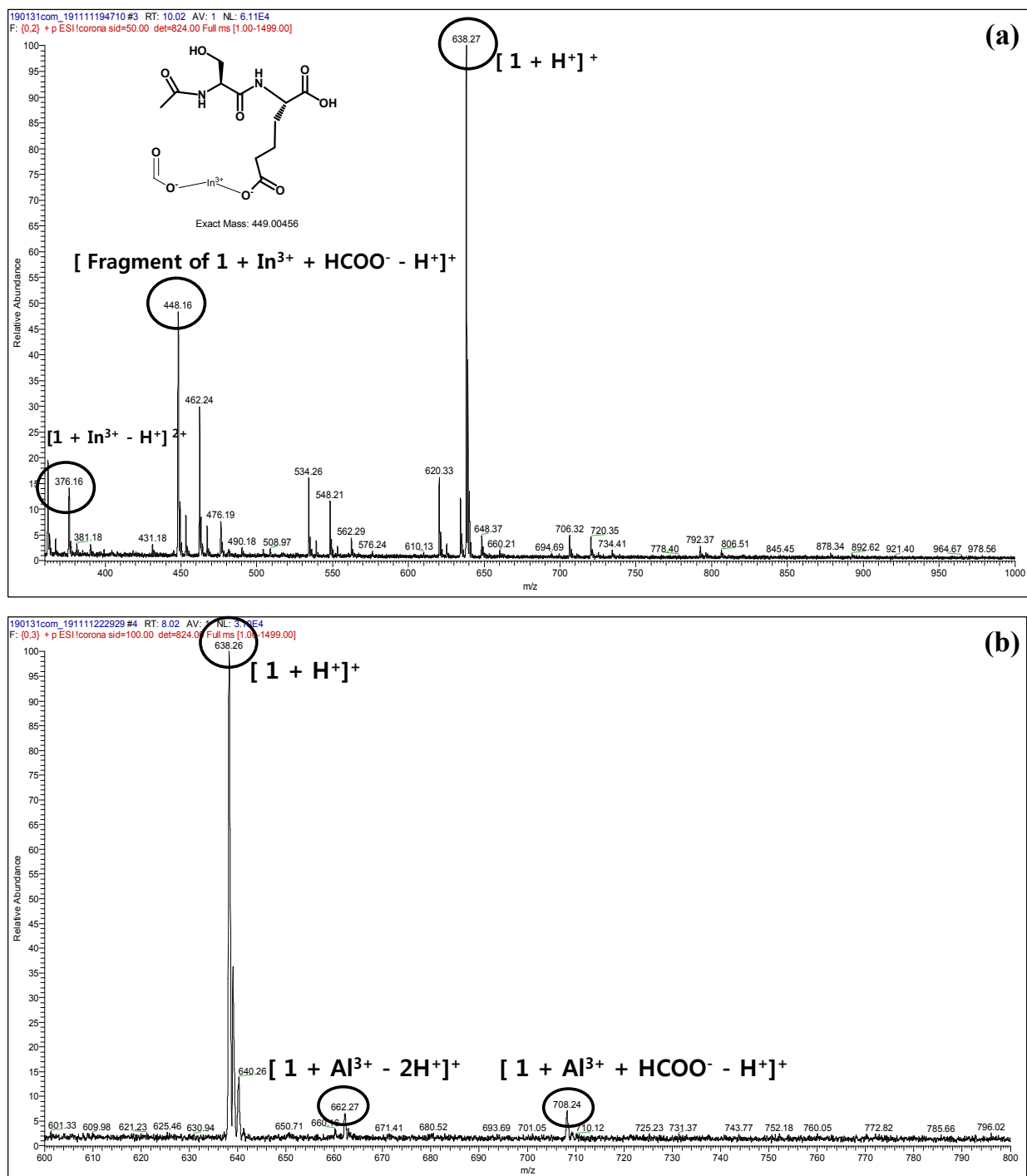
Figure S10. ESI-MS spectrum of **1** (20 μM) in the presence of (a) In^{3+} (100 μM) and (b) Al^{3+} (100 μM) in H$_2$O/CH$_3$CN (v/v = 9:1).
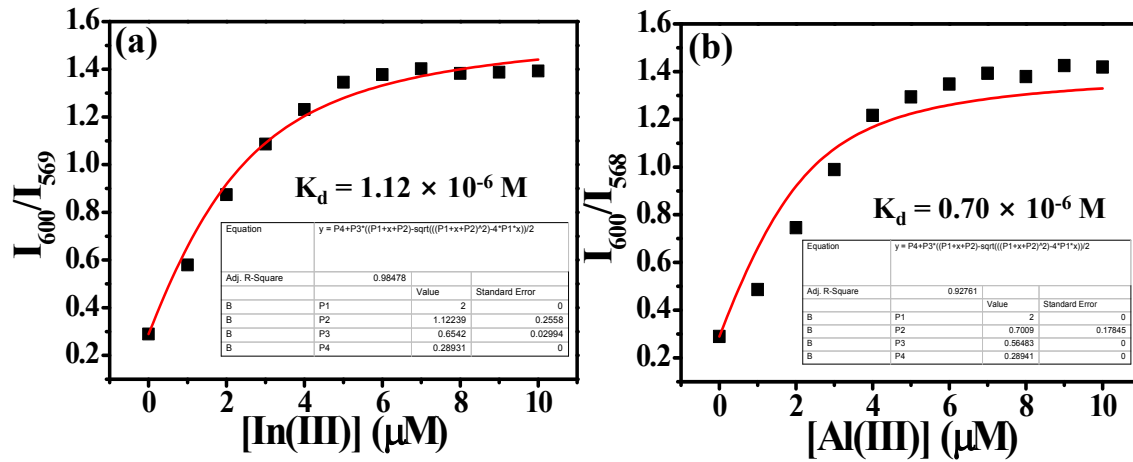
Figure S11. Non-linear least square fitting of the intensity ratio changes (I_{600}/I_{569}) and (I_{600}/I_{568}) as a function of (a) In^{3+} and (b) Al^{3+} by a 1:1 tight complex model.
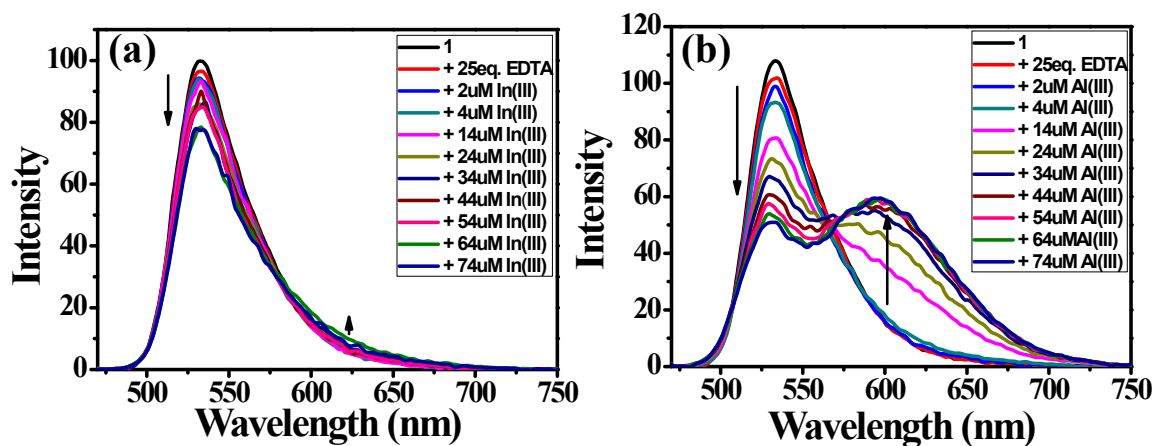
Figure S12. Fluorescence emission spectra of **1** (2 µM) with 25eq. of EDTA upon increasing concentration of (a) In(III) and (b) Al(III) (10 µM) in aqueous buffered solution (10 mM Hexamine, pH 5.0) containing 0.2% Acetonitrile (λ_{ex} = 450 nm)

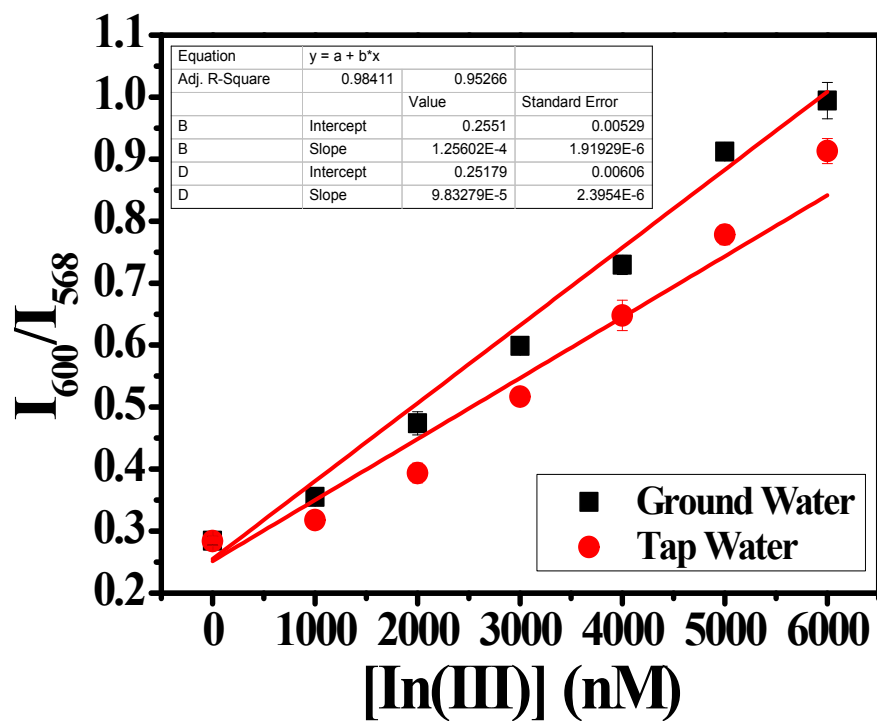| Equation	y = a + b*x		
Adj. R-Square	0.98411	0.95266	
		Value	Standard Error
B	Intercept	0.2551	0.00529
B	Slope	1.25602E-4	1.91929E-6
D	Intercept	0.25179	0.00606
D	Slope	9.83279E-5	2.3954E-6

Figure S13. Linear curve fitting of the emission intensity ratio (I_{600}/I_{568}) of **1** (2 μM) as a function of the concentration of In^{3+} in groundwater sample and tap water sample ($\lambda_{ex} = 450$ nm)

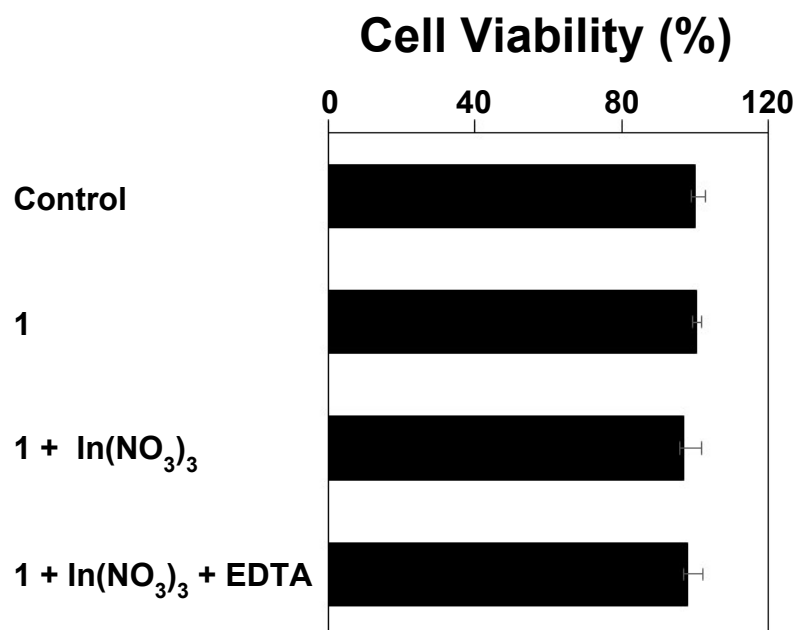# Cell Viability (%)

Figure S14. WST-1 assay for the viability of MG63 cells with **1**, **1** + In(NO$_3$)$_3$ and **1** + In (NO$_3$)$_3$ + EDTA for 24 h. The results are based on three separate WST-1 assays. The concentration of **1**, Al(NO$_3$)$_3$ and EDTA is 4 µM, 20 µM, and 100 µM, respectively.
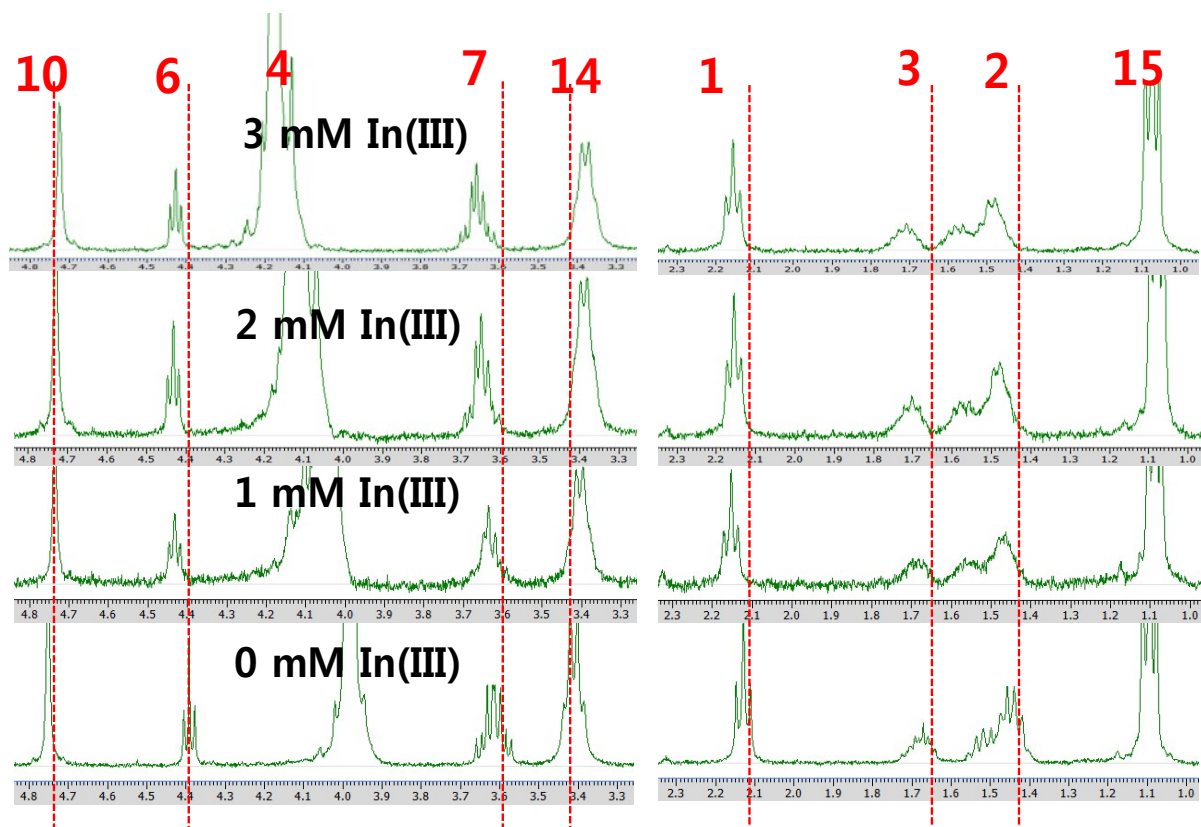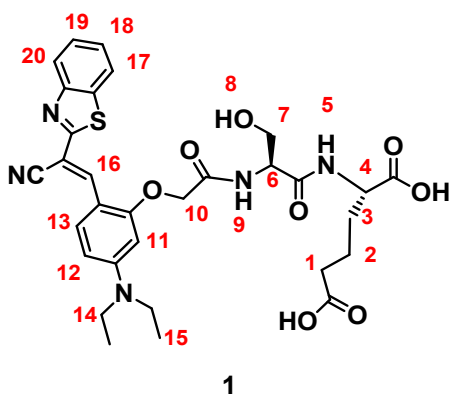
Figure S15. Partial ^{1}H NMR spectra (400 MHz) of **1** (3 mM) with increasing concentration of In^{3+} in DMSO-d_6/D$_2$O (v/v = 4:1) containing 10 mM ammonium formate.

References

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