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# Glycine–alanine conjugated macrocyclic lanthanide ion complexes as artificial ribonucleases

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**Abstract**—The lanthanide ion based macrocyclic complexes **1La**, **1Eu** and **1Yb**, were synthesised by the alkylation of *L*-alanine derived α-chloroamide, giving a tetrasubstituted GlyAla conjugated azamacrocycle using a 1,4,7,10-tetraazacyclododecane, followed by a complexation with either La(III), Eu(III) or Yb(III) triflate. Each complex gave rise to enhancements in the rate of hydrolysis of the phosphodiester of HPNP, an RNA mimic compound, at pH 7.4 and 37°C. For **1La**, the hydrolysis was shown to give dual pH behaviour, where within a narrow pH window the hydrolysis of HPNP was greatly enhanced between 6.5 and 7.0, whereas it decreased between 7.2 and 7.4. At higher pH, above 8, the rate was again greatly enhanced. We conclude that these enhancements are due to the formation of a hydrophobic cavity in **1** upon lanthanide ion complexation, in conjunction with the Lewis acid activation of the phosphate diester by the metal ion. These complexes thus mimic the nature of the active site of many ribonucleases. © 2002 Published by Elsevier Science Ltd.

Currently there is a great interest in the development of small organic compounds and coordination complexes that can mimic enzymatic reactions.[1,2] This is of particular importance in the development of supramolecular catalysts,[3] i.e. where small or medium size robust chemical assemblies, or functionalised coordination compounds are designed to participate in, or carry out, chemical transformations such as ester hydrolysis, oxidation, etc. of biological substrates.[4] We have been particularly interested in the use of lanthanide based macrocyclic complexes derived from cyclen (1,4,7,10-tetraazacyclododecane) as supramolecular devices.[5] Recently we have focused our research efforts on the development of kinetically stable lanthanide ion complexes as ribonuclease mimics for the cleavage of phosphodiester bonds of nucleic acids such as RNA.[6] Such enzymatic hydrolysis is thought to be due to the synergetic action of the several cofactors such as basic amino acids (that can participate in general acid–base catalysis),[7] divalent Lewis acid metal centres and/or metal ion bound water (hydroxy) molecules within the hydrophobic site of the enzymes.[7–9] We are particularly interested in designing these complexes so that the hydrophobic nature of the active site of ribonucleases is mimicked by incorporating appropriate cofactors[10] such as amines, amino acids and dipeptides into the basic structure of cyclen. With this in mind we set out to synthesise **1**, a GlyAla derivative (where Gly is part of the macrocyclic ring) and the La(III), Eu(III) and Yb(III) complexes **1La**, **1Eu** and **1Yb**, respectively, from cyclen by incorporating four amino esters (the 'pseudo' dipeptide GlyAla) as pendant arms into cyclen. We proposed that such modification would give rise to a possible increase in the rate of hydrolysis of the phosphodiester, since the dipeptide would form the wall of a hydrophobic cavity in **1La**, **1Eu** and **1Yb** after lanthanide ion complexation, and thus mimic the nature of the active site of ribonucleases. From an applications point of view, such lanthanide ion based ribonuclease mimics are highly desirable as therapeutics in gene therapy, as antisense agents, and as tools in molecular biology and genetics.[11]

The advantages of using cyclen based complexes are that they can be easily functionalised, the complexes are both kinetically and thermodynamically stable and they can adopt square antiprism geometry in both solution and the solid state,[12] which is of particular relevance to our design if hydrophobicity is to be achieved. Importantly, amide functionalised cyclen lanthanide complexes are known to have 1–2 water molecules associated with their structure, giving it a concave structure.[12,13] In this letter we describe the synthesis and characterisation of the ligand **1**, and the ribonuclease mimics **1La**, **1Eu** and **1Yb** as well as discussing the preliminary results of the hydrolysis of the RNA model compound 2-hydroxypropyl *p*-nitrophenyl phosphate

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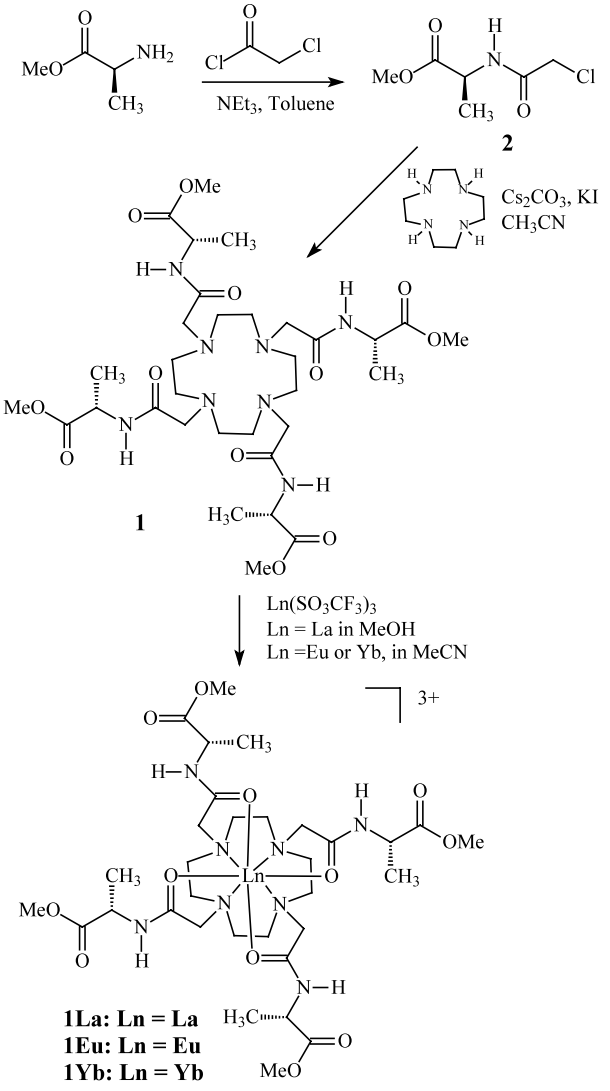
(HPNP) and a 23-mer RNA sequence from the GAG HIV gene, Scheme 2.

The synthesis of **1**, and the cationic complexes **1La**, **1Eu** and **1Yb** are shown in Scheme 1.[14] The α-chloroamide **2** was synthesised according to a modified published procedure[15] by adding a solution of chloracetyl chloride in toluene dropwise to a solution of *L*-alanine methyl ester and triethylamine in toluene at −20°C. This gave **2** in a 65% yield after aqueous acid work-up. The tetra-substituted ligand **1** was made in one step by reacting four equivalents of **2** with cyclen in dry $CH_3CN$, in the presence of 4 equiv. of $Cs_2CO_3$ and KI, at 80°C for 48 h. Following the removal of the inorganic solids by filtration, the solution was reduced in vacuum to give a solid that was treated with $CHCl_3$ followed by washing with saturated KCl and 1 M KOH solutions. Drying and subsequent removal of the solvent under reduced pressure gave **1** as a hydroscopic solid in a 49% yield. This compound was characterised using conventional methods. Characterisation by $^1H$ NMR ($CDCl_3$, 400 MHz) revealed that **1** had $C_4$ symmetry. The two ring protons of the cyclen and the protons of the adjacent α-methylene group (on the glycine pendant arms) were all chemically inequivalent, with the ring protons appearing as AB systems at 2.91 and 2.69 ppm with $J=10$ Hz. Similarly, the glycine methylene protons appeared at 3.27 and 3.12 ppm with $J=16$ Hz. The **1La**, **1Eu** and **1Yb** complexes were made by refluxing an equivalent amount of **1** with the corresponding lanthanide triflate salt in dry methanol for **1La** and in $CH_3CN$ for **1Eu** and **1Yb**. Precipitation in dry ether gave the complexes in yields of 58, 19 and 29%, respectively. The $^1H$ NMR of the paramagnetic **1Eu** complex is shown in Fig. 1, consisting of several resonances appearing at high field (−0.40 to −12.34 ppm) and a single signal at 24.09 ppm for the equatorial and the axial protons of the ring and the methyl protons of the glycine moieties. These signals indicate a mono capped square antiprism geometry in solution.[12,13] A minor isomer, a tricapped trigonal prism (ratio of 8:92) is also visible in the NMR with several smaller resonances showing at high field and a single resonance appearing at 51.3 ppm. The ESMS showed several peaks assigned to various forms of these complexes, balanced by the triflate anions. From these results we can predict that in solution the complexes possibly have the four arms forming the wall of a cavity with the metal ion sitting in the middle of the cavity. However we have been unable to grow suitable crystals of these complexes in order to verify their structure in the solid state to date.




**Scheme 1.** The synthesis of ligand **1** and the La(III), Eu(III) and Yb(III) complexes **1La**, **1Eu** and **1Yb**.

We evaluated the ability of **1** and its complexes to promote phosphodiester hydrolysis of HPNP (as a racemic mixture) by observing the changes in the UV–vis spectra of HPNP as a function of time at 37°C and pH 7.4 (50 mM of HEPES buffer). Cleaving HPNP (which absorbs at 300 nm) yields two new products: *p*-nitrophenolate (absorbing at 400 nm) and a cyclic phosphate, Scheme 2. Each measurement was carried out over several half-lifetimes.[16] The rate of the hydrolysis of HPNP ($k$) by **1Eu** at pH 7.4 was determined to be 0.0903 $h^{-1}$. This gives a half lifetime ($\tau_{1/2}$) of 7.7

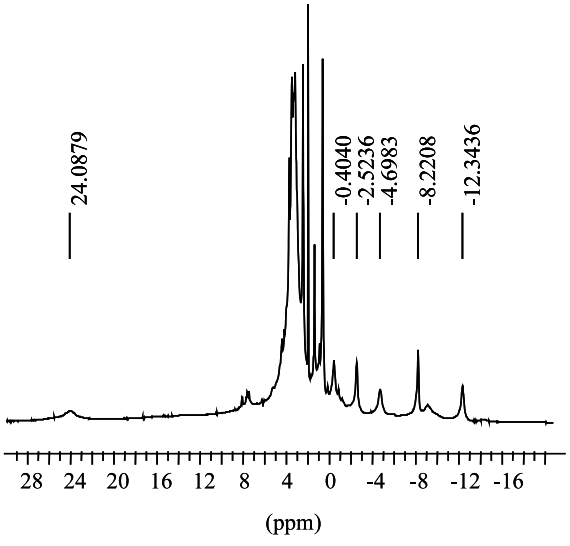
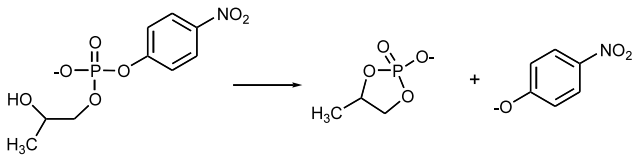



**Figure 1.** The $^1H$ NMR ($CD_3OD$, 400 MHz) at 20°C of **1Eu**.



**Scheme 2.** Hydrolysis of HPNP (absorbing at 300 nm) giving the cyclic phosphate and *p*-nitrophenolate, which absorbs at 400 nm.

hours,[†] which is a rate enhancement ($k_{obs}$) of ca. 752 [$k_{obs}$ is the ratio between $k$ and the rate of the 'uncatalysed' reaction, ($k_{unc}$) which has been measured to be 0.00012 $h^{-1}$, $\tau_{1/2}=5.78\times10^3$ h, at pH 7.4].[16] Under the same experimental conditions **1Yb** gave $k=0.058$ $h^{-1}$ or $\tau_{1/2}=12.0$ h, whereas **1La** gave $k=0.039$ $h^{-1}$ with $\tau_{1/2}=17.7$ h, which gives $k_{obs}=325$, which is rather slow.[†] No HPNP hydrolysis was observed by **1** under the same experimental conditions, indicating the presence of the lanthanide ion was necessary for hydrolysis to occur. Additionally, preliminary investigations have shown that **1Eu** efficiently induces cleavage at every base pair of a 23 mer-mRNA sequence from the GAG-HIV gene at pH 7.4 and 37°C after 4 h of incubation (this cleavage was not quantified). In comparison, **1** showed no such cleavage ability.

In comparison to the cleavage of HPNP by the above complexes, Morrow et al. have recently shown that a La(III) based cyclen amide complex (which lacks the extended 'walls' of **1La**) can cleave HPNP with $k=$ 0.058 $h^{-1}$ or $\tau_{1/2}\sim12$ h.[17] However, the corresponding Eu(III) complex was found to be inactive.[17] Our results indicate that the **1Eu** is quite efficient in cleaving HPNP and the 23-mer mRNA despite its rather bulky structure, which might shield the Lewis acid centre from the solvent environment. However, we were particularly interested in the 'lack of ability' of **1La** to promote hydrolysis of HPNP under these conditions, given the fact that the La(III) is known to be capable of promoting such hydrolysis effectively.[17] Because of this we decided to measure the hydrolysis of HPNP at different pHs (all other experimental conditions remained the same). After carrying out these experiments, we established that the hydrolytic cleavage by **1La** was extremely pH dependent. These results are shown in Fig. 2 (pH range 6–8). Each data point is the average value of two experimental runs within a 10% error. Examining Fig. 2 it can be seen that at a low pH (between 6 and 6.5) we were unable to determine the rate of hydrolysis accurately due to its extremely low reactivity. On the other hand, between pH 6.5 and 7.5 the rate of hydrolysis is particularly pH dependent, and a 'bell shape' curve is observed. Here **1La** is most active in promoting the hydrolysis with $k=0.147$ $h^{-1}$ or $\tau_{1/2}=$

[†] We have also made the *D*-analogue of **1** and the corresponding La(III) and Eu(III) complexes. These were found to cleave HPNP with rates that were within experimental errors of those shown for **1Eu** and **1La** above at pH 7.4. Since HPNP is used as a racemic mixture we are not seeing any diastereoselection for our systems at present. We are however, currently investigating these features.

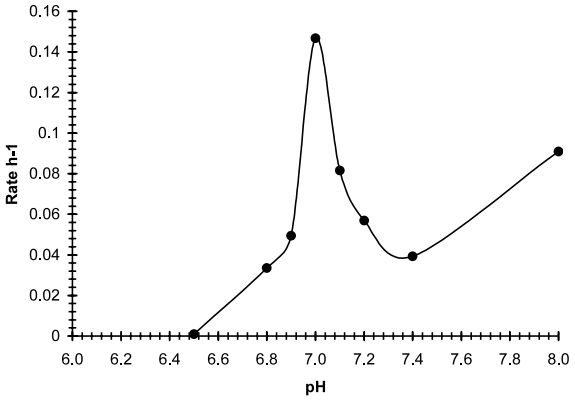



**Figure 2.** The changes in the rate of hydrolysis of HPNP by **1La** (in stoichiometric amounts) as a function of pH (50 mM HEPES buffer) at 37°C between pH 6 and 8.

4.6 h and $k_{obs}=1225$ at pH 7.0. Between pH 7.2 and 8 the hydrolysis is slower, but then is greatly improved (almost linearly) in more alkaline conditions, e.g. at pH 9.9, HPNP is hydrolysed with $k=0.400$ $h^{-1}$, $\tau_{1/2}=1.7$ h and $k_{obs}=3333$ uncorrected for background hydrolysis. To the best of our knowledge this is among the fastest hydrolyses using such kinetically stable cyclen based La(III) amide complexes. We did not evaluate the hydrolysis beyond this pH since background hydrolyses from the alkaline solution becomes active. It is interesting to see that **1La** shows such dual pH dependence, and it is remarkable to see that the complex is capable of hydrolysing HPNP within a very narrow window within the physiological pH range. We have not previously come across such clear dual pH dependence for our systems, and to the best of our knowledge **1La** is the first example of such a complex to exhibit such clear pH behaviour. Since La(III) has generally higher coordination requirements than Eu(III), it can be expected that **1La** could have an extra coordination site when compared to **1Eu**. Similar Eu(III) complexes are known to take up a single water molecule in their square antiprism geometry.[12,13] Consequently it could be expected that if **1La** is adopting such a structure in solution, it could have two water molecules associated with its structure. These could give rise to more efficient phosphodiester hydrolysis since: (i) one of these water molecules could be used to bind to the ester, whereas (ii) the second one would be available to act as a nucleophile or alternatively, deprotonate the 2′ group on the HPNP, making it more nucleophilic.[7] If one of these water molecules was, however, deprotonated one could deduce that the efficiency of at least one of these interactions could be greatly improved.[7] Hence, at higher pH both water molecules could be deprotonated, which would yield two metal bound hydroxy groups. These could further give enhanced binding and an increased rate of hydrolysis, which might explain the increased activity in the alkaline environment. However, our attempts to measure the p$K_a$s of these water molecules for **1La**, using potentiometric titration have not been conclusive. We are currently investigating these features more closely. With the aim of examining the possible binding of the phosphate diester to **1La** and correlate the binding affinity to the above trend, we

carried out a preliminary $^{31}P$ NMR titration in water. Upon adding diethylphosphate [$(CH_3CH_2O)_2PO_2^-$)] (which lacks the 2′ hydroxy group, and should thus be less susceptible to hydrolysis) to **1La**, a gradual shift was observed for the phosphorus signal upon binding to **1La**. However it took over 13 equiv. for the signal to achieve saturation. For **1Eu**, only 3 equiv. were needed to achieve saturation in the $^{31}P$ signal. Hence the binding of the phosphate diester to the metal centre is much weaker for **1La** than **1Eu**. We propose that similar binding preferences would be expected for HPNP. From the above results we can conclude that the complexes are quite efficient in promoting hydrolysis of HPNP and of mRNA. We deduce that even though the above complexes may suffer from steric hindrance due to the peptide walls, they give rise to the formation of a cavity that is hydrophobic. This, in combination with the metal bound water molecules, could give rise to increased rates of hydrolysis of HPNP. We thus conclude that the lanthanide complexes of **1** can be used to mimic the hydrophobic nature of the active site of ribonucleases. In summary, we have developed new types of lanthanide ion based complexes that mimic the hydrophobic cavity of ribonucleases. We found that when using La(III) the rate of hydrolysis was highly pH dependent, showing dual pH behaviour, where within the physiological pH range, the greatest efficiency was achieved at pH 7.0. We are currently investigating these features in greater detail.

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14. Compound **1**: calcd for $C_{32}H_{56}N_8O_{12}$: C, 51.60; H, 7.58; N, 15.04. Found: C, 51.52; H, 7.28; N, 14.74. Expected for $C_{32}H_{56}N_8O_{12}$: 745.4096 ($MH^+$). Found: 745.4058. Yield: 0.456 g (49%); $\delta_H$ ($CDCl_3$, 400 MHz): 7.9 (1H, d, $J=7.0$ Hz, N-H), 4.6 (1H, m, $J=7.0$ and 7.5 Hz, $CHCH_3CO_2CH_3$), 3.7 (3H, s, $CO_2CH_3$), 3.2 (2H, dd, $J=14$ and 16 Hz, $CH_2CO$-), 2.88 (4H, dd, $J=10$ Hz, cyc-$CH_2$s), 1.4 (3H, d, $J=7.0$ Hz, $CHCH_3CO_2CH_3$); $\delta_C$ ($CDCl_3$, 100 MHz): 173, 170, 59, 52.5, 51.9, 47, 17, 14; $m/z$: 745 ($M^+$), 768 ($MNa^+$); IR $\nu_{max}$ ($cm^{-1}$): 3384, 3048, 2954, 2825, 1745, 1667, 1539, 1455, 1291, 1212, 1163, 1102, 1055, 1012, 951, 898, 850, 757, 605, 502. Compound **1La**: expected: 883.3081 ($M^+$). Found: 883.3099. Yield: 53%; mp 262–265°C; $\delta_H$ (acetone, 400 MHz): 9.2 (1H), 4.6 (1H), 4.0 (1H), 3.7 (3H), 2.9 (5H), 1.5 (4H), 0.01 (2H); $m/z$: 294 ($M^+/3$), 308 ($MK^+/3$), 321 ($M2K^+/3$), 335 ($M3K^+/3$), 515 ($MTrif^+/2$), 1180 ($M2Trif^+$); IR $\nu_{max}$ ($cm^{-1}$): 3448, 2926, 2856, 1750, 1625, 1579, 1459, 1259, 1162, 1029, 956, 640, 577, 518. Compound **1Eu**: expected: 897.3230 ($M^+$). Found: 897.3273. Yield: 18.5%; mp 270–273°C; $\delta_H$ ($CD_3OD$, 400 MHz): 24.4 (1H, s, cyc), 2.5 (6H, s), 1.2 (3H, s), 0.5 (6H, s), −2.7 (2H, cyc-H), −4.3 (1H, cyc-H), −8.1 (2H, cyc-H), −8.9 (1H, cyc-H), −12.2 (1H, cyc-H); $m/z$: 299 ($M^+/3$); 448 ($M^+/2$); 523 ($Mtrif^+/2$);

IR $\nu_{max}$ ($cm^{-1}$): 3466, 2926, 2360, 1736, 1626, 1473, 1260, 1168, 1084, 1031, 958, 647. Compound **1Yb**: expected: 918.3406 (M). Found: 918.3374. Yield: 29%; mp 272–275°C; $\delta_H$ (acetone, 400 MHz): 16.8, 13.9, 10.64, 3.7, 2.9, −0.96, −1.5, −10.2, −22.3, −27.4; $m/z$: 306 ($M^+/3$), 459 ($M^+/2$), 533 ($Mtrif^+/2$); IR $\nu_{max}$ ($cm^{-1}$): 3446, 2925, 2360, 1743, 1637, 1459, 1260, 1163, 1084, 1031, 960, 913, 761, 639.

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16. The changes in the 400 nm absorption band were monitored using an Agilent Photodiode Array UV–vis spectrometer fitted to a circulating temperature controlled water bath, and water driven mechanical stirring. $k$ was determined by fitting these observed data by first order rate kinetics using *Biochemical Analysis Software for Agilent ChemStation*. Errors are within ±15%. The hydrolysis of HPNP in the absence of any 'catalyst' at pH 7.4 was evaluated by Breslow, R.; Huang, D.-L. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 4080.
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