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A Model for Zn^{II}-Containing-β-Lactamase: Synthesis, X-Ray Crystal Structure of a Zinc(II) Complex Bearing Thiol Group and Hydrolysis of Phosphate Diester

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Abstract—A novel N_3S_1 typed tripodal ligand bearing an SH group, *N*-(mercaptoethyl)-di(2-pyridylmethyl)amine, DPASH, was prepared and its zinc(II) complex, [Zn^{II}(DPAS)Cl], was structurally characterized by X-ray crystallography. [Zn^{II}(DPAS)Cl] promoted hydrolysis of sodium bis(*p*-nitrophenyl)hydrogenphosphate), BNP^-. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Zinc ion is biologically important and has been found in the active site of many enzymes such as carbonic anhydrase,[1] carboxypeptidase,[2] alkaline phosphatase,[3] and β-lactamases.[4] β-Lactamases, for example, are a family of enzymes that hydrolyze the antibiotic agents and metallo-β-lactamases have been recently recognized as potent threat in medicinal community because of their wide spectrum of substrates including carbapenems. More recently, X-ray crystallographic study of *B. fragillis* metallo-β-lactamase by Concha et al.[4c] has shown that this enzyme has a dinuclear zinc active site in which the first zinc is coordinated by the nitrogen atoms of His99, 101, and 162 and by a water/hydroxide with a tetrahedral geometry while the second one is coordinated by a nitrogen of His223, a carboxylate of Asp103, a thiolate of Cys181, and a water molecule and the water/hydroxide, which acts as a bridge between two zinc ions, with trigonal bipyramidal geometry. In this structure, the thiolate is thought to be important for enzymatic activity. Therefore, the structure of active site has been a topic of interest with respect to the discovery or design of the inhibitors against this enzyme in the fields of medicine and pharmacy.[5]

In a model study on Zn^{II}-containing-β-lactamase, Kimura et al.[6] have investigated the role of zinc(II) and the hydrolysis mechanism of β-lactam ring in *Bacillus cereus* β-lactamase II using a zinc(II) complex derived from macrocylic polyamine, 1,4,7,10-tetraazacyclododecane (cyclen). In the metallo-β-lactamase, the tetrahedral zinc center is recognized as the catalytic center but the role of the second trigonal bipyramidal zinc center remains to be answered.

In order to determine the role of sulfur and the function of zinc(II) ion as a Lewis acid, we synthesized an N_3S_1 typed tripodal ligand bearing a mercaptoethyl group, *N*-(2-mercaptoethyl)-di(2-pyridylmethyl)amine, **DPASH**. To our knowledge, only a few of such tripodal ligand with N_3S_1 donor set have been reported.[7] We described here the first synthesis of **DPASH**, where H denotes a dissociable proton, and of its zinc(II) complex, chloro{*N*-mercaptoethyl-di(2-pyridylmethyl)amine}zinc(II), [Zn^{II}(DPAS)Cl], and the ability of hydrolysis of diphosphoester, BNP^- along with that of dichloro{di(2-pyridylmethyl)amine = DPA}zinc(II) complex, [Zn^{II}(DPA)Cl_2][8] (Chart 1).

Results and Discussion

Syntheses

The synthetic routes of DPASH and [Zn^{II}(DPAS)Cl] are shown in Scheme 1. The tripodal ligand, **DPASH**,[9] was synthesized by the reaction of di(2-pyridylmethyl)amine[10] with 3 equiv of ethylene sulfide in dry toluene at 50 °C for 4 days under argon. The solvent was evaporated under reduced pressure and the resulting residue was purified by column chromatography with silica gel (eluent, AcOEt: *i*-PrOH = 1:1) to give **DPASH** as a yellow oil in 50% yield.

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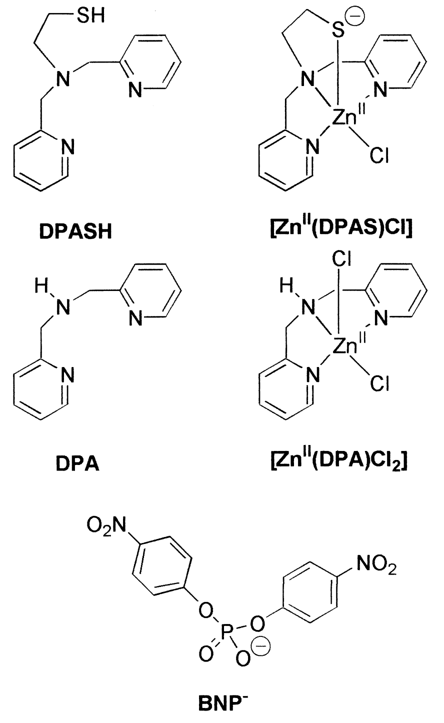


Chart 1.

The zinc(II) complex, [Zn^{II}(DPAS)Cl],[11] was prepared by mixing a solution of **DPASH** with equimolar amounts of $ZnCl_2$ and NaOH in methanol at room temperature. The insoluble materials formed were filtered off and the filtrate was concentrated in vacuo. The resulting residue was recrystallized from 1:1 methanol:water to give [Zn^{II}(DPAS)Cl] as colorless crystals in 45% yield.

The molar conductance at 25 °C of a 0.1 mM solution of the zinc(II) complex, [Zn^{II}(DPAS)Cl], in water is 3.1×10^{-2} S m^2/mol, indicating that a chloride ion might be displaced by a solvent water molecule to yield [Zn^{II}(DPAS)(H_2O)]$^+$ complex as a 1:1 electrolyte.

X-ray crystal structure

The structure of [Zn^{II}(DPAS)Cl] was determined by an X-ray analysis.[12] The ORTEP drawing of [Zn^{II}(DPAS)Cl] is shown in Figure 1. The complex is neutral molecule in the solid state. The geometry around zinc(II) is a trigonal bipyramid with τ^{13} value of 0.82 ($\tau=(\beta-\alpha)/60$, where β = Cl(1)-Zn-N(1); 167.59(9)° and α = S(1)-Zn-N(2); 118.68(10)°: τ is zero for an ideal square pyramid, whereas $\tau=1$ for an ideal trigonal bipyramid). The zinc atom is located above 0.409 Å from the trigonal plane defined by two pyridine nitrogen atoms, N(2) and N(3), and the thiolate ion S(1). The apical coordination sites are occupied by the tertiary amine N(1) and chloride ion Cl(1). This structure is different from that of the related zinc(II) complex, [Zn^{II}(DPA)Cl_2],[8] which takes a distorted square pyramid with τ^{13} value of 0.15. The average Zn-N$_{\text{pyridine}}$ (N(2) and N(3)) bond lengths of 2.122 Å are shorter than those found in [Zn^{II}(DPA)Cl_2][8] of 2.169 Å while the Zn-N(1) and Zn-Cl bond lengths of 2.313(3) and 2.337(1) Å respectively are longer than those of [Zn^{II}(DPA)Cl_2][8] (2.161(6) Å for Zn-N and 2.275(1) and 2.265(Å) for Zn-Cl). The Zn-S bond length of 2.303(1) Å is within normal range as compared to the other zinc complexes.[14] The angles of S(1)-Zn-N(2), S(1)-Zn-N(3), and N(2)-Zn-N(3) are nearly 120°, while the angle of Cl(1)-Zn-N(1) is 167.59(9)°. The conformations of five-membered chelate rings formed by coordination of thiol group to zinc are the same with either the δ,δ or λ,λ form. As a result, the dihedral angle between two pyridine rings increased from 14.05° for [Zn^{II}(DPA)Cl_2][8] to 47.57°.

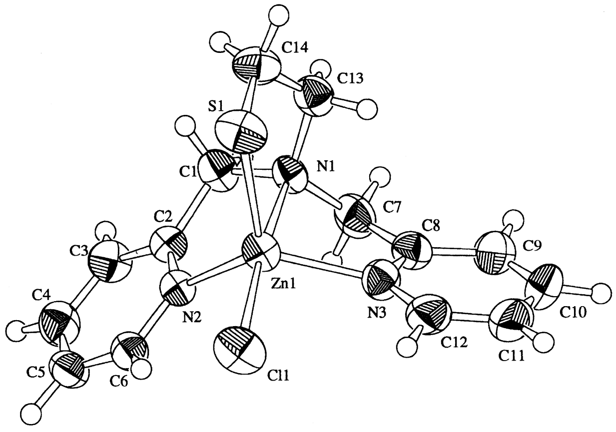
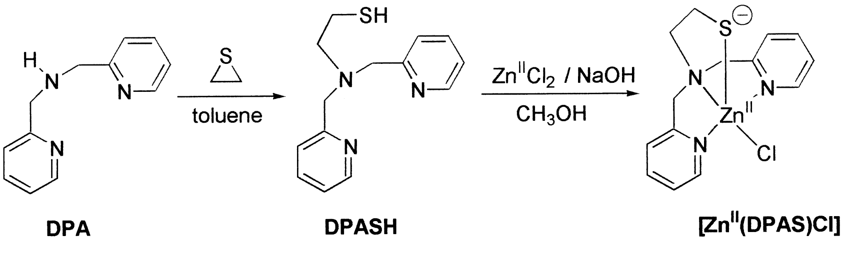


Figure 1. ORTEP drawing of [Zn^{II}(DPAS)Cl], showing with 50% probability thermal ellipsoids. Selected bond lengths (Å) and angles (°): Zn(1)-Cl(1) 2.337(1), Zn(1)-S(1) 2.303(1), Zn(1)-N(1) 2.313(3), Zn(1)-N(2) 2.113(3), Zn(1)-N(3) 2.130(4), Cl(1)-Zn(1)-S(1) 106.57(5), Cl(1)-Zn(1)-N(1) 167.59(9), Cl(1)-Zn(1)-N(2) 98.6(1), Cl(1)-Zn(1)-N(3) 97.0(1), S(1)-Zn(1)-N(1) 85.80(9), S(1)-Zn(1)-N(2) 118.7(1), S(1)-Zn(1)-N(3) 116.0(1), N(1)-Zn(1)-N(2) 75.6(1), N(1)-Zn(1)-N(3) 76.1(1), N(2)-Zn(1)-N(3) 115.0(1).

Potentiometric pH-titrations

The protonation constants (K_n) of DPASH were determined by potentiometric pH-titration of DPASH·3HCl (1 mM) at 25 °C and $I=0.10$ M ($NaNO_3$). A typical titration curve is shown in Figure 2a. The computed pK_a values ($=-\log K_1 \sim -\log K_4$) are 9.2 ± 0.2 (SH), 5.0 ± 0.1 (tertiary amine), and <3 (pyridines) and the pK_a value



Scheme 1.

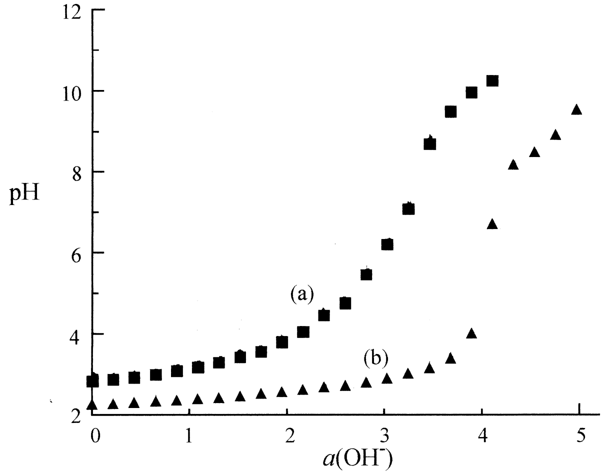


Figure 2. pH titration curves of triprotonated ligand DPASH·3HCl in the absence and presence of equimolar Zn^{II} at 25 °C and I = 0.10 M ($NaNO_3$). Key: (a) 1.0 mM DPASH·3HCl; (b) (a) + 1.0 mM $Zn^{II}(NO_3)_2 \cdot 6H_2O$.

of the thiol group is lower than that of ethanethiol (pK_a 10.61).[15] The 1:1 DPASH·3HCl-Zn^{II} titration curve is shown in Figure 2b where two inflections at $a = 4$ and 5 were observed. The first buffer region until $a = 4$ is ascribed to the loss of four protons from two pyridine nitrogens, a tertiary amine, and a thiol group. The second buffer region at $4 < a < 5$ represents the removal of a proton from the Zn^{II}-bound water molecule at the fifth site with a pK_a value of 8.6 ± 0.1, which is lower than that of $Zn^{II}(H_2O)_6^{2+}$ ($pK_a = 9.5$).[16] Under the same conditions, the pK_a of the coordinated water molecule of $[Zn^{II}(DPA)Cl_2]$ complex is 9.0 ± 0.1, indicating that thiolate anion in DPASH slightly lowers the pK_a of the Zn^{II}-bound water molecule and the zinc(II) is acting as Lewis acid.

Cleavage of phosphate diester

In order to determine whether hydrolysis of phosphodiester, BNP^-, is promoted by $[Zn^{II}(DPAS)Cl]$ or $[Zn^{II}(DPA)Cl_2]$, hydrolysis of BNP^- (15 mM) in 10% MeOH (v/v) aqueous solution of Zn^{II} complex (1.25 mM) at pH 6.0–10.5 (20 mM HEPES or CHES buffer) at 35 °C and I = 0.10 M ($NaNO_3$) was monitored by following the increase in the visible absorption at 400 nm due to the release of 4-nitrophenolate.[17] The initial rate increases linearly with the concentration of BNP^-. Therefore, v is expressed as eq (1).

$$v_{\text{init}} = k_{\text{obs}}[BNP^-] \qquad (1)$$

The observed initial rates, v_{init}, for $[Zn^{II}(DPAS)Cl]$ and $[Zn^{II}(DPA)Cl_2]$ at the concentration of $[BNP^-] = 15$ mM and the [Zn complex] = 1.25 mM are plotted as a function of pH (Fig. 3). Both reactions exhibited sigmoidal pH-rate profiles with inflection points at pH 8.9 for $[Zn^{II}(DPAS)Cl]$ and at pH 9.2 for $[Zn^{II}(DPA)Cl_2]$. These results are in good agreement with pK_a values obtained from potentiometric pH-titrations. At pH 10.0, $[Zn^{II}(DPAS)Cl]$ and $[Zn^{II}(DPA)Cl_2]$ gave rates of $(1.4 \pm 0.4) \times 10^{-9}$ Ms^{-1} and $(9.2 \pm 0.3) \times 10^{-9}$ Ms^{-1}. Under the same conditions, $Zn^{II}Cl_2$ gave $(1.7 \pm 0.3) \times 10^{-10}$ Ms^{-1}. Therefore, $[Zn^{II}(DPAS)Cl]$ and $[Zn^{II}(DPA)Cl_2]$ hydrolyzed BNP^- 8.4 and 55 times faster than $Zn^{II}Cl_2$, respectively.

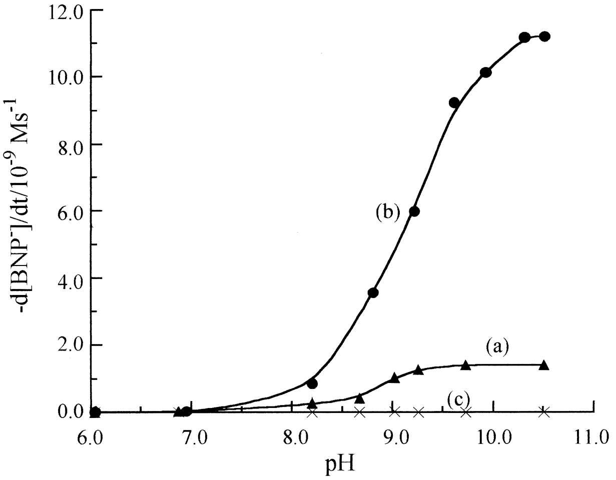


Figure 3. pH rate profile for the rate of hydrolysis of BNP^- (15 mM) at 35 °C and I = 0.10 M ($NaNO_3$) in 10% (v/v) MeOH: (a) 1.25 mM of $[Zn^{II}(DPAS)Cl]$; (b) 1.25 mM of $[Zn^{II}(DPA)Cl_2]$; (c) background BNP^-.

The rate of hydrolysis of both Zn^{II} complexes increased linearly with the concentration of the complexes at low concentration range but the magnitude of slope was gradually decreased at the higher concentration range as shown in Fig. 4. These results suggest that an equilibrium between monomers and a dimer exists in solution as shown in Scheme 2 and the monomer is probably an active species for hydrolysis. Such equilibrium in solution has been also observed in analogous Cu^{II} complex in which the thiol group in DPASH is replaced with an alcohol.[18]

If we assume that the monomeric Zn^{II} complex with hydroxide coordinated at the fifth site (M) is exclusively the catalytic active species, the concentration of M is expressed by

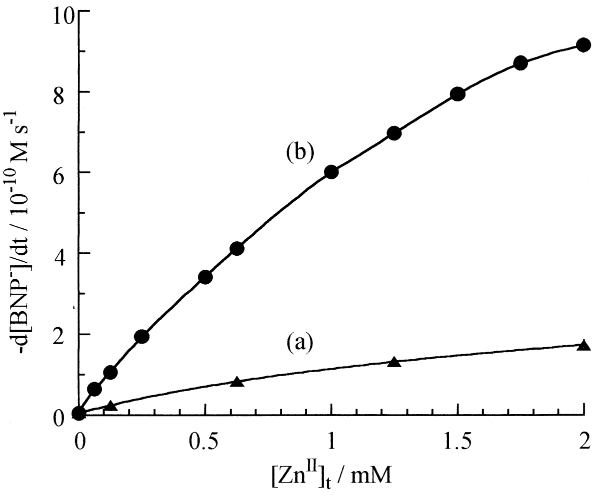
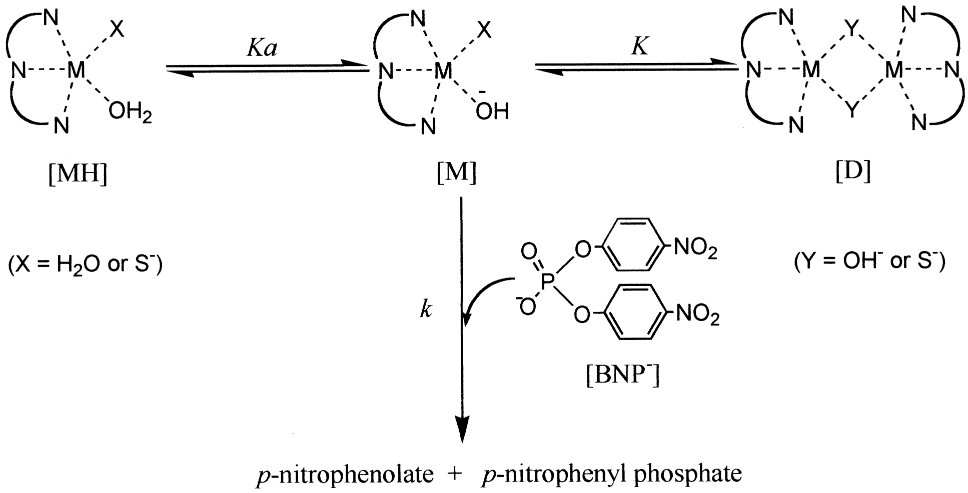


Figure 4. Dependence of the rate for the hydrolysis of BNP^- (1.25 mM) on the concentration of $[Zn^{II}(DPAS)Cl]$ (a) or $[Zn^{II}(DPA)Cl_2]$ (b) at 35 °C and I = 0.10 M ($NaNO_3$) in 10% (v/v) MeOH.



Scheme 2.

$$[M] = \left\{\left((1 + [H^+]/K_a)^2 + 8K[Zn^{II}]_t\right)^{1/2} - (1 + [H^+]/K_a)\right\}/4K$$

where $K = [\text{Dimeric species}]/[M]^2$, $K_a = [M][H^+]/[MH]$, and $[Zn]_t = [MH] + [M] + 2[\text{Dimeric species}]$. The rate expression can be written by

$$v = k_{obs}[BNP^-] = k\,[M][BNP^-]$$

and the pseudo-first order rate constant, k_{obs}, is given in eq (2), where $[Zn^{II}]_t$, $[H^+]$, K, and K_a represent the total zinc complex concentration, the proton concentration, apparent dimerization constant, and acid dissociation constant, respectively.

$$k_{obs} = k\left\{\left((1 + [H^+]/K_a)^2 + 8K[Zn^{II}]_t\right)^{1/2} - (1 + [H^+]/K_a)\right\}/4K \tag{2}$$

A non linear least-squares fitting[19] of the data using eq (2) gave K values of 762 ± 4 and 316 ± 6 M^{-1} and k values of $(1.5 \pm 0.2)\times10^{-4}$ and $(5.8 \pm 0.3)\times10^{-4}$ $M^{-1}s^{-1}$, and pK_a values of 9.1 ± 0.1 and 9.4 ± 0.2 for $[Zn^{II}(DPAS)Cl]$ and $[Zn^{II}(DPA)Cl_2]$, respectively. The curves based on these values are included as solid curves in Figure 4.

The dimerization constant of $[Zn^{II}(DPAS)Cl]$ with the mercaptoethyl group is about two times larger than that of $[Zn^{II}(DPA)Cl_2]$, indicating that the dimerization occurs readily as compared to $[Zn^{II}(DPA)Cl_2]$. $[Zn^{II}(DPAS)Cl]$ is about four times less reactive than $[Zn^{II}(DPA)Cl_2]$ due to the decreased overall charge or steric effect of mercaptoethyl substituent. Furthermore, these pK_a values obtained by fitting the concentration-rate profiles are in agreement with those obtained from pH-rate profiles.

Finally, in order to test whether Zn^{II} complex can hydrolyze a β-lactam antibiotic agent, nitrocefin, preliminary kinetics studies were carried out under the conditions; the concentrations of the complex and nitrocefin are 4.18 μM, 20 mM CAPS buffer (pH 9.0) at 35 °C and $I = 0.10$ M ($NaNO_3$). The obtained rates of hydrolysis of nitrocefin by Zn^{II} complexes are $(6.5 \pm 0.4)\times10^{-9}$ Ms^{-1} for $[Zn^{II}(DPAS)Cl]$ and $(25.2 \pm 0.2)\times10^{-9}$ Ms^{-1} for $[Zn^{II}(DPA)Cl_2]$ compared with a rate of $(3.4 \pm 0.2)\times10^{-9}$ Ms^{-1} for a control experiment. It seems that the intermolecular nucleophilic attack will not be enhanced since both Zn^{II}-bound water/hydroxide ion and carbonyl group of β-lactam ring of antibiotic agent can not simultaneously coordinate to Zn^{II} ion in $[Zn^{II}(DPAS)Cl]$.

In summary, a novel Zn^{II} complex bearing a mercaptoethyl group has been prepared and found to hydrolyze a diphosphoester, BNP^-. The rate of Zn^{II}-promoted hydrolysis of BNP^- increases with the increase in pH, indicating that zinc-bound hydroxide is an active species for the hydrolysis of BNP^-. The thiol group in DPASH molecule contributes to stabilization of the dimer in solution but decreases the hydrolytic activity, compared with non-substituted $[Zn^{II}(DPA)Cl_2]$ complex.

Finally, the major role of the thiolate coordination will be structural stabilization rather than enzymatic activity.

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9. ^{1}H NMR(CDCl$_3$): δ 1.62 (br, 1H), 2.68 (t, $J=5.5$ Hz, 2H), 3.82 (t, $J=5.5$ Hz, 2H), 3.86 (s, 4H), 7.16 (t, $J=5.5$ Hz, 2H), 7.54 (d, $J=7.9$ Hz, 2H), 7.66 (t, $J=6.1$ Hz, 2H), 8.52 (d, $J=5.3$ Hz, 2H). ^{13}C NMR (CDCl$_3$) δ: 22.5, 57.1, 60.2, 122.1, 123.1, 136.5, 149.1, 1593.2. MS(M$^+$): *m*/*z* 258.

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11. ^{1}H NMR (CD$_3$OD): δ 2.75 (t, $J=5.5$ Hz, 2H), 2.84 (t, $J=5.5$ Hz, 2H), 4.00 (AB, $J=5.5$ Hz, 2H), 4.25 (AB, $J=16.5$ Hz, 2H), 7.63 (d, $J=7.9$ Hz, 2H), 7.66 (t, $J=5.5$ Hz, 2H), 8.12 (t, $J=6.1$ Hz, 2H), 8.86 (d, $J=5.3$ Hz, 2H). Anal. calcd for $C_{14}H_{16}N_3S_1Cl_1Zn_1$: C, 46.81; H, 4.49; N, 11.70. Found: C, 46.85; H, 4.44; N, 11.47

12. Crystal data for [Zn^{II}(DPAS)Cl]: monoclinic, space group $P2_1/c$, $a=13.077(3)$, $b=7.338(3)$, $c=15.815(1)$ Å, $\beta=96.25(1)$ °, $V=1508.5(6)$ Å^3, and $Z=4$; 3900 measured reflections, 1906 with $I>3.0\sigma(I)$, 182 refined parameters, $R=0.038$, $R_w=0.029$. Intensity measurement: Rigaku AFC-7R diffractometer, Mo K_α radiation, graphite monochromator, ω-2θ scan, $2\theta_{max}=55$ °, $T=20\pm1$ °C. The structure was solved by direct methods. Non-hydrogen atoms were refined anisotropically and all the hydrogen atoms were included in the models at their calculated positions but were not refined. Atomic coordinates, bond angles, bond lengths and thermal parameters have been deposited at the Cambridge Crystallographic Data Center in CIF file. CCDC number: 144305.

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19. The second-order rate constant *k*, apparent dimerization constant, and dissociation constant were obtained by fitting the experimental data according to eq (2) using non-linear least-square curve fitting program (Kaleida Graph Inc.).