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Tirel, E.Y. and Williams, N.H. (2015) Enhancing phosphate diester cleavage by a zinc complex through controlling nucleophile coordination. Chemistry - A European Journal, 21 (19). 7053 - 7056. ISSN 0947-6539

https://doi.org/10.1002/chem.201500619

Enhancing Phosphate Diester Cleavage by a Zinc Complex Through Controlling Nucleophile Coordination

Emmanuel Y. Tirel and Nicholas H. Williams*[a]

Abstract: Metal ion complexes are the most effective artificial catalysts capable of cleaving phosphate diesters under mild aqueous conditions. A central strategy for making these complexes highly reactive has been to use ligand based alcohols that are coordinated to the ion, providing an ionised nucleophile under neutral conditions but at the expense of deactivating it. We have created a highly reactive Zn complex that is 350-fold more reactive than an alcohol analogue by preventing the nucleophile binding to the metal ion. This strategy successfully delivers the benefits of efficient nucleophile delivery without strongly deactivating the metal ion Lewis acidity nor the oxyanion nucleophilicity. Varying the leaving group reveals that the transition state of the reaction is much further advanced than the reaction with hydroxide.

The design and synthesis of metal ion complexes capable of efficient phosphate ester cleavage has received considerable attention, especially over the last two decades.[1-9] These compounds are the basis of the most effective artificial catalysts to date that are capable of functioning under mild aqueous conditions. However, the half-life of DNA at pH 7 and 25°C is estimated to be around 30 000 000 years[10], and no synthetic catalyst is able to accelerate this sufficiently and with enough selectivity to be used as therapeutics or laboratory reagents for manipulating nucleic acids. We report that using a well-positioned nucleophile that cannot coordinate to the metal ion can significantly enhance the reactivity of Zn complexes towards phosphate diesters that do not contain intramolecular nucleophiles.

Catalysts designed to cleave RNA can take advantage of the 2'-OH which is an efficient intramolecular nucleophile.[1] However, this mechanistic pathway is not possible for DNA-like substrates and the generally accepted mechanism involves the formation of a substrate-catalyst complex, which leads to product formation through nucleophilic attack at the phosphorous atom by a metal bound hydroxyl.[11] An effective way to further enhance the rate acceleration is to incorporate the nucleophile into the ligand structure itself **1**, so that a metal bound alkoxide attacks the phosphorous, and this has been the main strategy in designing efficient catalysts to date.[12,13] However, the cost of an increased concentration of anionic nucleophile at low pH is a decrease in intrinsic reactivity, and the overall combination of concentration and activity can lead to a decrease in reactivity.[14] Very recently, we reported the properties of a ligand that incorporated an acetal hydrate, **2**. The acetal hydrate allowed turnover and provided unexpectedly enhanced reactivity, which we proposed was due to the involvement of a non-metal ion bound nucleophile.[15] Following this line of enquiry, we sought ways of introducing a nucleophile into the ligand structure in a geometry that would prevent coordination to the metal centre to avoid reducing its nucleophilicity.

In this regard, oximes are promising. The coordination of the nitrogen to the metal centre leads to a close proximity between the oxygen atom and the incoming phosphate without deactivating the oxyanion by direct coordination to the cation nor reducing the Lewis acidity of the zinc ion. Oxime nucleophiles are also known to be reactive towards phosphate and carboxylic esters due to the alpha effect,[16-21] although coordination to the zinc ion presumably reduces the role of the N lone pairs in contributing to this effect. Recent work by Yatsimirsky *et al*. has highlighted that in reactions with carboxylate esters, coordination to Zn ions may enhance the nucleophilicity of oximate anions.[22] These reactions are not believed to involve initial coordination of the substrate to the metal ion before attack by the nucleophile, unlike the reactions of phosphate esters,[23] although Breslow and Chipman[24] has noted the potential value of an initial binding interaction so that the subsequent reaction occurs within the confines of a coordination complex. The constraint of delivering the nucleophile to a substrate bound to the metal ion of a complex differentiates the requirements of metal ion complexes designed to cleave phosphate esters from those aimed at cleaved carboxylic esters.[11] With these ideas in mind, we designed complex **3** which contains an oxime moiety in its structure and as shown in figure 1, the oxime oxygen atom is not coordinated to the zinc but is positioned close to the vacant binding sites of the Zn ion.

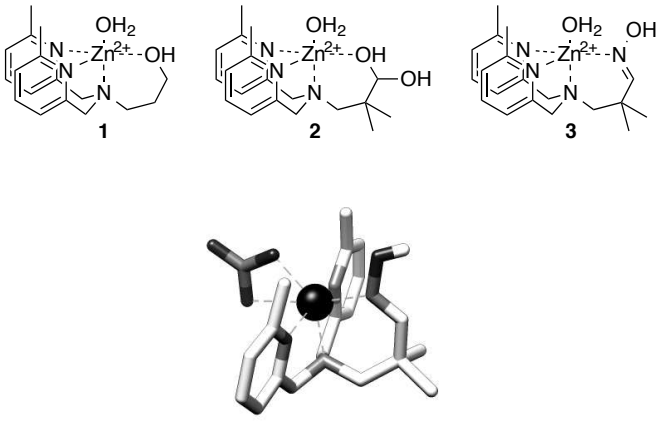

Figure 1. Zn complexes discussed in this study, and representation of the X-ray crystal structure of **3**.$(NO_3)_2$ (50% probability ellipsoids; non acidic hydrogen atoms and non coordinated nitrate omitted for clarity).

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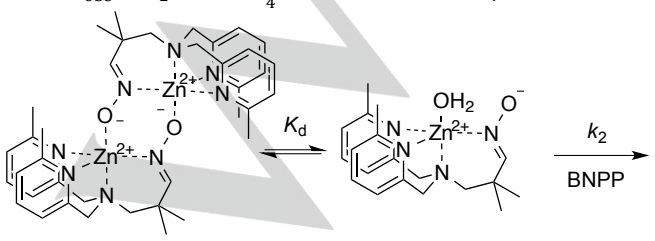
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201xxxxxx

We measured the reactivity of **3** towards the cleavage of bis-*p*-nitrophenyl phosphate (BNPP), which is a convenient model phosphate diester without an intramolecular nucleophile. The reactions were carried at 25 °C under aqueous conditions and the cleavage of BNPP was followed by the release of 4-nitrophenolate at 400 nm. Complex **3** is highly reactive and the half life of BNPP (0.05 mM) is about 20 min in the presence of 1 mM of complex **3** at pH 8.1.

Varying the concentration of complex **3** reveals a nonlinear relationship between the observed rate constants and concentration. The downward curvature that is observed could indicate saturation behaviour due to a substantial fraction of the substrate binding to **3**, and these data fit a 1:1 binding isotherm with a dissociation constant K_d = 12±1 mM for the complex between **3** and BNPP at pH 8.1 (figure S3). This value is surprisingly low as mononuclear zinc complexes usually have rather weak binding affinities with phosphate diesters in aqueous solution.[25,26] To estimate the affinity of **3** for phosphate diesters we measured how dimethyl phosphate inhibits the reaction. Figure S5 shows the normalized rate constant of the reaction catalysed by **3** with increasing concentration of DMP, and no inhibition can be observed up to 40 mM inhibitor. An observed K_d of 12 mM would have led to an 80% loss of reactivity at 40 mM DMP, and so this explanation for the curvature is not tenable. Using 4-nitrophenyl phosphate as an inhibitor (this substrate reacts much more slowly; see below) reveals a K_i of 7.5±0.8 mM at pH 8.1; typically, binding of phosphate monoester dianions to Zn complexes is about 1000 fold tighter than for diester monoanions, supporting the suggestion that **3** does not bind BNPP tightly.[26]

Hence, we explain this behaviour through the formation of an inactive dimer. A similar explanation has been proposed by Yatsimirsky *et al*.[27] when considering the activity of Zn complexes of 2,6-diacetylpyridine dioxime towards 4-nitrophenyl acetate in 24% aqueous ethanol. Dimerisation of metal ion complexes is common, especially with mononuclear copper complexes, and often leads to dihydroxy-bridged structures where substrate binding is prevented.[28] This behaviour is not observed for **1** or **2** which implies that the oxime plays a central role in the dimerization.[29] The dimerisation takes place mainly between pH 7.2 to 9.5 where the main species is the monodeprotonated complex as shown by the speciation derived from potentiometric titration (see SI). Equation 1, which is derived from scheme 1, was fit to the concentration profiles. The dissociation constant K_d = 9±1 mM is about an order of magnitude higher than observed by Yatsimirsky,[27] which is satisfactory agrement considering the change in solvent and the statistical factor due to the additional oximes.

$$k_{obs} = k_2 \frac{-K_d + \sqrt{K_d^2 + 8\,K_d\,[\mathbf{3}]_{tot}}}{4} \qquad \text{Equation 1}$$



Scheme 1. Formation of inactive dimer.

The pH dependence of k_2 reveals that the maximum rate constant is obtained between pH 7.5 and 9 with k_2^{max} = 0.80±0.08 M^{-1} s^{-1}, which is greater than the corresponding rate constants for **1** and **2** by 350 and 28 fold, respectively (figure 2). These data show good agreement with equation 2 (solid line), where the singly deprotonated species is the kinetically active ionic species. Potentiometric titration confirms the kinetic pK_as, which are not perfectly defined due to complex dissociation at low pH: under these conditions, ligand protonation competes with zinc binding. At pHs above 10, zinc hydroxide precipitation occurs.

$$k_{\mathrm{obs}} = k_2^{max}[\mathrm{ZnL}]\frac{K_a^1\,[\mathrm{H}^+]}{(K_a^1 K_a^2 + K_a^1[\mathrm{H}^+] + [\mathrm{H}^+]^2)} \qquad \text{Equation 2}$$

^{31}P NMR highlights a more complicated reaction mechanism than the simple phosphorylation of the ligand observed with **1**. Two pathways can be followed once the initial attack of the oxime nucleophile on the phosphate has occurred, and the dominant one depends on whether **3** or BNPP is in excess.

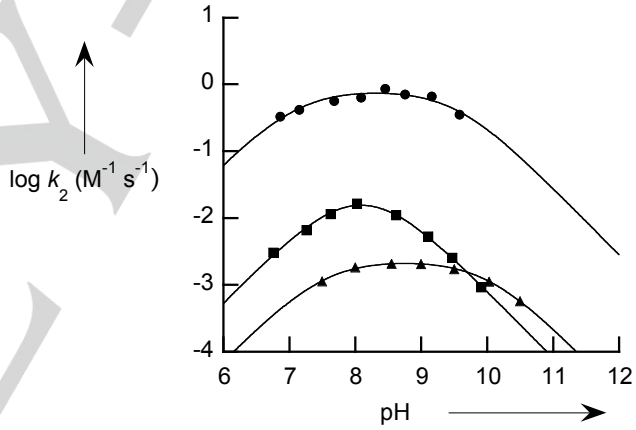


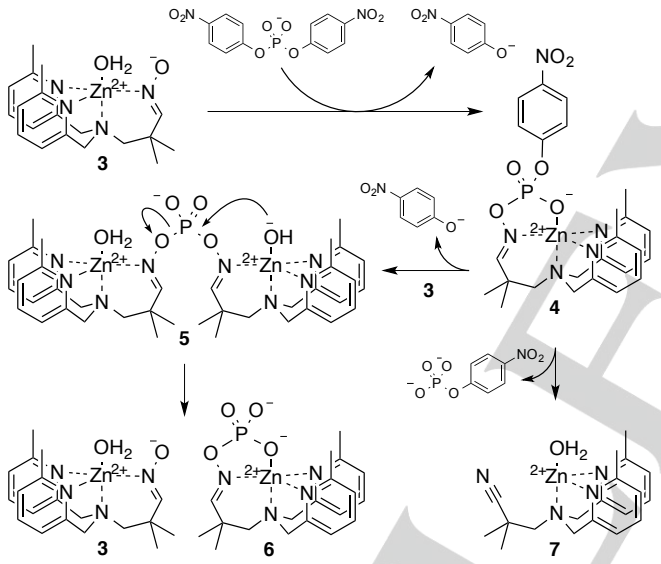
Figure 2. pH rate profile for the cleavage of BNPP; cleavage catalysed by **1** (squares)[15] and **2** (triangles)[15]; cleavage catalysed by **3** (circles) at 25 °C([buffer] = 0.05 M, *I* = 0.1 M ($NaNO_3$)). Solid lines are from fitting equation 1 to the data.

Table 1. Collected kinetic parameters from pH rate profiles.

Compound	pK_a^1	pK_a^2	k_2^{max} [M^{-1} s^{-1}]
1[15]	7.50±0.03	10.00±0.02	2.3±0.04 x 10^{-3}
2[15]	7.7±0.1	8.4±0.1	2.9±0.3 x 10^{-2}
3	7.02±0.02	9.57±0.02	8.0±0.8 x 10^{-1}

Under the conditions of the kinetic experiments (excess **3**), the release of two equivalents of 4-nitrophenolate can be monitored at 400 nm and fit to a first order equation. Monitoring the

reaction by ^{31}P NMR (at a 2:1 ratio of **3**:BNPP) shows the formation of an intermediate (**4**) and its subsequent conversion into mainly **6** (see SI). When a ligand based alcohol is the nucleophile, the resulting diester is a poor substrate for either intramolecular or intermolecular (by a second equivalent of Zn complex) displacement of the remaining good leaving group.[13] In this case, **4** is a good substrate for subsequent reaction and the second leaving group is efficiently expelled as well. We do not observe **5** directly, and assume that in this species, the intramolecular reaction is efficient. A competing pathway shows the decomposition of a small proportion of **4** into 4-nitrophenyl phosphate (*p*NPP) and a nitrile ligand (**7**). With an excess of substrate (at a 1:2 ratio of **3**:BNPP), only one equivalent of 4-nitrophenol is produced, because BNPP effectively competes with **4** as a substrate for **3**. This leads to an accumulation of **4**, which is relatively stable in the absence of **3**, and which decomposes more slowly through an elimination pathway to release *p*NPP and so overall BNPP is hydrolysed (although not catalytically). Preparative HPLC allowed for the separation and full characterisation of **6**, **7**, **3** and 4-nitrophenol in accord with this explanation (see SI).



Scheme 2. Reaction of **3** with BNPP.

The cleavage of 4-nitrophenyl monophosphate catalysed by **3** also results in formation of **6**, but $k_2 = 3.3\pm0.5 \times 10^{-3}$ M^{-1} s^{-1} at pH 8.1 which is around 200 fold slower than the overall reaction of BNPP (where $k_2 = 0.63$ M^{-1} s^{-1} under these conditions). Thus, the formation of **6** from BNPP does not proceed through *p*NPP as an intermediate. This rate constant is about 100 fold lower than reported by Lloyd and Cooperman[18] for the cleavage of N-methyl phosphorylimidazole (0.42 M^{-1} s^{-1} at 29.2 °C), but represents a 100 fold greater rate acceleration, as the uncatalysed rate of hydrolysis of *p*NPP dianion is about 10 000 fold slower than for phosphorylimidazole. The observation that **3**, a mononuclear zinc complex, catalyses the cleavage of *p*NPP is unusual as previous reports highlight the need of more complicated structures such as in micellar or supramolecular catalysts.[30,31]

Complex **3** is the most active mononuclear zinc complex to date regarding BNPP cleavage, and its high reactivity (k_2^{max} of 0.80 M^{-1} s^{-1}) makes it a promising catalyst for substrates which are less activated than BNPP. To study the dependence of the reactivity on the pK_a of the leaving group, a range of methyl aryl phosphate diesters were synthesised, with leaving groups ranging from 4-nitrophenol, pK_a = 7.14 to phenol, pK_a = 9.95. These substrates simplify the reaction pathway as only one cleavage event can take place even in the presence of an excess of complex, allowing the use of the initial rate method for the poorer leaving groups.

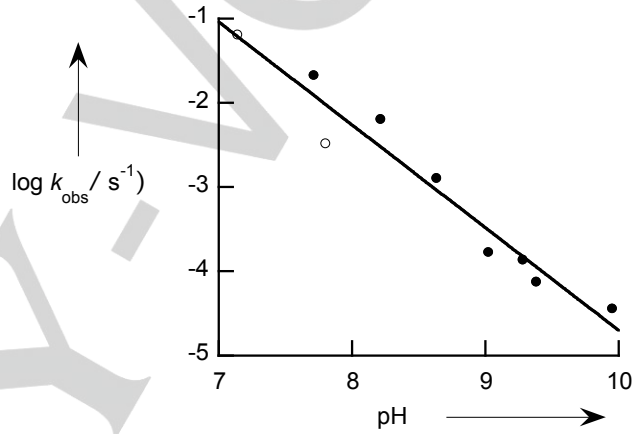


Figure 3. Leaving group dependence of the cleavage of methyl aryl phosphate diesters (4-NO_2 and 4-CN, open circle) catalysed by 2 mM complex **3**; pH 8.1, 50 mM buffer, 0.1 M ionic strength ($NaNO_3$), 25°C.

Linear least-squares fitting of the data (figure 3, solid line) leads to a β_{lg} of -1.2±0.1. This β_{lg} value is surprisingly high and suggests a transition state with substantial bond breaking with the leaving group. The equilibrium value for the reaction is β = 1.74, suggesting a Leffler index of 1.3/1.74 = 0.75 for this reaction (assuming a concerted process). For comparison, Zalatan and Herschlag reported a value of β_{lg} = -0.94±0.05 for the hydrolysis of methyl phenyl phosphate diesters[32], leading to a Leffler index of 0.54, significantly less advanced. It shows that stabilisation of the leaving group is key if the hydrolysis of less activated substrate and eventually DNA is to be achieved, and suggests that this interaction is not occurring with complex **3**.

If one does not include 4-NO_2 and 4-CN data points (open circles) in the fit, the value of β_{lg} is -1.3±0.1. Previous studies have suggested this is an appropriate treatment for the hydroxide promoted cleavage of methyl aryl diesters due to the strong mesomeric contribution to the stability of the product anions in 4-NO_2 and 4-CN.[32] A more thorough approach is to use a Yukawa-Tsuno analysis to fit these data[33] and yields values of ρ = 2.21±0.08 and r = 0.33 for the reaction of hydroxide with these diesters, and of ρ = 3.3±0.3 and r = 0.46 for the reaction catalysed by **3**. These parameters similarly

emphasis the more advanced nature of the transition state for the catalysed reaction.

In conclusion, we demonstrate that the use of a non-metal bound nucleophile is a valid and effective strategy for phosphate ester cleavage. Using an oxime as a nucleophile leads to an increased reactivity of 350-fold compared to a hydroxypropyl nucleophile, and **3** is the most reactive mononuclear Zn complex toward BNPP cleavage reported to date. Its maximal activity is 8 fold greater than an analogue of **1** where amino groups replace the 2-methyl groups on the pyridyl rings; and due to its lower first pK_a (7.0 cf. 7.9) about 40 fold more active at pH 7.[14] However, the high sensitivity of the reaction promoted by **3** to the pK_a of the leaving group shows that **3** provides much lower rate accelerations for diesters with poor leaving groups. We aim to discover whether combining the activating effects of the oxime nucleophile and secondary sphere interactions will create yet more reactive complexes that will be of practical utility in cleaving DNA. Our observations suggest that incorporation of functionality, such as additional metal ions or proton donors, will be necessary to develop complexes capable of cleaving substrates with alkoxy leaving groups.

Experimental Section

Kinetic experiments were carried out in water at 25 °C, 50 mM buffer (HEPES or CHES according to pH), *I* = 0.1 M ($NaNO_3$). A typical experiment was initiated by the addition of 50 µL of 1 mM BNPP in 50mM buffer and 0.1 M ionic strength to a 1 mL cuvette equilibrated at 25°C and containing 50µL of 20 mM **3** (dissolved in 50 mM buffer, *I* = 0.1 M ($NaNO_3$) and 900 µL of the same buffer. Reaction progress was monitored at 400 nm. Product analysis by ^{31}P NMR and preparative HPLC confirmed the course of the reaction. See supporting information for the synthesis of **3**, titration and speciation data, crystallographic details, observed rate constants and kinetic behaviour of **3** with BNPP, *p*NPP and in the presence of DMP and *p*NPP as inhibitors.

Acknowledgements

The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n° 238679.

Keywords: bioinorganic chemistry • DNA cleavage • enzyme models • kinetics • zinc

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Layout 2:

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*Emmanuel Y. Tirel, Nicholas H. Williams**

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Title

Enhancing Phosphate Diester Cleavage by a Zinc Complex Through Controlling Nucleophile Coordination

How to activate a nucleophile: Using an oxime as a ligand based nucleophile generates a Zn complex which is the most effective at cleaving DNA-like phosphodiesters reported so far. This nucleophile is held in close proximity to the substrate binding site, but the geometry prevents the nucleophile binding to the Zn ion and so stops their mutual deactivation.