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Binuclear Zn(II)–Zn(II) Complex from a 2-Hydroxybenzohydrazide-Derived Schiff Base for Selective Detection of Pyrophosphate

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Abstract

A hydroxybenzohydrazide-based Schiff base ligand was conveniently synthesized. Upon addition of Zn²⁺ cation, the ligand exhibited a high tendency to form a binuclear structure with a 2:2 ligand-to-zinc ratio, which was accompanied with a large fluorescence turn-on ($\lambda_{\text{em}} = 507 \text{ nm}$, $\phi_{\text{fl}} \approx 0.28$). The reactivity of zinc complex was examined by using different phosphate anions, which reveals a higher response to acid pyrophosphate anion. Detailed spectroscopic studies revealed that the pyrophosphate response is based on the ligand displacement mechanism.

Pyrophosphate ion (PPi) is an important anion, as it is the product of adenosine triphosphate (ATP) hydrolysis under cellular conditions.¹ Real-time detection of PPi can be achieved by using the enzymatic method to give bioluminescence,² which has been used in the commercial pyrosequence for DNA sequence.³ The enzymatic detection of PPi, however, involves a complicate detection scheme by converting PPi to ATP that is then used to generate bioluminescence signal.⁴ In an effort to search for a simpler detection method, there are significant interests to develop fluorescent chemosensors for PPi in recent years.^{5,6}

A binuclear core is formed when a chelating ligand holds two metal ions in close distance. Such binuclear cores are commonly found in metallenzymes,⁷ which are known to play an active role to catalyze the hydrolysis of esters such as phosphates.⁸ In the binuclear core, two metal centers are positioned closely to each other (with M–M distance of 3–5 Å). As a result, the two metal centers in the binuclear core work synergistically to bind a substrate, and the reactivity of one metal center is influenced by the other. The binuclear complexes consisting of Zn(II)–Zn(II) core is also the most commonly used strategy for selective recognition of PPi anion.^{9–18} Among the known examples, most binuclear complexes have a 1:2 ligand-to-metal ratio. Previous study has demonstrated that the benzoxazole ligand **1** (HL, $\lambda_{\text{em}} \sim 550 \text{ nm}$) tends to form binuclear complex **2** with the Zn(II)–Zn(II) distance of 3.16 Å ($\lambda_{\text{em}} \sim 459–478 \text{ nm}$).¹⁹ In this study, we report the synthesis of Schiff base **3** which

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Supporting Information. Experimental details for synthesis of **3**, its ¹H and ¹³C NMR data, and additional absorption and fluorescence spectra for **3** and its zinc complex. This material is available free of charge via the Internet at <http://pubs.acs.org>

has a similar array of chelating atoms (i.e. N–O–N) as **1** (seen from its complex **2**), providing a suitable binding environment for the formation of binuclear Zn(II)–Zn(II) core with a 2:2 ligand-to-metal ratio. In comparison with **1**, the ligand **3** contained additional phenolic and carbonyl groups, which provide additional zinc binding strength. Herein we report that the ligand **3** exhibits high tendency to form binuclear Zn(II)–Zn(II) complex **4**, giving strong green fluorescence. We then further examined the reactivity of **4** to different phosphate anions, which exhibited good selectivity for hydrogen pyrophosphate H₂PPI anion (H₂P₂O₇^{2–}), giving large response by turning-off the fluorescence.

Schiff base ligand **3** was conveniently synthesized by reaction of 2-hydroxy-5-methylisophthalaldehyde with the 2-hydroxybenzohydrazide Ar–CO–NHNH₂ in 90% yield by refluxing in ethanol for 2 hrs. In ethanol, the absorption peaks of ligand **3** (at 367 and 382 nm) were gradually decreased upon addition of Zn(NO₃)₂, which was accompanied with the formation of a new peak at 417 nm (Figure 1a). The observed bathochromic shift, in addition to a clear isobastic point (at 390 nm), indicated the formation of a zinc complex, as a consequence of removing the phenolic proton in **3**. Ligand **3** in ethanol was weakly fluorescent (orange in ethanol, λ_{em}~576 nm, φ_{fl} = 0.02). Addition of Zn(OAc)₂ (or Zn(NO₃)₂) to **3** shifted the emission peak to λ_{em} = 507 nm (Figure 1b), which was accompanied with a remarkable increase in fluorescence intensity (φ_{fl} = 0.28). The fluorescence signal was saturated when about 1.2 equiv of Zn²⁺ was added, suggesting the formation of 1:1 complex. The same crystalline product could be obtained by mixing ligand **3** and Zn(NO₃)₂ in either 1:1 or 1:2 ratio in DMSO/EtOH (5:1 by volume) solvent, showing the ligand's strong tendency to form the zinc complex **4** (ESI, Fig. S1).

Electrospray mass spectrometry showed that the reaction instantly formed the zinc complex, upon addition of Zn²⁺ cation to the solution of **3** in anhydrous ethanol (concentration at ~10 μM). The dimer-like complex L₂Zn₂ was found to be the major product (Figure 2b), whose isotope pattern matched the calculated spectrum for the dianion **4** (C₄₆H₃₄N₈O₁₀Zn₂, exact mass 986.0981). The crystal sample from “**3**+Zn” revealed nearly the same mass spectrum pattern (Figure 2c), where the detection of trace ligand indicated that the complexation was reversible. On the basis of detected 2:2 ligand-to-metal ratio, the structure of complex **4** was assumed to be similar to the structure of **2**.¹⁹ The results clearly indicated that ligand **3** had a high tendency to form the binuclear zinc complex **4**. Since the fluorescence response of **3** was saturated when about one equiv. Zn²⁺ cation was added (Figure 1b), the results consistently suggest that zinc complex **4** (2:2 ligand-to-zinc ratio) was predominant in the solution.

¹H NMR titration revealed additional information (Figure 3). When Zn²⁺ was added, the methyl proton at 2.36 ppm was gradually decreased, which was accompanied with a new signal at 2.46 ppm that was assigned to **4**. It appeared that some free ligand **3** was present in the solution even in excess Zn²⁺ cation at room temperature. The complex **4**, however, was relatively stable once it was formed in methanol, as the spectrum of crystal sample of **4** did not show any detectable amount of free **3**. Small amount of **3** could be observed when the crystal sample **4** was in DMSO (ESI Figure S2). The observation thus indicated that **4** was predominant in the ethanol solution at room temperature, which is consistent with the finding from the mass study (Figure 2).

IR spectra for **3** detected the absorption peak at 1619 cm^{-1} (Figure 4), which is within the stretching frequency ($1644\text{--}1610\text{ cm}^{-1}$) for C=N of substituted hydrozone.²⁰ The IR peak at 1647 cm^{-1} was assigned to aromatic C=O stretching. Complete disappearance of C=O groups in **3**-Zn complex indicated the absence of carbonyl group, as a consequence of Zn^{2+} binding. The observation strongly indicated that the participation of carbonyl in the cation binding, which led us to propose the structure of complex **4**, where the amide --C(=O)NH-- group was tautomerized to --C(OH)=N-- group. The assumption was supported by disappearance of the strong band at $\sim 1530\text{ cm}^{-1}$ (from **3**), which was characteristic C–N–H stretch-bend for noncyclic monosubstituted amide. IR spectrum of **4** exhibited two C=N bands at 1611 and 1626 cm^{-1} , in agreement with the formation of --C(OH)=N-- group. The assumption was also consistent with the ^1H NMR, where the doublet signal H_d of **3** at 7.9 ppm was shifted upfield to 7.7 ppm (Figure 3), as a consequence of zinc binding (to carbonyl) that would decrease the electron density of the aromatic ring (less aromatic deshielding effect). Molecular modeling study revealed that the Zn(II)–Zn(II) core in **4** was sandwiched between two ligands and was shielded by two pairs of “end phenols” (marked by A and A' in Figure 5 & ESI Fig. S18). Following repeated attempts, we finally obtained a good quality crystal whose structure verified that the carbonyl oxygen was connected to the zinc center as proposed in **4**. All the evidences thus convincingly pointed to that the binuclear compound **4** with L_2Zn_2 configuration was formed almost exclusively when ligand **3** reacted with Zn^{2+} cation. The finding is in sharp contrast to a structural very similar ligand¹⁸ where the phenol (not carbonyl) is reported to bind to the zinc cation to give $\text{L}\cdot\text{Zn}_2$ complex.

Response of L_2Zn_2 Complexes to PPI. Although the zinc complex was formed immediately in alcohol solution, the complex **4** was not stable in pure HEPES buffer ($\text{pH} = 7.2$) (ESI, Figure S3). The sensor response to anions, therefore, was examined in EtOH (Fig. 6 and ESI, Figure S5). UV-vis revealed that the absorption peak at 413 nm was gradually decreasing upon addition of H_2PPI (Figure 6a), indicating that the zinc complex was decomposed in ethanol solution. The fluorescence intensity of **4** at $\sim 500\text{ nm}$ was also decreasing upon addition of H_2PPI (Figure 6b). The resulting absorption and fluorescence spectra matched the spectra for **3**, indicating the release of free ligand **3** from the zinc complex.

Mass spectrum from the reaction mixture $\{\text{“3 + Zn(OAc)}_2\text{” + PPI}\}$ in ethanol showed a complicate pattern, which detected not only the free ligand **3**, but also zinc complexes **4** and **5** (Figure 7a). It was possible that the reaction of **4** with PPI in ethanol could initially form **6**, which could then react with EtOH solvent to give **5**. The observed mass isotope patterns **5a** (for $\text{C}_{27}\text{H}_{27}\text{N}_4\text{O}_7\text{Zn}_2$, exact mass 647.0306), **5b** (for $\text{C}_{27}\text{H}_{25}\text{N}_4\text{O}_8\text{Zn}_2$, exact mass 661.0255), and **5c** (for $\text{C}_{27}\text{H}_{23}\text{N}_4\text{O}_9\text{Zn}_2$, exact mass 675.0048) matched the calculated corresponding isotope patterns (ESI Figure S7). The detected **5** was assumed to occur via PPI adduct **6** (Scheme 2), as the binuclear complex **4** was stable in ethanol in the absence of PPI. Interestingly, the presence of 30% water in the reaction mixture greatly simplified the pattern, showing that the zinc complex **4** was almost completely consumed upon addition of one equiv. PPI. The adduct “**3** -Zn-PPI” was detected at $m/z\ 732.9058$, which matched the isotope pattern of **6** (for $\text{C}_{23}\text{H}_{19}\text{N}_4\text{O}_{12}\text{P}_2\text{Zn}_2$, exact mass $m/z\ 732.9058$) (Figure 7b). The

related adduct, dianion **7** (Calcd for $C_{23}H_{18}N_4O_{12}P_2Zn_2$, exact mass 731.8979), was also observed at m/z 365.9115. In addition, the mass spectrum detected a large peak at m/z 652.8777, which was attributed to **8** (for $C_{23}H_{18}N_4O_9PZn_2$, exact mass m/z 652.9400) that could be formed via hydrolysis of **6** or **7**.

On the basis of the spectral evidences, a plausible mechanism for the H_2PPi sensing, therefore, could be based on the ligand releasing mechanism (Scheme 3). Since the addition of PPi to **4** caused immediate spectral change, we assume that the binding of PPi to **4** released ligand **3** which was weakly fluorescent. Further interaction of **6** with PPi could also release **3**, accompanied with formation of $Zn_2(PPi)_2$. The assumption was consistent with the detection of **3** and related species **6–8** (Figure 7b).

1H NMR revealed the consistent evidence to support the proposed ligand-releasing mechanism. Addition of two equivalents of Zn^{2+} shifted most of the imine proton to ~ 7.80 ppm, indicating that majority of free ligand (at 7.71 ppm) was used to form the zinc complex **3-2Zn** (Figure 8). Subsequent addition of H_2PPi to the solution of **3-2Zn** reduced the population of the Zn^{2+} -bound imine (at ~ 7.80 ppm), while the population of imine in the free ligand (7.71 ppm) was increasing, clearly indicating that the H_2PPi binding event was associated with the release of free ligand. In contrast, addition of ATP to **3-2Zn** solution did not cause notable release of ligand **3** (see ESI Figure S13), showing that the complex **3-2Zn** was responding selectively to H_2PPi .

The selective response of sensor was examined by addition of different phosphate anions to the zinc complexes. Among the different phosphate anions used, only H_2PPi could completely quench the fluorescence of the zinc complexes (Figure 9). The remarkable selectivity to H_2PPi was not simply due to its acidity, as phosphoric acid KH_2PO_4 did not exhibit the same effect. It appeared that the sensing of H_2PPi involves a synergistic interaction of H_2PPi with two Zn^{2+} centers in **4**, where two protons are needed to produce each neutral non-fluorescent ligand **3**. The sensor's detection limit for H_2PPi was determined to be 2.7×10^{-9} M (2.7 nM) using $D = 3N/S$ (ESI Figures S14). The fluorescence intensity of **4** exhibited good linear response to H_2PPi over a wide concentration range (20 nM – 500 nM) (ESI Figures S15), indicating its potential application.

In conclusion, Schiff base ligand **3** could readily react with Zn^{2+} to form a binuclear $Zn(II)$ – $Zn(II)$ complexes, giving product **4** (with 2:2 ligand-to- Zn ratio) in solution. Evidences from 1H NMR, IR, and mass spectroscopy are constantly pointing to that the carbonyl of the amide group in the ligand **3** was binding to $Zn(II)$. In the ethanol solution, ligand **3** in ethanol was weakly fluorescent (orange in ethanol, $\lambda_{em} \sim 576$ nm, $\phi_{fl} = 0.02$). Addition of $Zn(OAc)_2$ to **3** led to strong green emission ($\lambda_{em} = 507$ nm, $\phi_{fl} = 0.28$), which was accompanied with notable spectral shift. The resulting zinc complexes showed intriguing selectivity to hydrogen pyrophosphate to turn-off the fluorescence, illustrating its potential for sensor applications. With the aid of mass spectroscopic study, the sensing mechanism was determined to be based on the reaction of complex **4** with pyrophosphate, which led to the release of ligand **3**. The study thus provides a simple template and valuable guide for future PPi sensor design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. UV-vis (a) and fluorescence spectra (b) of ligand **3** (10 μM) in EtOH upon addition of different equiv. of $\text{Zn}(\text{NO}_3)_2$. The inset in (b) shows the fluorescence response to the equiv. of zinc cation.

Fig. 2. ESI-MS spectra of ligand HL **3** (a) in EtOH, upon addition of 0.5 equiv $Zn(OAc)_2$ (b), and crystalline **4** (c). The inset in spectra (b) and (c) are experimental and simulated isotope pattern for mass peak $[L_2Zn_2-6H]^{2-}$.

Fig. 3.
 ^1H NMR titration of ligand **3** with different equiv. of $\text{Zn}(\text{NO}_3)_2$ in $\text{CD}_3\text{OD}/\text{DMSO}$ (5:1 by volume).

Fig. 4.

IR spectra of ligand 3 and 3-Zn complex in KBr. The peak at 1619 cm^{-1} is attributed to CH=N- stretching (associated with the substituted hydrozone), while the peak at 1647 cm^{-1} is to C=O stretching. The strong band at $\sim 1530\text{ cm}^{-1}$ (from 3) was attributed to characteristic C-N-H stretch-bend for noncyclic monosubstituted amide.

Fig. 5.

Left: Crystal structure of zinc complex **4** shows that the carbonyl oxygen atoms are bonding to zinc. Right: Geometry-optimized structure of **4** by using HyperChem software (molecular mechanics force field MM+), where the C, N, O and Zn atoms are in cyan, blue, red and grey colors, respectively. For clarity, one of the ligand is shown in orange color. The end and central phenyl rings are indicated by letters A and B, respectively.

Fig. 6. Fluorescence spectra of zinc complex **4** [prepared from **3** (10 μ M) + Zn^{2+} (10 μ M)] in EtOH upon addition of different equivalent of H_2PPI .

Fig. 7. ESI-MS spectrum of {**3** + Zn(OAc)₂} + H₂PPI in EtOH (a) and EtOH/H₂O (7:3 by volume) (b). The anion at m/z 652.8777 was attributed to negatively charged species **8**, while the anion at m/z 732.8384 was assigned to **6** (see also ESI, Fig S8–11).

Fig. 8.
 ^1H NMR titration of $[3 + 2\text{Zn}^{2+}]$ in CD_3OD with different equiv. of H_2PPi .

Fig. 9.

UV-vis (a) and Fluorescence spectra (b) of zinc complex **4** [prepared from **3** (10 μM) + Zn^{2+} (20 μM)] in EtOH upon addition of different anions (5 equiv). The image (c) shows the fluorescence color changes upon addition of ATP, ADP and H_2PPI to the zinc complex (excitation 365 nm).

Scheme 1.
Structure of ligand **1** and **3**, and their zinc complexes.

Scheme 2.

A plausible mechanism for the PPI sensor, where interaction of PPI with zinc centers frees the ligand **3** from the complexes.