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Cyclen based lanthanide ion ribonuclease mimics: the effect of pyridine cofactors upon phosphodiester HPNP hydrolysis

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Abstract—The cyclen based pyridine complexes **1Ln**–**3Ln** (Ln = La(III) and Eu(III)) were synthesised as metallo-ribonuclease mimics and their ability to hydrolytically cleave the phosphodiester of HPNP at 37 °C was investigated using UV–vis spectroscopy, whereas the binding of the substrate was evaluated using ^{31}P NMR and Eu(III)-luminescent measurements. In contrast **2La** gave rise to fast pH dependent hydrolysis of HPNP, with maximum efficiency at ca. pH 8.2, and with a half-lifetime of ~1 h, the **1Ln** and **3Ln** complexes were found to be inactive, emphasizing the importance of the nature of the pyridine isomer as a cofactor in the hydrolytic process.

Phosphoesters are structural motifs found in biological systems such as DNA and RNA. Nature has developed ribonucleases that hydrolytically cleave such esters in a controllable manner often thought to involve the synergic action of more than one metal ion.[1,2] Moreover, the active sites of such enzymes have basic amino acid residues, which in addition to metal coordination, act as cofactors that participate in general acid–base catalysis, helping to stabilise the transition state and assist leaving group departure. Unlike DNA, RNA has the 2′-hydroxyl group available as an internal nucleophile, making RNA much less stable in comparison to DNA. It is postulated that metal ions participate in the hydrolysis, either directly or indirectly, through Lewis-acid activation (via coordination to the phosphate anion), stabilisation of the transition state, or through the provision of nucleophiles in the form of metal-bound waters or hydroxyl-containing molecules.[1,2] We[3] and others[4] have developed synthetic nuclease molecules that can mimic the behaviour of these metallo-enzymes. Such mimics are potentially important for use in biotechnology as they can be utilised in the manipulation of genes, as structural probes, and in medicine as novel therapeutics for blocking gene transcription.[1,2,5] We have already focused our efforts on synthesising lanthanide ion-based ribonuclease mimics by incorporating dipeptides such as GlyGly and GlyAla into 1,4,7,10-tetraazacyclododecane (cyclen),[3] which gave large enhancements in the rate of hydrolysis of RNA phosphodiester mimics such as HPNP (2-hydroxypropyl-*p*-nitrophenylphosphate) **7**. Herein, we describe the synthesis and structural analysis of three new Ln(III) cyclen complexes **1Ln**–**3Ln** (Ln = La(III) and Eu(III)) where we have incorporated the three pyridine isomers onto the macrocyclic structure as part of the tetra-amide pendent arms. The main objectives were to investigate the role of the pyridine moieties (which can give rise to a hydrophobic cavity)[3,6] in conjunction with the lanthanide ion centre in the hydrolysis and to demonstrate the potential role of these molecules as ribonuclease mimics or artificial enzymes using non-'natural' metal ions.

The synthesis of **1**–**3** is shown in Scheme 1, commencing with the α-chloroamides **4**–**6**. The precursor **5** was formed in 98% yield by reacting chloroacetyl chloride with 3-aminopyridine in acetone at 0 °C, in the absence of any base. However, the synthesis of **4** and **6** was only successful in DCM at 0 °C using triethylamine as base.

Keywords: Ribonuclease mimics; Lanthanides; Eu(III); La(III); Hydrolysis; HPNP; Cyclen.
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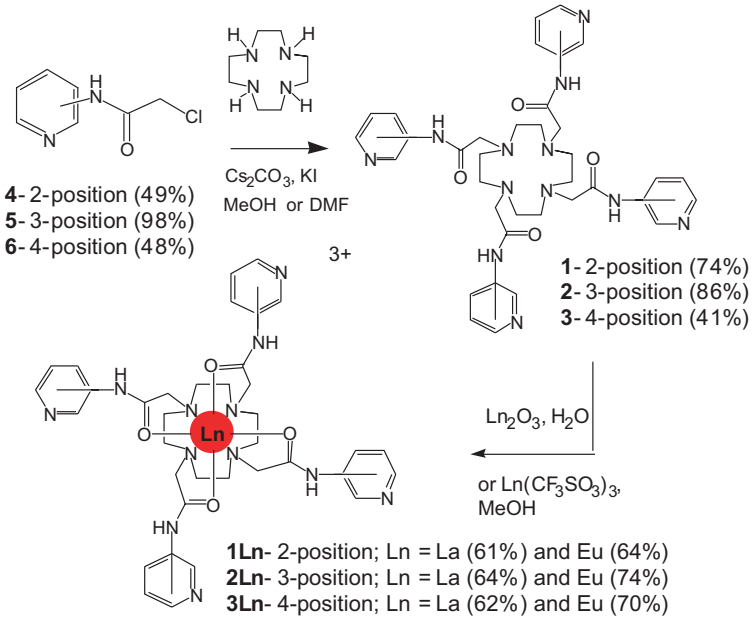


Scheme 1. Synthesis of **1**–**3** and the corresponding lanthanide ion complexes **1Ln**–**3Ln**.

The next step involved the incorporation of these arms into the macrocycle. For **1** and **3** the desired products were obtained using 5.5 equiv of **4** and **6**, respectively, in the presence of cyclen, Cs_2CO_3 and KI in DMF (80 °C for 64 h). The products were purified by precipitation from dry $CHCl_3$ yielding only the desired tetra-substituted cyclen derivatives **1** and **3** in 74% and 86% yields, respectively. However, the synthesis of **2** under these conditions was not successful. It was isolated as a pale orange powder in 41% yield, by reacting **5** (5.5 equiv) and cyclen in refluxing MeOH in the presence of Cs_2CO_3 and KI for five days. This yielded both the tri- and tetra- (**2**) substituted cyclen derivatives, which were separated by gradient elution on alumina [CH_2Cl_2:MeOH·NH_3 (1–20%)]. The ^{1}H NMR spectra of **1**–**3** showed the presence of C_4 symmetry for all these complexes, for example, for **2** (400 MHz, DMSO-d_6) the methylene protons on the acetamide arms and the cyclen ring protons appeared as singlets at 3.20 and 2.72 ppm, respectively, with the pyridine protons appearing at 8.51, 8.16 and 7.03 ppm, while the amide proton appeared at 10.39 ppm. Similar results were seen for **1** and **3**. The corresponding La(III) and the Eu(III) complexes of **1** and **3** were formed in water from either La_2O_3 or Eu_2O_3, respectively, under reflux. After filtration, the solvent was removed under reduced pressure, giving oils that were redissolved in the minimum amount of MeOH followed by trituration using $CHCl_3$. This yielded **1Ln** and **3Ln** (Ln = La and Eu) in ca. 60% yields. This method was, however, unsuccessful for **2La** and **2Eu** (as well as the Gd(III) complex **2Gd** which was also prepared) which were formed by reacting **2** with $La(CF_3SO_3)_3$ and $Eu(CF_3SO_3)_3$ in refluxing MeOH followed by precipitation from $CHCl_3$, giving **2La** and **2Eu** in 75 and 65% yields, respectively.[7]

The ^{1}H NMR spectra of all the Eu(III) complexes showed, as expected, that the resonances for the equatorial and the axial protons of the cyclen ring and the pendent arms were substantially shifted due to the presence of the paramagnetic Eu(III) ion. For **2Eu** these appeared (in D_2O, 400 MHz) at 16.35, 8.08, 7.18, 4.61, 3.16, 1.59, 1.03, −1.31, −4.21 and −10.36 ppm, respectively. We also determined the hydration state (q), the number of metal-bound water molecules for the europium complexes, as this would confirm the coordination number of the complexes in solution. For all of these, a q-value of ca. 1 was determined, Table 1. We propose that this geometry would give rise to a concave structural motif as previously discussed.[3] We were able to grow single crystals of **2La**, **2Eu** and **2Gd** as colourless needles, by slow evaporation of water solutions. The structure of **2La** is shown in Figure 1. It is known that such La(III)–cyclen complexes usually adopt a 10 coordination geometry.[6,8,9] This was found to be the case for **2La**, which clearly showed the concave nature of the complex, with the La(III) ion centrally located, coordinating to the four nitrogen atoms of the cyclen ring and four oxygen atoms of the carboxylic amides, giving a disordered geometry. Furthermore, the complex had the ninth coordination site occupied by a metal-bound water molecule, and in the tenth coordination site was a triflate anion.[8] We predict that in water this second

Table 1. Determination of the hydration state of the Eu(III) complexes[a,†]

Complex	k (ms^{-1}) H_2O	τ (ms) H_2O	k (ms^{-1}) D_2O	τ (ms) D_2O	q (±0.5)
1Eu	1.74	0.36	0.60	1.66	1.09
2Eu	2.60	0.38	1.07	0.94	1.18
3Eu	2.56	0.39	1.41	0.71	0.90

[a] Determined using $q^{Eu} = 1.2[(1/\tau_{H_2O} - 1/\tau_{D_2O}) - 0.25 - 0.075x]$ ($x = 4$ for **1**–**3**).[10]

[†] We were unable to determine the q-value for the La(III) complexes using this method.[6,8,9]

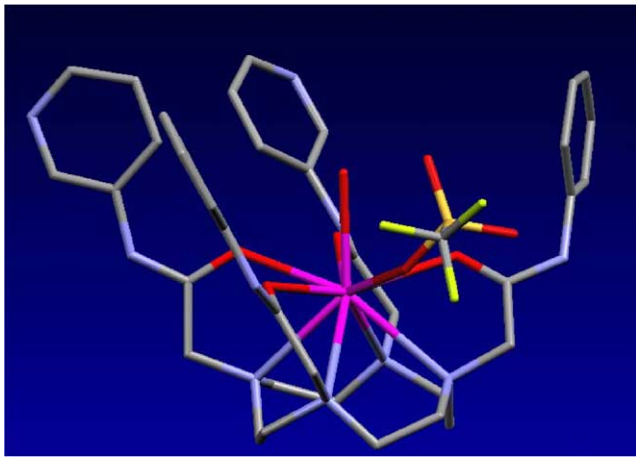


Figure 1. X-ray crystal structure of **2La** showing the 10 coordinate environment of the La(III) complex. Hydrogen atoms, solvent and non-coordinated triflate anions have been removed for clarity.

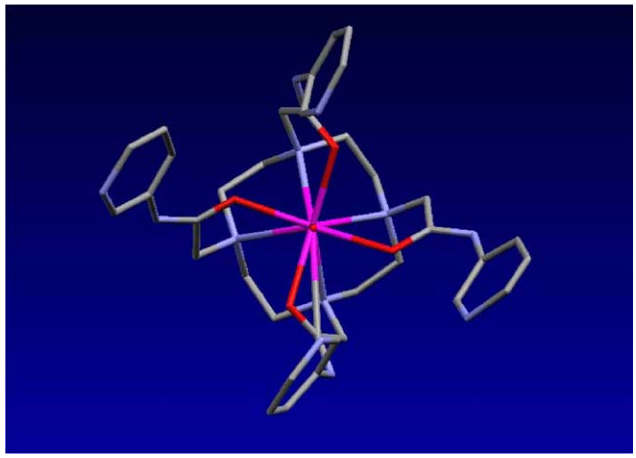
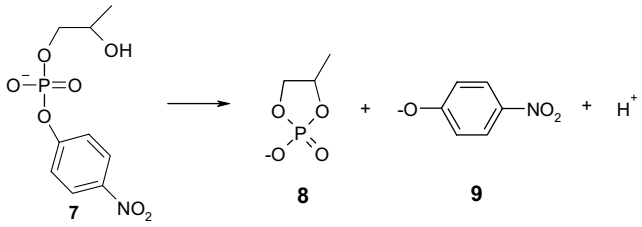


Figure 2. X-ray crystal structure of **2Eu** ($\Delta(\lambda\lambda\lambda\lambda)$) showing the nine coordinate environment of the Eu(III) complex. Hydrogen atoms and triflate anions have been removed for clarity.

coordination site is occupied by the solvent, due to fast H_2O exchange, giving rise to the diaqua species. As shown later, the presence of these metal-bound water molecules is vital to the activity of the ribonuclease mimic and towards phosphodiester hydrolysis.[1–4,10] In comparison to these results, the X-ray crystal structure of **2Eu**, Figure 2, showed a typical monocapped square antiprism geometry, with the enantiomeric conformation $\Delta(\lambda\lambda\lambda\lambda)$ where the water molecule was in the axial position and the overall coordination number of Eu(III) was nine. The X-ray crystal structure of the **2Gd** complex was isostructural, supporting the results of the solution studies which showed that these complexes have a single bound water molecule.

In order to investigate the ability of these complexes to promote phosphodiester hydrolysis, we used the RNA mimic compound HPNP, **7**, Scheme 2. The rate constant of hydrolysis of HPNP can be followed by observing the appearance of *p*-nitrophenolate **9** at 400 nm, upon hydrolysis (HPNP absorbs at 300 nm), Scheme 2.[‡] The resulting absorption changes at 400 nm were fitted to give the rate constants k_{obs}. Under these conditions, **2La** was found to promote hydrolysis of **7** with a k_{obs} of 0.189 (±0.003) h^{-1} and a $\tau_{1/2} \sim 3.7$ h. This is a rate enhancement of 1600.[§] In direct contrast to these results, neither **1La** nor **3La** gave rise to any significant hydrolysis at this pH. We also evaluated the hydrolysis of **2Eu** under identical conditions, which gave $k_{obs} = 0.078$ (±0.0028) h^{-1} and $\tau_{1/2} = 8.9$ h. When these measurements were repeated in the absence of either complexes



Scheme 2. The hydrolysis of HPNP.

no measurable HPNP hydrolysis was observed over 48 h, indicating that the rate enhancement was indeed due to the hydrolysis of the substrate by these complexes.

To investigate the hydrolytic ability of **2La** and **2Eu** further, we evaluated the hydrolysis of **7** as a function of pH. These results, where k_{obs} is shown as a function of pH, are shown in Figure 3. For both complexes, the hydrolysis was too slow to evaluate in acidic media below pH ~ 6. The overall results demonstrate that for both complexes some hydrolysis is observed at around

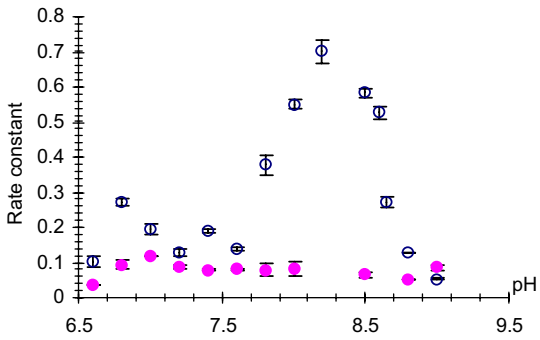


Figure 3. The changes in the rate constant of hydrolysis of HPNP for **2La** (blue open circles) and **2Eu** (pink circles) as a function of pH. The error bars are determined from two set of measurements.

[‡] All initial kinetic experiments were carried out at 37 °C, over several half-lifetimes, using 4.32×10^{-7} mol of HPNP (giving 0.18 mM) and 50 mM HEPES buffer to maintain constant pH. A solution containing 4.32×10^{-7} mol of the corresponding lanthanide complex was added and the reaction monitored over 16 h, with constant stirring. Measurements were recorded with either an Agilent 8453 or a Cary 50 Scan spectrophotometer, both fitted to a circulating temperature controlled water bath and mechanically stirred. Errors are within ±10%.

[§] $k_{ucat} = 0.00012\ h^{-1}$.[14]

pH 7.4, and that for **2Eu** the hydrolysis becomes pH independent above pH 7.5, Figure 3. However, for **2La** it is clear that the hydrolysis is significantly pH dependent in alkaline media, as a 'bell-shaped' curve is observed. Here, **2La** has highest activity at ca. pH 8.2, $k_{obs} = 0.71\ (\pm 0.014)\ h^{-1}$ and $\tau_{1/2} = \sim 1$ h, which is a rate enhancement of $\sim 4.8 \times 10^3$. We attribute this 'bell-shape' nature to the presence of two metal-bound water molecules in **2La** versus one for **2Eu**, as the main difference is the higher coordination environment of the La(III) complex. This consequently affects the nature of the cavity in **2La**, Figure 1 versus Figure 2, and we would expect that such a structural difference would also exist in solution though we have no direct evidence for that at present. These results clearly demonstrate, since only **2Ln** is able to promote significant hydrolysis of HPNP, that minor structural modifications have an enormous effect on the hydrolytic activity of the complex as neither **1La** nor **3La** gave rise to such enhanced hydrolysis at pH ~ 8. This could possibly be due to the different affinities of these complexes for the substrate, caused either by their ability to bind the substrate, or any resulting intermediates/leaving groups, through hydrogen bonding and/or base–acid catalysis.[11] Moreover, the resonance stabilisation of any deprotonated amides can also have an effect here, and we are currently investigating that possibility in greater detail. The fact that the hydrolysis is slower above pH 8.5 suggests that the second water molecule becomes deprotonated, giving the dihydroxy species that prevents the binding of the substrate to the metal centre. Hence, the observed enhancements in k_{obs} between pH 7.5 and 8.5 are not due to background hydrolysis (or a buffer effect), but to the synergic action of Lewis-acid activation of the substrate and nucleophilic activation of the 2-hydroxy nucleophile.

We propose a possible mechanism for **2La** where both the metal-bound water molecules participate in the hydrolytic process, Figure 4.[3,12] We propose that deprotonation of one of these water molecules would give rise to a metal-bound hydroxy group that could function as a nucleophile activator. This would give rise to subsequent deprotonation of the 2-hydroxypropyl group in HPNP, after coordination of HPNP through the phosphate anion to the metal ion, and tandem expulsion of the second water molecule. The resulting activated nucleophile could then attack the metal-bound/coordinated phosphodiester, which would produce a five-coordinate phosphorane intermediate, which would break down to give a cyclic metal bound phosphate and **9**. Finally, the cyclic phosphate would be released by ligand exchange with water. For the Eu(III) complex, only one water molecule is present in the complex and if the second water molecule is necessary for hydrolysis to take place, then the rate constant should be independent of pH. This was indeed found to be the case as shown in Figure 3.

To test if the substrate was binding to the metal complexes, we carried out ^{31}P NMR binding studies in H_2O using **2Eu** and **2La**, and barium diethylphosphate $[(CH_3CH_2O)_2PO_2^-]$ (DEP), which lacks the 2′-hydroxyl group. For **2Eu** the phosphorus resonance was shifted upon coordination, demonstrating fast exchange on the NMR timescale. For **2La**, the changes were, however, found to be in slow exchange, with a signal that became saturated after addition of ca. 1 equiv of DEP. We also evaluated the changes in the *q*-value for the Eu(III) complex in the presence of HPNP and DEP. Whereas the *q*-value was affected for DEP, it remained constant, for example, ~1, for HPNP, suggesting that the water was not displaced by the phosphodiester. We also observed the changes in the Eu(III) emission of **2Eu** as a function of HPNP and DEP. For the former, the emission remained constant, whereas for DEP it increased in intensity. This suggests that HPNP was not binding, whereas DEP did. We are evaluating these effects in greater detail.

In summary, we have synthesised three new cyclen-based pyridine isomers and their corresponding La(III) and Eu(III) complexes. Of these only the 3-isomer (**2Ln**) gives rise to hydrolysis of HPNP, demonstrating that the pyridine isomer plays a pivotal role in

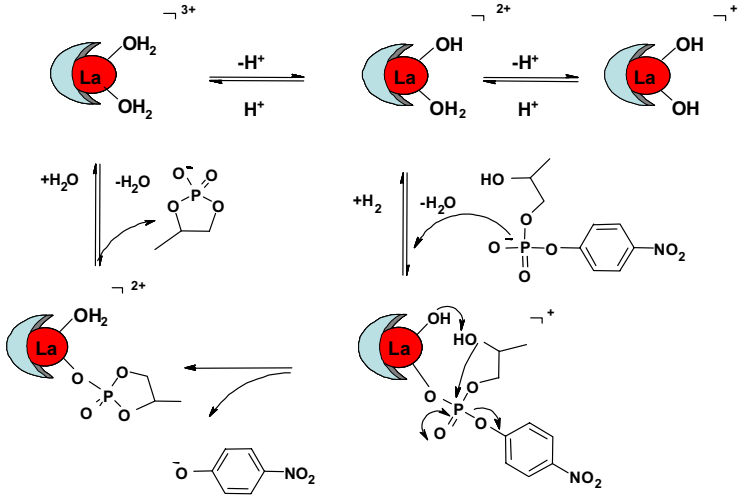


Figure 4. The proposed mechanism for the hydrolysis of HPNP by the La(III) complex **2La** (see text for further details).

promoting HPNP hydrolysis. We are currently evaluating these features in greater detail.¶

X-ray crystallography: Data were collected on a Bruker SMART diffractometer using the SAINT-NT[13a] software with phi/omega scans. Although the structure of **2La** was unambiguously established, the quality of the crystals and significant disorder within the pyridine moieties unfortunately led to a rather poor refinement ($R_1 \sim 13\%$). A crystal was mounted onto the diffractometer at low temperature ca. 120 K. The structure was solved using direct methods and refined with the SHELXTL program package.[13b] Additional material available from the Cambridge Crystallographic Data Centre comprises relevant tables of atomic coordinates, bond lengths and angles, and thermal parameters (CCDC Number: CCDC 265791).

N-Pyridin-3-yl-2-[2,7,10-tris-(pyridin-3-ylcarbamoylmethyl)-1,4,7,10-tetraazacyclododec-1-yl]-acetamide **2**: Calculated for $C_{36}H_{44}N_{12}O_4{\cdot}H_2O{\cdot}CHCl_3$: C, 52.53; H, 5.60; N, 19.86. Found: C, 52.23; H, 5.21; N, 19.87; Calcd for $C_{36}H_{45}N_{12}O_4$: [M+H] *m/z* (ES^+): 709.3687. Found: 709.3653; ^1H (DMSO-d_6, 400 MHz), δ 10.39 (br s, 4H, NH), 8.51 (s, 4H, CCHN), 8.16 (t, $J = 6.0$ Hz, 8H, NC*H*CH), 7.03 (s, 4H, NCHC*H*CH), 3.20 (s, 8H, CH_2), 2.72 (16H, s, NCH_2CH_2N); ^{13}C (DMSO-d_6, 100 MHz), δ 172.2, 143.0, 140.1, 163.0, 127.3, 124.1, 56.8, 52.3, 51.6; *m/z* (ES^+): 709.2 $(M+H)^+$, 731.2 $(M+Na)^+$; IR ν_{max} (cm^{-1}) 3385, 3218, 3170, 3066, 2969, 2828, 1696, 1548, 1483, 1305, 1204, 1106, 950, 805, 706. **2La**: Calcd for $C_{36}H_{44}N_{12}O_4La$: [$(M)^{3+}$ peak] *m/z* (ES^+): 847.2672. Found: 847.2654; ^1H (D_2O, 400 MHz), δ 8.46, 8.11, 7.82, 7.1, 3.8, 3.6, 2.9, 2.7, 2.4; *m/z* (ES^+): 423.02 $(M)^{2+}$, 497.99 $(M+Trif)^{2+}$; IR ν_{max} (cm^{-1}) 3463, 3284, 1651, 1486, 1282, 1030, 963, 639. **2Eu**: Calcd for $C_{36}H_{44}N_{12}O_4Eu$: [$(M)^{3+}$ peak] *m/z* (ES^+): 861.2821. Found: 861.2827; ^1H (D_2O, 400 MHz), δ 16.35, 8.08, 7.18, 4.61, 3.16, 1.59, 1.03, −1.31, −4.21, −10.36; *m/z* (ES^+): 430.17 $(M)^{2+}$, 505.21 $(M+Trif)^{2+}$, 1159.37 (M+2Trif); IR ν_{max} (cm^{-1}) 3463, 3284, 1651, 1486, 1282, 1168, 1030, 963, 639.

Acknowledgements

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¶ Preliminary investigation has also shown that even though **1Eu** did not cleave HPNP, it efficiently cleaved a 23 mer-mRNA sequence from the GAG-HIV gene at pH 7.4 and 37 °C after 4 h of incubation. We have not quantified this hydrolysis.

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