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Spiropyran-Based Fluorescent Anion Probe and Its Application for Urinary Pyrophosphate Detection

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In recent decades, numerous spiropyran derivatives have been designed and utilized for optical sensing of metal ions. However, there is still less research on spiropyran-based anion sensors. In this work, a new spiropyran compound (L) appended with a pendant bis(2-pyridylmethyl)amine was synthesized and used in fluorescent sensing of pyrophosphate ion (PP_i) in aqueous solution. The molecular recognition and signal transduction are based on the cooperative ligation interactions and the ligation-induced structural conversion of the spiropyran, which leads to a significant change in the photophysical property of the spiropyran. In an ethanol/water solution (30:70, v/v) at pH 7.4, ligation of L with Zn^{2+} causes an intense fluorescence emission at 620 nm at the expense of the original fluorescence at 560 nm. Once PP_i was introduced, interaction between PP_i and the L–Zn^{2+} complex leads to full quenching of the 620 nm band emission which was concomitant with recovery of the 560 nm band emission, and the fluorescence intensity ratio, F_{560}/F_{620}, is proportional to the PP_i concentration. Under the optimum condition, the L–Zn^{2+} complex responds to PP_i over a dynamic range of 1.0×10^{-6} to 5.0×10^{-4} M, with a detection limit of 4.0×10^{-7} M. The fluorescence response is highly selective for PP_i over other biologically related substrates, especially the structurally similar anions, such as phosphate and adenosine triphosphate. The mechanism of interaction among L, Zn^{2+}, and PP_i was primarily studied by ^1H NMR, ^{31}P NMR, and HRMS. To demonstrate the analytical application of this approach, the PP_i concentration in human urine was determined. It was on the order of 3.18×10^{-5} M, and the mean value for urinary PP_i excretion by three healthy subjects was 62.4 μmol/24 h.

Anions play an important role in a wide range of chemical and biological processes. Among the interesting biologically functional inorganic anions, the multiphosphorylated species, such as phosphate ion and pyrophosphate ion ($P_2O_7^{4-}$, PP_i), are particularly important, because they play a key role in a wide range of critically biological processes ranging from information processing to energy storage and transduction in organisms.[1] For example, PP_i is the product of adenosine triphosphate (ATP) hydrolysis under cellular conditions, which is at the heart of important biochemical pathways such as DNA polymerization and synthesis of cyclic adenosine monophosphate (AMP) catalyzed by DNA polymerase and adenylate cyclase, respectively.[2−4] Also, PP_i is a natural component that is produced, degraded, and transported by a specialized mechanism in mammalian cells, which might have a physiological function related to crystallization processes.[5]

In recent decades, a number of molecule probes for phosphates have been developed,[6−8] a few for PP_i.[9] Early in 1994, Czarnik et al. reported the pioneering work in which an anthracene derivative bearing polyamine groups was used as a PP_i probe in aqueous solution.[10] The appropriate distance between the two polyammonium sites permits cooperative PP_i chelation but not phosphate chelation. Recent advances in PP_i sensing included designs of various synthetic fluorescent molecules bearing guanidinium,[11] amide,[12] or imidazolium[13,14] units as the PP_i receptors. Most of them display moderate to high affinity

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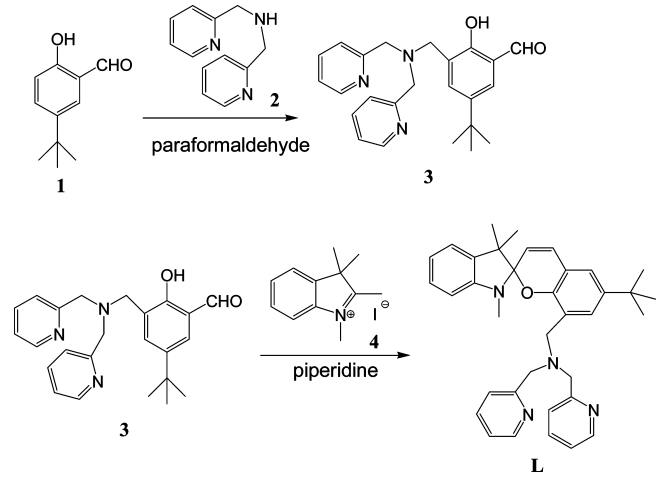
constants for PP_i in organic media but exhibit low affinities in aqueous media, because they rely on hydrogen bonding or electrostatic interactions for recognition of the analyte. These interactions are, however, attenuated drastically in highly polar media such as water because of the competing solvation effect. An alternative approach to efficient binding of PP_i in aqueous media is metal coordination interaction. For example, a Cd^{2+}−cyclen−coumarin complex[15] and a series of bis(2-pyridylmethyl)amine (Dpa)−Cu^{2+}[16] and −Zn^{2+} derivatives[17−21] were developed as highly selective fluorescent and colorimetric PP_i probes in water. These metal−ligand coordinative interactions display their superiorities to hydrogen bonding or electrostatic interaction in a polar environment. For example, they can be highly energetic as a result of the strong ligand field stabilization energy effects that may be induced by coordination.[22]

Spiropyrans make up one of the fascinating photochromic families that undergo reversible structural isomerization between a colorless spiropyran form and a colored merocyanine upon either light, heat, or chemical stimulus.[23,24] In recent decades, numerous spiropyran derivatives have been designed and utilized for optical sensing of metal ions,[25−31] a few for neutral molecules, such as nucleobases,[32] amino acids,[33−35] and DNA.[36] Recently, a spiropyran derivative was used as a colorimetric CN^- probe via the nucleophilic addition of CN^- to the opened spiropyran nucleus.[37] The probe is highly selective and sensitive for CN^- and functions in aqueous media. We for some time have been interested in the design and application of these photochromic probes in a sensor approach. We have constructed Cu^{2+} fluorescent sensors via the fluorescent inner filter effect and direct fluorescence detection with spiropyran[30,31] and developed a spiropyran-based colorimetric approach for cysteine.[34] More recently, we have designed a double-spiropyran probe for GSH via multipoint electrostatic interaction.[35]

Scheme 1. Preparation of Spiropyran L



We report here the design of a spiropyran-based fluorescent probe for PP_i. The underlying anion recognition scheme is derived from a specific metal coordination interaction. The probe spiropyran compound [**L** (Scheme 1)] bears one pendant Dpa arm which can universally form a complex with Zn^{2+} in an aqueous solution.[17−21] Because bis(Zn^{2+}−Dpa)-based receptors exhibit a high affinity for PP_i in aqueous solution, we expect that the **L**−Zn^{2+} complex would cooperatively create an anion binding site. Bringing two Zn^{2+}−Dpa moieties into the presence of PP_i allows the two spiropyran nucleases to form an intermolecular structure that gave a new optical response to the analyte.

At the same time, from the point of view of analytical chemistry, although the design of molecular probes and sensors for PP_i has attracted interest among researchers,[10−21] less attention has been paid until now to realizing a practical application for a real-time assay of the PP_i concentration in a genuine sample. To the best of our knowledge, there is no fluorescent sensing approach reported for the determination of the PP_i concentration in biological fluids. The PP_i concentrations were usually determined on the basis of chromatographic separation,[38,39] chemical reaction,[40,41] and an enzymatic technique.[42,43] They

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suffer from drawbacks such as being discontinuous, being time-consuming, and, moreover, not allowing the direct determination of PP_i. Since there is growing demand for monitoring of PP_i online during biotechnical processes, reversible chemical sensing probes are in great demand. Therefore, development of a new fluorescent sensor which displays high selectivity for PP_i versus ATP and phosphates and functions in a real-time assay of the PP_i concentration in biological fluids has become our goal.

EXPERIMENTAL SECTION

Chemicals and Apparatus. All chemicals were obtained from Aldrich or Sigma. We obtained the stock solution of 1.0×10^{-3} M spiropyran **L** by dissolving the material in ethanol. All stock solutions of metal ions were prepared from analytical grade nitrate salts or chloride salts and were dissolved in doubly distilled water, and those of anions were prepared from analytical grade sodium salts. We obtained the work solutions by successively diluting the stock solutions with 0.01 M HEPES buffer (pH 7.4). Other chemicals were of analytical reagent grade and used without further purification.

Proton magnetic resonance spectra were recorded at 400 MHz, and carbon spectra were recorded at 100 MHz on an Invoa-400 (Invoa 400) spectrometer with tetramethylsilane (TMS) as the internal standard. *J* values are given in hertz. The ^{31}P NMR spectra of PP_i in the absence and presence of **L** or the **L**$-Zn^{2+}$ complex were recorded with the Invoa-400 spectrometer at 298 K. The samples were placed into a 4 mm broadband/^1H dual-frequency magic-angle-spinning probe head, and the spectra were recorded at 400 MHz. The chemical shifts were referenced relative to external 85% H_3PO_4 (0 ppm). Low-resolution mass spectra were recorded at 50−70 eV by fast atomic bombardment on a Finnigan MAT SSQ-710 mass spectrometer. High-resolution mass spectra (HRMS) were recorded on a Q-Star Pulsar I instrument (Applied Biosystem/PE Sciex). UV−visible absorption spectra were recorded on a Hitachi (Kyoto, Japan) U-3010 UV/vis spectrophotometer. Fluorescence emission spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer. Data processing was performed on a personal computer with Sigmaplot version 9.0.

Synthetic Procedure. Spiropyran derivative **L** was synthesized according to the sequences summarized in Scheme 1.

Compound ***3.*** Bis(2-pyridylmethyl)amine (**2**) (0.47 g, 2.4 mmol) was added to glacial acetic acid (8 mL) while being stirred in a round-bottom flask and soaked in an ice bath. After 15 min, paraformaldehyde (0.10 g, 3.3 mmol) was added. Then, 5-*tert*-butyl-2-hydroxybenzaldehyde (**1**) (0.36 g, 2 mmol) dissolved in glacial acetic acid (2 mL) was added to the reaction mixture. The mixture was refluxed for 5 h. After the mixture was cooled to room temperature, a 2 M NaOH solution was added until the pH of the mixture became slightly basic. Then the mixture was poured into water (20 mL) and extracted with CH_2Cl_2 (3 × 30 mL). The combined organic layers were dried and purified on a silica gel column using a PE/EA mixture (1:3) as the eluting solvent to yield **3** as a colorless oil (0.20 g, 25% yield): ^1H NMR (270 MHz, $CDCl_3$) δ 1.19 (s, 9H), 3.66 (d, 1H, J = 16.5), 3.75 (d, 1H, J = 16.5), 3.82 (s, 4H), 7.07 (m, 2H), 7.29−7.59 (m, 6H), 8.46 (d, J = 4.8, 2H), 10.34 (s, 1H); HRMS calcd for $C_{24}H_{28}N_3O_2$ $[M + H]^+$ 390.2181, found 390.2165.

Spiropyran ***L.*** To a mixture of *N*-methyl-2,3,3-trimethylindolenine iodide (**4**)[30] (0.15 g, 0.5 mmol) and piperidine (0.1 mL, 1 mmol) in absolute ethanol (8 mL) was added **3** (0.16 g, 0.4 mmol) in ethanol (1 mL). The mixture was refluxed for 16 h. After the reaction was complete, the solvent was removed with a rotary evaporator under vacuum. The crude product was purified with a silica gel column using a PE/EA mixture (2:3) as the eluting solvent to yield **L** as a colorless thick oil (0.14 g, 65% yield): ^1H NMR (270 MHz, $CDCl_3$) δ 1.16 (s, 3H), 1.27 (s, 9H), 1.33 (s, 3H), 2.66 (s, 3H), 3.42 (d, J = 12.8, 1H), 3.57 (d, J = 12.8, 1H), 3.67 (s, 4H), 5.63 (d, J = 10.0, 1H), 6.45 (d, J = 10.0, 1H), 6.74−7.20 (m, 6H), 7.39−7.56 (m, 6H), 8.43 (d, J = 4.3, 2H); HRMS calcd for $C_{36}H_{40}N_4OH$ $[M + H]^+$ 545.2094, found 545.2079.

Spectrophotometric Titrations and Association Constant. Because of the somewhat low solubility of **L** in water, both the UV and fluorescence titrations were conducted in a 30% ethanol/water (v/v) solution via addition of a few microliters of a work solution of the metals or anions to 2.0 mL of 4.0×10^{-5} M **L** or **L**$-Zn^{2+}$ complex with a quartz cell (cross section of 1.0 cm × 1.0 cm). The addition was limited to 100 μL so that the volume change was insignificant. The fluorescence emission spectra were recorded by excitation at the maximal absorption wavelength as determined by UV titrations. To determine the stoichiometry and association constant, *K*, the obtained intensities of **L** at 620 nm were analyzed by using the relations that are established with the formation of an *n*:*m* host−guest complex.[44]

$$\frac{\alpha^n}{1-\alpha} = \frac{1}{nK} \times \frac{1}{[\mathbf{L}-Zn^{2+}]_T^{n-1}[PP_i]^m} \quad (1)$$

where *K* is the association constant for the association of the **L**$-Zn^{2+}$ complex with PP_i and $[\mathbf{L}-Zn^{2+}]_T$ is the total concentration of the **L**$-Zn^{2+}$ complex in the system. The α term is the ratio of the concentration of the free **L**$-Zn^{2+}$ complex (has not been bound to PP_i) to the total concentration of $[\mathbf{L}-Zn^{2+}]_T$. α can be determined from the fluorescence changes of the **L**$-Zn^{2+}$ complex in the presence of different concentrations of PP_i:

$$\alpha = \frac{F - F_{min}}{F_0 - F_{min}} \quad (2)$$

where *F* is the fluorescence intensity at 620 nm in the presence of different concentrations of anion and F_0 and F_{min} are the limiting values of the fluorescence intensity at zero anion concentration and at a final (plateau) anion concentration, respectively. From eqs 1 and 2, one can see that the functional relationships between the fluorescence intensity and the PP_i concentrations depends on the stoichiometric ratio of the **L**$-Zn^{2+}$ complex to PP_i. The experimental data could be fitted to eqs 1 and 2 by changing the ratio of *m* to *n* and adjusting the equilibrium constant, *K*.[44]

Determination of the Fluorescence Quantum Yield (Φ). For measurement of the quantum yield of **L**, the ethanol solution of **L** was adjusted to an absorbance of ~0.05. The emission spectra

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were recorded with an excitation wavelength of 522 nm, and the integrated areas of the fluorescence-corrected spectra were measured. The quantum yields were then calculated by comparison with rhodamine B as the reference using the following equation:[45,46]

$$\Phi_F = \frac{I}{I_R}\frac{A_R}{A}\left(\frac{n}{n_R}\right)^2\Phi_R \quad (3)$$

where Φ_F is the quantum yield, I is the integrated area under the fluorescence spectrum, A is the absorbance, n is the refractive index of the solvent, and R refers to the reference fluorophore, rhodamine B. A Φ_R of 0.97 in ethanol was used as the reference quantum yield.[47]

Treatment and Analysis of the Urine Sample. The urine sample was collected in 6 M HCl (approximately 0.05 mL of HCl per 50 mL of sample) and purified using a chromatographic column with an inner diameter of 8 mm containing 0.25 g of activated carbon.[48]

Then 0.8 mL of a solution of four parts 1 M perchloric acid and one part 0.08 M ammonium molybdate was added to 3.5 mL of the decolorized urine sample, and after the solution had been thoroughly mixed, 0.4 mL of 0.1 M triethylamine hydrochloride (pH 5.0) was introduced. The final acid normality was 0.5. The resultant cloudy green precipitate was allowed to form for 5 min. After centrifugation at 28000*g* (counts per second) for 10 min, the clear supernatant solution was poured into another tube and centrifuged again at 40000*g* for 15 min.[49]

To precipitate all the Mg^{2+}, a small quantity of a suitable concentration of NaOH was introduced into the sample to increase the pH of the solution to >12. The resulting ivory-white colloidal precipitate was thus formed. After centrifugation at 30000*g* for 15 min, the clear supernatant was sucked into a calicle to adjust the pH to 7.4 with HCl. (It is important that no precipitate be carried over into the next step. If necessary, the supernatant should be centrifugated once more.) Then 1.0 mL of the treated urine sample was added to 1.0 mL of the reagent mixture (ethanol solution of 4.0×10^{-5} M **L**−Zn^{2+}), and the fluorescence spectrum of the resultant mixture was recorded.

RESULTS AND DISCUSSION

Design Concept. The sensing of an anion in an aqueous solution requires a strong affinity for the anion as well as the ability to convert the target recognition event into a measurable signal. In general, a sensory molecule would involve the covalent linking of a receptor domain to the signaling unit. The two components are intramolecularly connected together so that the binding of the target analyte can induce significant changes in the photophysical properties of the signaling unit.[6,7] First, to realize PP_i recognition in an aqueous solution, one needs to choose an acceptor molecule that can strongly bind PP_i. It is known that the Zn^{2+}−Dpa complex can be a good acceptor for PP_i in a neutral aqueous solution, and the oxygen atoms coordinate to zinc ion as the fourth or fifth ligand.[17−21] Therefore, a Dpa moiety was introduced into the spiropyran molecular backbone as the anion acceptor. Second, the effectiveness of this approach involves transduction of a molecular recognition event to a measurable signal. We have devoted ourselves to the development of spiropyran derivatives as the signal display units to report the molecular recognition events.[25,31] We envisioned that the binding of an anion to the Zn^{2+} complex of **L** would alter the interaction of the spiropyran with the metal ion and thus trigger a change in the optical readout of the spiropyran backbone.

Metal Ion Binding Studies of L in Aqueous Solution. Prior to being applied in the fluorescence sensing of anions, the binding interaction of **L** with divalent metal ions was first studied by UV−vis absorption and fluorescence spectroscopy. The photophysical property of a spiropyran molecule is related to the chemical structure of the spiropyran nucleus and surrounding media, such as the solvent polarity and the external substrate complexation.[24] Spiropyran **L** is soluble in DMSO, ethanol, CH_2Cl_2, etc. Although **L** is nondissoluble in water, a clear homogeneous aqueous solution at a low concentration can be formed in an 80% water/ethanol (v/v) mixture if **L** is first dissolved in ethanol and water is then added. This relatively high solubility is due to the formation of few polar mero components of the probe induced by water. To ensure that **L** is completely dissolved, a 70% water/ethanol (v/v) system was employed to investigate the photochromic behavior in aqueous solution.

In general, nonpolar solvents favor the closed spiro form of a spiropyran molecule, while polar solvents produce the opened mero form. Surprisingly, in ethanol/water mixtures with different compositions, **L** (4.0×10^{-5} M) invariably remains colorless; almost no detectable absorption could be observed in the visible region, suggesting the nearly complete absence of the merocyanine component. Irradiation of the ethanol/water solution of **L** for 30 min with UV light caused no significant difference in the absorption spectra, reflecting the fact that **L** does not undergo photoisomerization in the solvent. This observation is distinctly different from the photochromic behavior of the common 6′-nitro-substituted spirobenzopyrans, which could be attributed to the electronic and steric effects of the substituent.[23] We anticipate that the presence of the Dpa moiety in **L** is crucial in blocking the thermal isomerization of the spiropyran to the planar merocyanine structure, presumably because of the steric and electronic repulsion effect. The relatively higher solvent and light stationary properties of the closed form of **L** in water are advantageous for exploiting it as a probe in aqueous solution media.

Another way to transform the spiro form to the mero form is metal chelation. The complexation of a metal center with the opened mero form is the driving force for opening spiropyran. The formation of the metal−merocyanine complex of **L** is greatly dependent on the nature of the metal center. The ability of **L** to bind various metal ions was studied by visual examination of the metal-induced color and UV−visible absorption spectral changes in the ethanol/water solution. The nitrate salts of Mg^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Zn^{2+}, Mn^{2+}, Pb^{2+}, Ni^{2+}, and Cu^{2+} ions were used.

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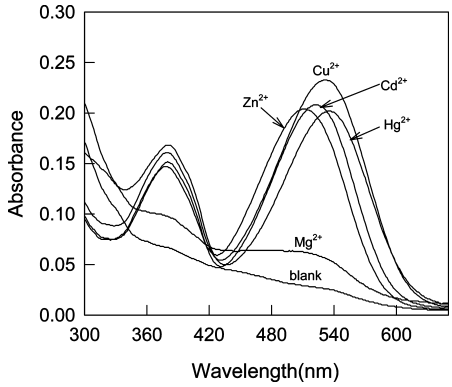

Figure 1. UV−visible absorption spectra of 5.0×10^{-5} M **L** in an ethanol/water solution in the presence of 5.0×10^{-5} M Mg^{2+}, Hg^{2+}, Cu^{2+}, Cd^{2+}, or Zn^{2+}.

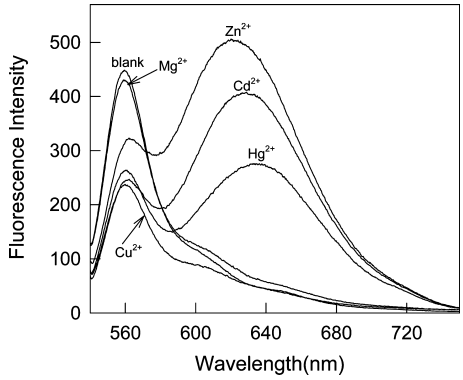

Figure 2. Fluorescence emission spectra of 5.0×10^{-5} M **L** (λ_{ex} = 522 nm) in an ethanol/water solution in the presence of 5.0×10^{-5} M Mg^{2+}, Hg^{2+}, Cu^{2+}, Cd^{2+}, or Zn^{2+}.

All titration studies were conducted using a fixed concentration of the ligand at 5.0×10^{-5} M. The absorption spectra of **L** upon addition of 1 equiv of the metal ions are shown in Figure 1. The ethanol aqueous solution of free **L** is colorless and has no absorption in the visible range. No absorption spectra or color change was observed upon addition of Mg^{2+} or Ca^{2+} to the ethanol/water solution of **L**. However, upon addition of heavy metal ions, such as Hg^{2+}, Cu^{2+}, and Zn^{2+}, the solutions clearly became pink and displayed clear absorption peaks in the region of 420−600 nm (Figure 1), reflecting formation of the opened merocyanine forms.

The fluorescence spectroscopic character of **L** was then explored. In our studies, the spiro form of **L** was found to be highly fluorescent in the ethanol/water aqueous solution, which displays its emission in the range of 535−600 nm. The quantum yields of **L** in ethanol were determined to be 0.13 (Φ_F). Figure 2 shows the fluorescence response curves of **L** toward different metal ions, which were obtained at room temperature when the metal ion was added to the ethanol/water solution of **L** for 5 min. As shown in Figure 2, there was no change when Mg^{2+} or Ca^{2+} was added to the sensor solution, while Cu^{2+} showed strong fluorescence quenching of the 560 nm emission intensity of **L**. We found that Zn^{2+} and Hg^{2+} exhibited not only obvious quenching of the 560 nm emission peak but also a new emission band at a long wavelength of 620 nm. Both the fluorescence and absorption[50] changes of **L** are sensitive to different concentrations of Zn^{2+}. Upon addition of Zn^{2+} to the solution of **L**, the fluorescence intensity at 560 nm gradually decreased but that at 620 nm greatly increased through a well-defined iso-emission point at 570 nm (Figure 3). When the Zn^{2+} concentration was ~0.04 mM, the emission maximum shifted to 620 nm. The stoichiometric ratio of **L** to Zn^{2+} was estimated to be 1:1 with an association constant of (log *K*) of 5.4.[50]

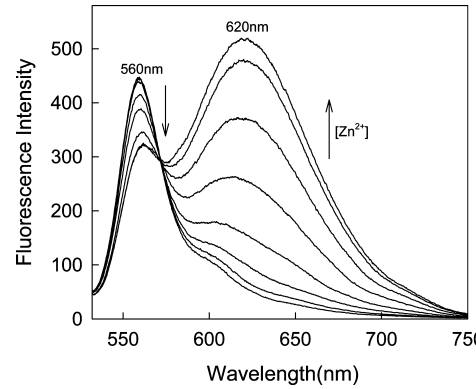

Figure 3. Changes in fluorescence emission spectra (λ_{ex} = 522 nm) of 5.0×10^{-5} M **L** upon addition of different concentrations of Zn^{2+}. The arrows indicate the signal changes with the increases in Zn^{2+}concentration (0, 2.0×10^{-7}, 5.0×10^{-7}, 1.0×10^{-6}, 2.0×10^{-6}, 5.0×10^{-6}, 1.0×10^{-5}, and 2.0×10^{-5} M).

We proposed that fluorescence quenching of **L** by metal ions contributes to the proximity of the metal to the unpaired electrons of the ligand which leads to spin−orbit coupling and intersystem crossing.[51−53] The observed enhancement of the new long wavelength emission peak of **L** at 620 nm is due to not only an increase in the concentration of the opened form of **L** but also the binding of Zn^{2+} to **L** which could induce a conformation restriction, and subsequently change the intramolecular charge transfer (ICT) from the phenolate oxygen to the electron-deficient quaternary nitrogen center.[54,55] The reduction in the level of ICT (effectively unquenching) affords a dramatic enhanced and red-shifted wavelength fluorescence as a function of the Zn^{2+} concentration.

Fluorescence Sensing of PP_i. Figure 4 shows the fluorescence emission spectral changes of **L** in a 30% ethanol/water (v/v) solution at pH 7.4 in the presence of metal ions and PP_i. The fluorescence emission of free **L** is hardly changed upon addition of 2.0 equiv of PP_i, indicating there is little interaction between **L** and the anion. On the other hand, upon addition of the same amount of PP_i to an ethanol/water solution containing **L** and Zn^{2+}, the emission at 620 nm greatly decreases while the 520 nm emission band increases. In the presence of Hg^{2+} or Cd^{2+} in the ethanol/water solution, similar spectroscopic behavior was obtained, whereas the response sensitivity for PP_i is lower

(50) For details, see the Supporting Information.

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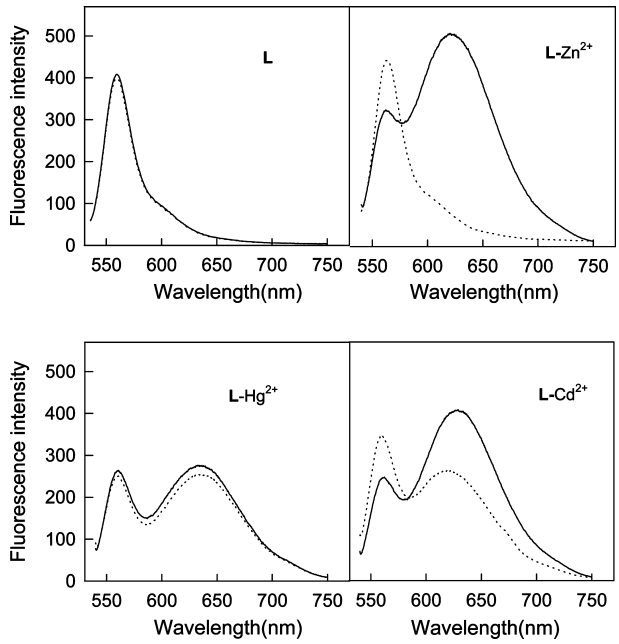

Figure 4. Fluorescence emission spectra of **L** and its metal complexes with Cd^{2+}, Hg^{2+}, or Zn^{2+} in the absence (—) and presence of PP_i (•••). The concentrations of the respective species are as follows: [**L**] = [metal ion] = 5.0×10^{-5} M, and [PP_i] = 1.0×10^{-4} M.

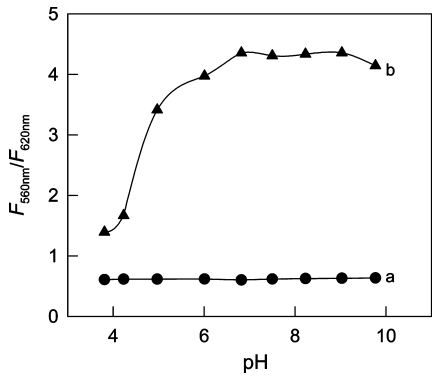

Figure 5. Fluorescence intensity ratio−pH profile for titrations of the ethanol aqueous solution containing 5.0×10^{-5} M **L**−Zn^{2+} complex in the absence (a) and presence (b) of 5.0×10^{-5} M PP_i. The excitation wavelength was 522 nm.

than that in the presence of Zn^{2+}. These results demonstrate that the interaction of **L** with PP_i is highly dependent on metal ion and Zn^{2+} could promote the binding of **L** with the anion, which constitutes the basis for fluorescence sensing of PP_i in aqueous solution.

Because the formation of the opened mero form of a spiropyran can be induced by H^+,[24] and considering that PP_i can form a complex with H^+, it is important to first test the pH dependence of the fluorescence sensing behavior of the **L**−Zn^{2+} complex for PP_i. Figure 5 depicts the fluorescence intensity ratio of the **L**−Zn^{2+} complex as a function of pH in the absence and presence of PP_i. One can see that in the absence of PP_i the fluorescence intensity ratio of the **L**−Zn^{2+} complex was hardly influenced in the pH range of 4.0−10.0. In the presence of PP_i, the fluorescence changes of **L** at both 560 and 620 nm are not significantly pH-dependent and the F_{560}/F_{620} value remains constant between pH 5.0 and 9.0. In a strong acidic solution

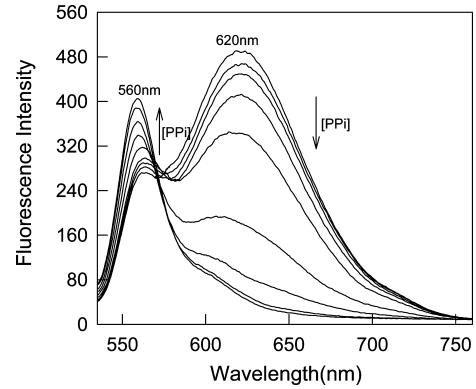

Figure 6. Fluorescence emission spectra (λ_{ex} = 522 nm) of 5.0×10^{-5} M **L**−Zn^{2+} complex in the ethanol aqueous solution at pH 7.4 upon addition of different concentrations of PP_i. The arrows indicate the signal changes with increases in PP_i concentration (0, 1.0, 2.0, 5.0, 10, 20, 50, 100, and 200 μM).

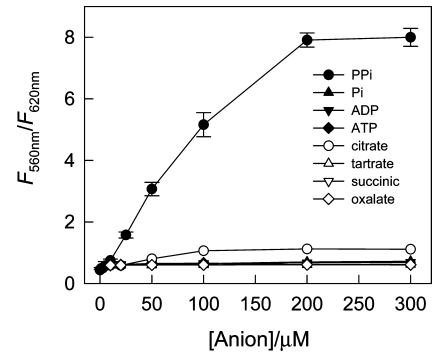

Figure 7. Changes in the fluorescence intensity of **L** at 560 nm with respect to that at 620 nm in the ethanol aqueous solution (pH 7.4) upon addition of different concentrations of anions. The excitation wavelength was 522 nm.

(pH <4.5), PP_i is converted to its protonated form, which weakens its interaction with Zn^{2+}, while at relatively higher pH values, Zn^{2+} may form a complex with OH^-, which in turn weakens its formation of a complex with the anion and **L**. Thus, the pH range of 5.0−9.0 is practical for PP_i sensing.

Both the sensitivity and response range of the system depend on the amount of probe and metal ion presented in the solution. To realize a highly sensitive response to PP_i, we optimized the Zn^{2+} amount, at which the concentration of **L** remains constant (5.0×10^{-5} M) and that of Zn^{2+} changes. With a molar ratio of metal to **L** of <1.0, both the sensitivity (response slope) and response range increase with an increase in the concentration of Zn^{2+}, and with a further increase in the concentration of Zn^{2+}, no obvious change in the response feature could be observed.[50]

Figure 6 shows the typical fluorescence response of a buffered ethanol/water solution (pH 7.4) containing the **L**−Zn^{2+} complex to increasing concentrations of PP_i. Under the optimum condition, a dramatic increase in the fluorescence intensity at 560 nm and a decrease in that at 620 nm are observed. Figure 7 shows the change in the intensity ratio, F_{560}/F_{620}, as a function of PP_i concentration. F_{560}/F_{620} increases linearly in the PP_i concentration range of 1.0×10^{-6} to 1.0×10^{-4} M ($R^2 = 0.9963$). The detection limit that is taken to be 3 times the standard derivation of a blank solution is 4.0×10^{-7} M. The results suggest that the proposed approach is potentially appropriate

Table 1. Fluorescence Signal Changes of the L−Zn^{2+} Complex at 560 and 620 nm (I_{560}/I_{620}), Anion Complex Constants (K), and Response Selectivities of L to Anions[a]

species	R (= I_{560}/I_{620})	K [$(M)^{-2}$]	response selectivity[b]
L−Zn^{2+}	0.6096	–	–
L−Zn^{2+} with PP_i	6.493	2.37×10^8	1
L−Zn^{2+} with H_2PO_4	0.6204	–[c]	–[c]
L−Zn^{2+} with ADP	0.6033	–[c]	–[c]
L−Zn^{2+} with ATP	0.6273	–[c]	–[c]
L−Zn^{2+} with citrate	1.0628	5.01×10^3	3.46×10^6
L−Zn^{2+} with tartrate	0.6106	–[c]	–[c]
L−Zn^{2+} with succinic	0.5949	–[c]	–[c]
L−Zn^{2+} with oxalate	0.5979	–[c]	–[c]

[a] The concentration of anion in each case was 5.0×10^{-5} M. [b] Response selectivity = $(K_iR_i)/(K_{PP_i}R_{PP_i})$. [c] The association constant could not be obtained with eqs 1 and 2 because the fluorescence intensity changes of the **L**−Zn^{2+} complex at 560 and 620 nm are too small in the presence of the anions.

for quantification of PP_i in physiological fields.[49] By following the fluorescence intensity change at 620 nm and a curve fitting analysis, we estimated the stoichiometry of the **L**−Zn^{2+} complex with PP_i to be 2:1; the corresponding association constant (log K) is 8.6.[50]

One important characteristic of a sensor is its specific response to the species to be measured versus that to other coexisting substrates present in solution. Since the potential application of this approach is for the analysis of PP_i concentration in biological samples, the selectivity toward biologically relevant anions is of particular concern. To define the fluorescence responses of the **L**−Zn^{2+} complex to PP_i, complexation experiments with a variety of biological related anions were performed in ethanol/water solutions. The fluorescence intensity ratios, I_{560}/I_{620}, are plotted against the increasing amount of different anions, as shown in Figure 7. Although the **L**−Zn^{2+} complex also responds to citrate at a concentration of $>1.0 \times 10^{-4}$ M, the value of I_{560}/I_{620} is substantially smaller than that caused by PP_i.[50] The association constant for the association of the **L**−Zn^{2+} complex with citrate was determined to be 5.01×10^3 $(M)^{-2}$, which is ca. 4.37×10^4-fold smaller than that of the **L**−Zn^{2+} complex with PP_i, whereas the binding constants of other anions with the **L**−Zn^{2+} complex could not be determined because the corresponding changes in fluorescence emission at the two wavelengths were too small. The anion selectivity factor of PP_i for citrate, which was evaluated by comparing the association constant and the fluorescence signal change at 560 and 620 nm (KR),[56] is ca. 2.89×10^5. Here K is the association constant and R the fluorescence intensity ratio ($R = I_{560}/I_{620}$) of the **L**−Zn^{2+} complex in the presence of 5.0×10^{-5} M anions. The selectivity factors of the **L**−Zn^{2+} complex for the anions studied are in the following order: $PP_i \gg$ citrate $\gg$ AMP $\approx$ ADP $\approx$ tartrate $\approx$ ATP $\approx P_i \gg$ succinic $\approx$ oxalate (Table 1). The spectroscopic response of the **L**−Zn^{2+} complex to other inorganic anions shows no influence on either the selectivity or sensitivity of PP_i detection. More importantly, a titration of PP_i in the presence of potential biologically interfering substrates (1.0×10^{-3} M Na^+, K^+, and Ca^{2+} and 1.0×10^{-4} M Cl^-, phosphate ions, and citrate) gives a response curve almost superimposable with that obtained solely in the presence of PP_i.[50] Our detailed findings clearly indicate that the **L**−Zn^{2+} probe is not only insensitive to other anions or cations but also selective for PP_i in their presence, which is important and crucial in validating the method to meet the selectivity requirements of the PP_i assay in physiological fields.

Anion Binding Reversibility and Response Time. For a practical working sensor to be employed in the detection of a specific analyte, the limit of quantification, selectivity, reversibility, and response time are all important aspects. The **L**−Zn^{2+} complex has been demonstrated to be sensitive and selective for the detection of PP_i. Consequently, it is necessary to investigate the reversibility and response time of the system. In light of the photosensitivity of the metal complex of spiropyrans, we determined the real-time records showing the extent of complexation between the **L**−Zn^{2+} complex and PP_i in the ethanol/water solution by plotting the 620 nm band fluorescence emission as a function of time.[50] The formation of the metal complex proceeds very quickly, and the fluorescence intensity of the complex reaches the maximum ~30 s after it has been stirred and remains constant afterward when **L** and Zn^{2+} are mixed together. For sensing PP_i, the response time depends on the concentration of PP_i, as the time required to reach the equilibrium increases with an increase in the PP_i concentration. Among all studied concentrations, stable readings can be obtained within 3−6 min. From the fluorescence intensity changes with time, one could also realize that the PP_i−ligand complex of the **L**−Zn^{2+} species is reasonably stable, and irradiation of the metal anion complex with visible light did not change the emission intensity at 620 nm.

We subsequently studied the chemical reversibility of the anion binding of the **L**−Zn^{2+} complex in the ethanol/water solution. Because of the high affinity of Mg^{2+} for PP_i,[57] we anticipated that addition of Mg^{2+} will sequester PP_i, liberating the **L**−Zn^{2+} complex. With this intention, 1 equiv of the Mg^{2+} solution was added to the PP_i complex of **L**−Zn^{2+} species, which visibly turned the colorless solution back to the initial pink color, and the fluorescence intensity fully recovered.[50] The chemically regenerated **L**−Zn^{2+} complex can then take part in another anion binding event. In contrast to the facile photochemical reversibility demonstrated by 6′-nitro-substituted spiropyrans,[24] this result reveals that the anion binding of the **L**−Zn^{2+} complex is chemically reversible but immune from the influence of photochemical stimuli.

Study of the Interaction Mechanism. We studied the complexation of **L** with Zn^{2+} and PP_i by ^1H NMR and ^{31}P NMR in methanol-d_4 and a D_2O mixture to provide some insight into the interaction mechanism. The ^1H NMR spectrum of **L** was largely affected upon addition of 1 equiv of Zn^{2+} to the methanol-d_4/D_2O solution of **L** at room temperature. The ^1H NMR spectrum of free **L** exhibits the typical ^1H signals of spiropyrans: two singlets at 1.08 and 1.31 ppm, assigned to the magnetically nonequivalent methyl protons, and a singlet at 2.55 ppm, ascribed to the *N*-methyl protons of the indoline

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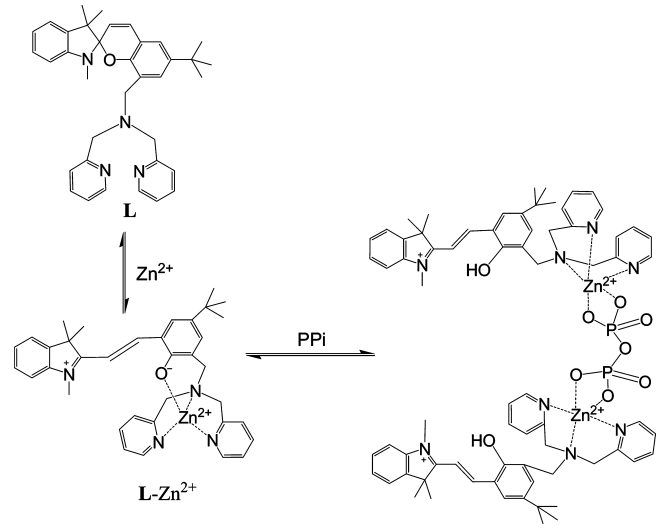
rings. When an equimolar amount of Zn^{2+} was added, the two singlets of methyl protons disintegrated into one singlet at ~1.47 ppm, indicating they have become magnetically equivalent because of the planar structure of the fully conjugated merocyanine form. Also, downfield shifts of the vinyl protons at 5.64 and 6.42 ppm and *N*-methyl protons of the indoline rings were observed after the addition of Zn^{2+}. In addition, the interaction of Zn^{2+} with **L** caused the most pronounced downfield shifts of proton signals of methylene on the Dpa group (~3.85 ppm) and aromatic protons of the pyridine moiety (~7.70 and 8.64 ppm) (data not shown). As the vinyl protons are very sensitive indicators of ring opening,[58] the results primarily indicate that Zn^{2+} forms a complex with the opened mero form of **L** and the interaction may occur through the phenolate hydroxyl group and the Dpa moiety. On the other hand, there was no change when PP_i was added to the methanol-d_4/D_2O solution of **L**, but that of the **L**−Zn^{2+} complex. The ^{31}P NMR spectrum of PP_i exhibited one single phosphorus peak at −5.17 ppm,[50] and addition of **L** caused no shift in the phosphorus resonance. However, a downfield shift to −2.51 ppm for PP_i was observed when it was mixed with 0.5 equiv of the **L**−Zn^{2+} complex.

The quantitative combinations of **L** with Zn^{2+} and the **L**−Zn^{2+} complex with PP_i were further confirmed by their HRMS.[50] In an ethanol aqueous solution containing a 1:1 mixture of **L** and Zn^{2+}, a peak at m/z 609.3 assigned to the **L**−Zn^{2+} complex was found. In addition, a peak at m/z 1399.4 corresponding to [2(**L**−Zn^{2+}) + PP_i + 6H] is clearly observed in the HRMS spectrum when Zn^{2+} and PP_i are both added to the solution of **L**. The HRMS results corroborated well with the proposition that Zn^{2+} forms a complex with the mero form of **L** which provides an avenue for subsequent selective binding with PP_i.

These corroborative results indicate that the interactions of PP_i with the metal ion would result in the formation of a bridged dimer complex of the ligand via the cooperative combination of Zn^{2+} with both **L** and PP_i. We thus infer that the metal center may combine with both one PP_i molecule and two **L** molecules to form a 2:1:2 (Zn^{2+}:PP_i:**L**) ternary assembly complex. The proposed structure of the anion ligand complex is shown in Scheme 2. Spiropyran **L**, metal ion Zn^{2+}, and PP_i combined together through the interactions between nitrogen atoms of the Dpa group in **L** and the P═O group in PP_i with Zn^{2+}.

Determination of PP_i Concentrations in Human Urine. We demonstrated the potential application of the appraoch by using it in the detection of trace levels of PP_i in human urine. Urinary PP_i plays an important role in the formation of calcigerous stones because it represents a major inhibitor of urinary crystallization.[59,60] Experimental evidence has suggested that PP_i can act as an inhibitor of renal calculi formation, and pharmacological treatments that tend to increase the urinary level of PP_i have been proposed to prevent relapsed urolithiasis.[61] Assessment of its urinary excretion may therefore be useful in the laboratory investigation of any patient with

Scheme 2. Formation of the Zn^{2+} Complex of L (L−Zn^{2+}) and the Ternary Complex Containing L, Zn^{2+}, and PP_i



urolithiasis. The classic method for determination of the urinary level of PP_i is usually an enzyme assay.[62−64] Although this method is very selective, it does not allow the direct determination of the level of PP_i. The same limitation holds for the multistep coupled enzyme assays. The development of a new analytical methodology for the direct assay of PP_i content in urine becomes necessary.

The analysis of PP_i in urine is commonly conducted in the presence of metal ions, anions, urea, and proteins. Following the described procedure, the selectivity of the sensor for PP_i in the presence of the interfering species in urine was first investigated by addition of different amounts of the interfering agent to the samples containing 5.0×10^{-5} M PP_i. The tolerance limit was taken as the concentration causing an error of ±5% in the determination of the level of PP_i. The results are summarized in Table 2. As one can see, most substances existing in urine such as K^+, Na^+, Ca^{2+}, Cl^-, SO_4^{2-}, $H_2PO_4^-$, urea, uric acid, and BSA showed no remarkable interference in the determination of the level of PP_i even at high concentrations. Since PP_i has an intrinsic binding affinity with Mg^{2+},[56] the introduction of the metal ion at a concentration of $>1.0 \times 10^{-5}$ M revealed an obvious influence on the **L**−Zn^{2+} fluorescence quenched by PP_i.

Finally, the PP_i concentrations of three urine samples were measured using the calibration curve. The PP_i concentration determined in urine samples varied from 3.8×10^{-5} to 4.3×10^{-5} M. The mean value for urinary PP_i excretion by three healthy subjects was 62.4 μmol/24 h, which is agreement with the values obtained with another method.[65] We also found that the PP_i concentration of the morning excretion sample is higher than that of the noon excretion sample or the night excretion sample. To reach a conclusion about the reliability of the proposed method, we also analyzed urine samples with low additional levels of PP_i, and the recoveries were 95−106% (Table 3).

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Table 2. Effect of Different Interfering Agents on the Fluorescence Intensity of the L−Zn^{2+} Complex

interfering agent	concentration (M)	fluorescence change $\Delta F = (F_1 - F_2)$[a]	relative signal change value (%) $(\Delta F/F_1 \times 100)$
K^+	0.1	1.41	4.26
Na^+	0.1	0.90	2.71
Ca^{2+}	0.1	1.17	3.52
Mg^{2+}	1.0×10^{-5}	−1.00	−3.03
Zn^{2+}	0.001	0.77	2.33
Fe^{2+}/Fe^{3+}	0.005	−0.82	−2.03
Cl^-	0.1	0.65	1.96
SO_4^{2-}	0.1	0.15	0.46
$H_2PO_4^-$	0.05	0.39	1.17
urea	1.0×10^{-3}	1.16	3.50
uric acid	1.0×10^{-3}	−0.89	−2.69
BSA	2.0 mg/mL	0.76	2.28

[a] F_1 and F_2 are the fluorescence intensities (λ_{ex} = 522 nm, and λ_{em} = 620 nm) of the L−Zn^{2+} complex interacting with the 5.0×10^{-5} M PP_i solution without and with interfering agents, respectively.

Table 3. PP_i Concentrations in Urine and Recoveries

	PP_i			
sample	added (M)	found (M)	recovery (%)	RSD (%)[a]
urine 1	0	3.29×10^5	−	1.9
	1.0×10^5	4.56×10^5	106	1.8
urine 2	0	3.60×10^5	−	1.6
	2.0×10^5	5.31×10^5	95	2.0
urine 3	0	2.65×10^5	−	2.2
	5.0×10^5	7.55×10^5	98	2.0

[a] Mean values of three measurements.

To further verify the biological application of the L−Zn^{2+} complex toward PP_i, the real-time assay of PP_i hydrolysis catalyzed by pyrophosphatase, a Mg^{2+}-dependent[66] hydrolase that serves to drive the reaction depending upon PP_i release by further hydrolysis to phosphate anion, was conducted. To do this, an ethanol/water buffer solution containing 5×10^{-5} M L−Zn^{2+} complex, 50 μM PP_i, and 1.0 μM $MgCl_2$ was prepared and used for the measurements of hydrolysis kinetic curves of PP_i at different pyrophosphatase concentrations. Inorganic PPase from baker's yeast was added, and after a brief mixing period (5 s), the fluorescence intensity at 620 nm was monitored as a function of time. As shown in Figure S11 of the Supporting Information, the pyrophosphatase-catalyzed PP_i hydrolysis caused distinct fluorescence enhancement over time until a steady value was reached. Thus, we proved that the activity of inorganic pyrophosphatase, which hydrolyzes the pyrophosphate, could be monitored by using the L−Zn^{2+} complex. Therefore, the L−Zn^{2+} complex could become the preferred anion sensor for PP_i in many biological and analytical applications.

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CONCLUSION

In conclusion, a new spiropyran-based fluorescent sensing approach for PP_i has been developed. Anion recognition and signal transduction are based on the cooperative ligation interactions and structural conversion of the spiropyran, which leads to a significant change in photophysical property of the spiropyran. To the best of our knowledge, this is the first spiropyran-based fluorescent anion probe reported. Compared with the known fluorescent sensors for PP_i, the sensor described here possesses three remarkable features. First, it displayed extreme specificity toward PP_i in aqueous solution even in the presence of high concentrations of competing anions. Second, it is worth noting that one key feature of the L−Zn^{2+} complex is its long wavelength excitation and emission, making it potentially applicable to biological sensing and screening in vivo. Finally, the PP_i quantification could be achieved by fluorescent ratiometry, which provides more precise measurements to normalize the variation in measuring conditions of factors such as path length, photobleaching, and dye concentration. Although we demonstrate here the feasibility of determining the PP_i content of urine, we expect that the design strategy would help to extend the development of highly selective spiropyran-based fluorescent sensing approaches to other analytes of interest.

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SUPPORTING INFORMATION AVAILABLE

Absorption and fluorescence response curves of **L** with metal ions and anions, ^1H NMR, ^{13}C NMR, and HRMS spectra of **L**, and ESI of **L** with the addition of zinc ion and PP_i. This material is available free of charge via the Internet at http://pubs.acs.org.

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