



## Phosphorus, Sulfur, and Silicon and the Related Elements



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### Hydrolysis of phosphate esters in aqueous solution promoted by cobalt(III)-tetraamine metallohydrolases: Chromatographic and spectroscopic studies

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#### ABSTRACT

A combination of chromatographic and spectroscopic (Q-ToF/MS; FTIR) studies were conducted to further understand hydrolytic processes in reactions of phosphate mono- and phosphodiesters with model Cobalt(III)-tetraamine metallohydrolases of the type  $[CoL(OH)(OH_2)]^{2+}$ ;  $L = (en)_2$ ,  $(tn)_2$ ; en = 1,2-diaminoethane, tn = 1,3-diaminopropane.

High resolution mass spectroscopic (HRMS) analysis of the reaction fragments indicates that the monoester substrates undergo initial coordination to the metal center followed by an intramolecular hydroxyl attack on phosphorus, a process that is concerted with liberation of the bound alcoholic leaving group. The  $Co^{III}(tn)_2$  complex displays twice as more reactivity towards both mono- and phosphodiesters compared to the  $(en)_2$  analog under similar conditions, a behavior that is discussed in terms of the structural differences in the coordinated amine ligands. Plausible mechanisms for Co(III)-promoted hydrolysis of both the mono- and phosphodiesters are proposed.

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Metallohydrolase; UHPLC; Q-ToF/MS; phosphate monoester; phosphodiester; hydrolysis

#### **GRAPHICAL ABSTRACT**



### Introduction

Chemical reactions that involve the formation or cleavage of P-O bonds in phosphate esters are of fundamental prominence in life processes.<sup>[1]</sup> The transfer of phosphoryl groups from one entity to another is of crucial importance in nature. Indeed, the regulation of virtually all cellular processes including the biosynthesis of macromolecules such as proteins, RNA and DNA, energy production, signal transduction and the control of protein activity is dependent upon the phosphorylation and dephosphorylation of various different biomolecules.<sup>[2,3]</sup> The reaction mechanisms that lead to the cleavage of the phosphoester bond are divided into two major classes; those mechanisms that proceed via the hydrated  $PO_3^{-}$  anion (metaphosphate) are called *dissociative* (Equation 1), whereas mechanisms that require formation of an intermediate or transition state with pentacovalent phosphorus (phosphorane) belong to the family of associative mechanisms (Equation 2).[1,4,5]

$$HPO_4^{2-} \rightleftharpoons PO_3^{-} + OH^{-}$$
(1)

$$HPO_4^{2-} + OH^{-} \rightleftharpoons H_2PO_5^{3-}$$
(2)

The hydrolysis of phosphate esters is largely complicated by the possible involvement of neutral or charged substrate species including monoanionic and dianionic phosphate esters. Furthermore, these substrates can be attacked by H<sub>2</sub>O and OH<sup>-</sup> nucleophiles in solution.<sup>[6-8]</sup> Several plausible mechanisms have been advanced in the literature for Phosphoryl Transfer Reactions (PTRs).<sup>[9-11]</sup> The potential for both solvent and substrate-assisted pathways, which involve proton transfer to the phosphoryl oxygens, further complicates the interpretation of available experimental and computational tools.<sup>[12]</sup> These structural (X-ray structure analysis), spectroscopic (UV-vis; 31P NMR) and computational (Linear Free Energy Relations, LFER's; Kinetic Isotope Effects, KIE's; Entropic Effects - EF) tools have been collectively used to study the reaction pathways and the accumulated data has provided details of [PO<sub>3</sub>]<sup>-</sup> transfer at atomic resolution.<sup>[9,12,13]</sup>

Recently, we reported<sup>[14]</sup> on the uncatalyzed hydrolysis reactions of the phosphate monoesters, phenyl phosphate (PP) and 4-nitrophenyl phosphate (NPP), and the phosphodiesters, diphenyl phosphate (DPP) and bis-4-nitrophenyl

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phosphate (BNPP) probed by chromatographic and spectroscopic techniques. Here, we demonstrated, for the first time and using High Resolution Mass Spectrometry (HRMS; Q-ToF-MS), that under the experimental conditions of pH 7.0;  $H_2O_1$  25 °C, the monoesters react dissociatively to yield the free monomeric metaphosphate,  $[PO_3]^- - m/z$  78.9976,  $(E_r = -0.0266 \text{ Da})$ . It was further demonstrated that in the absence of an unhindered nucleophile, this reactive species is transferred to the solvent, water, with concerted proton transfer to the phosphate, producing predominantly othorphosphoric acid,  $H_3PO_4 - m/z$  97.9954,  $(E_r = -0.0014 \text{ Da})$ . These findings were found to be consistent with reported theoretical studies (KIE's, LFER's) that have been reported for aqueous reactions of these compounds.<sup>[13,15]</sup> As a continuation of our investigation on phosphate ester hydrolysis, we report here the hydrolysis of some phosphate mono- and phosphodiesters (PP, NPP, DPP and BNPP) promoted by phosphatase models of Co(III) the type; cis- $[N_4Co(OH)(OH_2)]^{2+}$ ,  $N_4 = (en)_2$ - Compound 1;  $(tn)_2$ -Compound 2, (en = 1,2-diaminoethane; tn = 1,3-diaminopropane). Co(III) artificial metallonucleases stabilized by tetraamine ligands and that are analogous to compounds 1 and 2, such as  $Co^{III}$ -tren (tren = tris- 2- aminoethyl amine),  $Co^{III}$ -trpn (trpn = tris- 3-aminopropyl amine) and  $Co^{III}$ cyclen (cyclen = 1,4,7,10- tetraazacyclododecane), have been the subject of extensive research and they pass the test as the most suitable models for ATPase and phosphatases.<sup>[16-18]</sup> They have been identified as some of the most active agents, providing up to  $10^7$ - fold rate enhancement compared to the uncatalysed reactions. Most studies have focused on their hydrolytic efficiency on phosphate monoand phosphodiesters with good leaving groups such as p-NPP and bis-dinitrophenyl phosphate, BDNPP, including phosphoanhydrides.<sup>[19-21]</sup>

Model systems based on Co(III) are especially advantageous in that metal coordination sites open for phosphate substitution can be limited thus permitting studies of a type not readily available using labile cations such as Mg(II), Ca(II), Zn(II), Cu(II) and Mn(II).<sup>[22,23]</sup> Co(III) provides the opportunity for a detailed mechanistic analysis in that isolated reaction species have defined metal ion-substrate interactions. The relative substitutional inertness of Co(III) complexes allows unambiguous analysis of the reaction mixtures. Moreover, the Co(III) system has been chosen as a functional model in order to take advantage of the high positive charge of Co(III) to activate substrates and/or nucleophiles.<sup>[23,24]</sup>

Hydrolysis studies involving the selected phosphate mono- and phosphodiester model compounds such as NPP and BNPP, respectively, constitute significant reactions for understanding the reactivity of Co(III) metalloenzymes and metal complexes in biological systems. BNPP remains the most suitable DNA model since Co(III) complexes give comparable rate enhancements for the hydrolysis of phosphate esters with good or poor leaving groups. NPP is the most researched model substrate for phosphate monoester hydrolysis; it is known to have slower hydrolysis rates in comparison to bisphosphates thus allowing more accurate kinetic data to be accumulated.<sup>[24–26]</sup> The present study focusses on developing a more qualitative (HRMS) and quantitative (UV-Vis) picture in metalion-promoted phosphate ester hydrolysis that would hopefully produce a detailed experimental trend in a group of selected phosphate esters with activated and inactivated leaving groups. Qualitative and quantitative data on metal ionsubstrate reactivity profiles was collected using a combination of chromatographic (ultra high performance liquid chromatography; UHPLC) and spectroscopic techniques (Quadrupole-time-of-flight-mass spectrometry - Q-ToF-MS; ultraviolet-visible spectrometry - UV-Vis, DAD and Fourier Transform Infrared Spectrometry – FTIR).

### **Results and discussion**

Characterisation of the metal complexes was conducted by FTIR and significant vibrational modes are depicted in Table 1. As expected, the vibrational spectra of the complexes display a striking similarity, particularly in those bands associated with direct bonding to the metal center such as v(M-N), v(M-O) and the coordinated water/ hydroxyl stretch, v(M-H<sub>2</sub>O/OH). Vibrations that are affected by the character of the amine ligand such as the v(CH<sub>2</sub>) and v(NH<sub>2</sub>) stretches show some slight differences. All these assignments were made by reference to available literature data on analogous Co(III) complexes.<sup>[15,26-29]</sup>

The neat Co(III) complexes,  $[N_4Co(OH)(H_2O)]^{2+}$ ,  $N_4 = (en)_2$ ;  $(tn)_2$ , were subjected to chromatography and spectroscopy at the conditions of the experiment in order to understand their dissociation profiles. It is expected that in solution, the enzyme model complexes would lose the water ligand rather than the hydroxyl ligand and in the case of the  $Co^{III}$ -(en)<sub>2</sub> complex (MW = 210.123 Da), this view was supported by a low intensity peak at m/z = 191.9952, which is associated with the loss of the water ligand ( $E_r$ , Da = 0.1128). A similar behavior was observed in the case of the  $Co^{III}$ -(tn)<sub>2</sub> complex (MW = 238.177 Da) where loss of the water ligand gave rise to a peak in the mass spectrum at m/z = 220.9595, giving an error of -0.7975 Da in relation to the calculated exact mass of 220.162 Da. With reference to similar bidentate amine ligand complexes of Co(III), the observed vibrational data, coupled with their solution dissociation profile are in agreement with the cis-arrangement of the ligands around the metal center, leaving the water and hydroxyl ligands to occupy the axial positions.<sup>[15,30-33]</sup>

# The reaction profile of phosphate monoesters (PP, NPP) with the enzyme models – qualitative analysis

The reaction profile of phosphate monoesters (PP, NPP) with the cobalt metallohydrolases at various cobalt to substrate ratio (1:1, 2:1, 3:1 and 20:1) were conducted in aqueous medium at pH 7.0 and under ambient conditions  $(23 \pm 2.0 \,^{\circ}\text{C})$ . Reactions were monitored at the predetermined time intervals of t = 30 min (after adjusting the pH to 7.0), 60, 90, 120, 150 and 180 min. Qualitative data revealed that reactions at the 3:1 Co(III):substrate ratio contained the

Table 1. Significant vibrations of the	enzyme model complexes.						
			Signifi	cant FTIR Vibrations (cm <sup>-</sup>	( <sub>1</sub>		
Complex	(M-N)	γ (C–N)	γAssymNH <sub>2</sub>	y SymNH <sub>2</sub>	γ (CH-sp <sup>3</sup> )	у (M–O)	γ (M-H <sub>2</sub> O/OH)
[Co(en) <sub>2</sub> (H <sub>2</sub> O)(OH)] <sup>2+</sup>	628 m	2032 w; 1632 s	1375 m	1113 sb	2925 sb	490 w	3200–3700 sb
Other Vibrational Assignments	3482 - 3410 m/b 3204: 3113 w	- U[H <sub>2</sub> O] 1)[OH]					
	2925/2853 s/d —	- sp <sup>3</sup> C–H stretching.					
	1636/1622 w/d –	<ul> <li>C–N stretching – Aliphatic Amine</li> </ul>					
	1050/1020 s/t —	Aliphatic C–N stretching					
	630m — Co-N s	tretching					
	541w — Co-O si	tretching					
[Co(tn) <sub>2</sub> (H <sub>2</sub> O)(OH)] <sup>2+</sup>	628 m	2032 w; 1639 s	1377 m	1110 sb	2911 sb	490 w	3200-3700 sb
Other Vibrational Assignments	3550/3420 s/b —	- U[H <sub>2</sub> O]					
1	3300/3247 m —	U[OH] — chelated –OH group					
	2930s/2853 s —s	p3 C–H stretching					
	1644/1620 s — C	-N stretching — Aliphatic Amine					
	630 m — Co-N	stretching					
	543 w Co-O stre	tching					
Abbreviations: $b = broad$ ; $m = mediurent beta = broad$	m; s = strong; w = weak; sb	= strong, broad; $d = doublet$ ; $t = trip$	let.				

most reactive species in solution and therefore all accumulated qualitative chromatographic and spectroscopic data concentrated on the 3:1 E:S reaction medium. The 20:1 E:S reaction medium was used to confirm the identification of MS fragment ions and it was found that no significant difference in qualitative data existed in reactions conducted at the 3:1 versus those at the 20:1 Co(III):substrate ratios.

Figure 1 shows the negative electrospray total ion chromatogram (TIC) of the aqueous reaction between  $\text{Co}^{\text{III}}$ -(en)<sub>2</sub> and the monoester substrate, NPP. The corresponding chromatographic and spectroscopic data for the  $\text{Co}^{\text{III}}$ -(en)<sub>2</sub>-PP reaction is included in the supplemental material.

A common feature in the chromatographic eluent analysis of the Co<sup>III</sup>-(en)<sub>2</sub>-monoester reactions is that below the retention time ( $t_R$ ) of 0.50 min, the identifiable fragments are the conjugate alcohols and the four phosphorus oxyacids, hypophosphoric acid ( $A_1$ ; MW = 161.974 Da), pyrophosphoric acid ( $A_2$ ; MW = 177.973 Da), orthophosphoric acid ( $A_3$ ; MW = 97.994 Da) and the dibasic phosphoric/phosphorus acid ( $A_4$ ; MW = 81.995 Da).

The extracted mass spectra (in negative ionization mode) depicting the identification of these molecular species in the reactions of  $\text{Co}^{\text{III}}$ -(en)<sub>2</sub> with NPP are shown in Figures 2 and 3. A similar reaction profile was observed in the reactions of the  $\text{Co}^{\text{III}}$ -(tn)<sub>2</sub> system with the monoester substrates and therefore the corresponding chromatographic and mass spectral data are not included in this manuscript but are available as supplemental material ( $\text{Co}^{\text{III}}$ -(tn)<sub>2</sub>/NPP reaction mixtures).

The nitrophenolate conjugate ( $O_2N-PhO^-$ ) appears at m/z 138.0732 (MW = 138.102 Da;  $E_r = 0.0288$ ); peaks at m/z160.9801 and 177.9051 are attributed to the two phosphorus oxy-acids, hypophosphoric acid (MW = 161.974 Da;) $E_r = 0.9939$ ) and pyrophosphoric acid (MW = 177.973 Da;  $E_r = 0.0679$ ), respectively. A similar pattern is observed in the extracted mass spectra in reactions of the PP substrate with CoN<sub>4</sub>. In both substrates, the most abundant of the oxy-acids is othorphosphoric acid,  $H_3PO_4$  ( $t_R = 0.465 \text{ min}$ ) and appears as a base peak at m/z 98.9970  $(MW = 97.994 \text{ Da}; E_r = -1.0030)$ . The peak at m/z 82.9952 in the extracted mass spectra of the Co<sup>III</sup>(en)<sub>2</sub> - NPP reaction mixtures is associated with the dibasic, phosphorus/ phosphonic acid,  $H_3PO_3$  (MW= 81.995 Da;  $E_r = -1.0002$ ). All these assignments are common for the monoesters and in their reactions with the Co<sup>III</sup>-(en)<sub>2</sub> and Co<sup>III</sup>-(tn)<sub>2</sub> metallohydrolases. They have been confidently assigned to the respective fragments at an average error of  $\sim 0.7575$  Da.

The actual hydrolysis reaction can be considered to occur in the retention time region of  $t_R = 1.0$  to 4.5 min for the monoesters. Focussing on the reaction between the Co<sup>III</sup>-(en)<sub>2</sub> system and NPP, significant fragment ions identified in reaction mixtures in this region are shown in Figures 4 and 5.

The reactivity of Co(III) complexes is largely influenced by isomeric configurations and acid-base equilibria as depicted in Equations 3 and 4. Therefore in solution, each of the Co(III) complexes exists in three different oxidation states with the Co(III)-aquo-hydroxo form being the active



Figure 1. Negative Electrospray TIC of Co<sup>III</sup>-(en)<sub>2</sub>- NPP, pH 7.0; H<sub>2</sub>O, 25 °C.



Figure 2. Extracted MS Spectrum of Co<sup>III</sup>(en)<sub>2</sub> - NPP at 0.270 min.

species under conditions of the present experiment (pH 6.8-7.2).<sup>[23,33-36]</sup>

$$\begin{array}{rl} N_4 Co(OH_2)_2 \rightarrow & N_4 Co(OH)(OH_2) & + & H^+ \\ pH \, < \, 6.0 & pH \, \sim \, 6.0 \, - \, 8.0 \end{array} \tag{3} \label{eq:2.1}$$

$$\begin{array}{rrr} N_4 Co(OH)(OH_2) & \rightarrow & N_4 Co(OH)_2 + & H^+ \\ pH \, > \, 8.0 \end{array} \tag{4}$$

In solution, inactive trans- $[Co(L)_2(OH)(OH_2)]^{2+}$  (L = en, tn) isomerizes to the cis-isomer rapidly ( $t_{1/2}$  =1s, 25 °C) and completely so that under the present experimental conditions (pH 6.8 – 7.2), the chemistry of Co<sup>III</sup>(en)<sub>2</sub> and Co<sup>III</sup>(tn)<sub>2</sub> can be attributed exclusively to the hydrolytically active cis-isomers.<sup>[23,36,37]</sup>

Mass spectral data for the Substrate Fragments in the Reactions of PP/NPP with  $CoN_4$ ,  $N_4 = (en)_2$ ;  $(tn)_2$  show



Figure 3. Extracted MS Spectrum of Co<sup>III</sup>(en)<sub>2</sub> - NPP at 0.463 min.



Figure 4. Major lonic Fragments in the Reaction of Co<sup>III</sup>(en)<sub>2</sub> with NPP, pH 7.0, 25 °C.

liberation of the conjugate alcohols as depicted in Figure 6. The underlying mechanism for the reaction is believed to undergo similar patterns of reactivity observed for aqueous dissociation profile of the neat hydroxoaqua,  $[N_4Co(OH)(OH_2)]^{2+}$  complexes, which dissociate via the loss of the water ligand to yield the coordinatively unsaturated  $N_4Co^{III}(OH)$  species.

Under the experimental conditions, the monoesters exist in aqueous solution in their dianionic form,<sup>[36–38]</sup> the detected monodentate Co(III)-phosphato complexes result from the

substitution of the water ligand for phosphate through coordination of one of the non-bridging oxygen atoms of the ester to the metal center to form complexes  $C_1$ ,  $C_4$ ,  $C_6$  and  $C_{10}$ . This observation is in agreement with data from the aqueous dissociation of the enzyme models coupled with reported work on similar Co(III) complexes, that in solution, formation of the monodentate CoN<sub>4</sub>(OH)–phosphato complexes,  $C_1$ ,  $C_4$ ,  $C_6$  and  $C_{10}$  proceeds via the slow, rate limiting substitution of the monoester substrates for H<sub>2</sub>O in the reactive aquohydroxo complex.<sup>[37–39]</sup>



Figure 5. Major lonic Fragments in the Reaction of  $Co^{III}(en)_2$  with PP, pH 7.0, 25  $^\circ C.$ 



Figure 6. Significant Co  $^{III}$  – Substrate Fragments in the Reactions of PP/NPP with CoN<sub>4</sub>, N<sub>4</sub> = (en)<sub>2</sub>; (tn)<sub>2</sub>.

Liberation of the nitrophenolate/phenolate conjugates from the hydroxo-(p-nitrophenyl/phenylphosphato) (tatraamine) cobalt(III) complexes is believed to be fast and yields the bidentate (phosphato)(tetraamine)Co(III) 4-membered ring chelate, C<sub>2</sub> (m/z 268.8761;  $E_R = 1.1949 \text{ Da}$ ) for the Co<sup>III</sup>-(en)<sub>2</sub> – NPP reaction (Figure 4). These reactions proceed via intramolecular attack of the metal-coordinated hydroxyl (HO<sup>-</sup>) group on the phosphorus center, a route that is favored by entropic effects over the alternative intermolecular attack by the solvent, H<sub>2</sub>O.<sup>[23,38,39]</sup> Moreover, intramolecular attack by the Co<sup>III</sup>-coordinated hydroxyl nucleophile is favored since it is 10<sup>5</sup> – 10<sup>6</sup> more nucleophilic than uncoordinated hydroxide.<sup>[36]</sup> From mass spectral analysis, it is detected that the 4membered ring chelate, C<sub>2</sub> is highly susceptible to H-bonding at the bridging oxygen atoms to yield the N<sub>4</sub>Co<sup>III</sup>– $\mu$ (OH)<sub>2</sub>–P<sub>i</sub> complex, C<sub>3</sub> (Figure 4). Such a structural arrangement conforms to that of natural enzymes which are normally bridged in the enzyme active sites by either the hydroxyl groups from amino acid residues or by –OH nucleophiles. Hydrogen bonding at the Co<sup>III</sup>–O–Pi bridge assists the metal ion by providing additional electrostatic stabilization including activation and orientation of the substrate.<sup>[39–41]</sup>

Reactions involving intramolecular attack by coordinated hydroxyl ion have previously been reported for a number of analogous Co(III) complexes.<sup>[37-39]</sup> Sargeson and coworkers<sup>[37]</sup> conducted 18O-labelling studies in the hydrolysis of coordinated p-NPP the in complex, cis-[Co(en)<sub>2</sub>(OH)O<sub>3</sub>POC<sub>6</sub>H<sub>4</sub>NO<sub>2</sub>], Complex A, which is an equivalent of Compounds C1, C4, C6 and C10 shown in Figure 6. Complex A was synthesized and fully characterized afterwhich hydrolysis reaction with NPP was carried out in the pH range of 7-14 and monitored using 31P NMR and UV-vis spectroscopy. These workers detected that intramolecular 18O-labeled metal-coordinated <sup>-</sup>OH initially yielded a five-coordinate phosphorane which rapidly decayed to the  $[Co(en)_2PO_4]$  chelate. In addition, the labeled 18O was detected bonded between Co and the P atom, suggesting direct transfer from the attacking <sup>-</sup>OH nucleophile. When the same experiment was conducted using unlabeled complex but in 18O-labeled water, it was demonstrated that only 16% of the 18O label was incorporated into inorganic phosphate. These findings clearly demonstrated that the hydrolysis proceeds through an intramolecular Co(III)hydroxide attack on the phosphorus atom.<sup>[37]</sup>

Further supporting evidence for intramolecular attack by coordinated hydroxide was demonstrated using known experimental markers and mathematical models such as Kinetic Isotope Effects (KIE's). Cleland and coworkers<sup>[40]</sup> the analogous used Co(III) complexes,  $\left[\text{Co(en)}_2(\text{OH})(\text{OH}_2)\right]^{2+}$ and  $[Co(NH_3)_5(OH)(NH_2)]$ to determine KIE's on the hydrolysis of Co(III)-coordinated p-NPP. The experimental approach was through the measurement of mass ratios in liberated p-NP and also in dissociated p-NPP. In this way, these workers were able to calculate isotope effects on both the hydrolysis and dissociation processes. It was observed that the hydrolysis process

Table 2. Isotope Effects (%) on Reactions of Cobalt(III)-Coordinated p-Nitrophenyl Phosphate.  $\space{[40]}$ 

Reaction, Complex	<sup>15</sup> K	Bridge <sup>18</sup> K	Non-bridge <sup>18</sup> K
Hydrolysis:			
Co(en) <sub>2</sub>	0.21	2.13	0.06
$Co(NH_3)_5$	0.12	0.98	0.45
[Co(cyclen)] <sub>2</sub>	0.16	2.07	0.57
Dissociation:			
Co(en) <sub>2</sub>	-0.02	0.63	1.35
$Co(NH_3)_5$	-0.02	0.11	1.67

involves attack of cis-coordinated  $OH/NH_2$  on phosphorus with a concerted release of the nitrophenolate moiety.

Table 2 presents the calculated isotope effects from these studies, focusing on the reactions of Co(III)-coordinated p-NPP:

With reference to Table 2, evidence of intramolecular hydroxide attack/nitrophenolate release is indicated by the large primary 18O isotope effect (18O-bridge) of 2.13%, indicating that the P-O bond is largely cleaved in the transition state (TS). The 0.21% 15N isotope effect demonstrates considerable electron delocalization at the leaving group substituent. In addition, it was concluded that the relatively small secondary isotope effect (<sup>18</sup>O non-bridge) of 0.06% suggests a slightly associative TS. By contrast, the smaller primary 18O isotope effect of 0.98% in the [Co(NH<sub>3</sub>)<sub>5</sub>] complex suggests a more associative mechanism with no intramolecular activity, reflecting the different properties of the OH and  $^{-}NH_{2}$  moieties as nucleophiles . In the case of the cyclen complex, hydrolysis isotope effects are similar to those of the Co(en)<sub>2</sub> system which can be attributed to the cis-OH attack on the phosphorus center<sup>[40]</sup>.

On the basis of the observed qualitative, spectroscopic data coupled with supporting evidence from related studies involving analogous Co(III)-tetraamine complexes, the proposed mechanism for the reaction of the selected monoesters with the  $N_4Co^{III}$  complexes of  $(en)_2$  and  $(tn)_2$  may be proposed to proceed via the following sequential pathway:

In the proposed mechanism for  $[N_4Co(OH)(OH_2)]^{2+}$ promoted hydrolysis of PP and NPP, the metal ion plays a dual role; as a Lewis acid and also as a template for the reaction. From spectroscopic data, it is believed that the intramolecular hydroxyl attack on phosphorus (Step I) and the phenolate leaving group departure is a concerted process that leads to the 4-membered, bidentate phosphorane intermediate, (3). In the case of the bidentate, 4-membered intermediate, (3), chelate-ligand geometry and basicity control metal ion reactivity, phosphate ligand substitution and the initial hydrolytic step.<sup>[39,41,42]</sup> Relief of ring strain on the phosphorus atom in the 4-membered ring complex, (3), may be thought to provide a driving force for the subsequent solvent/H2O attack and generation of the hydrolysis products and the  $N_4Co^{3+}$  enzyme residues. On attack by the water solvent, the cyclic phosphorane intermediate decomposes exclusively by exocyclic Co-O- cleavage to yield the hydrolysis products.<sup>[42-44]</sup>

Observations from the present work are that solvent attack at the phosphorus of the strained 4-memberered ring system does not lead to the formation of the starting intermediate, monodentate Co(III)-tetraamine phosphato complexes, C<sub>1</sub>, C<sub>4</sub>, C<sub>6</sub> and C<sub>10</sub> (Figure 6). Such a pathway would result in the possible regeneration of the starting aquohydroxo(tetraamine)cobalt(III) complex,  $[N_4Co(OH)(OH_2)]^{2+}$  and production of phosphoric acid. Whilst phosphoric acid is the major hydrolysis product from the present study, the starting CoN<sub>4</sub> complexes have not been detected and they are not regenerated under conditions of the present experiments.

Previous studies by Horn and coworkers<sup>[44]</sup> reveal that the unique attribute of the metal complex in these



**Figure 7.** Major lonic Fragments in the Reaction of Co<sup>III</sup>(en)<sub>2</sub> with BNPP, pH 7.0, 25 °C.



Figure 8. Major Ionic Fragments in the Reaction of Co<sup>III</sup>(en)<sub>2</sub> with BNPP, pH 7.0, 25 °C.

hydrolysis reactions is its ability to generate a higher concentration of the reactive nucleophile at a much lower pH than would be possible. It is further noted that the pH for which the rate of the intramolecular process reaches a maximum is shifted into the physiological range (pH 6.8–7.2), which is a result of potential significance to the mechanism of action of the enzyme *E. coli* Alkaline Phosphatase (AP)<sup>[44,45]</sup>.

# The reaction profile of phosphate diesters (DPP, BNPP) with the enzyme models – qualitative analysis

As observed in the reactions of monoesters with the  $CoN_4$ systems, the corresponding diester reactions for DPP and BNPP follow the same reactivity profile in that hydrolysis is detected in the retention time region of 1.0–4.5 min. Significant ionic fragments detected in the reactions of



Figure 9. Major Ionic Fragments in the Reaction of Co<sup>III</sup>(en)<sub>2</sub> with BNPP, pH 7.0, 25 °C.



Figure 10. Major lonic Fragments in the Reaction of Co<sup>III</sup>(en)<sub>2</sub> with BNPP, pH 7.0, 25 °C.

BNPP with the  $Co^{III}$ -(en)<sub>2</sub> system are depicted in Figures 7–10. A similar reaction profile was obtained for the reactions of the  $Co^{III}$ -(tn)<sub>2</sub> system with the diseters and for purposes of focusing on the discussion of the observed diester reaction results, the corresponding chromatographic and spectroscopic data for the  $Co^{III}$ -(tn)<sub>2</sub> – BNPP reaction are included in the supplemental material.

In the total ion chromatograms (TIC's) of the diester reactions with the  $CoN_4$  enzyme models, retention times in the region  $t_{R=}$  0.10–0.47 min represent the area where the conjugate alcohols and the polymeric phosphorus oxyacids

(A<sub>1</sub>, hypophosphoric acid; A<sub>2</sub>, pyrophosphoric acid) are detected. The chromatographic peak in the region 0.45–0.48 min is the area where othorphosphoric acid predominates, always detected as a base peak at m/z 98.9970 (MW = 97.994). The dibasic phosphonic acid, A<sub>4</sub>, m/z 82.8963 (M/W = 81.995) is also detectable in this region.

The hydrolysis reactions of the diesters may best be described in terms of the observed initial dissociation products, monophosphate and the alcoholic conjugate fragments. In the  $Co^{III}$ -(en)<sub>2</sub> – BNPP reaction mixtures, the dissociated monoester and the alcoholic leaving group fragments can be



**Scheme 1.** Proposed Mechanism for the Reaction of PP/NPP With  $[N_4Co(OH)(OH_2)]^{2+}$ ;  $N_4 = (en)_2$ ,  $(tn)_2$ ; Ar = Phenolate/Nitrophenolate.

detected in the solution mixtures of the BNPP substrate and the enzyme system, signifying that aqueous dissociation of the substrate occurs prior to the hydrolysis process. With reference to the previously reported uncatalyzed aqueous reactions of the diesters<sup>[14]</sup>, the dissociated NPP monoester fragment could be detected in reaction solutions of BNPP (protonated or deprotonated form), an indication that it is one of the solution dissociation products following the release of the first equivalent of the conjugate alcohol. Extracted mass spectral data indicated that the monoanion of the deprotonated molecular ion in both DPP and BNPP is detectable as a base peak in the reaction mixtures of these substrates: DPP = 249.0294 ( $E_r = 0.1576$ ); BNPP = 339.0928 ( $E_r = 0.0812$ ). Since the deprotonated molecular anion is detected together with the dissociation products, pNP and pNPP in the reactions of BNPP, it is evident that in the two possible reactions in solution, which are, dissociation and hydrolysis, the dissociation of the diester to the monoester and the alcoholic conjugate occurs first. Therefore it is safe to say that it is this dissociated monoester product that subsequently coordinates to the Co(III) center to complete the hydrolysis process which facilitates the release of the second equivalent of the conjugate alcohol as depicted in scheme 1.

These observations are in direct contrast to earlier views by Chin and coworkers <sup>[17,43]</sup> who studied the hydrolysis of BNPP promoted by Co(III) complexes of the type; cis-[(L)Co(OH)(OH<sub>2</sub>)]<sup>2+</sup>, L=cyclen, tren, trpn. These workers proposed a mechanism involving coordination of the diester monoanion to the Co(III) center via the non-bridging oxygen atom, followed by ring closure through intramolecular



Scheme 2. BNPP Hydrolysis Promoted by cis -[(L)Co(OH)(OH2)]<sup>2+</sup>, L = cyclen, tren, trpn<sup>[17,43]</sup>.

hydroxide attack at phosphorus. Hydroxide attack at phosphorus is reported to be concerted with the cleavage of one of the P–O–R bonds, as shown in Scheme 2.

Under the present experimental conditions, such a mechanism does not account for the observed monodentate Co(III)-phosphato fragments detected in the reactions of DPP and BNPP with the  $Co^{III}$ -(en)<sub>2</sub> and  $Co^{III}$ -(tn)<sub>2</sub> model systems (C<sub>4</sub> - Figure 8). Therefore this mechanism cannot be applicable under conditions of the present study since no coordinated diester monoanion complexes in the form of DPP/BNPP–Co(III) fragments could be detected in any of the reaction mixtures using accurate mass measurements.

Therefore from the foregoing spectroscopic evidence, the diester monoanions are not involved in the hydrolysis reaction but undergo initial dissociation to yield the corresponding monoester and one equivalent of the conjugate alcohol. The monoester fragments may be protonated but it is believed that under conditions of the present experiment, the dianionic form is predominant and readily coordinates to the Co(III) center to yield the monodentate Co(III)-phosphato complex,  $C_4$ , similar to monodentate phosphato species detected in the case of monoester reactions with the enzyme models (refer to Figure 6).

The detection of compounds  $C_4$  and  $C_{10}$  (BNPP reactions with  $CoN_4$ ) in the mass spectra provides sufficient evidence that the dissociated monoester products, NPP and PP are responsible for coordination to the Co(III) center before any second equivalent of the conjugate alcohol can be released. Thus as is the case with the monoester reactions reported earlier, attack of the metal-coordinated hydroxyl nucleophile at the phosphorus center results in the formation of the  $Co^{III}$ – $\mu$ (O)<sub>2</sub>–phosphato, four-membered ring chelates (Compound C<sub>2</sub>, Figure 9).

As mentioned earlier, compounds  $C_2$  and  $C_7$  are detected to be highly susceptible to hydrogen bonding at the bridging oxygen atoms. This, coupled with the ring strain at the  $Co^{III}$ - $\mu$ (OH)<sub>2</sub>-P center presents an ideal situation for the hydrolysis process to progress. From spectroscopic data and reports from similar work involving analogous Co(III) tetraamine complexes,<sup>[36,37]</sup> it is believed that ring strain is released through exocyclic Co-O-ring opening leading to the hydrolysis products and some enzyme residues.

In the present study, the 4-membered Co(III)-phosphato chelates postulated by other workers<sup>[37]</sup> are essentially represented by compound C<sub>2</sub> (Figure 9). Based on data from the present studies, and with reference to studies involving other analogous Co(III) complexes, it is believed that Co(III)-bound hydroxyl attack and the P–O–L<sub>g</sub> bond cleavage are concerted processes and determine steps towards the formation of the Co(III)- $\mu$ (O<sub>2</sub>)-phosphato, 4-membered ring chelate.<sup>[38,39]</sup> X-ray studies of similar Co(III)-tetraamine

complexes reveal that the preferred angle at P in the 4membered ring chelate is 108–109° and provided the chelate spans an axial-equatorial position, the strain remains considerably less.<sup>[39,45]</sup>

The influence of hydrogen bonding at the bridging oxygen atoms deserves some comment. In a recent study involving the Co(III) complexes,  $cis-[Co(cyclen)(H_2O)_2]^{3+}$ and  $[Co(tren)(H_2O)_2]^{3+}$ , Basallote and coworkers<sup>[41]</sup> demonstrated that the formation of mono- and bis-hydroxobridged species in solution is relevant for understanding their speciation and reactivity. It was further shown that in the reactivity of biologically relevant polyphosphates and inorganic phosphate, P<sub>i</sub>, outersphere hydrogen bonding interactions remain a dominant factor for the hydrolysis process. Thus in view of the obtained experimental evidence, the present work demonstrates that coordination of a phosphate substrate to a Co(III) center that is positioned cis- to a hydroxo ligand results in an enhanced rate of cleavage of the ester. The study further demonstrates the efficacy of metal-coordinated –OH ion as a powerful nucleophile in the intramolecular pathway for cleaving phosphate esters under neutral pH conditions.

Therefore, on the basis of the accumulated spectroscopic data that is supported by relevant analogous studies involving Co(III) complexes, the mechanism for the Co(III)-promoted aqueous hydrolysis of the phosphodiesters, DPP and BNPP can be demonstrated as shown in Scheme 3.

#### **Conclusion and future considerations**

It has been demonstrated in the present study that the preparation of the Co(III) model compounds comes with minimal challenges yet with good yields under favorable laboratory conditions. The complexes are kinetically robust thereby allowing the characterization of all the species present in solution. The kinetics and mechanism of the observed cobalt(III) complex substitution reactions is easily understood, a knowledge that has facilitated the identification of mechanisms involved in complex-promoted phosphate ester hydrolysis.

In the practical context, the present study has permitted the identification of experimental techniques that may facilitate future investigations of similar reactions. It has been demonstrated that the UHPLC-QToF method of analysis offers numerous advantages over other researched methods such as the UV-Vis and NMR spectroscopic techniques. The challenge with other techniques is that they offer limited knowledge from direct experimental investigations, particularly on the mechanism of the cleavage of the phosphate esters promoted by metal complexes. This is due to lack of



Scheme 3. Proposed Mechanism for the Reaction of DPP/BNPP With  $[N_4Co(OH)(OH_2)]^{2+}$ ;  $N_4 = (en)_{2r}$   $(tn)_2$ ; Ar = Phenolate/Nitrophenolate.

direct observations and first hand evidence during the reaction which results in uncertainties of the binding mode of the substrate-metal complexes and interactions between the phosphate esters and the metal complexes. The UHPLC-QToF procedure adopted in the present study permits separation and detection of charged species in solution (negative and positive); it is therefore convenient and should have general applications to metal-ion complex-promoted catalytic reactions, in general. Using this technique, we have been able to demonstrate that hydroxoaquotetraaminecobalt(III) complexes are capable of hydrolyzing organophosphorus compounds with a clear identification of critical reaction intermediates. The catalytic nature of the reaction is derived from the relatively rapid hydrolysis of the intermediate phosphate- cobalt(III) complexes to yield inorganic phosphates as the predominant products.

It was further demonstrated that on their own, the model compounds,  $[N_4Co(OH)(OH_2)]^{2+}$ ,  $N_4 = (en)_2$ ;  $(tn)_2$ , undergo spontaneous dissociation in aqueous medium and at pH 7.0 via the release of the water ligand to yield the coordinatively unsaturated N<sub>4</sub>Co-OH species. Therefore, under the current experimental conditions and in the  $[N_4Co(OH)(OH_2)]^{2+}$ promoted hydrolysis of the monoesters, PP and NPP, the reaction is initiated by the substitution of the water ligand for the monoester to the cobalt(III) center via one of the non-bridging oxygen atoms in a monodentate fashion. From spectroscopic data, it was demonstrated that this is followed by the intramolecular attack of the Co(III)-cis-coordinated hydroxyl ligand on phosphorus which is believed to be a concerted process with the departure of the alcoholic phenolate leaving group. This results in the formation of a strained, 4-membered, bidentate phosphorane intermediate, where chelate-ligand geometry and basicity control metal ion reactivity, phosphate ligand substitution and the initial hydrolytic step. Relief of ring strain on the phosphorus atom in the 4-membered ring chelate provides a driving force for the subsequent solvent/H<sub>2</sub>O attack. In the process, the cyclic phosphorane intermediate decomposes exclusively by Co–Oexocyclic cleavage to yield the hydrolysis products and some  $N_4Co^{3+}$  enzyme residues.

One of the key reaction elements that has been demonstrated in the present study is that in aqueous medium, phosphate diesters first undergo solution dissociation to yield the monophosphate and one equivalent of the conjugate alcohol before they can embark on any hydrolysis process involving the  $[N_4Co(OH)(OH_2)]^{2+}$  model systems. This signifies that under the experimental conditions, the energy barrier towards the dissociation pathway is favored to that of the hydrolysis pathway via the non-bridging oxygen atom of the diester. Once the dissociation step has been achieved, the hydrolysis reaction simply follows that observed in the case of monoesters and can be summarized as follows:

- 1. Rate-limiting substitution of the phosphate ester for the water ligand in the hydroxoaquo Co(III) complexes;
- Fast, intramolecular attack of Co(III)-coordinated hydroxide on phosphorus leading to the formation of a strained, 4-membered ring chelate, a process that is concerted with the liberation of the conjugate leaving group alcohol;
- 3. Intermolecular attack by the solvent water on the phosphorus of the 4-membered ring chelate to yield the hydrolysis products and some enzyme residues;
- 4. The rate at which the potent M–OH nucleophile attacks the phosphorus center depends, amongst other factors, on the number and nature of electron withdrawing substituents attached to the bridging O-atom of the substrate, and, on the nature of the N<sub>4</sub> ligand coordinated to the Co(III) ion;
- 5. The hydrolysis pathway does not lead to the generation of the starting hydroxoaquo (tetraamine)cobalt(III) complex.

#### **Experimental**

#### Materials and methods

Synthetic reagents including the organophosphorus substrates (PP, DPP, NPP and BNPP; Sigma-Aldrich) and all other reagents used were either analytical reagent grade or the purest available commercially and were used without further purification. Standard solutions of both the metal complexes and the substrates were freshly prepared before any reactions were attempted, otherwise solutions were generally kept refrigerated.

pH measurements were performed on a Jenway 3520 pH meter equipped with a combination electrode and connected to a temperature probe (Lasec). Instrument calibration was performed using standard buffer solutions (pH 4.0 and pH 7.0). Deionised water was purified by continuously passing through a dual filtration system; Purite Pre-treat 8/Purite

Puripac PP8 cartridges using a Purite Select Water Purification System (Lasec).

Hydrolysis studies for qualitative analysis were conducted on a STEM RS 600 Electrothermal Reaction Station equipped with a magnetic stirring devise and a temperature probe (Lasec).

In the preparation of samples for FTIR analysis, reaction aliquots (6–8 mL) were separately transferred into glass tubes and evaporated to dryness using a centrifugal evaporator. Equipment parameters: **Centrifuge:** RVC 2–33 IR, T°C set at  $30 \pm 3$  °C, 900 rpm; **Freeze-drier:** Martin Christ Freeze Drier Alpha 2–4 LSC Plus, Vacuum set at  $20 \pm 1$  mbar, T°C set at  $-80 \pm 5$  °C. Solid samples were then reconstituted as mulls in liquid paraffin between KBr discs and the instrument resolution was maintained at  $4 \text{ cm}^{-1}$ . Characteristic vibrational bands were assigned by comparison with spectral data of similar Co (III) coordination compounds.<sup>[15,27,43]</sup>

Preliminary kinetic studies of the reaction mixtures were conducted using the UHPLC-QToF instrument, monitoring the depletion of the substrate against formation of the product at the respective m/z values. Confirmatory kinetic studies were conducted *in-situ* inside the cuvette using the UV-Vis method by monitoring the release of the conjugate alcohols (pNP at 405 nm in the reactions of NPP and BNPP; phenol (Ph) at 290 nm in the reactions of PP and DPP).

### Synthesis of the enzyme models: $[co(tn)_2(OH)(H_2O)]^{2+}$ and $[co(en)_2(OH)(H_2O)]^{2+}$

The synthetic route leading to the preparation of the Co(III)-hydroxoaquotetraamine complexes followed the initial synthesis of the intermediate, sodium triscarbonatocobaltate (III) trihydrate, Na<sub>3</sub>]Co(CO<sub>3</sub>)<sub>3</sub>].3H<sub>2</sub>O, developed by *Bauer and Drinkard*.<sup>[46]</sup> The intermediate has certain advantages as a precursor for the preparation of a representative group of Cobalt (III) coordination compounds in that it is very reactive under mild conditions and it is fairly stable on storage, if kept dry and away from UV rays.

The intermediate was converted to the respective perchlorate salts using established literature procedures.<sup>[23,28,29]</sup> Conversion of the perchlorate salts to the hydroxoaquo complexes was achieved by the addition of 6 M perchloric acid (2.5 mmol) to 1 mmol of finely divided carbonato species. The mixture was then stirred in an aspirator vacuum for 30 min at  $50 \pm 0.1$  °C to liberate CO<sub>2</sub>. The resulting purple solution was then diluted with deionized water (10–15 m $\ell$ ) and the pH adjusted to 7.0 ± 2.0 using varying concentrations of NaOH or HClO<sub>4</sub>.

Liquid samples of the hydroxoaquo complexes were evaporated to dryness under vacuum and kept in a dessicator, in the dark until ready for use.

# Protocol of the hydrolysis reactions – qualitative analysis

The substrate solution (6 mL; 5 x  $10^{-3}$  M) was transferred into a thermostated reaction vessel equilibrated at

 $25 \pm 0.1$  °C. The enzyme solution (6 mL:  $5 \times 10^{-2}$ M) was transferred, drop-wise followed by the addition of the solvent, deionized water (18 m $\ell$ ). The mixture was then stirred continuously for about 30 min after which the pH of the solution was adjusted to  $7.0 \pm 0.2$  using varying concentrations of NaOH or HClO<sub>4</sub> (0.05 M, 0.10 M, 0.25 M, 0.5 M, 0.75 and 1.0 M). After pH adjustment, the first aliquot was drawn for analysis (t = 0 min). Subsequent aliquots were then drawn at the predetermined time intervals of 30 min, 60 min, 90 min, 120 min and 180 min. Aliquots were then prepared for analysis using Q-ToF-MS, DAD-UV-Vis and FTIR techniques.

# Protocol of the hydrolysis reactions – quantitative analysis

Initial kinetic studies were conducted using the UHPLC-QToF method by tracking substrate depletion against product formation. This method had to be abandoned due to clogging of the HPLC pumping system presumably from insoluble Co(III)- substrate residues from the reaction mixtures.

The UV-Vis method was then followed using the Thermo-Scientific Multiscan Go Version 100.4 instrument equipped with a SkanIt Software (Version 3.2). In this method, basis for selecting the right software parameters were set by initially conducting spectral scanning of all the substrates and expected reaction products in the range 200 to 600 nm. This was done to confirm  $\lambda_{max}$  values of the substrates and reaction products. Using the instrument software, at least three (3) kinetic loops were programed with 1 x loop consisting of 1000 readings at a kinetic interval of 10 s. Photometric settings were put at two (2)/dual wavelengths; one corresponding to the reactant substrate and the other corresponding to the expected phenolate product (300 nm and 405 nm for NPP/BNPP reactions; 270 nm and 290 nm for PP/DPP reactions, respectively).

The hydrolysis reaction was initiated inside the cuvette by first transferring the substrate solution ( $\sim 10^{-3}$  M; 2.0 mL) followed by the enzyme solution ( $\sim 10^{-2}$  M; 2.0 mL) in a 1:10, S:E stoichiometric ratio, assuming the hydroxoaqua complex is the only reactive species under the experimental conditions; pH 7.0, 25 °C, aqueous medium. Each kinetic loop accumulated data related to changes in absorbance over time whilst photometrically monitoring substrate depletion against product formation.

### Instrumental techniques

# UHPLC-QToF-MS (agilent 1290 UHPLC coupled to an agilent G6550 QToF)

The QToF instrument is preceded by liquid phase separation in the UHPLC, a technique that is highly amenable to the compounds considered for the present study. The compounds are water soluble and do not require derivatization prior to separation. The instrument is capable of identifying more low abundance compounds with a higher degree of confidence and in complex mixtures. QToF-MS combines high full spectral sensitivity with high mass resolution, allowing the mass of any ionizable component in the sample to be accurately measured. The system can provide a notable amount of chemical information in a single experiment; this being facilitated by the interface with UV or Diode Array Detection (DAD) which provides additional information that is useful if molecules are not readily ionizable or missed by the QToF method. Besides providing accurate mass measurements of the deprotonated molecular anions, the instrument in full scan mode provides accurate masses of their collision induced dissociation (CID) products.

### UV-Vis Spectroscopy (Thermo- Scientific Multiscan Go Version 100.40/Skanlt Software Version 3.2)

UV-vis studies were designed to examine the effects of long reaction times between the Co(III) complex and the substrate and also to assess the effects of the reaction medium on substrate depletion versus alcoholic leaving group formation. In conducting *in-situ* kinetic studies, substrate and enzyme solutions were separately transferred into a 5.0 m $\ell$  cuvette and the reaction monitored over at least three (3) kinetic runs. Substrate-enzyme reactions were carried out under pseudo-first order conditions with a large excess ( $\sim$ x10) of the Co(III) complex over the phosphate ester. Observed first norder rate constants were obtained by fitting the first 3 half-lives of the reaction according to the first order kinetics equation,  $\ln[A]_t = \ln[A]_0 - k_{obsd}$ . t. The half-lives of the reactions were determined using the expression  $t_{1/2} = \ln 2/k$ .

Data from these studies is available as supplementary material.

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