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Highly Effective Fluorescent and Colorimetric Sensors for Pyrophosphate over $H_2PO_4^-$ in 100% Aqueous Solution

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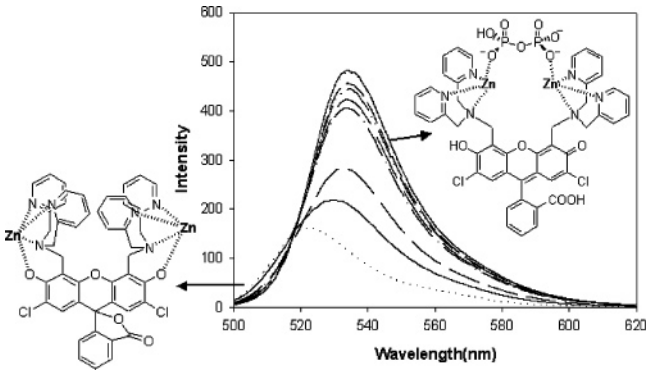

This study demonstrated that Zinpyr-1·Zn^{2+} acts as a fluorescent and colorimetric sensor for pyrophosphate at pH 7.4. In addition, Zinpyr-1·Cu^{2+} and DIARB-1·Cu^{2+} complexes were found to act as selective fluorescent sensors for pyrophosphate. Furthermore, the chemosensors Zinpyr-1·Zn^{2+} and Zinpyr-1·Cu^{2+} show highly selective and ratiometric fluorescence changes for pyrophosphate compared with $H_2PO_4^-$.

Anions play an important role in various chemical and biological processes. Accordingly, there has been a great deal of effort devoted to the development of abiotic receptors for anionic species.[1] Sensors based on the anion-induced changes in fluorescence are particularly attractive on account of their simplicity and the high detection limit of the fluorescence.[1,2] In particular, pyrophosphate can be a biologically important target because it is the product of ATP hydrolysis under cellular conditions.[3] Furthermore, the detection of released pyrophosphate has been examined as a real-time DNA sequencing method.[4] However, there are few reports on pyrophosphate-selective fluorescent chemosensors.[5] Recently, Hong et al. reported an azophenol-based fluorescent pyrophosphate sensor in water, which also contained di(2-picolyl)-amine (DPA) units bound by Zn^{2+}.[5b] Hamachi et al. recently reported anthracene derivatives with two Zn^{2+}-DPA units as novel fluorescent chemosensors for phosphorylated peptides and ATP.[6]

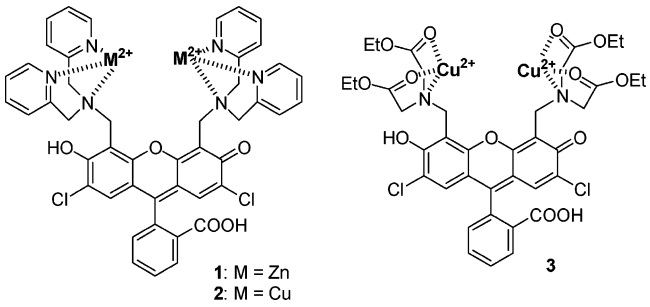

FIGURE 1. Structures of Zinpyr-1·Zn^{2+} complex (**1**), Zinpyr-1·Cu^{2+} complex (**2**), and DIARB-1·Cu^{2+} complex (**3**).

This paper reports the use of Zinpyr-1·Zn^{2+} as a fluorescent and colorimetric sensor for pyrophosphate at pH 7.4. Zinpyr-1·Cu^{2+} and DIARB-1·Cu^{2+} complexes also display selective fluorescent changes for pyrophosphate over $H_2PO_4^-$. Furthermore, the Zinpyr-1·Zn^{2+} complex (**1**) and Zinpyr-1·Cu^{2+} complex (**2**) act as ratiometric fluorescent chemosensors for pyrophosphate that can be used in aqueous solutions.

Zinpyr-1 was synthesized following a procedure reported elsewhere.[7] The treatment of Zinpyr-1 with either $Zn(NO_3)_2$ or $Cu(NO_3)_2$ in $CH_3CN-THF-MeOH$ afforded the Zinpyr-1·Zn^{2+} complex (**1**) or Zinpyr-1·Cu^{2+} complex (**2**), respectively, as orange powders. A diethyl iminodiacetate fluorescein (DIABR-1, **5**) was synthesized using the Mannich reaction between 2′,7′-dichlorofluorescein (**4**) and the iminium ion condensation of product of formaldehyde and diethyl iminodiacetate with a 61% yield (Scheme 1). The DIARB-1·Cu^{2+} complex (**3**) was obtained

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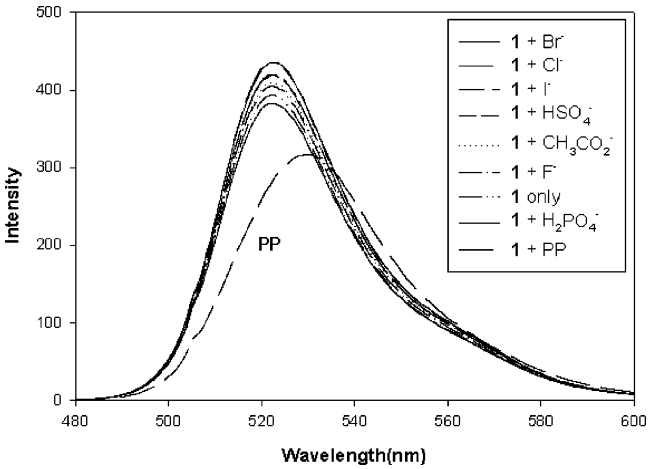
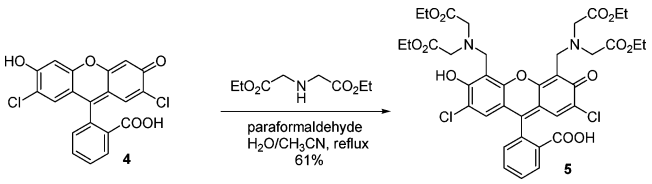


FIGURE 2. Fluorescence emission changes of **1** (1 μM) upon addition of tetrabutylammonium salts of HSO_4^-, CH_3COO^-, I^-, Br^-, Cl^-, F^-, $H_2PO_4^-$, and hydrogen pyrophosphate (100 equiv, 100 μM) at pH 7.4 (20 mM HEPES) (excitation at 504 nm).

SCHEME 1. Synthesis of Compound 5



using a methodology similar to that described for complex **2**. The ^1H and ^{13}C NMR spectra of complexes **1** and **5** are shown in Supporting Information.

Figure 2 shows the fluorescence emission changes in complex **1** upon the addition of HSO_4^-, CH_3COO^-, I^-, Br^-, Cl^-, F^-, $H_2PO_4^-$, and pyrophosphate. The fluorescence spectra were obtained by excitation of the fluorescein fluorophore at 504 nm. Both the excitation and emission slits were 5 nm. As shown in Figure 2, there was a unique change in the emission spectrum upon the addition of pyrophosphate. When pyrophosphate was added, the UV absorption spectra of complex **1** showed a bathochromic shift (~13 nm) similar to that observed in the fluorescence spectra (Figure 3). A colorimetric change of **1** with pyrophosphate is explained in Figure 5, Supporting Information. Again, there was no significant change in the UV spectrum when $H_2PO_4^-$ was added. Figure 4 shows the fluorescence titration results of the Zinpyr-1·Zn^{2+} complex (**1**) with pyrophosphate at pH 7.4 (20 mM HEPES) at an excitation wavelength of 517 nm. The emission maximum of complex **1** gradually shifted from 523 to 534 nm upon the addition of pyrophosphate, and chelation enhanced fluorescence (CHEF) effects (~150%) were observed. When complex **1** was excited at 504 nm after adding pyrophosphate, a red shift (~12 nm) and chelation enhanced fluorescence quenching (CHEQ) effects (~30%) were observed (Figure 6, Supporting Information). The excitation spectrum of **1** with pyrophosphate also displayed similar ratiometric changes, which is explained in Figure 7, Supporting Information. The Zinpyr-1·Cu^{2+} complex (**2**) showed a similar red shift (~11 nm) and CHEF effects (~25%) when pyrophosphate was added (Figure 5). The UV absorption spectra of complex **2** showed a rather smaller bathochromic shift

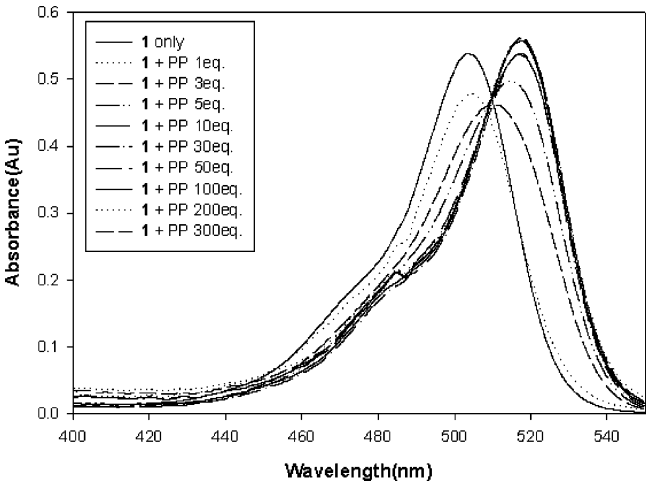


FIGURE 3. UV titrations of compound **1** (0.1 mM) with tris(tetrabutylammonium) hydrogen pyrophosphate at pH 7.4 (20 mM HEPES).

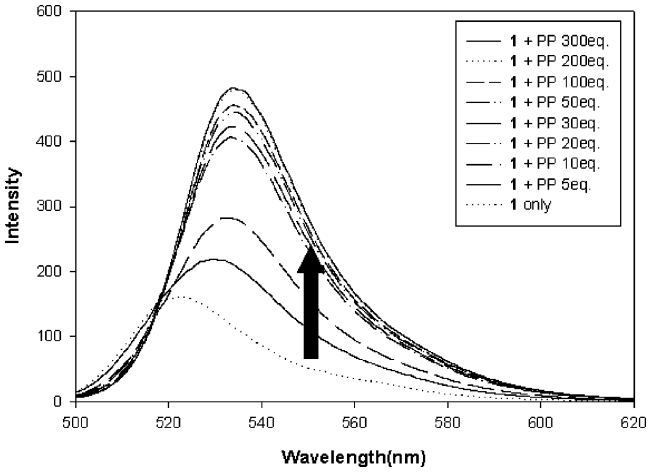


FIGURE 4. Fluorescent titrations of compound **1** (1 μM) with tris(tetrabutylammonium) hydrogen pyrophosphate at pH 7.4 (20 mM HEPES) (excitation at 517 nm).

(~3 nm) compared to that of **1** when pyrophosphate was added. The fluorescence titration curves of complex **1** and **2** with pyrophosphate are shown in Figure 8, Supporting Information, utilizing their ratiometric changes. However, there was almost no change in both the λ_{max} and the fluorescence intensity when $H_2PO_4^-$ was added to the complex **1** (Figure 9, Supporting Information). The fluorescent titration spectrum of complex **3** with pyrophosphate is also explained in Figure 10, Supporting Information. Unlike complexes **1** and **2**, there were only fluorescent emission enhancements without any significant changes in the λ_{max}. From the fluorescence titration, the association constants of complexes **1**, **2**, and **3** were observed to be 98 400, 168 000, and 6 400 M^{-1}, respectively (errors < 10%).[8]

With the Zn^{2+}-DPA-anthracene receptor reported by Hamachi et al., the decrease in the cationic character of the pyridine rings of DPA induced by the binding of the

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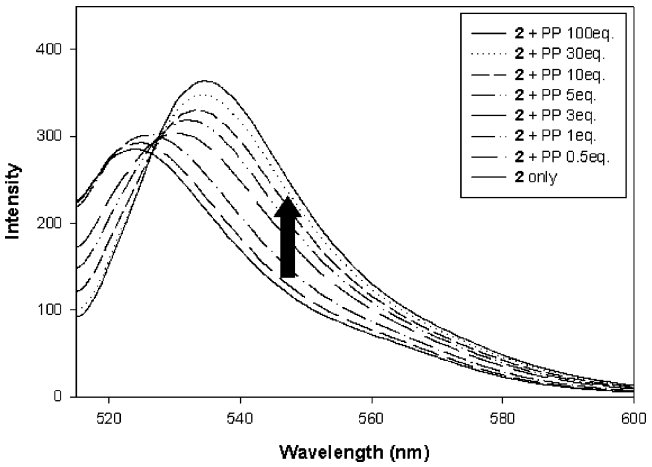


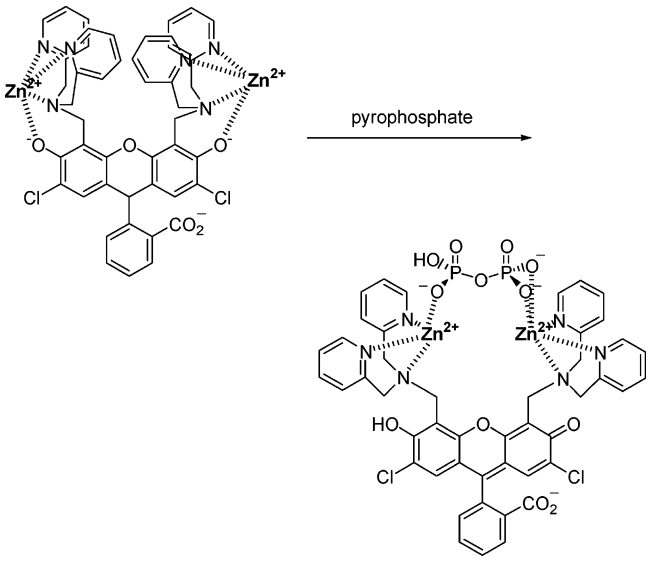
FIGURE 5. Fluorescent titrations of compound **2** (1 μM) with tris(tetrabutylammonium) hydrogen pyrophosphate at pH 7.4 (20 mM HEPES) (excitation at 512 nm).

phosphate anion to a zinc center was suggested as a reasons for the suppression of PET quenching.[6] In addition, the red shift observed in this study can be explained on the basis of the reports by Hong et al.[5b,9] and Lippard et al.[7b] In Hong's work,[9] a similar red shift of the λ_{max} in the UV spectrum of the azaphenol-DPA based receptor was observed when pyrophosphate ions were added. Hong et al. reported similar results in their azaphenol-DPA-naphthalene based receptor.[5b] The red shifts in the UV and emission spectra were observed when pyrophosphate was added to Hong's Zn-azapenol-DPA-naphthalene host. This was explained by a weakening of the bond between the *p*-nitrophenylazo phenolate oxygen and Zn^{2+}, which induced a more negative charge.[5b] The crystal structure of Zinpyr-1·Zn^{2+} (perchlorate salts) reported by Lippard and Tsien revealed that Zn^{2+} coordinates not only with the DPA ligands but also with the two phenoxides on the fluorescein moiety.[7b] Therefore, the red shift of complexes **1** or **2** upon the addition of pyrophosphate can be explained in a manner similar to Hong et al's reports.[5b,9] Scheme 2 shows the proposed mechanism of ratiometric changes in complex **1** upon the addition of pyrophosphate. The Job plot for the binding between complex **1** and pyrophosphate shows a 1:1 stoichiometry (Figure 11, Supporting Information). In the electrospray ionization (ESI) mass spectrum, a peak at *m/z* 1126.9 which corresponds to [**1** + **PP**]$^+$ was clearly observed (Figure 12, Supporting Information). The reason for the absence of red shifts in the emission spectra of complex **3** with pyrophosphate is not clear at this moment.

Upon the addition of 1 equiv of complex **1** in DMSO-d_6, the chemical shifts due to the two different pyrophosphorus compounds in hydrogen pyrophosphate moved from −4.7 to −7.9 ppm and 3.9 to 1.9 ppm, respectively (Figure 13, Supporting Information), which indicates that complex **1** directly interacts with the phosphate sites. The protons in the pyridine moiety (C-6′, next to the nitrogen) shifted from 8.563 to 8.665 in DMSO-d_6−D_2O (4:1, v/v) upon the addition of 1 equiv of pyrophosphate (Figure 14, Supporting Information).

Complexes **1**, **2**, and **3** certainly have important advantages over the pyrophosphate-selective fluorescent chemosensors reported so far. Both the emission and excitation wavelengths are suitable for biological applications. In addition, all fluorescent changes can be monitored in a 100% aqueous solution at pH 7.4. Most importantly, complexes **1** and **2** are ratiometric fluorescent sensors. A ratiometric sensor allows a calibration curve to be determined in vitro, which is independent of the sample conditions, e.g., the concentration of the sensor, etc.

SCHEME 2. Proposed Mechanism for the Binding Mode of Complex 1 with Pyrophosphate



In conclusion, complexes **1**, **2**, and **3** act as fluorescent sensors for pyrophosphate at pH 7.4. The fluorescent chemosensors **1** and **2** exhibited highly selective and ratiometric fluorescence changes for pyrophosphate compared with $H_2PO_4^-$.

Experimental Section

Zinpyr-1·Zn^{2+} (1). Zinpyr-1 was synthesized following a published procedure[1] in a yield of 45%. To a solution of Zincpyr-1 (550 mg, 0.67 mmol) in CH_3CN−THF (5:1, 30 mL) was added dropwise 112 mM $Zn(NO_3)_2$ in MeOH (11.96 mL, 1.34 mmol).[2] After stirring for 30 min at room temperature, the precipitate was filtered and washed with cold CH_3CN to give Zincpyr-1·Zn^{2+} (539 mg, 67%) as an orange powder: ^{1}H NMR (DMSO-d_6) δ 8.64 (t, 4H, J = 4.5 Hz), 8.16 (d, 1H, J = 7.0 Hz), 7.77−7.99 (m, 6H), 7.43−7.57 (m, 6H), 7.35 (d, 2H, J = 7.5 Hz), 7.15 (d, 1H, J = 7.0 Hz), 6.61 (s, 2H), 4.34 (m, 12H); ^{13}C NMR (DMSO-d_6) δ 174.5, 166.6, 155.4, 155.1, 154.8, 148.2, 141.2, 140.9, 133.5, 131.3, 128.1, 125.4, 125.2, 124.3, 124.0, 111.7, 111.2, 59.8, 50.9; MALDI TOF $C_{46}H_{36}Cl_2N_6O_5$·2Zn·2NO_3 *m/z* = 1136.8 (M)$^+$.

Zinpyr-1·Cu^{2+} (2). Zinpyr-1·Cu^{2+}(**2**) was synthesized using $Cu(NO_3)_2$ in a similar way as described for Zinpyr-1·Zn^{2+}(**1**): LRMS (FAB) *m/z* = 949.1 [(M + H)$^+$ − 2NO_3^-]. Anal. Calcd for $C_{46}H_{35}Cl_2Cu_2N_8O_{11}$: C, 51.45; H, 3.29; N, 10.44. Found: C, 51.08; H, 3.08; N, 10.25.

DIABR-1 (5). Diethyl iminodiacetate (1.52 mL, 8.68 mmol) and paraformaldehyde (0.224 g, 7.47 mmol) were combined in 20 mL of CH_3CN and refluxed for 30 min. 2,7-Dichlorofluorescein (**4**) (1.00 g, 2.49 mmol) in 30 mL of CH_3CN−H_2O (1:1) was added to the solution, and the reaction mixture was refluxed for 24 h. The CH_3CN was removed, and the product and the residual water were triturated with 30 mL of boiling ethanol. After cooling to room temperature, 5 mL of ether was added to

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the solution. The product was precipitated at −4 °C and filtered on a frit (1.21 g, 61%): ^{1}H NMR ($CDCl_3$) δ 8.06 (d, 1H, J = 6.8 Hz), 7.69 (quintet, 2H, J = 7.4 Hz), 7.20 (d, 1H, J = 6.2 Hz), 6.69 (s, 2H), 4.30−4.53 (d, 4H, J = 14 Hz), 4.21 (q, 8H, J = 7.1 Hz), 3.54 (s, 8H), 1.28 (t, 12H, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ170.6, 169.0, 155.9, 151.6, 148.6, 135.6, 130.6, 128.3, 127.3, 125.8, 124.3, 117.9, 110.9, 109.9, 83.0, 61.6, 54.5, 49.1, 31.2, 14.4; HRMS (FAB) m/z = 825.1805 $(M + H + Na)^+$, calcd for $C_{38}H_{40}Cl_2N_2O_{13}Na$ = 825.1805.

DIARB-1·Cu^{2+} (3). DIARB-1·Cu^{2+}(**3**) was synthesized in a similar way as described for Zinpyr-1·Cu^{2+}(**2**): MALDI TOF $C_{46}H_{36}Cl_2N_6O_5$·2Cu·2NO_3 m/z = $(M)^+$. Anal. Calcd for $C_{38}H_{39}Cl_2Cu_2N_4O_{19}$: C, 43.31; H, 3.73; N, 5.32. Found: C, 43.61; H, 3.48; N, 5.19.

Preparation of Fluorometric Anion Titration Solutions. Stock solutions (1 mM) of the tetrabutylammonium salts of pyrophosphate and $H_2PO_4^-$ in 20 mM HEPES (pH 7.4) were prepared. Stock solutions of hosts (0.01 mM) were also prepared in 20 mM HEPES (pH 7.4). Test solutions were prepared by placing 4−40 μL of the probe stock solution into a test tube, adding an appropriate aliquot of each metal stock, and diluting the solution to 4 mL with 20 mM HEPES (pH 7.4).

For all measurements, excitation was at 504 nm and emission was measured at 522 nm. Both excitation and emission slit widths were 5 nm.

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Supporting Information Available: NMR spectra, fluorescence spectra, and ^{31}P NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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