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Analogues of Zinpyr-1 Provide Insight into the Mechanism of Zinc Sensing

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Three compounds structurally related to the fluorescent zinc sensor Zinpyr-1 (ZP1) have been synthesized and characterized. In each of these ZinAlkylPyr (ZAP) analogues, an alkyl group (methyl, benzyl) replaces one of the metal-binding picolyl moieties in ZP1. The methyl-for-picolyl substitutions in ZAP1 and ZAP2 have a negligible effect on the optical spectrum of the fluorophore but elevate the quantum yields ($\Phi = 0.82$ (ZAP1), 0.74 (ZAP2)) to values near that of Zn^{2+}-saturated ZP1 ($\Phi = 0.92$). The benzyl-for-picolyl substitution in ZAP3 similarly enhances the quantum yield ($\Phi = 0.52$) relative to that of metal-free ZP1 ($\Phi = 0.38$). As previously observed for methylated ZP1 sensors, methylation of the 6-position of the pyridyl ring diminishes the emission by lowering both the molar extinction coefficient and the quantum yield. Although these new ZAP compounds cannot detect Zn^{2+} fluorimetrically at neutral pH, complexation of Zn^{2+} does occur, as evidenced by sizable changes in the optical spectra. The ZAP1–3 probes can detect Zn^{2+} fluorimetrically at pH 9, indicating that proton-induced background emission obscures any Zn^{2+}-induced fluorescence at pH 7. The tertiary amine groups in ZAP1–3 are less basic than those in ZP1, which implies that the additional pyridine rings are responsible for the emissive response to Zn^{2+} at pH 7.0.

Introduction

The Zinpyr (ZP) family of fluorescent zinc sensors has found application in the study of biological stores of chelatable Zn^{2+}.[1−5] The sensors are composed of two types of modules: a fluorescein-derivative reporter and either one or two metal-binding units. The traits that enable these molecules to function as sensors have been largely unexplored, and the connection between their molecular structures and photophysical properties remains poorly understood. Many fluorescein-based zinc sensors result from modification of the bottom ring, and the influence of bottom ring substituents on their photophysical properties has been extensively studied.[6−12] Fluorescein derivatives with nonhalide functionalities installed on the xanthenyl ring are more rare.[1−4,13−17]

Our laboratory has been interested in developing sensors with weaker affinities for Zn^{2+} in order to evaluate estimates of its concentration at synapses involving glutamatergic hippocampal neurons, which may reach values as high as 300 μM.[18,19] Probes with dissociation constants (K_d values)

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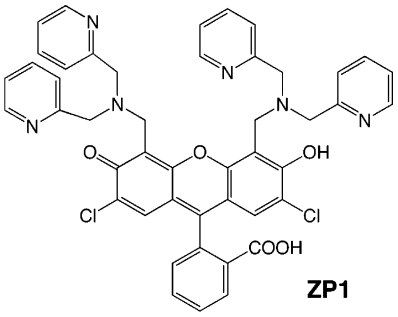
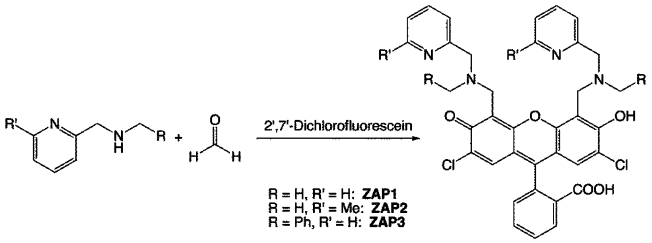
Scheme 1



Scheme 2



in the micromolar to millimolar range could be used in conjunction with nanomolar probes to estimate the amount of Zn^{2+} in cellular and extracellular regions more precisely than is currently possible. This objective necessitates finding viable alternatives to the commonly used dipicolylamine metal ion binding group.[1−4,20,21]

One possible way to increase the K_d value is to eliminate chelating atoms from the metal-binding modules. This strategy has been previously used to develop the QZ and Zinspy sensor families[15,16] and has a greater impact on the K_d value than installing steric bulk near the chelating atoms.[9,17] Presented here are the syntheses of three analogues of Zinpyr-1 (ZP1, Scheme 1) with alkyl groups substituting for two of the four picolyl arms (Scheme 2). The ZinAlkyl-Pyr (ZAP) compounds are similar to ZP1 in that they share 2′,7′-dichlorofluorescein as the parent fluorophore, enabling comparison to one of the most extensively characterized probes in the Zinpyr family.[4,20]

Experimental Section

Reagents. Acetonitrile (MeCN) was dried over 3 Å molecular sieves. Ethanol (EtOH), methanol (MeOH), diethyl ether, chloroform ($CHCl_3$), 2′,7′-dichlorofluorescein (DCF), and paraformaldehyde were purchased from Aldrich and used as received. *N*-Methyl-1-(pyridin-2-yl)methanamine[22] and *N*-methyl-1-(6-methylpyridin-2-yl)methanamine[23] were synthesized as described in the literature and distilled prior to use. *N*-Benzyl-1-(pyridin-2-yl)methanamine was prepared as reported previously.[24]

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Materials and Methods. Octadecyl-functionalized silica gel (reverse phase, RP18) was used for analytical thin-layer chromatography (TLC). NMR spectra were acquired on a Varian 500 MHz spectrometer and referenced to internal probe standards for both ^1H and ^{13}C NMR. IR spectra were collected on an Avatar 360 FT-IR instrument. Melting points were measured with a Mel-Temp apparatus. The pH values of solutions were determined with an Orion glass electrode that was calibrated with three standards prior to each use. Fluorescence spectra were obtained on a Photon Technology International fluorescence spectrophotometer. UV/vis spectra were acquired on a Cary 1E spectrophotometer. High-resolution mass spectrometry was performed by staff at the MIT Department of Chemistry Instrumentation Facility.

Syntheses. 2-(2,7-Dichloro-6-hydroxy-4,5-bis((methyl(pyridin-2-ylmethyl)amino)methyl)-3-oxo-3*H*-xanthen-9-yl)benzoic acid (ZAP1). *N*-Methyl-1-(pyridin-2-yl)methanamine (0.139 g, 1.14 mmol) and paraformaldehyde (0.037 g, 1.23 mmol) were refluxed in 3 mL of MeCN for 30 min. A slurry of DCF (0.137 g, 0.342 mmol) in 4 mL of MeCN/H_2O (1:1) was added, and the mixture was refluxed for 24 h. The MeCN was removed. The residue was dissolved in 3 mL of boiling EtOH which was gradually cooled to precipitate the red product (0.107 g, 47%). mp: 189 °C (dec). ^1H NMR ($CDCl_3$, 500 MHz): δ 2.44 (6 H, s), 3.96 (4 H, d, $J = 6.0$ Hz), 4.12 (4 H, dd, $J_1 = 19.0$ Hz, $J_2 = 14.5$ Hz), 6.62 (2 H, s), 7.20 (1 H, d, $J = 7.5$ Hz), 7.27 (m, obscured by solvent peak), 7.40 (2 H, d, $J = 7.5$ Hz), 7.67−7.77 (5 H, m), 8.05 (1 H, d, $J = 7.5$ Hz), 8.65 (2 H, d, $J = 4.5$ Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 42.41, 53.39, 62.95, 110.22, 110.44, 117.44, 123.10, 123.72, 124.20, 124.85, 125.64, 127.31, 127.72, 130.42, 135.48, 137.39, 148.38, 149.54, 151.65, 156.34, 156.39, 169.01. FT-IR (KBr, cm^{-1}): 3395 (w), 3057 (w), 1761 (s), 1634 (s), 1610 (s), 1587 (s), 1480 (m), 1411 (m), 1356 (m), 1266 (m), 1215 (m), 1091 (s), 956 (w), 947 (m), 905 (m), 884 (s), 780 (m), 760 (s), 715 (s). HRMS (ESI): Calcd for MH^+: 669.1671. Found: 669.1667.

2-(2,7-Dichloro-6-hydroxy-4,5-bis((methyl((6-methylpyridin-2-yl)methyl)amino)methyl)-3-oxo-3*H*-xanthen-9-yl)benzoic acid (ZAP2). *N*-Methyl-1-(6-methylpyridin-2-yl)methanamine (0.236 g, 1.77 mmol) and paraformaldehyde (0.053 g, 1.76 mmol) were refluxed in 4.5 mL of MeCN for 30 min. A slurry of DCF (0.218 g, 0.544 mmol) in 8.5 mL of MeCN and 6.5 mL of H_2O was added, and the resultant mixture was refluxed for 24 h. The MeCN was removed. The residue was dissolved in 5 mL of EtOH and cooled to −25 °C to precipitate a red solid. This material was purified by chromatography on an alumina column with 5% MeOH/$CHCl_3$ as an elutant and isolated as a red solid (0.182 g, 48%). mp: 172 °C (dec). ^1H NMR ($CDCl_3$, 500 MHz): δ 2.45 (6 H, s), 2.60 (6H, s), 3.92 (4 H, s), 4.10 (4 H, dd, $J_1 = 21.5$ Hz, $J_2 = 14.0$ Hz), 6.61 (2 H, s), 7.11 (2 H, d, $J = 7.5$ Hz), 7.19 (3 H, m), 7.62 (2 H, t, $J = 7.5$ Hz), 7.70 (3 H, m); 8.04 (1 H, d, $J = 6.9$ Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 24.49, 42.49, 53.39, 63.09, 110.25, 110.36, 117.43, 120.62, 122.73, 124.20, 125.62, 127.33, 127.68, 130.40, 135.46, 137.57, 148.36, 151.68, 155.67, 156.35, 158.55, 169.05. FT-IR (KBr, cm^{-1}):3410 (s), 3055 (m), 2957 (s), 2926 (s), 2854 (m), 1761 (w), 1684 (w), 1632 (m), 1576 (m), 1362 (w), 1283 (w), 1097 (w), 788 (w). HRMS (ESI): Calcd for MH^+: 697.1984. Found: 697.1987.

2-(4,5-Bis((benzyl(pyridin-2-ylmethyl)amino)methyl)-2,7-dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (ZAP3). *N*-Benzyl-1-(pyridin-2-yl)methanamine (0.506 g, 2.55 mmol) and paraformaldehyde (0.078 g, 2.59 mmol) were refluxed in 10 mL of MeCN for 30 min. A slurry of DCF (0.339 g, 845 mmol) in 12 mL of a 1:1 mixture of MeCN/H_2O was added, and the resultant mixture was refluxed for 24 h. The MeCN was evaporated to yield a crude

red residue. The residue was dissolved in 10 mL of EtOH and cooled to yield the product as a pinkish-red powder (0.377 g, 54%). mp: 226 °C (dec). $R_f = 0.10$ (RP, MeOH). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.81 (4 H, dd, $J_1 = 33$ Hz, $J_2 = 13$ Hz), 3.91 (4 H, d, $J = 14.5$ Hz), 4.15 (4 H, s), 6.63 (2 H, s), 7.19 (d, $J = 7.5$), 7.30 (15 H, m), 7.69 (4 H, m), 8.05 (1 H, d, $J = 7.5$ Hz), 8.67 (2 H, d, $J = 4.5$ Hz). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 49.87, 58.34, 58.49, 110.45, 111.02, 117.51, 122.90, 123.70, 124.23, 125.56, 127.29, 127.77, 127.99, 128.87, 129.54, 130.37, 135.43, 136.70, 137.37, 148.68, 149.17, 151.65, 156.09, 156.86, 169.03. FT-IR (KBr, cm^{-1}): 3487 (w), 3066 (w), 3030 (w), 2899 (w), 2851 (w), 1754 (s), 1626 (m), 1589 (m), 1571 (w), 1465 (s), 1397 (m), 1365 (s), 1252 (m), 1211 (s), 1095 (s), 1028 (w), 897 (m), 871 (m), 764 (m), 754 (m), 700 (s). HRMS (ESI): Calcd for MH^+: 821.2297. Found: 821.2304.

Spectroscopic Methods. Aqueous solutions were prepared with Millipore water. Stock solutions (1−2.00 mM) of the dyes in DMSO were prepared, separated into aliquots, stored at −25 °C, and thawed in the dark immediately before use. The aliquots were periodically checked for decomposition products via TLC (RP, 100% MeOH) and were discarded upon the first sign that impurities had formed. All spectroscopic data were recorded at room temperature (22 °C) in a pH 7.0 buffer solution of 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) and 100 mM KCl unless noted otherwise. Quantum yields were measured relative to fluorescein in 0.1 M NaOH ($\Phi = 0.95$)[25] by a previously described method.[20]

To measure the K_d values of ZAP1−3 complexes with Zn^{2+}, aliquots of 10 mM $ZnCl_2$ were added to solutions of the sensors in pH 7.0 buffer. The optical spectrum was acquired immediately after each addition. The full data set was then analyzed with the singular decomposition value program Specfit. Reported values are the average of at least three measurements.

To measure pK_a values, fluorescence emission spectra over the 515−700 nm range were acquired at numerous points from pH 12.0 to 2.0. A 0.5 μM solution of dye in an aqueous solution containing 11 mM KOH and 100 mM KCl was prepared, and its pH and emission were measured. The pH was then lowered gradually by addition of HCl solutions as described previously.[20] The integrated emission areas for each data point were plotted against pH and fit to the nonlinear model previously reported.[20]

Results

Syntheses. All three compounds presented can be synthesized in two steps from commercially available materials (Scheme 2). The first step prepares the secondary amine precursor. The second step in the syntheses connects 2 equiv of the secondary amine to DCF through a Mannich reaction, analogous to the preparation of ZP1 from dipicolylamine and DCF.[4] Both ZAP1 and ZAP2 substitute methyl groups in place of two ZP1 picolyl subunits. The ZAP2 compound is further modified from ZP1 by the installation of methyl groups on the 6-positions of the remaining pyridine rings. Substituting benzyl groups for two of the picolyl groups in ZP1 yields ZAP3. The products are precipitated from EtOH solutions, with only ZAP2 requiring chromatography for purification. The yields of the Mannich reaction step range from 47% to 54%. DMSO and $CHCl_3$ solutions of ZAP3 appear to decompose over several hours, as assessed by TLC and ^{1}H NMR spectroscopy, respectively. The fluorescent signal of pH 7.0 aqueous solutions of ZAP3 decreases by ~5% per hour, which is slow enough to allow quantitative photophysical measurements. The ZAP1 and ZAP2 dyes are more stable in solution, although stock solutions will discolor over days at room temperature.

Table 1. Spectroscopic Data for ZAP1−3 and ZP1

	absorption		emission	
compound	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	λ_{max} (nm)	Φ
ZAP1	506	83 000	525	0.82
Zn−ZAP1	500	54 600		
ZAP2	508	78 000	527	0.74
Zn−ZAP2	503	57 800		
ZAP3	510	80 400	527	0.52
Zn−ZAP3	504	56 800		
ZP1	515	79 000	531	0.38
Zn_2−ZP1	507	78 000	527	0.92

Photophysical Properties. Table 1 summarizes the photophysical properties of ZAP1−3 and lists values for the free and Zn^{2+}-bound forms of ZP1 for comparison. ZAP1 and ZAP2 both resemble the zinc-saturated ZP1 dye in all four listed parameters. The molar extinction coefficient for the 510 nm band of ZAP3 decreases to 23700 M^{-1} cm^{-1} as the concentration of the solution is increased beyond 1 μM, consistent with compound aggregation. This sensor also has the lowest quantum yield among the ZAP series of compounds.

pH-Dependent Behavior. The pH-dependent emission of the three ZAP compounds is shown in Figure 1. Each ZAP compound undergoes a large increase in emission between pH 10 and 7 and diminishes in fluorescence intensity below pH 5. The solutions cannot be made sufficiently acidic to yield a full turn-off in fluorescence; dye dilution and the reversibility of the Mannich reaction in strongly acid solutions compromise data acquired at low pH. The pK_a values of the reaction associated with the loss of emission are estimated as 2.0 (ZAP1), 2.7 (ZAP2), and 3.2 (ZAP3). The protonation events between pH 10 and 7 can be modeled more accurately, with the pK_a values being 8.30 (ZAP1), 8.33 (ZAP2), and 7.88 (ZAP3).

Zinc Response. Upon the addition of Zn^{2+}, the optical spectrum of each compound undergoes a slight change, most significantly manifest by a 5−10 nm blue shift of the maximum absorption band (λ_{max}, Figure 2). The intensity of the absorption decreases by ~30% as the concentration of the metal ion increases. The emission intensities of ZAP1−3 do not increase upon addition of Zn^{2+} at pH 7.0. The data suggest that the fluorimetric responses of the ligands to Zn^{2+} are not influenced by the metal counteranion but are strongly affected by pH. In 50 mM PIPES, 100 mM KNO_3, pH 7.0 buffer, the addition of $Zn(NO_3)_2$ does not enhance the emission of ZAP1. At pH 9, each ZAP sensor is much less fluorescent than it is at pH 7, with $\Phi = 0.13$ (ZAP1), 0.14 (ZAP2), and 0.10 (ZAP3). Subsequent addition of $ZnCl_2$ in this basic medium leads to large enhancements in fluorescence for ZAP1−3 (Figures 3, S4, S5). With 200 μM added Zn^{2+}, the quantum yields increase to 0.39 (ZAP1), 0.40 (ZAP2), and 0.27 (ZAP3). At this higher pH value, the maximal increase in the integrated emission is 2-fold for ZAP1 and ZAP2 and 5-fold for ZAP3.

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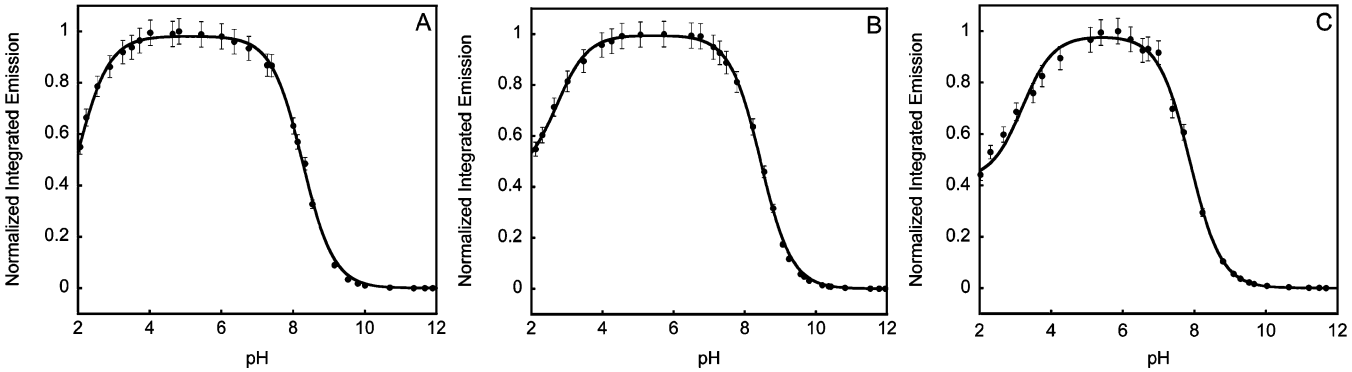


Figure 1. Normalized integrated emission response versus pH for ZAP1 (A), ZAP2 (B), and ZAP3 (C). The fit to the model[20] is shown for each compound.

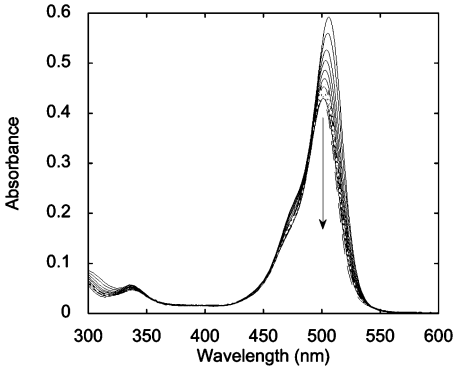


Figure 2. Addition of 10 1.0 μL aliquots and one 50 μL aliquot of 10 mM $ZnCl_2$ to a 5.5 μM solution of ZAP1 in 100 mM KCl, 50 mM PIPES, pH 7.0 buffer, such that the final concentration of Zn^{2+} is 0.2 mM. The dye is diluted by less than 2% over the titration.

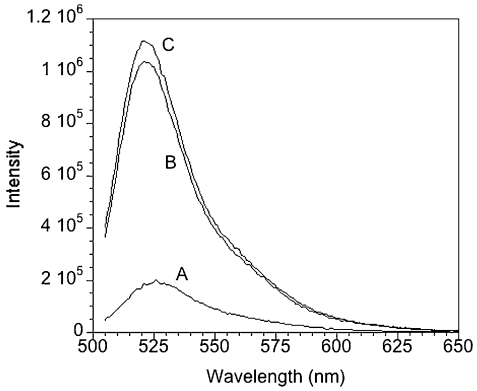


Figure 3. Emission of a 1.0 μM sample of ZAP3 in 100 mM KCl, 50 mM CHES, pH 9.0 buffer with 500 nm excitation. (A) No $ZnCl_2$ added. (B) 10 equiv of $ZnCl_2$ added. (C) 20 equiv of $ZnCl_2$ added.

Discussion

The ZAP dyes are synthesized and isolated in a straightforward manner from DCF and dialkylamines under conditions similar to those used to prepare ZP1 from DCF and dipicolylamine.[4] The syntheses from this study extend earlier work,[4,14,16] demonstrating that it is possible to prepare a number of potential chemosensors from secondary amines and DCF in one simple step.

The ZAP1−3 and ZP1 probes share the same DCF chromophore; consequently, most of their photophysical characteristics are similar. Both the peak absorption and the peak emission fall within narrow 10 nm wavelength ranges. At pH 7.0, the wavelengths of maximal absorption and emission and the quantum yield of ZAP1 and ZAP2 more closely resemble those of the zinc-saturated ZP1 than those of the metal-free sensor. Methylation of ZAP1 to yield ZAP2 reduces both the molar extinction coefficient and the quantum yield of the dye, consistent with results found for methylated versions of ZP1.[17] The benzyl substitution in ZAP3 more significantly impacts the optical and fluorimetric features. The benzyl groups appear to promote sensor aggregation because higher-concentration samples of ZAP3 (>2 μM) absorb more weakly than similarly concentrated solutions of ZAP1, ZAP2, or ZP1. The quantum yield is markedly lower than those of ZAP1 and ZAP2 as well, but is not outside the range of observed quantum yields for xanthenyl ring-derivatized fluoresceins.[2−4,15−17,26] All but one of the compounds described in ref 14 have lower quantum yields, for these molecules were designed to adopt a conformation that facilitates quenching from tethered aniline functionalities. The outlier, a control compound that lacks the aniline groups, has a quantum yield approximately the same as those of ZAP1 and ZAP2.[14]

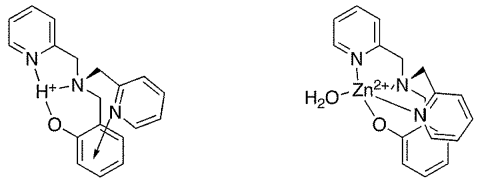
Unlike ZP1, none of the ZAP dyes exhibits enhanced emission in the presence of Zn^{2+} at pH 7.0. Noticeable shifts in the optical spectra of the three compounds upon the addition of $ZnCl_2$ reveal that Zn^{2+} is coordinating to the probes. The magnitudes of the overall optical changes are sufficient to allow the calculation of dissociation constants for ZAP1−3 through singular value decomposition of spectrophotometric Zn^{2+} titrations. ZAP1 and ZAP3 have nearly identical K_d values of 10.2 (±1.4) μM and 9.3 (±0.7) μM, respectively. The 6-methyl substituents on the pyridine rings of ZAP2 have a sizable impact on its binding affinity for Zn^{2+}; the dissociation constant of the 1:1 Zn^{2+}/ZAP2 complex is 109 (±20) μM. The 10-fold increase in the K_d value from ZAP1 to ZAP2 is comparable to the 5-fold rise between ZP1 and Me_2ZP1 but much less than the 200-fold change between Me_2ZP1 and Me_4ZP1.[17]

Analyses of the fluorescence intensity as a function of pH suggest that ZAP1−3 are almost completely protonated at pH 7.0 (Figure 1). The pK_a values obtained from the fits are consistent with protonation at the tertiary amines in the molecule. When not bound to a proton or a diamagnetic metal ion, these amines are believed to quench the fluorescence by a photoinduced electron-transfer mechanism,[27,28] thereby accounting for the reduced emission at higher pH. The

response to H^+ appears to be quantitatively similar to that induced by Zn^{2+} for these compounds, such that the emission of the monoprotonated sensor is equivalent to that of the 1:1 Zn^{2+}/ligand complex. These data suggest that Zn^{2+}-responsive probes appropriate for ambient conditions could be prepared if the pK_a values of the tertiary amines were lowered by judicious chemical modification. The Zinspy (ZS) sensors previously synthesized by our laboratory support this hypothesis. The pK_a values of the protonated tertiary amines in ZS1 and ZS2 are both 7.7, slightly lower than those of the ZAP sensors; both probes exhibit a weak fluorimetric response to Zn^{2+}.[16]

The pK_a values fluorimetrically measured for ZAP1−3 (pK_a = 8.30, 8.33, 7.88) are slightly lower than that observed for ZP1 (pK_a = 8.4).[20] The additional pyridyl units in ZP1 relative to the ZAP series appear to be key to the Zn^{2+}-induced response of the sensor. It is possible that ZP1 binds protons through an ON_2 coordination (Scheme 3), leaving a free pyridine to participate in fluorescence quenching analogous to that observed for aniline.[14] If Zn^{2+} were bound through a similar ON_3 coordination set in solution as that revealed by solid-state studies,[20] neither the pyridines nor the tertiary amine of the metal-binding group would be available to disrupt the emission. At pH 7, the tertiary amines of ZP1 may thus have a lesser impact on the sensing capability than previously thought.[1,3,4] It may be possible, therefore, to prepare zinc sensors without chelating amines; such a design element would circumvent much of the acid−base behavior that complicates interpretation of the zinc response of several currently used probes.

Scheme 3. Proposed ZP1 Sensor Coordination for H^+ and Zn^{2+}[a]



Second Pyridine not Coordinated, Available to Quench Fluorescein

Second Pyridine Coordinated, Unavailable to Quench Fluorescein

[a] The majority of the fluorescein component of ZP1 has been omitted for clarity.

Summary

The new fluorescein derivatives described here provide an important insight into the turn-on mechanism of the Zinpyr family of sensors. Their high basicities preclude them from fluorimetrically signaling the presence of Zn^{2+} at pH 7.0. The pK_a values of the emission-quenching tertiary amines are slightly lower than that of ZP1, indicating that the pyridine rings of ZP1 and related sensors have a greater contribution to their zinc responses than was previously understood.

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Supporting Information Available: Fits used to calculate dissociation constants; fluorimetric responses of ZAP1 and ZAP2 to Zn^{2+} at pH 9.0; and ^1H NMR and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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