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Dizinc Phosphohydrolase Model Built on a *m*-Terphenyl Scaffold and Its Use in Indicator Displacement Assays for Pyrophosphate Under Physiological Conditions

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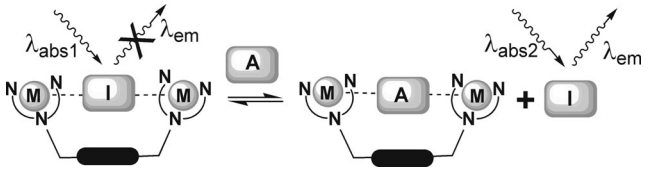
A dinucleating ligand {1,3-bis[2-(di-2-picolylaminomethyl)-phenyl]benzene, **L2**} built on a *m*-terphenyl scaffold was prepared. The dissociation constants for the dizinc complex of **L2** (Zn_2**L2**) binding to phosphate, pyrophosphate and commercially available complexometric indicators were determined under physiological pH [10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer at pH 7.4]. Colorimetric and fluorescence-based indicator displacement assays with selectivity for pyrophosphate over other anions were achieved with Zn_2**L2** as the receptor component. These assays show good response characteristics for quantification of pyrophosphate concentrations as low as 2.5×10^{-6} M, suggesting their utility for measuring pyrophosphate levels in synovial fluid.

Introduction

The indicator displacement assay (IDA, Scheme 1) strategy is a simple and increasingly popular approach to colorimetric and fluorescent chemosensing.[1,2] Early IDAs were developed to recognize a variety of anions,[3–16] including inorganic phosphates,[15,17] and have even been adapted to challenging problems such as nitric oxide (NO) genesis by living cells[18–20] and nerve-agent detection.[21] The interplay between IDA receptor, indicator, target analyte, and potentially interfering species in the sample must be carefully controlled in order to achieve the desired sensitivity and selectivity. Numerous multipodal and compartmental complexes have served as IDA receptors.[22–26] The design of such receptors can be inspired by enzyme active sites known to bind target analytes. Dizinc phosphohydrolase models, for example, are attractive candidates for detection of inorganic phosphates or phosphorylated biomolecules such as proteins or phospholipids, which are important biological contributors in metabolic and signaling pathways.[27] Pyrophosphate (PP_i) is of particular interest to us because synovial fluid [PP_i] is a diagnostic marker used to differentiate osteoarthritic conditions wherein calcium pyrophosphate depositions form between joints,[28] and because there are already numerous phosphate (P_i) sensors relative to PP_i sensors.[29]



Scheme 1. General indicator displacement assay strategy in which the receptor is a bimetallic complex (I = indicator, A = analyte). This representation assumes indicator emission enhancement upon displacement.

Our previous work on IDAs[30,31] included a study[30] which built on extensive work by others who established *m*-xylylene scaffolded dizinc complexes as preeminent receptors for phosphate derivatives under physiologic conditions.[16,22,32,33] Zn_2**L1** (the product of Zn^{2+} addition to aqueous **L1**, Scheme 3), for example, has a Zn–Zn distance (3.0 Å)[34] somewhat less than that in phosphotriesterase (3.5 Å), which binds with high specificity to P_i derivatives,[35] whereas Zn_2**L1** and related complexes bind both P_i and PP_i.[36,37] We tested Zn_2**L1** as an IDA receptor with various commercial indicators, but the similar binding affinity of P_i and PP_i to Zn_2**L1** made their differentiation difficult.[30] The Zn–Zn distance should play a key role in analyte preference. The dependence of phosphoester hydrolysis activity on Zn–Zn distance in phosphohydrolase models has been studied employing, for example, xylyl, naphthyl,

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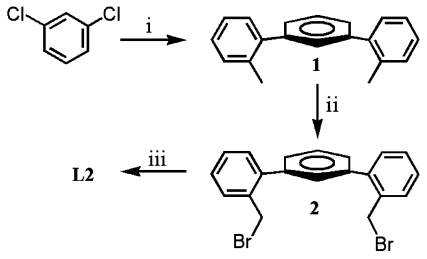
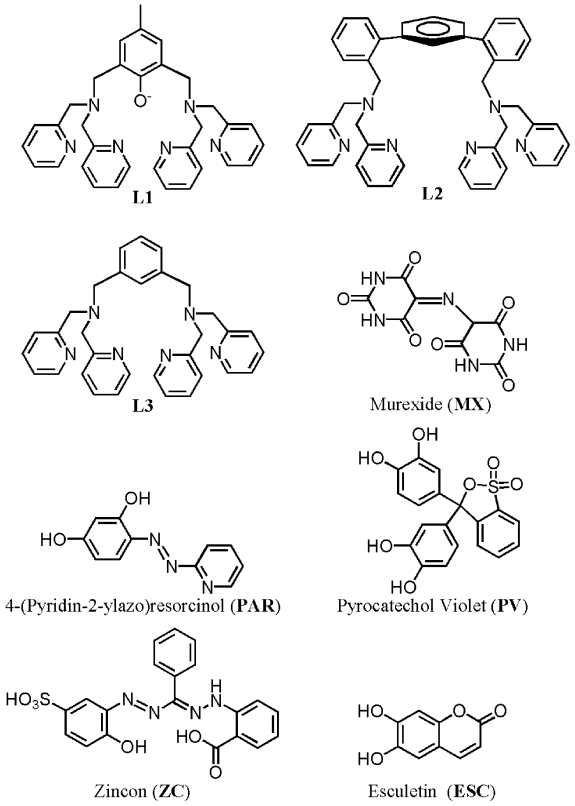
anthryl, and biphenyl spacers between Zn-chelating moieties.[38] Although these spacers have provided some functional models, an emerging theme in bimetallic enzyme modeling has been to position metals within a steric pocket to exert control over metal–substrate interactions. This same strategy could prove useful for developing sensors as well, because steric coordination control may alter analyte selectivity and affinity. Notable bulky ligands for enzyme modeling include *m*-terphenyl carboxylates, particularly for diiron and dicopper enzyme models.[39–42] This established success of *m*-terphenyl scaffolds for supporting bimetallic model complexes led us to explore translocation of the metal-ligating units from their typical position on the central aryl ring onto the flanking rings, i.e. at the benzylic sites of **1** (Scheme 2). This substitution pattern could provide useful dinucleating ligands for a variety of enzyme models for both catalytic and chemosensor applications. Herein we discuss a dizinc complex of one such *m*-terphenyl-scaffolded ligand (**L2**, Scheme 3) and report its utility as the receptor component of IDAs that are selective for PP_i at physiological pH. Binding and selectivity of **L2** complexes are compared with those of two related dinucleating ligands (**L1** and **L3**) that have been used in indicator displacement assays in biological contexts.



Scheme 2. Preparation of **L2**: i. 1) 1.1 equiv. *n*BuLi, –78 °C; 2) 3.5 equiv. 2-tolylmagnesium bromide, Δ; 3) H^+. ii. *N*-bromosuccinimide, benzoyl peroxide, $CHCl_3$, Δ. iii. bis(2-picolyl)amine, triethylamine, THF.

Results and Discussion

There is only one example of a *m*-terphenyl scaffold supporting a bimetallic complex in which ligands are only present on the flanking aryl rings (rather than on the central ring), a bimetallic palladium complex with a Pd–Pd distance of 3.6 Å.[43] Molecular modeling, however, suggested that such a scaffold is capable of supporting M–M distances relevant for bimetallic enzyme modeling (typically 3–5 Å).[44] We therefore sought to incorporate ligands for binding biologically relevant metals, particularly zinc, onto the *m*-terphenyl scaffold. The bis(2-picolyl)amino unit was selected for our initial study on the basis of its strong affinity for Zn^{2+} and its widespread use in previous models.[22] Ligand **L2** was readily prepared (Scheme 2) from known precursor **2**[45] in one step through a procedure analogous to that used to prepare **L1**.[46] The Zn_2**L2** complex used in absorption spectroscopic studies was prepared in situ in HEPES buffer (10 mM, pH 7.4), following the procedure reported for **L1**.[30] The binding strength and optical changes of commercial indicators (Scheme 3) upon binding to Zn_2**L2** were probed to reveal any differences from *m*-xylylene scaffolded analogues. Data from absorption spectroscopic titrations were used to calculate dissociation constants (K_d, Table 1) by a modification of the Benesi–Hildebrand method (Benesi–Hildebrand plots are provided in the Supporting Information).[47] Binding data for several indicators with Zn_2**L2**, as well as for *m*-xylylene-based Zn_2**L1** (which has a bridging phenolate) and Zn_2**L3** (lacking a bridging ligand) are provided in Table 1. It is worth noting that the measured affinity of indicators for Zn^{2+} complexes is several orders of magnitude lower than that of Zn^{2+} for a dipicolylamine receptor,[48–50] precluding the possibility that the indicators may be demetallating the receptor. Despite anticipated differences in *average* Zn–Zn distances, a similar range of K_d values were found for all three complexes (Table 1). The similarities are presumably due to the flexibility of the **L2** and **L3** scaffolds, which allows access to a range of Zn–Zn distances in order to optimize binding interactions with a particular indicator.



Scheme 3. Binucleating ligands **L1**–**L3** and complexometric indicators tested for displacement by inorganic phosphates. Only one protonation state/resonance structure is provided for each.

Competitive binding analysis of absorption data from titration of indicator-Zn_2**L2** complexes with P_i and PP_i revealed K_d for P_i (3.1×10^{-5} M) and PP_i (2.4×10^{-6} M) on approximately the same order of magnitude as those reported for Zn_2**L1** complexes (9.1×10^{-6} M and 1.5×10^{-6} M for P_i

Table 1. Absorption data and photos demonstrating the range of colorimetric responses observed in free, bound and pyrophosphate-displaced states. λ_{free} is the absorption maximum for the indicator alone and λ_{Zn2L2} is the absorption maximum for the indicator when bound to the Zn_2**L2** complex. The photos under each absorption wavelength is of the color observed in the cuvettes by eye at 5×10^{-5} M indicator concentration. All data are for solutions in 1 mM HEPES buffer at pH 7.4. Absorption spectra for these experiments are provided in the Supporting Information.

Indicator	K_d	Δλ	λ_{free}	λ_{bound}	Displacement		PP_i selectivity	
	μM	nm	nm	nm	Pi	PPi	**L1**	**L2**
ARS	50	20	517	537	Y	Y		
BPR	990	36	554	590	N	Y	X	X
MB9	85	25	527	552	Y	N	X	
MX	28	58	522	464	Y	Y		
PAR	13	81	414	492	N	Y		X
PV	99	229	438	667	N	Y		X
ZC	200	96	465	561	N	Y	X	X

[a] Y = yes, N = no. Indicator is considered to be displaced when there is >50% return in absorbance at λ_{bound} to the absorbance at that wavelength for the unbound form.

and PP_i, respectively).[51] It is notable that although PP_i is bound equally well by both dizinc species, Zn_2**L2** binds P_i three times less strongly than does Zn_2**L1**. The reduced affinity of Zn_2**L2** for P_i presumably stems from the larger Zn–Zn separation accessible using the *m*-terphenyl scaffold vs. that provided by the *m*-xylylene spacer and anchoring of zinc centers by the μ-phenolate in **L1**.

The enhanced preference for binding PP_i over P_i suggested that IDAs with improved selectivity for PP_i should be possible using **L2**. Four commercial complexometric indicators previously shown[30] to exhibit notable differences in their absorption spectra between free and dizinc-bound states were tested (Table 1). All of these indicators provide IDAs selective for PP_i over P_i and other simple anions (no displacement of indicator is observed upon addition of excess F^-, Cl^-, Br^-, I^-, AcO^-, NO_3^-, CO_3^{2-} and HSO_4^-). The zinc centers in Zn_2**L2** are not anchored by an intramolecular bridging ligand (cf. **L1**), so the ability of Zn_2**L2** to expand and accommodate a larger anion such as triphosphate may be envisioned. Addition of one equiv. of triphosphate, however, did not significantly displace any of the indicators. Because biological or environmental assays are targeted end uses of these IDAs, other potentially interfering bipodal biological anions were also tested. Biomolecules such as glutamate and aspartate are the most obvious candidates for testing in this regard due to their similarity in size (i.e., distance between anionic atoms) to PP_i and previous reports that these anions can displace indicators from bimetallic receptors.[11] None of the indicators were displaced from Zn_2**L2** to any appreciable extent by glutamate or aspartate, indicating that Zn_2**L2** maintains selectivity for phosphates over carboxylates, a property previously noted for xylylene-bridged dizinc analogues.[32]

Although colorimetric assays are convenient for assessing analyte presence visually, fluorescent sensors exhibiting emission enhancement in response to analytes are considerably more sensitive and thus preferred for biological studies. Esculetin (**ESC**, Scheme 3), a catechol-derivatized fluorescent indicator previously used in IDAs,[19] was thus tested for detection of PP_i by fluorescence spectroscopy. Esculetin is effectively bound by Zn_2**L2** ($K_d = 3.85 \times 10^{-5}$ M), and the **ESC**-Zn_2**L2** complex is only 25% as emissive as free esculetin. Near full restoration of emission to that of the free indicator was achieved upon addition of 1.5 equiv. PP_i (Figure 1), corresponding to a 4.1-fold emission enhancement. The fluorescent turn-on IDA using **ESC**-Zn_2**L2** has a modest sensitivity limit (based on 5% increase in integrated emission) estimated at 2.5×10^{-6} M. This is, however, within the level required for detection of biologically relevant [PP_i] found in synovial fluid (8.6×10^{-6} M to 15.9×10^{-6} M), which is elevated to higher concentrations in arthritis patients.[28,52] Because clinical methods for measuring synovial [PP_i] rely most commonly on radiological methods and may not readily differentiate phosphate from pyrophosphate,[28,53] a sensitive fluorescence assay could eventually prove more economical and convenient for some practical applications. Once PP_i selectivity was confirmed in the presence of 5 equiv. P_i per PP_i in solution, the viability of the Zn_2**L2** IDA for providing quantitative data on [PP_i] was examined. Calibration curves constructed from absorption (Figure 2, a) and fluorescence (Figure 2, b) spectroscopic data indicate a linear response as a function of [PP_i] in HEPES buffer at physiological pH. The operational range obtainable from absorption (on the order of 10^{-4} M) vs. fluorescence (on the order of 10^{-6} M) data emphasizes the approximate two orders of magnitude greater sensitivity of the later method; however, both techniques appear well suited for quantitative measurements.

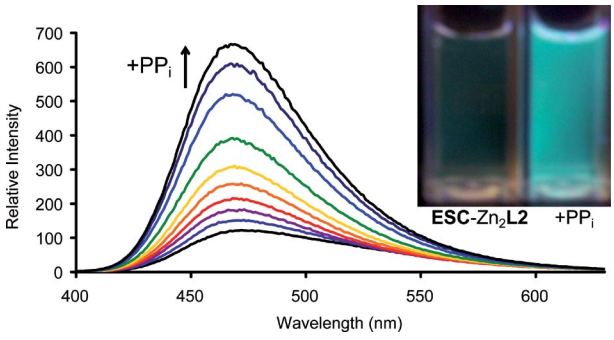


Figure 1. Change in emission intensity of **ESC**-Zn_2**L2** (2.5×10^{-5} M in pH 7.4 HEPES) as up to 1.5 equiv. PP_i were added (λ_{ex} = 380 nm). The inset demonstrates the visual change in emission (λ_{ex} = 365 nm).

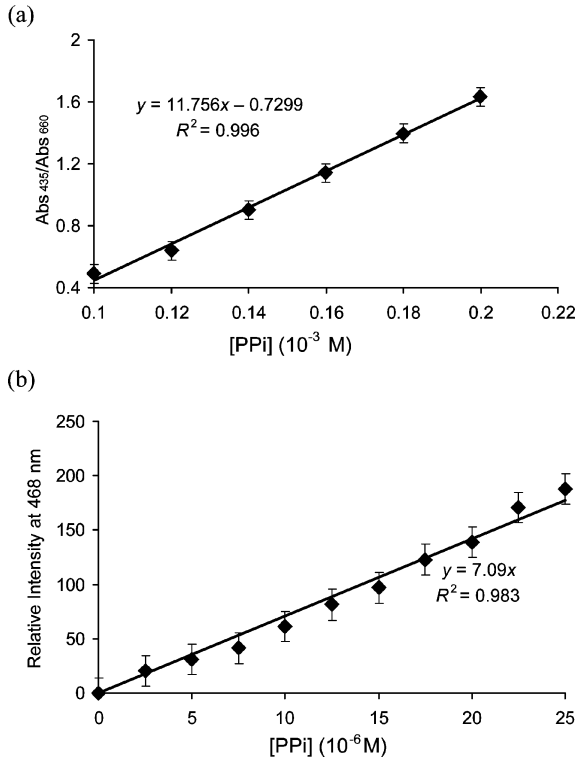


Figure 2. Calibration curves for [PP_i] measured via displacement of pyrocatechol violet measured by ratiometric absorption spectroscopy (a) and displacement of esculetin measured by increase in emission intensity of fluorescence spectra (b) in HEPES buffer (pH = 7.4). In both graphs, diamonds with error bars are original data points and the black line is a linear fit of the data. Equations of linear fits and corresponding R_2 values are also provided on each graph. Error bars represent error associated with absorption (a) and fluorescnece intensity readings (b).

Conclusions

To summarize, a binucleating ligand utilizing a *m*-terphenyl scaffold has been prepared. The dizinc complex of this ligand is a suitable receptor for PP_i at physiologic pH, affording improved selectivity over P_i in IDAs vs. related systems. IDAs that employ ratiometric absorption changes and fluorescence emission enhancement have both been developed and provide linear responses to pyrophosphate at physiological pH. Although tests reported herein have been ex vivo, the fluorescence assay provides sensitivity appropriate for monitoring biologically relevant pyrophosphate concentrations. Efforts are underway to elaborate the *m*-terphenyl scaffold for additional applications in chemosensing and enzyme modeling.

Experimental Section

Reagents and General Methods: All reagents were obtained from Aldrich Chemical Co., TCI America, Alfa Aesar, Fischer Scientific, Mallinckrodt, Baker and Adamson, or MP Biomedicals, LLC and used as received. Solvents were purified by passage through alumina columns under N_2 using an MBraun solvent purification system. Literature procedures were employed for the preparation of 2,2′′-dimethyl-*m*-terphenyl (**1**)[54] and 2,2′′-bis(bromomethyl)-1,1′:3′,1′′-terphenyl (**2**).[45] All ^1H and ^{13}C NMR spectra were obtained with a Bruker Avance 300 spectrometer with operational parameters set at 300 MHz for ^1H nuclei and 75 MHz for ^{13}C nuclei. All spectra were collected at 20 °C and referenced to either tetramethylsilane (δ = 0 ppm) as an internal standard or the residual solvent peak. A Corning CHEK-MITE pH 25 sensor interfaced with a Symphony Ag/AgCl pH electrode was used for measuring the pH of HEPES buffer, which was calibrated to the target pH value of 7.4 by the addition of NaOH (aq.). All absorption and fluorescence spectra were collected at 20 °C using a Cary 50 spectrophotometer for absorbance, and a Cary Eclipse fluorescence spectrophotometer for photoluminescence. Samples were prepared in poly(methylmethacrylate) cuvettes (1.0-cm path lengths) from Starna Cells, Inc.

Preparation of 2,2′′-Bis[(2,2′-dipicolylamino)methyl]-1,1′:3′,1′′-terphenyl (L2): A stirred solution of **2** (0.630 g, 1.51 mmol) was initially prepared in THF (10 mL, anhydrous) and cooled to 0 °C in an ice water bath under N_2. Separately, a solution of triethylamine (0.460 g, 4.56 mmol) and bis(2-picolyl)amine (0.600 g, 3.01 mmol) was prepared in anhydrous THF (5 mL). The solution containing triethylamine and bis(2-picolyl)amine was added dropwise via syringe to the stirring 2,2′′-bis(bromomethyl)-1,1′:3′,1′′-terphenyl solution, resulting in an orange mixture. The solution was removed from the ice water bath and allowed to slowly warm to room temperature where it was permitted to continue stirring under N_2 and ambient temperature for approximately 48 h, over which time the solution turned from orange to brown. The solution was filtered to remove precipitated triethylammonium bromide, and the filtrate was concentrated in vacuo to yield a dark brown oil. About 50 mL of dichloromethane was added and the organics washed with satd. $NaHCO_3$(aq.) (50 mL × 3). The organic layer was collected, dried with Na_2SO_4 and concentrated under reduced pressure to yield a red oil. Approximately 10 mL of acetone and two drops of concentrated HCl were added to the crude product. The solution was allowed to stand at room temperature for 12 h, over which time a white microcrystalline solid formed. The solid was collected by filtration, rinsed with acetone, and dried in vacuo to yield the target compound (0.270 g, 51%) as a hygroscopic off-white powder; m.p. 122–126 °C. ^1H NMR (300 MHz, $CDCl_3$): δ = 8.47 (d, J = 7 Hz, 4 H), 7.81 (d, J = 7 Hz, 2 H), 7.56–7.08 (m, 22 H), 3.76 (s, 8 H), 3.72 (s, 4 H) ppm. ^{13}C NMR ($CDCl_3$): δ = 159.7, 148.9, 142.3, 141.2, 136.4, 136.3, 130.3, 129.6, 127.9, 127.8, 127.5, 126.8, 122.6, 121.8, 60.1, 56.0 ppm. $C_{44}H_{41}N_6O$ (**L2**·H_2O) (669.85): calcd. C 79.85, H, 6.24. N, 12.70; found C 79.96, H 5.96, N 12.58.

Absorption Spectroscopic Titration of Indicators with Zn_2L2: Indicator stock solutions (50 mL, 5×10^{-5} M) were individually prepared in a buffered solution of HEPES (10 mM, pH 7.4) and used for all titration experiments. A solution of Zn_2**L2** (1.5 mM, 10 mL) was prepared in 1:4 acetonitrile/HEPES (10 mM, pH 7.4) and utilized for all titration experiments. For delineation of respective spectral responses from each indicator due to the introduction of Zn_2**L2**, 3 mL of the stock indicator solution was added to a cuvette and the resulting spectrum was recorded in the absence of Zn_2**L2**. Subsequently, the indicator solution was titrated in situ with 10 μL aliquots (0.10 equiv.) of the Zn_2**L2** solution; individual spectra were recorded following the addition of each Zn_2**L2** aliquot until a total of at least10 aliquots (1 equiv.) had been added. Titrations were performed in identical fashions for all indicators under consideration. Resulting absorption titration spectra for each indicator are provided in the Supporting Information.

Determination of Indicator/Zn_2L2 Dissociation Constants: Spectral data were used to estimate dissociation constants through a variation of the Benesi–Hildebrand method prescribed by Hammond.[47] Plots of $(\Delta Abs)^{-1}$ vs. $[Zn_2\mathbf{L2}]^{-1}$ were constructed for each series of indicator/receptor complex titration data, followed by a regression analysis of the data by a linear fit function. Benesi–Hildebrand plots, along with the corresponding linear regression functions and coefficients of determination (R_2), are provided in the Supporting Information.

Absorption Spectroscopic Titration of Indicator/Zn_2L2 Complex with Phosphorous-Containing Analyte: Indicator (5×10^{-5} M) and Zn_2**L2** (1.5 mM) solutions were used for indicator displacement assay titrations. Solutions of sodium hydrogen phosphate (1.5 mM, 5 mL) and sodium pyrophosphate decahydrate (1.5 mM, 5 mL) were prepared in HEPES buffer (10 mM, pH 7.4). Initially 3 mL of an indicator solution and 100 μL of the Zn_2**L2** solution (1:1 stoichiometric equivalence) were added to a cuvette, followed by recording of the respective spectrum. For determination of the ability of the selected anion (phosphate or pyrophosphate) to displace the indicator from the indicator/Zn_2**L2** complex and concomitantly form an anion/Zn_2**L2** complex, the indicator/Zn_2**L2** solution was titrated in situ with 10 μL aliquots (0.100 equiv. relative to both the indicator and Zn_2**L2**) aliquots of the anion solution. Individual spectra were recorded following the addition of each anion aliquot until a total of 20 aliquots had been added to the indicator solution (resulting in a 2:1:1 anion/indicator/Zn_2**L2** stoichiometry). Titrations were iterated in the same manner for all indicator/analyte combinations. Absorption spectra resulting from the titration of each indicator-Zn_2**L2** complex with PP_i are provided in the Supporting Information.

Response of Indicator-Zn_2L2 to Other Anions: Indicator-Zn_2**L2** solutions were prepared as described above for absorption spectroscopic studies, and up to 10 equiv. of NaF, NaCl, NaBr, NaI, NaO_2CCH_3, NaP_3H_{10}, Na_2SO_4, $NaNO_3$, Na_2CO_3 sodium glutamate, or sodium aspartate was added to each cuvette, followed by collection of an absorption spectrum. None of these samples exhibited significant changes in absorption spectra.

Fluorescence Titration of Indicator-Zn_2L2 Complex with Phosphorous-Containing Analyte: Fluorescence experiments were executed in a manner similar to that described above for absorption spectroscopic experiments, with the exception that initial indicator concentrations of 2.5×10^{-5} M were used.

Supporting Information (see also the footnote on the first page of this article): ^{1}H and ^{13}C NMR spectra, absorption spectra, fluorescence spectra and Benesi–Hildebrand plots.

Acknowledgments

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