Supporting Information

New Strategy for Quantifying Biological Zinc by a Modified Zinpyr Fluorescence Sensor

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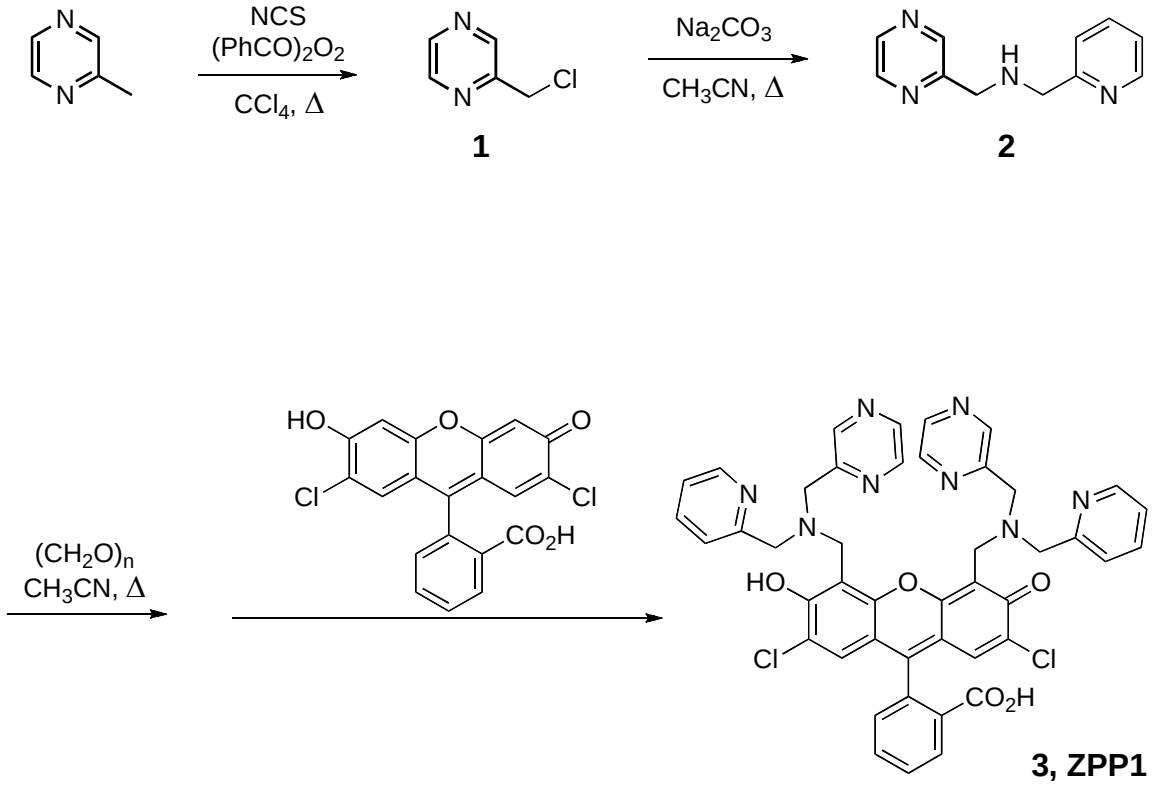
Contents

1. Synthesis of ZPP1.

All reagents were purchased and used as received. Merck F254 silica gel-60 plates were used for analytical thin-layer chromatography (TLC) and silica gel-60 (230-400 mesh) for flash chromatography. ^{1}H and ^{13}C NMR spectral analyses were performed with a Varian 300 MHz or Bruker 400 MHz spectrometer. ^{1}H chemical shifts were referenced to internal solvent peaks. The synthesis of ZPP1 is described below and outlined in Scheme S1. 2-Chloromethylpyrazine (**1**) was prepared by a modified literature method.[1,2] The Mannich reaction follows a method described elsewhere.[3,4]



Scheme S1. Synthesis of ZPP1

2-Chloromethylpyrazine (1). 2-Methylpyrazine (15.1 g, 160 mmol) and benzoyl peroxide (0.45 g, 1.1 mmol) were added to a suspension of *N*-chlorosuccinimide (24.1 g, 180 mmol) in carbon tetrachloride (700 mL). The mixture was refluxed for 24 h under argon and then cooled to room temperature. The solvent was removed by rotary evaporation, and the residue was separated by flash chromatography (silica, 1:1 to 2:3 pentane/diethyl ether) to give the product as a clear, thin oil (8.36 g, 41% yield). Because **1** decomposes rapidly at room temperature, it was immediately used in the subsequent reaction. ^{1}H NMR (CD_2Cl_2, 300 MHz): δ 8.69 (1H, d), 8.53 (1H, q), 8.49 (1H, d), 4.57 (2H, s).

(2-Picolyl)(pyrazin-2-ylmethyl)amine (2). 2-Chloromethylpyrazine (**1**, 1.43 g, 11 mmol) and 2-picolylamine (5.00 g, 46 mmol) were combined with activated sodium carbonate (14.5 g, 140 mmol) in anhydrous acetonitrile (75 mL) and stirred at 82 ºC under an N_2 atmosphere for 20 h. The reaction was cooled to room temperature and filtered through Celite, and the solvent was removed by rotary evaporation. Flash chromatography (silica, 200:1 to 20:1 dichloromethane/methanol) gave the secondary amine as a dark-yellow oil (1.39 g, 63% yield). ^{1}H NMR (CD_2Cl_2, 300 MHz): δ 8.64 (1H, d), 8.52 (1H, dq), 8.48 (1H, q), 8.42 (1H, d), 7.65 (1H, td), 7.34 (1H, dt), 7.16 (1H, m), 3.99 (2H, s), 3.96 (2H, s), 3.42 (1H, bs). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 159.18, 155.19, 149.32, 144.26, 143.99, 143.07, 136.47, 122.26, 122.03, 54.98, 52.75. MS (ESI) calcd for $M+H^+$, 201.11; found, 200.9.

***9-(o-Carboxyphenyl)-2,7-dichloro-4,5-bis[(2-picolyl)(pyrazin-2-ylmethyl)-aminomethyl]-6-hydroxy-3-xanthanone* (ZPP1, 3)**. A portion of (2-picolyl)(pyrazin-2-ylmethyl)amine (**2**, 98.9 mg, 0.49 mmol) and paraformaldehyde (15.3 mg, 0.51 mmol) were combined in freshly distilled acetonitrile (3 mL) and refluxed at 90 ºC under N_2 for 30 min. 2',7'-Dichlorofluorescein (DCF, 74.6 mg, 0.19 mmol) was suspended in acetonitrile and water (3 mL each) and added dropwise to the mixture. The reaction was refluxed for an additional 16 h at 100 ºC, during which time the color changed from bright orange to deep red. The solvents were removed by rotary evaporation, and the residue was thoroughly dried under vacuum and dissolved in a minimal volume of boiling ethanol. The product was precipitated at 0 ºC and filtered. A thorough wash of the

precipitate with ice-cold water and diethyl ether afforded pure ZPP1 as a pale pink powder (91.3 mg, 60% yield). Melting point: 206.1 – 208.3 °C. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.60 (2H, dq), 8.57 (2H, d), 8.54 (2H, td), 8.46 (2H, d), 8.01 (1H, m), 7.85 (3H, m), 7.75 (1H, m), 7.43 (2H, d), 7.35 (2H, dt), 6.54 (2H, s), 5.76 (1H, s), 4.18 (4H, s), 4.09 (4H, s), 4.05 (4H, s). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 156.81, 155.32, 153.29, 151.33, 148.60, 147.93, 144.88, 143.85, 143.48, 137.42, 135.87, 130.54, 126.63, 126.02, 125.09, 124.10, 123.50, 122.83, 116.20, 111.81, 109.90, 58.43, 56.75, 48.96. HRMS (ESI) calcd for $M+H^+$ = 825.2102; found, 825.2112.

2. X-ray Crystal Structure of ZPP1.

Intensity data were collected on a Bruker SMART APEX CCD diffractometer with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å), controlled by a Pentium-based PC running the SMART software package.[5] A single crystal of ZPP1 was mounted on the tip of a glass fiber, coated with paratone oil, and cooled to 110 K under a stream of N_2 maintained by a KRYO-FLEX low-temperature apparatus. The structure of ZPP1 (**3**) was solved by direct methods and refined on F^2 by using the SHELXTL-97 software[6] incorporated in the SHELXTL software package.[7,8] An empirical absorption correction was applied by using the SADABS program.[9] All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were either located directly from a difference Fourier map or assigned to idealized positions with a thermal parameter equivalent of 1.2 (non-methyl hydrogen atoms) or 1.5 (methyl hydrogen atoms) times the thermal parameter of the atom to which they were attached.

A single crystal of ZPP1 was obtained by vapor diffusion of water into a DMSO solution. ZPP1 (**3**) crystallizes with two molecules of water and two molecules of DMSO as colorless plates. One water molecule was modeled with an occupancy factor of 50%,

while the other one has full occupancy and is hydrogen-bonded to two nitrogen donor atoms from the zinc-binding units. Both DMSO solvent molecules are disordered. The sulfur atom of one DMSO moiety was modeled as disordered over two positions (90:10) and the second molecule of DMSO was modeled as disordered over three positions (47:31:22). In both cases, the disordered molecules were refined with the help of similarity restraints. Four hydrogen atoms, which are involved in hydrogen bonding, were located from the difference Fourier map. Two of them are bound to the oxygen atoms of the hydroxyl groups (O1 and O3) the other two are located on the oxygen atom (O1W) of one of the water molecules.

The crystal structure of ZPP1 (**3**), including the hydrogen-bonded water molecule, is shown in Figure S1. Crystallographic information, selected bond lengths and angles, and a summary of distances between hydrogen bond donor and acceptor atoms are displayed in Tables S1–S3, respectively.

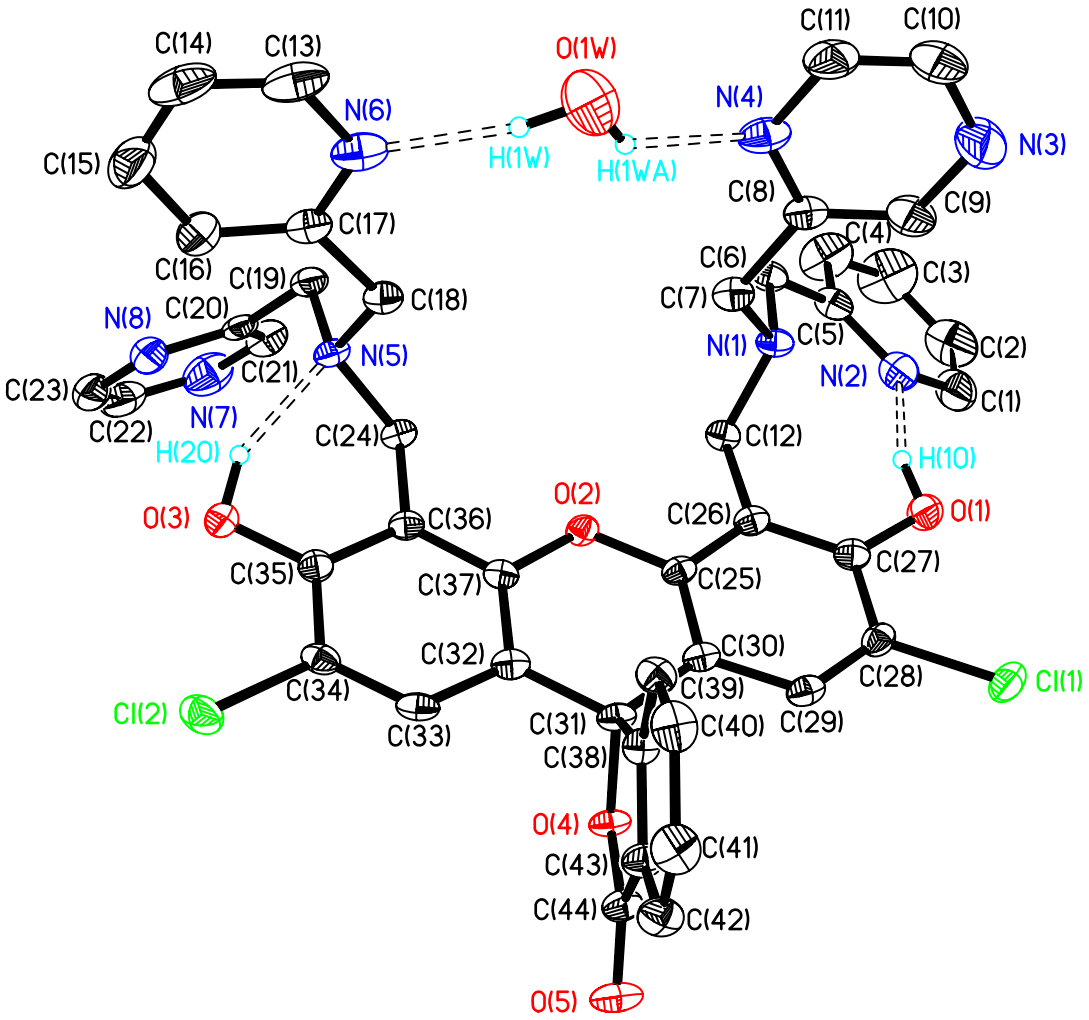


Figure S1. ORTEP diagram of ZPP1 (**3**) showing 40% probability thermal ellipsoids on all non-hydrogen atoms.

Table S1. Summary of X-ray Crystallographic Data for ZPP1 (**3**).

	ZPP1·1.5H_2O·2DMSO
Empirical formula	$C_{48}H_{48}N_8O_{8.50}Cl_2S_2$
Formula weight	1015.96
Crystal System	Triclinic
Space group	$P\bar{1}$
a (Å)	13.207(2)
b (Å)	13.372(2)
c (Å)	16.028(3)
α (deg)	83.814(3)
β (deg)	76.198(3)
γ (deg)	60.728(3)
V (Å^3)	2397.8(8)
Z	2
$\rho_{calc,}$ g/cm^3	1.396
Temperature (K)	110
μ (Mo Kα), mm^{-1}	0.287
θ range, deg	2.17 to 26.37
Crystal size (mm)	0.40 x 0.15 x 0.05
Total no. of data	37063
No. of unique data	9775
No. of parameters	707
Completeness to theta	99.6 %
max, min peaks, e/ Å^3	0.800 and -0.623
Goodness-of-fit on F^2	1.052
R_1 (%)	6.74
wR_2 (%)	15.64

[a] $R_1 = \sum||F_o|-|F_c||/\sum|F_o|$, [b] $wR_2 = \{\sum [(F_o^2-F_c^2)/ \sum [w(F_o^2)^2]\}^{1/2}$

Table S2. Summary of Distances (Å) between Hydrogen Bond Donor and Acceptor Atoms in the X-ray crystal Structure of ZPP1 (**3**).

Bond Lengths	(Å)
O(3)–H(2O)...N5	2.679(3)
O(1)–H(1O)...N2	2.774(4)
O(1W)–H(1W)...N6	2.888(4)
O(1W)–H(1WA)...N4	2.941(4)
O(1)–H(1O)...N1	3.032(3)
O(3)–H(2O)...N8	3.378(3)

Atoms are labeled as indicated in Figure S1.

Table S3. Selected Bond Lengths (Å) and Angles (Deg) for ZPP1 (**3**).

Bond Lengths	(Å)	Bond Angles	(Deg)
N(2)–C(1)	1.339(4)	C(25)–O(2)–C(37)	119.7(2)
N(2)–C(5)	1.330(4)	C(31)–O(4)–C(44)	111.55(19)
N(3)–C(9)	1.343(4)	O(1)–C(27)–C(26)	124.0(3)
N(3)–C(10)	1.350(5)	O(3)–C(35)–C(36)	121.5(2)
N(4)–C(8)	1.333(4)	C(12)–N(1)–C(7)	111.2(2)
N(4)–C(11)	1.325(4)	C(12)–N(1)–C(6)	110.4(2)
N(6)–C(13)	1.337(4)	C(18)–N(5)–C(19)	110.3(2)
N(6)–C(17)	1.352(4)	C(18)–N(5)–C(24)	110.0(2)
N(7)–C(21)	1.347(5)		
N(7)–C(22)	1.312(5)		
N(8)–C(20)	1.343(4)		
N(8)–C(23)	1.342(4)		

Atoms are labeled as indicated in Figure S1.

3. Fluorometric Studies

The experimental procedures of fluorometric studies, including quantum yield measurement, pH titration and metal selectivity test, have been reported in detail previously.[3,4]

3.1. pK_a Measurement

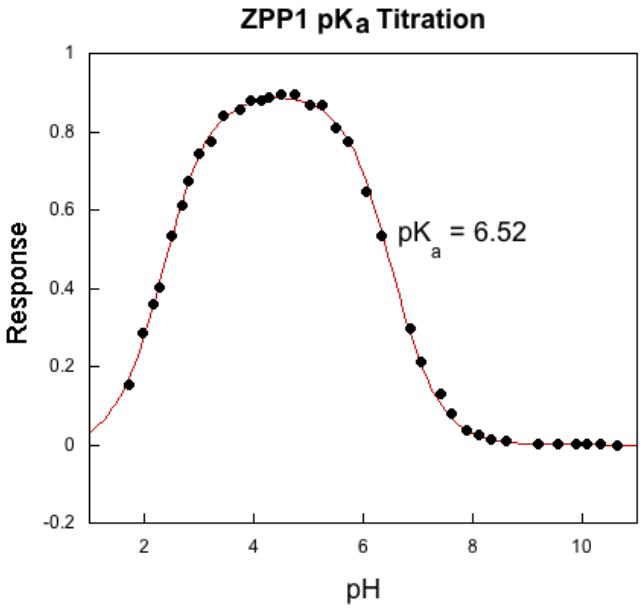


Figure S2. pH fluorescence titration data (dots) of ZPP1 and the calculated fitting curve with pK_a = 6.52. The fluorescence response was normalized to the fluorescence intensity at pH 10.34, 0.001. The increase in fluorescence intensity as the pH is lowered from 10 to ~ 5 reflects protonation of the quenching units. The subsequent decrease in fluorescence, as the pH is further lowered to ~ 2, is typical of this family of sensors and attributed to protonation of the fluorescein moiety.

3.2. Metal Ion Selectivity

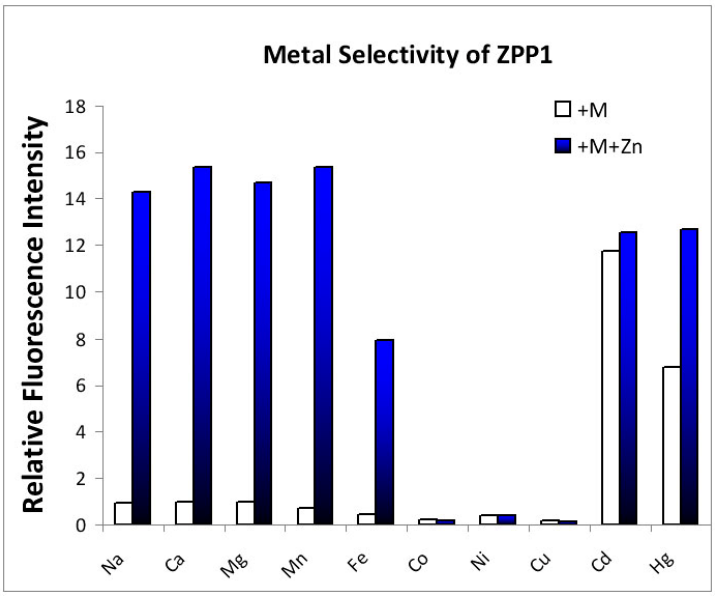


Figure S3. Relative fluorescence response of 5 μM ZPP1 to 50 μM concentrations of different divalent metal ions (open bars), and subsequent addition of 50 μM Zn(II) (blue bars) in pH 7 PIPES buffer (50 mM, 100 mM KCl).

3.3. Zinc Titration

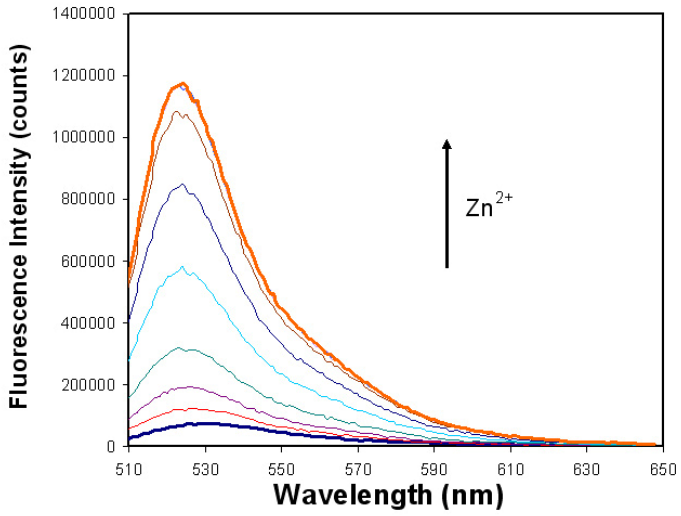


Figure S4. Fluorescence response of 5 μM ZPP1 upon addition of increasing amounts of zinc(II) chloride (0 μM, 1.7 μM, 3.3 μM, 5.0 μM, 6.7 μM, 8.3 μM, 10.0 μM, 11.7 μM and 13.3 μM) in pH 7 buffer solution (50 mM PIPES with 100 mM KCl).

4. Data Fitting Procedure

The fitting for fluorescence zinc titration data was performed by adopting a method described by Kneeland, et al..[10] Initial K_1 and K_2 approximations were used with the Newton-Raphson scheme in order to estimate the amount of free zinc in solution at each point in the titration curve. The equations applied are:

$$[\text{ZPP1}] + [\text{Zn}] \overset{K_1}{\rightleftharpoons} [\text{ZPP1-Zn}_1]$$

$$[\text{ZPP1-Zn}_1] + [\text{Zn}] \overset{K_2}{\rightleftharpoons} [\text{ZPP1-Zn}_2]$$

$$K_1K_2[\text{Zn}]^3 + K_1(2K_2[\text{ZPP}_1]_t - K_2[\text{Zn}]_t + 1)[\text{Zn}]^2 + (K_1[\text{ZPP}_1]_t - K_1[\text{Zn}]_t + 1)[\text{Zn}] - [\text{Zn}]_t = 0$$

where $[\text{ZPP1}]_t$ and $[\text{Zn}]_t$ refer to the total concentrations of ZPP1 and Zn(II) added in the solution, respectively. Microsoft Excel[11] was used to solve the cubic equation and to obtain estimates of free zinc in solution at each point. The values of free zinc obtained

were applied in the following equations in order to determine the amount of each species present.

$$[\text{ZPP1-Zn}_1] = \frac{K_1[\text{ZPP}_1]_t[\text{Zn}]}{K_1K_2[\text{Zn}]^2 + K_1[\text{Zn}] + 1)}$$

$$[\text{ZPP1-Zn}_2] = \frac{K_1K_2[\text{ZPP}_1]_t[\text{Zn}]^2}{K_1K_2[\text{Zn}]^2 + K_1[\text{Zn}] + 1)}$$

$$[\text{ZPP1}] = [\text{ZPP}_1]_t - [\text{ZPP1-Zn}_1] - [\text{ZPP1-Zn}_2]$$

These values were used to determine a calculated fluorescence intensity using the equation

$$Fluorescence_{\text{calc}} = E_{\text{ZPP1}}[\text{ZPP1}] + E_{\text{ZPP1-Zn}}[\text{ZPP1-Zn}_1] + E_{\text{ZPP2-Zn}}[\text{ZPP1-Zn}_2]$$

The *E* values are defined as proportionality constants,[12] which are used to define the total fluorescence intensity. The proportionality constants used in this equation were initially determined by estimations based on the measured fluorescence data. The fluorescence intensity can also be determined by the equation

$$Fluorescence = 2.3I_0\phi\varepsilon bc$$

where I_0 is the excitation radiation intensity, ϕ is the fluorescence quantum yield, ε is the molar absorptivity, b is the path length, and c is the molar concentration.

The solver function of Excel was then used to minimize the difference between the calculated and measured fluorescence intensities. The solver function uses an iterative process to minimize or maximize the value of one cell in Excel.[11] The values set as variables are K_1, K_2, $E_{\text{ZPP1-Zn2}}$, $E_{\text{ZPP1-Zn1}}$. The solver function was run until an appropriate fitting was obtained.

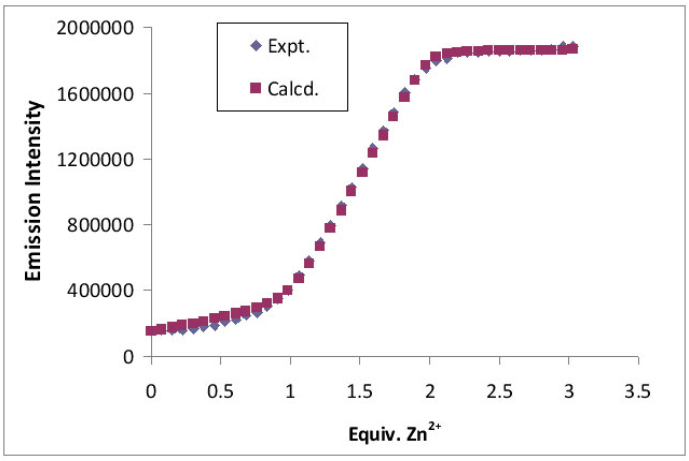


Figure S5. Experimental and calculated fluorescence response of ZPP1 (5 μM) upon addition of Zn(II) in a pH 7 PIPES buffer solution (50 mM) with 100 mM KCl.

5. Cell Culture and Fluorescence Microscopy

5.1. Cell Culture. Min6 cells were cultured in 25 cm^2 flasks containing Dulbecco's Modified Eagle's Serum (DMEM; Invitrogen) with 10% fetal bovine serum (Hyclone, Logan, UT). Prior to imaging, cells were passed into P35G-1.5-14C poly-D-lysine coated culture dishes with glass bottom (MatTek) and allowed to grow for 48 h. The cells were grown to ~ 50% confluence and incubated with 25 μM of **ZPP1** for 24 h. Cells were washed twice with PBS buffer and then incubated with Hoechst 33258 before being imaged in both green and blue channels. TPEN (50 μM) was added to the plate while on the microscope stage, and allowed to incubate for 5 min. The cells were then imaged again.

5.2. Fluorescence Microscopy. The cell fluorescence imaging experiments were performed with a Zeiss Axiovert 200M inverted epifluorescence microscope, equipped with a Hamamatsu EM-CCD digital camera C9100 and a MS200 XY Piezo Z stage (Applied Scientific Instruments, Inc.). An X-Cite® 120 metal-halide lamp (EXFO) was used as the light source. The fluorescence images were obtained by using a 63× oil immersion objective lens. The microscope was operated with Volocity software (Improvision).

6. Zinc Quantification

Min-6 cells were grown in a 75 cm^2 flask to approximately 90% confluence. The cells were then washed once with 7 mL of DMEM without phenol red and allowed to incubate at room temperature for 5 min. The media was then removed and saved as a control. An additional 7mL of DMEM without phenol red, supplemented with 50 mM KCl and 20 mM glucose, was then added. The cells were incubated in this mixture at room temperature for 5 min, and the media was again removed and saved. The cells in the flask were then treated with trypsin to enable counting of the cells.

A titration was performed using the media (3 mL) that had been saved by adding small portions of ZPP1. The fluorescence was recorded after each addition. In order to quantify the amount of zinc released, the fluorescent levels of the DMEM before treatment with KCl and glucose were subtracted from the fluorescent levels of the DMEM after treatment, and the resulting values were plotted (Figure S6).

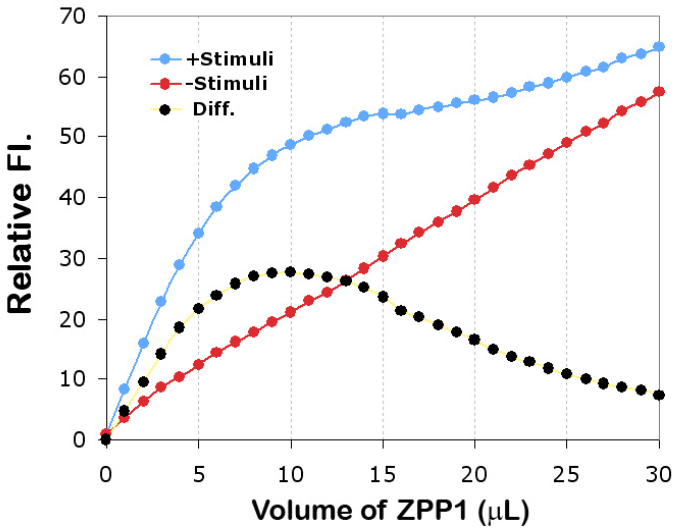
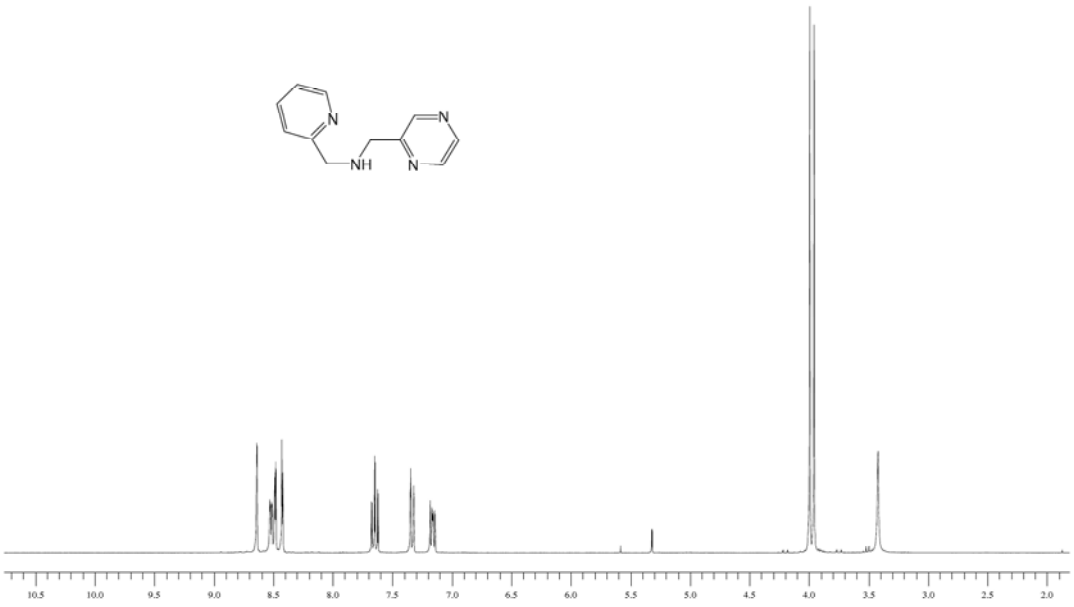
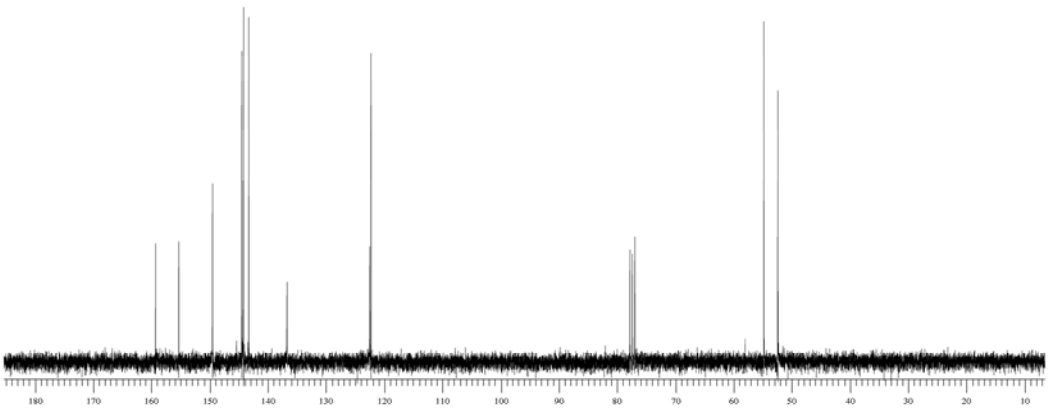
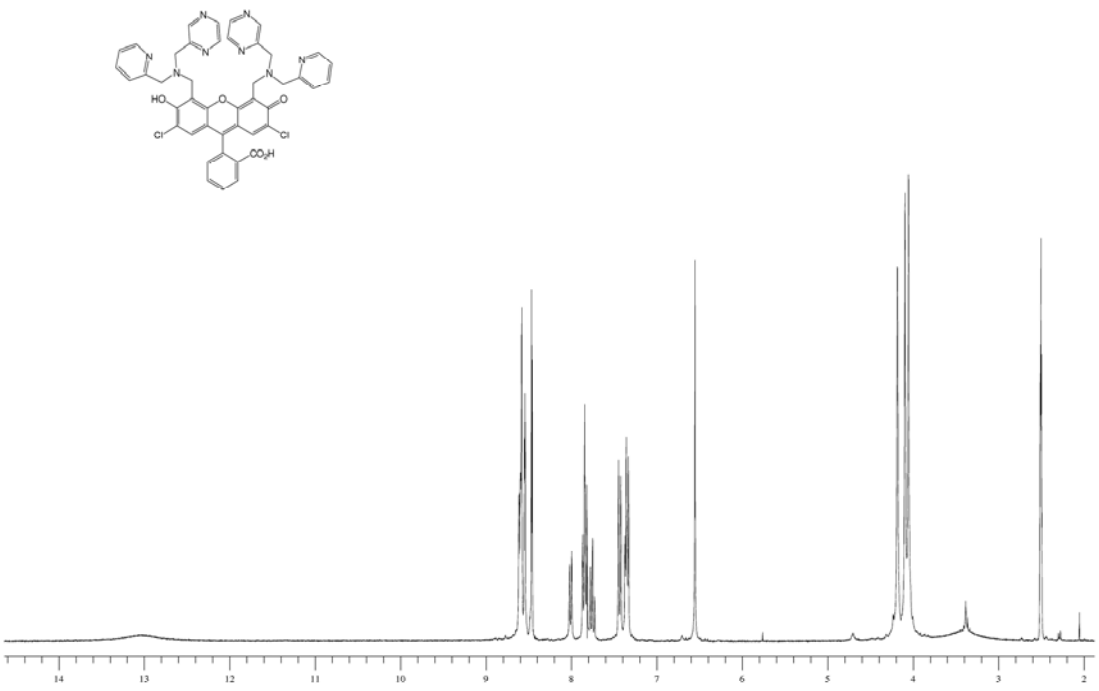


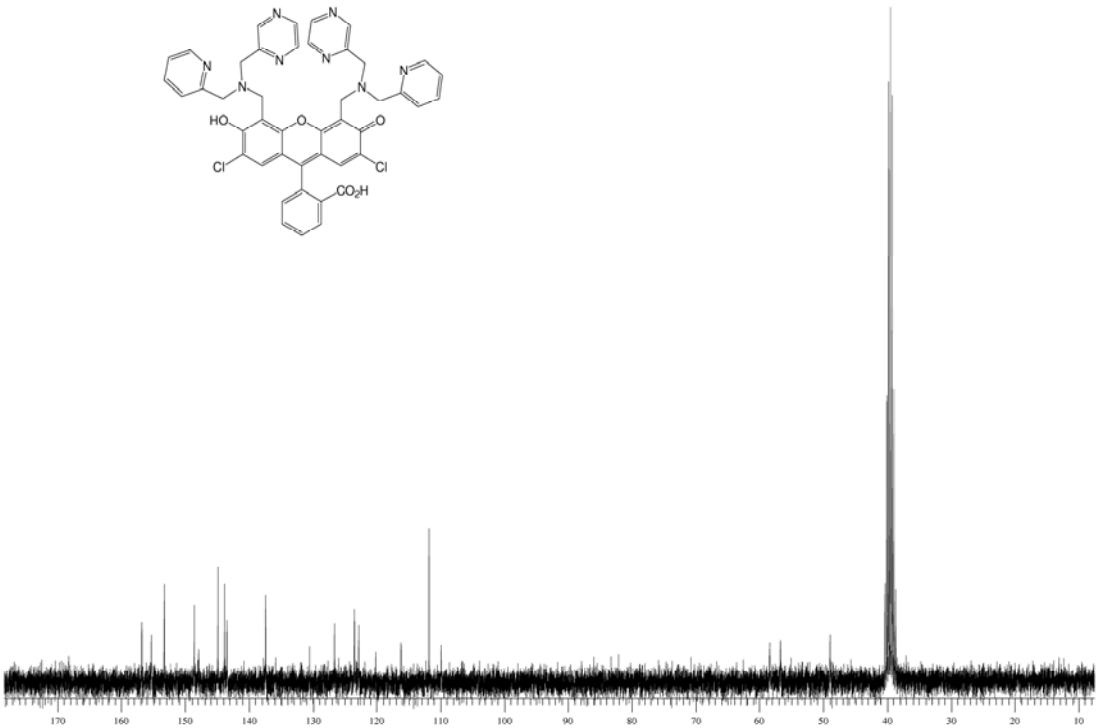
Figure S6. The Min6 cell medium after KCl/glucose stimulation was titrated with ZPP1 (blue) followed by fluorescence spectroscopic analysis. The zinc responsive curve (black) was obtained after the subtraction of the background fluorescence of zinc-free ZPP1 (red).

7. NMR Spectra









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