J. Chem. Sci., Vol. 117, No. 2, March 2005, pp. 175–178.

Biocatalysis by metallated cyclotriphosphazenes: $L_2Zn(NO_3)_2$ {L = *spiro*-$N_3P_3[O_2C_{12}H_8][N(CH_3)NH_2]$} as a synthetic phosphoesterase and nuclease

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Abstract. Catalytic activity of $[L_2.Zn][NO_3]_2$ (L = *spiro*-$N_3P_3[O_2C_{12}H_8][N(CH_3)NH_2]$) towards the hydrolysis of two phosphodiesters, [bis(*p*-nitrophenyl)phosphate, bNPP] and [2-(hydroxypropyl)-*p*-nitrophenyl phosphate, hNPP] has been examined. While the rate of hydrolysis of the former is accelerated over a million-fold, the rate of hydrolysis of the latter also is enhanced considerably. Detailed kinetic evaluation of these reactions has been carried out and all the kinetic parameters including the Michaelis–Menten parameters are reported. The catalyst $[L_2.Zn][NO_3]_2$ has also been found to be an effective nuclease. Relaxation of supercoiled plasmid DNA, pBR322, occurs in presence of $[L_2.Zn][NO_3]_2$ without the need for any exogenous reagents.

Keywords. Cyclophosphazene hydrazides; metalated cyclophosphazenes; zinc complexes; phosphate ester hydrolysis; DNA cleavage.

1. Introduction

Cyclophosphazenes are an important class of inorganic heterocyclic ring systems containing a $[N=PR_2]$ repeating unit. They are excellent precursors for the construction of multi-site coordination ligands due to their robust framework and reactive periphery.[1–4] Thus, the replacement of the P–Cl bond in $N_3P_3Cl_6$ by appropriate substituent-containing donor atoms can afford a library of diverse ligand systems. We have carried out extensive work on pyrazolyl cyclophosphazenes, $N_3P_3(3,5\text{-}Me_2Pz)_6$ and *gem*-$N_3P_3Ph_2(3,5\text{-}Me_2Pz)_4$.[3,5] Subsequently, we have incorporated the pyrazolylcyclotriphosphazene structural motif as a pendant group in a cross-linked polymer, CPPL, and have shown its utility in binding Cu(II) and Zn(II).[6–8] Recently, we demonstrated that cyclophosphazene hydrazides are excellent ligands for transition metal ions.[9] In this paper we report the catalytic activity of $L_2Zn(NO_3)_2$ {L = *spiro*-$N_3P_3[O_2C_{12}H_8][N(CH_3)NH_2]$}(**1**). In order to test the potential of **1** as a homogeneous catalyst we have chosen to use it in the hydrolysis of phosphate esters and in DNA cleavage experiments. Phosphate ester hydrolysis plays a very important role in energy metabolism and in various cellular signal transduction pathways in biological systems.[10] A number of phosphoesterases require two or more metal ions for their catalytic activity in these reactions. In recent years, there has been considerable interest in the design of synthetic models that can function as catalysts for this biologically important reaction.[11–16] In view of this we have evaluated **1** as a catalyst in the hydrolysis reaction of two substrates: a phosphodiester, [*bis*(*p*-nitrophenyl)phosphate, bNPP], and an RNA model phosphodiester, [2-(hydroxypropyl)-*p*-nitrophenyl phosphate, hNPP]. In order to test whether **1** would also be useful as a catalyst for the cleavage of natural substrates, we have chosen to study the cleavage of pBR322, a supercoiled plasmid DNA. These results are presented in this paper.

Dedicated to the memory of the late Professor Bhaskar G Maiya
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2. Experimental

2.1 *Synthesis of $L_2Zn(NO_3)_2$ {L = spiro-$N_3P_3[O_2C_{12}H_8][N(CH_3)NH_2]_4$} (**1**)*

Compound **1** was synthesized as reported previously.[9,17]

2.2 *Kinetics*

All hydrolytic reactions were performed in duplicate, in centrifuge tubes thermostated at 40°C unless

otherwise mentioned. The assay mixture contained 3 ml of the substrate prepared in 0·01 M N-ethylmorpholine (pH 8·0), in 80% aqueous buffer (20% DMF). The reference cell contained substrate solution without compound **1** to correct the background hydrolysis. The concentration of compound **1** was kept at 1 mM for all assays. Control experiments revealed that unmetalated ligand failed to assist phosphate ester cleavage reaction. The progress of cleavage reactions was monitored spectrophotometrically (Shimadzu UV-160) from the time-dependent release of *p*-nitrophenolate anion (l_{max} = 400 nm, $å = 1{\cdot}65 \times 10^4\ M^{-1}\ cm^{-1}$). Michaelis–Menten parameters were determined using the corresponding Lineweaver–Burk plots. Pseudo-first-order rate constants were obtained from $\ln A_\infty/A_\infty - A_t$ vs time plots. All hydrolytic reactions were performed at least over eight half-lives for each of the substrates.

2.3 *pBR322 hydrolysis assay*

All cleavage reactions were performed in 20 ìl of sodium cacodylate buffer (10 mM, pH 7·5, 35°C). Supercoiled pBR322 (0·16 g) and compound **1** (1 mM) were mixed and incubated at 35°C. The reactions were terminated by adding gel-loading buffer (100 mM EDTA, 50% glycerol in Tris-HCl, pH 8·0, 5 ìl) followed by arresting the reaction in liquid nitrogen and storing at –20°C. Samples (6 ìl) were loaded on 0·7% agarose gel containing ethidium bromide (1 ìg/1 ml) and electrophoresced for 1 h at constant current (70 mA), in 0·5 × TBE (*tris*(hydroxymethyl)aminomethane : triboric acid : EDTA (1 : 1 : 0·2)) buffer. The gel was destained for 4 h in distilled water before imaging on PC-interfaced Bio-Rad Gel Documentation System 2000.

3. Results and discussion

3.1 *Hydrolysis studies of bNPP and hNPP with L_2Zn (**1**)*

Reaction of $N_3P_3[O_2C_{12}H_8][N(CH_3)NH_2]_4$ (L) with $Zn(NO_3)_2$. afforded $[L_2Zn][NO_3]_2$ (**1**). Synthesis and structural characterization of **1** have been reported by us previously.[9,17] Two molecules of the cyclophosphazene hydrazide are involved in coordination to the metal ion. Each cyclophosphazene binds to the zinc ion through a ring nitrogen atom and two non-geminal NH_2 (hydrazine) nitrogen atoms. The coordination geometry around zinc is approximately octahedral. The full details of the X-ray crystal structure of **1** are found elsewhere.[9] The molecular structure of the compound is as shown in chart 1.

The hydrolysis reactions of two model substrates, *bis*(*p*-nitrophenyl)phosphate (bNPP) and 2-hydroxypropyl-*p*-nitrophenyl phosphate (hNPP) (chart 2) were studied to screen the utility of **1** in phosphoesterase activity. While bNPP is a model for the phosphodiester substrate, hNPP is a phosphodiester as well as an RNA model in that the 2′–OH position of the latter is structurally mimicked. Hydrolysis of these substrates aided by **1** was done in 80% aqueous (20% DMF) 0·01 M N-ethylmorpholine buffer at pH 8·0, by the time-dependent release of *p*-nitrophenolate anion at 400 nm ($e = 1{\cdot}65 \times 10^4\ M^{-1}\ cm^{-1}$).

Rate constants for the hydrolysis of these substrates by **1** were determined under pseudo-first-order conditions. The hydrolysis proceeded with remarkable rate enhancements for bNPP and hNPP over the respective uncatalysed reactions. We used 10 mM of bNPP and 3 mM of hNPP as substrate concentrations to determine the pseudo-first-order rate constants. The pseudo-first-order rate constants (k_{obs}) were determined from $\ln A_\infty/A_\infty - A_t$ versus time plots (figures 1 and 2). For bNPP, a 2-million fold rate enhance-

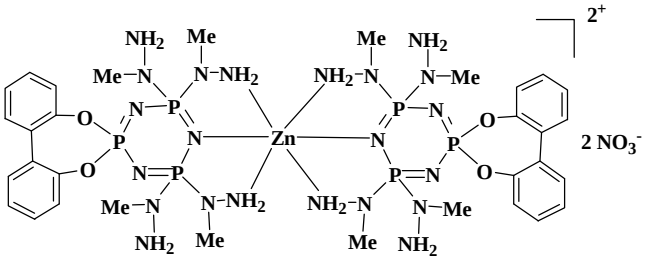

Chart 1. The molecular structure of the compound **1**.

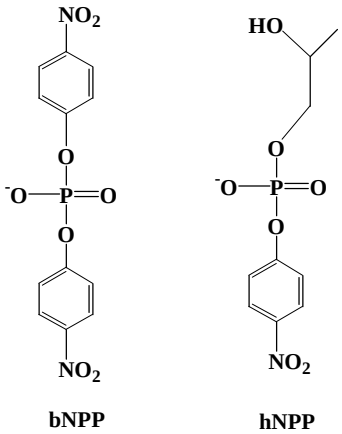

Chart 2. Structures of bNPP and hNPP.

ment was observed in comparison to the uncatalysed reaction. These results compare well with the synthetic phosphotases (for the hydrolysis of bNPP).[18–20] The rate enhancement for hNPP, was 4×10^3 times that of the uncatalysed reaction (table 1).

The Michaelis–Menten constant (K_m), maximal velocities (V_{max}) and turnover number (k_{cat}) were derived for both the substrates from the corresponding Lineweaver–Burk plots, $1/[S]$ vs $1/V_i$ (figures 3 and 4). Kinetics for the hydrolysis of bNPP and hNPP were determined in 80% N-ethylmorpholine buffer (pH 8·0) (20% DMF) at 40°C and 30°C respectively. The other experimental conditions relating to the data plotted in these figures are summarized in table 2. The K_m, V_{max}, and k_{cat} for bNPP hydrolysis were

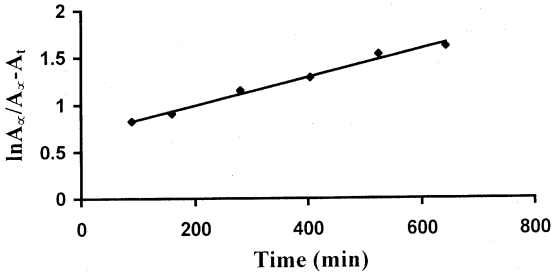

Figure 1. Plot of $\ln A_\infty/A_\infty - A_t$ vs time for the hydrolysis of bNPP.

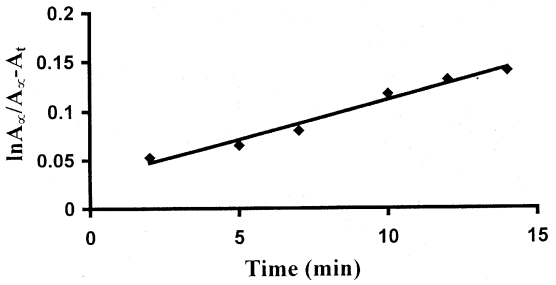

Figure 2. Plot of $\ln A_\infty/A_\infty - A_t$ vs time for the hydrolysis of hNPP.

Table 1. Pseudo-first-order rate constants[a] for bNPP and hNPP hydrolysis catalyzed by **1**.

Substrate	k_{obs} (min^{-1})	k_{uncat} (min^{-1})	k_{rel}
bNPP	$1{\cdot}492 \times 10^{-3}$	$7{\cdot}8 \times 10^{-10}$	$1{\cdot}923 \times 10^{6}$
hNPP	$7{\cdot}999 \times 10^{-3}$	$1{\cdot}979 \times 10^{-6}$	$4{\cdot}042 \times 10^{3}$

[a]All hydrolytic reactions were performed in duplicate in 0·01 M 80% aqueous N-ethylmorpholine buffer (pH = 8·0) (20% DMF), in centrifuge tubes thermostatted at 40°C for bNPP and 30°C for hNPP. Concentration of bNPP was 10 mM, hNPP was 3 mM and of **1** was 1 mM and the total reaction volume was 3 ml. Reference cell contained substrate solution without **1**

4·28 mM, $3{\cdot}55 \times 10^{-5}$ mM min^{-1} and $3{\cdot}52 \times 10^{-5}$ min^{-1}. The corresponding values for hNPP hydrolysis were found to be 1·09 mM, $29{\cdot}15 \times 10^{-4}$ mM min^{-1} and $29{\cdot}24 \times 10^{-4}$ min^{-1} respectively (table 2).

To determine the effect of pH over bNPP hydrolysis, a broad range of pH was used in the hydrolysis experiments. Figure 5 (pH vs k_{obs}) clearly indicates that the rate of reaction slowly increases with increase in the pH of the buffer, with the maximum rate being attained at pH 8·3. Subsequently, the rate decreases with increasing the pH, and the overall plot resembles a bell shape curve.[21–23]

The efficient catalytic activity of **1** towards the model phosphate esters prompted us to evaluate its

Table 2. Michaelis–Menten parameters[a] for bNPP and hNPP substrates with **1**.

Substrate	K_m (mM)	V_{max} (mM min^{-1})	k_{cat} (min^{-1})
bNPP	4·28	$3{\cdot}55 \times 10^{-5}$	$3{\cdot}52 \times 10^{-5}$
hNPP	1·09	$29{\cdot}15 \times 10^{-4}$	$29{\cdot}24 \times 10^{-4}$

[a]All hydrolytic reactions were performed in duplicate in 0·01 M, 80% N-ethylmorpholine buffer (pH = 8·0) (20% DMF), in centrifuge tubes thermostatted at 40°C for bNPP and 30°C for hNPP hydrolysis. Concentrations for bNPP were 3–10 mM, and for hNPP concentrations were 0·31–0·72 mM, concentration of **1** was 1 mM in 3 ml of total reaction volume. The reference cell contained substrate solution without **1** for correcting the background hydrolysis

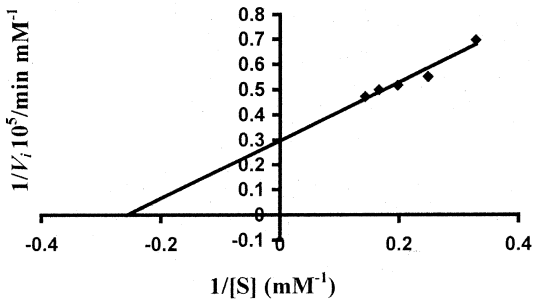

Figure 3. Lineweaver–Burk plot for bNPP hydrolysis.

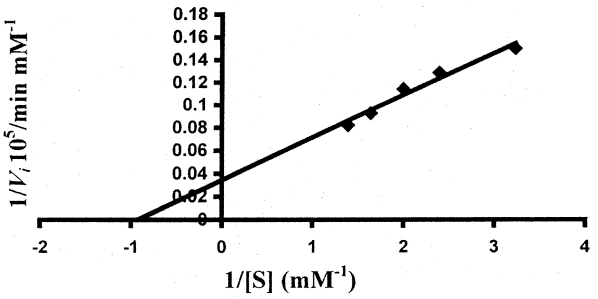

Figure 4. Lineweaver–Burk plot for hNPP hydrolysis.

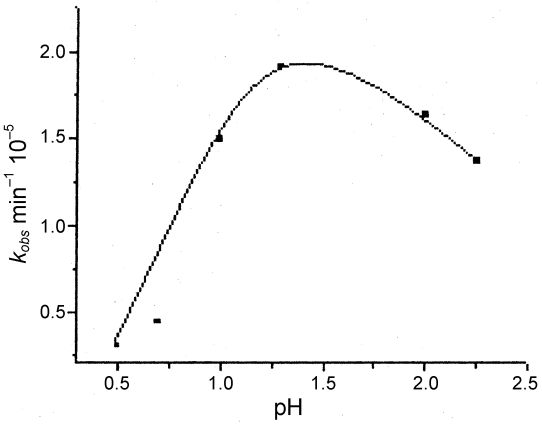

Figure 5. pH vs k_{obs} plot for the hydrolysis of bNPP by compound **1**.

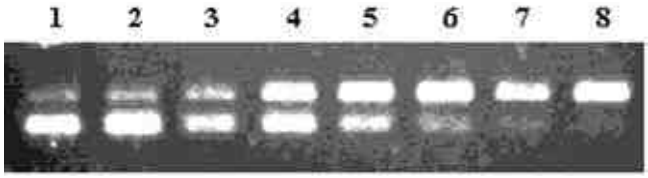

Figure 6. pBR322 cleavage with **1** (lane 1: DNA alone; lane 2: DNA (10 h); lane 3: DNA + **1** (1 h); lane 4: DNA + **1** (2 h); lane 5: DNA + **1** (4 h); lane 6: DNA + **1** (6 h); lane 7: DNA + **1** (8 h); lane 8: DNA + **1** (10 h)).

activity for cleavage of the natural phosphodiester substrate, pBR322 supercoiled DNA. Time course experiments were performed to find the optimal time for DNA hydrolysis by using 1 mM of compound **1**, in the *absence* of exogenously added co-oxidants. Figure 6 indicates the gradual conversion of supercoiled plasmid DNA (form I) to the linearized form (form III). This process is completed in 10 h at 35°C. Although the mechanistic details of the DNA cleavage have not yet been fully elucidated, based on literature precedents, we believe that the zinc complex **1** catalyses DNA cleavage through the hydrolytic pathway. It is well documented in the literature that zinc complexes catalyse DNA cleavage preferentially through the hydrolytic pathway.[24]

4. Conclusion

We demonstrate, for the first time, the utility of metalated cyclophosphazenes for biocatalysis. Thus, the zinc complex {*spiro*-$N_3P_3[O_2C_{12}H_8][N(CH_3)NH_2]_4Zn\}\{NO_3\}_2$ (**1**) has been found to be quite effective as a homogeneous catalyst and functions as a phosphoesterase as well as a nuclease. Further experiments are in progress to evaluate other cyclophosphazene-based metal complexes for this activity.

Acknowledgement

We thank the Council of Scientific and Industrial Research, New Delhi for financial support.

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