Fluorescent Probes

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Bis(Dpa-Zn^{II}) Appended Xanthone: Excitation Ratiometric Chemosensor for Phosphate Anions

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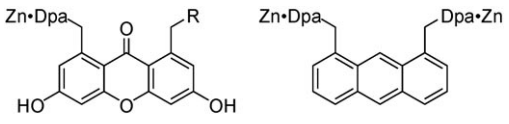
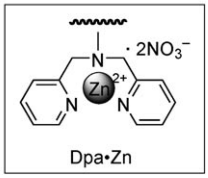
The development of molecular-recognition and sensing systems for biologically important anions has received considerable attention in recent years.[1] Among various anions, phosphate derivatives, which include nucleoside pyrophosphates such as adenosine triphosphate (ATP),[2] inorganic pyrophosphate,[3] and phosphoproteins,[4] are significant sensing targets because of their pivotal roles in biological systems. For example, ATP is known not only as a universal energy source but also as an extracellular signaling mediator in many biological processes,[5] and protein phosphorylation is recognized as a ubiquitous regulatory mechanism of signal transduction cascades in living cells.[6] In recent years, several chemosensors that exhibit a rather strong binding affinity for the phosphate derivatives in aqueous medium were reported,[2d,e,3a,c,4] although their bioanalytical application in biological systems has not yet been sufficiently developed.[2e,3c,7]

The detection of a specific anion by using the emission or excitation change at two different wavelengths provides a significant advantage over conventional measurement at a single wavelength because such a dual excitation/emission system enables a ratiometric detection of an analyte, thus allowing precise and quantitative analysis and imaging even in complicated systems. In contrast to the relatively high number of reports of fluorescent ratiometric sensors for cations such as Ca^{II} or Zn^{II},[8] there are few reports of ratiometric anion sensors.[2e,9] Herein, we report a new fluorescent sensory system that is capable of the ratiometric detection of phosphate anions under neutral aqueous conditions. A new chemosensor, **1**–2Zn^{II}, which comprises a xanthone fluorophore that bears two 2,2′-dipicolylamine (Dpa)–zinc(II) moieties as a phosphate-binding site, effects a change in the corresponding fluorescence excitation spectrum at three wavelengths upon binding to phosphate species based on a unique sensing mechanism, that is, a phosphate anion induced coordination rearrangement of the Zn^{II} ions.



1-2Zn^{II}: R = Dpa•Zn
2-Zn^{II}: R = H

3-2Zn^{II}



The syntheses of the chemosensor **1**–2Zn^{II} and the control monodentate complex **2**–Zn^{II} are described in the Supporting Information.[10] The structure of the binuclear zinc(II) complex **1**–2Zn^{II} with chloride counterions has been investigated by X-ray crystallographic analysis (Figure 1):[11] Complex **1**–2Zn^{II} is a dimeric complex connected by two bridging chloride ions, Cl(1), in the solid state. Interestingly, in the asymmetric half unit, one Zn(1) center coordinates to the carbonyl oxygen atom of the xanthone fluorophore from the upper side of the xanthone ring, in which the Zn(1)–O(1) bond length is 2.18 Å and the C(12)-O(1)-Zn(1) bond angle is 125.07°. The Zn(1) atom is also bound by the three nitrogen atoms of the Dpa unit and by both chloride anions in a square-bipyramidal six-coordinate environment. In contrast, the Zn(2) atom, which is positioned on the lower side of the xanthone ring, is bound to the Dpa unit and the two chloride

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author. Dpa = 2,2′-dipicolylamine.

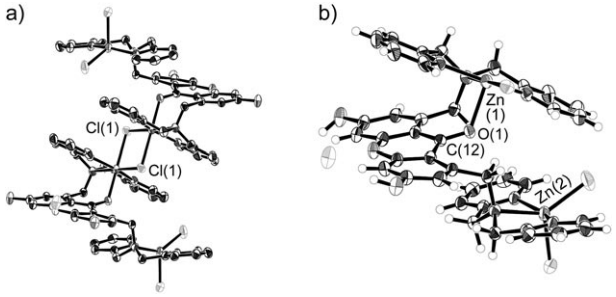


Figure 1. ORTEP drawing of the binuclear Zn^{II} complex [**1**–2 Zn^{II}]Cl_4: a) dimer complex; b) an asymmetric half unit of the complex. Disordered solvent molecules are omitted for clarity.

anions to form a tetragonal-pyramidal five-coordinate environment.

The absorption maximum of **1**–2 Zn^{II} is observed at 397 nm, and when the complex is excited at 397 nm, the emission maximum is 447 nm. The fluorescence quantum yield of **1**–2 Zn^{II} is high ($\Phi = 0.61$) under neutral aqueous conditions (50 mM HEPES, 50 mM NaCl, pH 7.2). Figure 2a shows the excitation spectral response of **1**–2 Zn^{II} upon addition of ATP. The changes in excitation with increasing concentration of ATP occur at three wavelengths (322

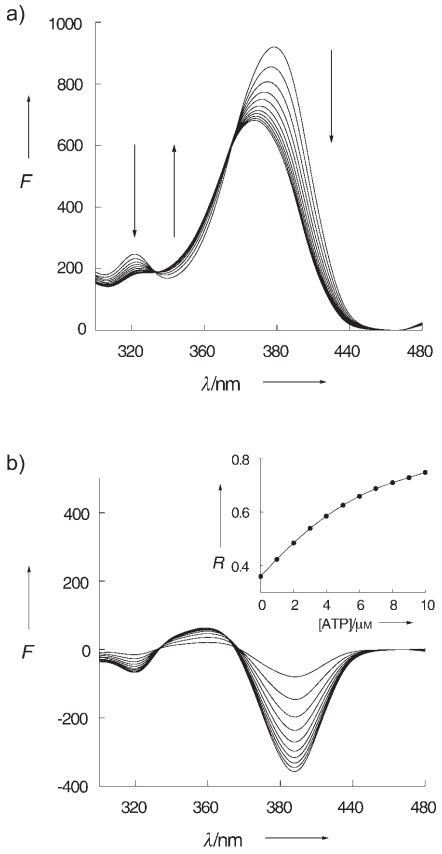


Figure 2. a) Change in the excitation spectrum of **1**–2 Zn^{II}; b) difference spectrum versus the initial state upon addition of ATP. *F* refers to the fluorescence intensity. Inset: plot of the fluorescence intensity ratio *R* (*F* at 360 nm/*F* at 407 nm; 3 μM **1**–2 Zn^{II} in 50 mM HEPES, 50 mM NaCl, pH 7.2, [ATP] = 0–10 μM, λ_{em} = 480 nm, 25 °C).

(down), 360 (up), and 407 nm (down)) with two isoemission points at 332 and 377 nm (Figure 2b). The plot of the fluorescence intensity at 407 nm (inset) shows saturation behavior with increasing ATP concentration; curve-fitting analysis gives an affinity constant of $4.2 \times 10^5\,M^{-1}$ for ATP. An almost identical affinity value was obtained by the analysis of the intensity ratio *R* (*F* at 360 nm/*F* at 407 nm) within an appropriate detection range ($R_{max}/R_{min} \approx 2$), which indicates that ATP is detected in an excitation ratiometric manner (Figure 2b, inset). The binding of **1**–2 Zn^{II} with ATP was also evaluated by isothermal titration calorimetry (ITC). The ITC data indicate that the binding is an exothermic, entropy-driven process ($\Delta H = -3.83$ kcal mol^{-1} and $T\Delta S = 4.19$ kcal mol^{-1}; see the Supporting Information) and give a binding constant $K_{app} = 7.5 \times 10^5\,M^{-1}$ ($n = 0.83$), which is almost identical to that obtained from the fluorescence titration.

The sensing selectivity of **1**–2 Zn^{II} for various biologically important phosphate species was examined by ratiometric excitation titration (Table 1). The chemosensor **1**–2 Zn^{II}

Table 1: Apparent binding constants K_{app} of chemosensor **1**–2 Zn^{II} to phosphate species as determined by fluorescence titration.

Anion[a]	K_{app} [M^{-1}][b]	Anion[a]	K_{app} [M^{-1}][b]
ATP	4.2×10^5	HPO_4^{2-}	1.8×10^3
GTP	1.0×10^6	AcO^-	[c]
ADP	2.8×10^5	SO_4^{2-}	[c]
AMP	5.0×10^2	NO_3^-	[c]
cAMP	[c]	HCO_3^-	[c]
UDP-Gal	2.8×10^2		

[a] Phosphate species with sodium counterions. ATP = adenosine-5′-triphosphate, GTP = guanosine-5′-triphosphate, ADP = adenosine-5′-diphosphate, AMP = adenosine-5′-monophosphate, cAMP = adenosine-3′,5′-cyclic monophosphate, UDP-Gal = uridine-5′-diphosphogalactose. [b] Conditions: 50 mM HEPES, 50 mM NaCl, pH 7.2, λ_{em} = 480 nm, 20 °C. [c] As the fluorescence change was scarcely observed, no binding constant was obtained.

showed strong affinities ($K_{app} > 10^5\,M^{-1}$) towards nucleoside pyrophosphates such as ATP, GTP, and ADP, whereas binding was relatively weak for the HPO_4^{2-} ion and monophosphate derivatives such as AMP. **1**–2 Zn^{II} also sensed a pyrophosphate diester (UDP-Gal) with a weak affinity, and a change in fluorescence was scarcely noticeable with a monophosphodiester (cAMP). A change in fluorescence was not induced upon addition of millimolar concentrations of other anions such as acetate, sulfate, nitrate, and carbonate, thus indicating that **1**–2 Zn^{II} is a selective fluorescent chemosensor for phosphate anions, especially for polyanionic nucleoside pyrophosphate species.[2,12]

Subsequently, we explored the sensing mechanism of the changes in the excitation spectrum of **1**–2 Zn^{II}. In a UV/Vis titration experiment of the metal-free ligand **1** with Zn^{II} ions in aqueous methanol, a stepwise spectral change occurred during complexation with two equivalents of Zn^{II}. The addition of one equivalent of Zn^{II} induced an absorbance decrease at 328 nm and the concomitant increase of a newly emerged peak at 376 nm with an isosbetic point at 357 nm (Figure 3a), while the subsequent addition of more than one equivalent of Zn^{II} caused an increase at 397 nm and a

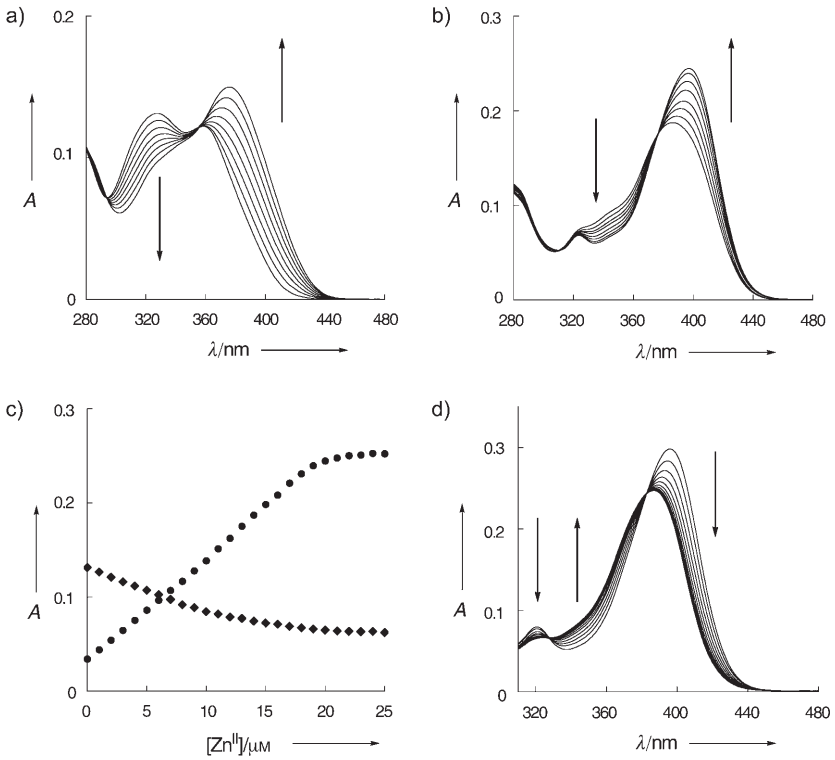


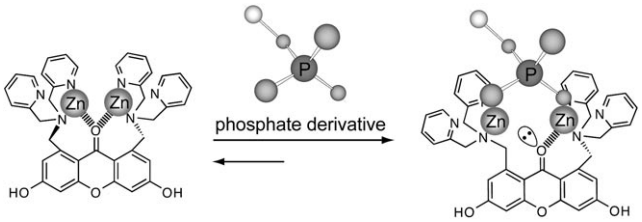
Figure 3. a–c) Zinc(II)-induced change in UV absorption of the ligand **1** (10 μM) upon addition of a) 0–0.7 and b) 1.3–2.0 equivalents of Zn^{II}. c) The plot of the UV absorption at 397 (●) and 328 nm (◆) of **1** (10 μM) in the zinc(II) titration (50 mM HEPES, 50 mM NaCl, pH 7.2/MeOH = 1:1, 25 °C). d) UV spectral change of **1**–2Zn^{II} upon addition of ATP (10 μM **1**–2Zn^{II} in 50 mM HEPES, 50 mM NaCl, pH 7.2, [ATP] = 0–20 μM, 25 °C).

decrease of 335 nm with an isosbetic point at 376 nm (Figure 3b).[13] The observed stepwise change in absorption can be reasonably ascribed to the coordination of both Zn^{II} ions within the molecule to the carbonyl oxygen atom of **1**, which should perturb the electronic properties of the xanthone fluorophore.[14] Interestingly, the absorption spectral change was also induced upon binding of **1**–2Zn^{II} to ATP. As shown in Figure 3d, the absorption maximum of **1**–2Zn^{II} at 397 nm decreases with an isosbestic point at 383 nm upon binding to ATP, the change of which is almost identical to the three signal changes observed in the ATP titration monitored by fluorescence excitation spectroscopy (Figure 2a).

It should be pointed out that the spectral changes induced by ATP absorption are in a direction opposite to the change in UV absorption observed in the process for the complexation of the second Zn^{II} center by **1** (Figure 3b). These results suggest that the excitation spectral change of **1**–2Zn^{II} upon binding to ATP is ascribed to the breaking of the coordinative bond between the xanthone carbonyl oxygen atom to the second Zn^{II} center.

This bond-breaking was also confirmed by a ^{13}C NMR spectroscopic study.[14b] The signal corresponding to the carbonyl carbon atom of the metal-free ligand **1** (10 mM in [D_6]DMSO) shifted downfield from $\delta = 177.9$ to 181.5 ppm in **1**–2Zn^{II}; the latter signal shifted upfield to $\delta = 178.9$ ppm upon adding one equivalent of NaH_2PO_4. A control chemosensor, mononuclear xanthone **2**–Zn^{II}[15] did not change its fluorescence upon adding phosphate species such as ATP or Na_2HPO_4 (data not shown), which suggests that **1**–2Zn^{II} binds to the phosphate anion with both Zn^{II}–Dpa sites as previously reported for the anthracene-type chemosensor **3**–2Zn^{II}.[4c] By combining these results, the excitation ratiometric change of binuclear **1**–2Zn^{II} upon phosphate binding can be reasonably explained as follows: in the resting state, both Zn^{II} centers are coordinated by the carbonyl oxygen atom of the xanthone fluorophore (Scheme 1). When a phosphate derivative is bound, the relatively labile coordination bond between one of Zn^{II} centers and the carbonyl oxygen atom is cleaved by the strong interaction of the metal ion with the phosphate, which results in the excitation change of three signals of the xanthone fluorophore. However, the tight coordination of the carbonyl oxygen atom to the other Zn^{II} center, which is observed in the X-ray crystallographic study of **1**–2Zn^{II}, remains intact in the phosphate complex such that this zinc atom interacts less strongly with the phosphate (Scheme 1).[16]

In conclusion, we have developed a new fluorescence chemosensor, **1**–2Zn^{II}, for phosphate species, which displays excitation change of three signals, thus enabling the ratiometric detection of phosphate species under neutral aqueous conditions. Our experimental data strongly support that the ratiometric excitation change is ascribed to rearrangement of the coordination induced by phosphate binding. We now envision bioanalytical applications of this chemosensor for real-time monitoring of an enzymatic reaction involving nucleoside pyrophosphates in vitro.[2e,3c,7]



Scheme 1. Phosphate anion-induced coordination rearrangement of **1**–2Zn^{II} upon binding to a phosphate derivative.[16]

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[11] Crystals of **1**–2 Zn^{II} with chloride counterions grew as colorless prisms by recrystallization from MeOH. A crystal suitable for X-ray crystallography was immersed in cryoprotectant oil (Paratone-N, Hampton Research) directly from the crystal solvent system, to avoid efflorescence, and immediately mounted on a goniometer head by using a CryoLoop (0.2–0.3 mm, Hampton Research) with cooling (120 K). X-ray diffraction data were collected on a Rigaku RAPID-HR diffractometer with a 2D area detector with graphite-monochromatized $Mo_{K\alpha}$ radiation ($\lambda = 0.7107$ Å). Crystal data for [**1**–2 Zn^{II}]Cl_4·*n*MeOH: $C_{39}H_{34}Cl_4N_6O_{10}Zn_2$, $M_r = 1019.30$, monoclinic, space group $P2_1/n$, $a = 12.947(2)$, $b = 12.711(2)$, $c = 27.948(4)$ Å, $V = 4581.2(1)$ Å^3, $\beta = 95.070(6)°$, $Z = 4$, $\rho_{calcd} = 1.478$ g cm^{-3}, $\mu = 1.340$ mm^{-1}, crystal size $0.10 \times 0.10 \times 0.30$ mm^3, 74011 collected, 37732 unique. $R1 = 0.076$ ($I > 2\sigma(I)$), $wR2 = 0.241$ ($I > 3\sigma(I)$), GOF = 1.517. All calculations were performed by using CrystalStructure software package (Rigaku). Direct methods (SIR2002) were used for the structure solution. The structure was refined by a full-matrix least-squares procedure by using observed reflections based on F^2. All non-hydrogen atoms were refined with anisotropic displacement parameters, and hydrogen atoms were placed in idealized positions with isotropic displacement parameters relative to the connected non-hydrogen atoms and refined in riding geometries. CCDC-602702 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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[13] In the zinc(II) titration of the metal-free ligand **1** (0.5 μM) under the same conditions, the fluorescence emission at 447 nm largely increased ($\lambda_{ex} = 397$ nm), and the change was almost complete upon addition of two equivalents of $Zn(NO_3)_2$. The plot of the emission intensity gave a biphasic titration curve, which was analyzed by nonlinear curve-fitting analysis to estimate the first and second zinc(II) complexation constant to be $> 10^7$ and 1.2×10^7 M^{-1}, respectively.

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[15] In the zinc(II) titration of the metal-free ligand **2** (10 μM in 50 mM HEPES, 50 mM NaCl, pH 7.2/MeOH 1:1) the UV/Vis spectrum changed in an almost identical manner with that observed in **1** up until the addition of one equivalent of zinc(II) (Figure 3a) with an isosbestic point at 333 nm, thus indicating the coordination of zinc(II) to the carbonyl oxygen atom of the ligand **2**. However, further spectral change was not induced upon addition of an excess amount of zinc(II) ions, as observed in the case of **1** (Figure 3b). These results also support that **1**–2 Zn^{II} forms a complex with coordination between both zinc(II) centers and the carbonyl oxygen atom.

[16] In the binding of **1**–2 Zn^{II} with nucleoside pyrophosphates such as ATP, it was proposed that, unlike the monophosphate species, the two phosphate groups simultaneously interact with the Zn^{II}–Dpa sites of **1**–2 Zn^{II}. This theory was supported by a ^{31}P NMR spectroscopic study, in which the signals corresponding to β and γ phosphate of ATP observed at $\delta = -21.1$ and -8.4 ppm shifted to $\delta = -17.7$ and -4.2 ppm, respectively, upon addition of one equivalent of **1**–2 Zn^{II}, whereas the resonance corresponding to the α-phosphate observed at $\delta = -9.7$ ppm scarcely shifted. Similar shifts were also observed in the case of the two phosphates of ADP upon **1**–2 Zn^{II} binding. Such a multipoint binding mode involving the two phosphate groups may reasonably explain the strong binding affinity of **1**–2 Zn^{II} for nucleoside pyrophosphates.

