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Delayed lanthanide luminescence sensing of aromatic carboxylates using heptadentate triamide Tb(III) cyclen complexes: the recognition of salicylic acid in water†

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The coordinately unsaturated terbium complexes Tb.1 and Tb.2 possess two labile metal-bound water molecules that can be displaced upon metal chelation to aromatic carboxylic anions such as salicylic acid in water, which gives rise to large enhancements in the Tb(III) luminescence.

The use of fluorescence detection[1] for the sensing and physiological monitoring of cations,[2] anions[3] and neutral molecules has been extensively investigated.[4] For *in vivo* sensing, it has been shown to be essential to use fluorophores that absorb and emit at long wavelengths, or have long lived excited states. This is to prevent poor signal-to-noise ratio caused by short-lived (~ns) background emission or autofluorescence and light scattering from the active biological environment.[5] One way of overcoming these drawbacks is the use of delayed lanthanide luminescence.[6] The attractiveness of such metal ion emission is the occurrence of long-lived excited states (in the ms range), long emission wavelengths (500–700 nm), and line-like emission bands (10–30 nm bandwidth) under ambient conditions.[6] Using Eu(III) and Tb(III) based cyclen (1,4,7,10-tetraazacyclododecane) complexes we have made both luminescent sensors and switches for several cations and neutral molecules.[7] Since lanthanide ions are generally considered to be photophysically inert ($\varepsilon \sim 10\ M^{-1}\ cm^{-1}$), we have covalently incorporated into these complexes antennae, or chromophores whose function is to populate the exited states of the lanthanide ions indirectly ($^5D_0$ and $^5D_4$ for Eu(III) and Tb(III), respectively) *via* energy transfer.[5–7] Recognition at these receptor sites consequently modulates the energy transfer process causing the Eu(III) or Tb(III) emission to be '*switched on*'.[6,7] Concurrently, Parker *et al.* have developed lanthanide luminescent sensors for anions such as bicarbonate using coordinated unsaturated complexes with covalently bonded chiral antennae.[8] In these, the oxy-anions displayed quenching of metal bound water molecules, which caused the lanthanide ion emission to be 'switched on'. Inspired by this work, we were interested in developing new types of luminescent chemosensors for aromatic carboxylic acids, such as *N*,*N*-dimethylaminobenzoic acid (**3**) and salicylic acid (**6**), with biological concentration of *ca.* 0.4 mM. Here, the sensing action would be due to enhanced sensitisation of the lanthanide ion excited state by *the aromatic acid itself* upon displacement of the labile metal-bound water molecules. The attractiveness of such complexes is that they do not have covalently attached antennae, and as such are photophysically silent prior to the anion recognition. Hence, only upon coordination of the chromophore, *e.g.* the aromatic anion, and the formation of a ternary complex, would give rise to an effective population of the excited state.[9]

The heptadentate tri-arm ligands **1** and **2** were made in a one-pot synthesis from cyclen and the corresponding α-chloroacetamide (1 : 3.1) in CH_3CN and purified by tituration or flash column chromatography for **1** and **2**, respectively. The corresponding cationic complexes **Tb.1**, **Tb.2**, **Eu.1** and **Eu.2** were made upon complexation to triflate salts of Tb(III) and Eu(III), respectively, in refluxing dry CH_3CN (ESI†). Due to the high coordination requirements of the lanthanide ions (usually 9–10), the heptadentate lanthanide ion complexes have vacant coordination sites that are occupied by solvent molecules such as water.[10] The presence of two water molecules was confirmed by X-ray crystallography for both **Eu.1** and **Eu.2**, the latter being shown in Fig. 1.‡ As far as we know, these are the first crystal structures of heptadentate tri-arm amide cyclen complexes showing two metal bonded water molecules.[10] The structure shows that the ion is coordinating to the four nitrogens of the cyclen structure and the three oxygens of the amides, with N···Eu distances of 2.610(7), 2.625(6), 2.655(6) and 2.647(6) Å and O···Eu distances of 2.341(5), 2.364(5) and 2.378(5) Å. The Eu···O1W and Eu···O2W distances were measured to be 2.418(5) and 2.421(5) Å with a O1W–Eu–O2W bite angle of 72.20(18)°.

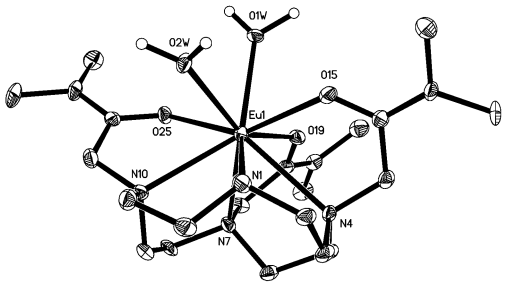
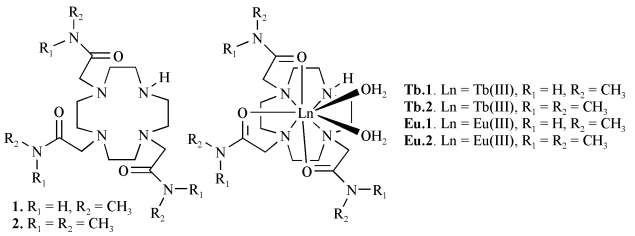


Fig. 1 Diagram of **Eu.2** showing the two coordinated water molecules with atomic displacement parameters at the 30% level. Hydrogen atoms have been omitted from the ligand for clarity.

The ability of **Tb.1**, **Tb.2**, **Eu.1** and **Eu.2** to bind and recognise the aromatic carboxylic acids **3**, **6** and **7**, the ester **4** and the ketone **5**, was investigated in water, in both pH 7.4 TRIS and pH 7.4 HEPES buffers, and in the presence of 0.1 M of tetramethylammonium chloride to maintain constant ionic strength. In a typical experiment, 17 μM solutions of the complexes were titrated with several potential sensitisers (chromophores) **3**–**7** and the changes in the lanthanide emission spectra and in lanthanide excited state lifetimes monitored. All of these sensitisers have triplet state energies[11] of *ca.* 22000–26000 cm^{-1} which is close to that found for Eu(III) $^5D_0$ (E = 17200 cm^{-1}) and Tb(III) $^5D_4$ (E = 20500 cm^{-1}),[7] suggesting that energy transfer from their excited states to the



† Electronic supplementary information (ESI) available: experimental section, Fig. S1, Table S1. See http://www.rsc.org/suppdata/cc/b2/b204888d/

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excited state of the lanthanide ions is feasible.[7b] As expected none of the above complexes were emissive in the absence of these chromophores indicating that the complexes were *photophysically silent* at low concentration (when excited at 300 nm). However, it was possible to determine the hydration number q,[6,7] for all the complexes by measuring their excited state lifetimes in H_2O and D_2O at high concentration. All of these gave a q value of *ca.* 2, indicating that two water molecules were coordinating to the metal centres in solution.

Initially the ability of the complexes to recognise **3** was evaluated in water. Of the above four complexes, only **Tb.1** and **Tb.2** became emissive upon titrating with **3**, when excited at 300 nm. For **Tb.2** the emission at 491, 548, 587 and 622 nm was *'switched on'* with luminescent enhancement factors of *ca.* 680, the largest changes being in the 548 nm band for the $^5D_4 \rightarrow {}^7F_5$ transition. For **Tb.1** the changes were somewhat smaller, *ca.* 220. When these measurements were carried out in buffered solutions and high ionic strength, large emission enhancements were also observed as shown in Fig. 2 for **Tb.2**, but the $^5D_4 \rightarrow {}^7F_3$ transition was not observed at 622 nm. For **Tb.2** the lifetimes of the excited state of **Tb.2** in the presence of 0.40 mM of **3** was measured to be 1.79 and 1.59 ms in D_2O and H_2O, respectively, which gives $q \approx 0$ (using $q = 5[(1/\tau)_{H_2O} - (1/\tau_{D_2O}) - 0.06]$[6] See ESI†), *i.e.* both water molecules had been displaced upon coordination of the carboxylic acid to Tb(III). By plotting the changes in the Tb(III) emission at 548 nm as a function of the concentration of **3** (as $-\log[\mathbf{3}]$) a bell-shaped curve[7a] was observed (see insert in Fig. 2). Examination of this curve showed that the emission was 'switched on' from $-\log[\mathbf{3}]$ *ca.* 5.8 to 4 (*ca.* 2 log units), which is an indication of 1:1 binding and simple equilibrium [an estimated $\log \beta = 5 (\pm 0.1)$ can be deduced from these changes between $-\log[\mathbf{3}] = 5.8$–4]. However, between $-\log[\mathbf{3}] = 4$–3.4 the emission was 'switched off'. No pH changes were observed upon titrating **Tb.2** with **3** (in buffered solution) indicating that that this was not due to protonation of **3** and concomitant dissociation of the ternary complex. We believe that this 'switching off' is rather due to self-quenching but we are currently investigating these features more closely. Upon titrating **Tb.2** using **4** or **5**, no Tb(III) emission was observed. However, the addition of 0.4 mM of **3** to a solution of **Tb.2** and **4** (3 mM) gave rise to large enhancements in the Tb(III) emission, indicating the selective recognition and binding of **3** to the Tb(III) ion and the formation of a ternary complex structure. These results suggest that efficient binding and population of the Tb(III) $^5D_4$ excited state is only possible if the metal bounded water molecules are removed upon binding.[8]

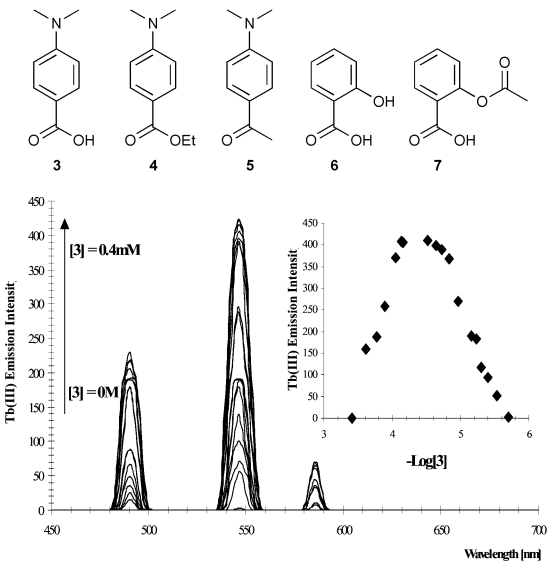

Fig. 2 The change in the Tb(III) emission in **Tb.2** upon addition of **3** in water when excited at 300 nm under physiological conditions. *Insert*: The changes in the Tb(III) emission at 548 nm as a function of $-\log[\mathbf{3}]$.

The effect on the Tb(III) emission upon binding of salicylic acid **6** ($\lambda_{ex.} = 296$) and its acetate ester **7** (Aspirin®) under physiological conditions was also investigated. As described above, the emission from **Tb.2** was highly dependent on the concentration of **6**, with a larger order of magnitude enhancement in the Tb(III) emission (see ESI†). The enhancement factor for **Tb.1** was somewhat smaller. However, unlike that seen for **3**, only two emission bands at 491 and 548 nm were observed. As for **3**, the binding of **6** ($-\log[\mathbf{6}]$) to **Tb.2** gave a bell-shaped dependence from $-\log[\mathbf{6}] = 2.6$–4.5 when measured at 548 nm ($\log \beta \sim 4.5$ in the range of 3.5–4.9, see ESI†). In contrast, the binding of **7** to **Tb.2** was *not* observed, *i.e.* no Tb(III) luminescence was observed. We propose that this lack of binding and hence sensitisation, is due to steric effects since **7** might be too large to approach the metal ion centre efficiently. Therefore **Tb.2** is an excellent chemosensor for **6**, the biologically active form of **7** under physiological conditions. We predict that upon recognition of **3** and **6**, the oxygen atoms of the aromatic carboxylates bind to the Tb(III) ion, forming a four-membered ring chelate, but a similar (five-membered) binding mode has been reported by Dickins *et al.* for lactate[12] (see also ref. 8) However, it is also possible that **6** binds to **Tb.2** *via* a one of the coarboxylate oxygens and the phenolic oxygen in a six-membered chelate. This is currently under investigation.

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Notes and references

‡ Data were collected on a Bruker SMART diffractometer using the SAINT-NT[13] software with phi/omega scans. A crystal was mounted on to the diffractometer at low temperature (*ca.* 120 K). The structure was solved using direct methods and refined with the SHELXTL program package.[13] *Crystal data* for **1**: $C_{23}H_{49}EuF_9N_7O_{16}S_3$, $M = 1098.83$, triclinic, space group $P1$, $a = 8.7196(19)$, $b = 13.147(3)$, $c = 18.427(4)$ Å, $\alpha = 95.805(3)$, $\beta = 97.220(3)$, $\gamma = 104.013(3)°$, $U = 2014.3(7)$ Å^3, $Z = 2$, $\mu = 1.828$ mm^{-1}, $R_{int} = 0.0788$. A total of 23924 reflections were measured for the angle range $2 < 2\theta < 58°$ and 9229 independent reflections were used in the refinement. The final parameters were $wR2 = 0.1659$ and $R1 = 0.0546$ [$I > 2\sigma I$]. CCDC reference number 186595. See http://www.rsc.org/suppdata/cc/b2/b204888d/ for crystallographic data in CIF or other electronic format.

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Rapid hydrolytic cleavage of the mRNA model compound HPNP by glycine based macrocyclic lanthanide ribonuclease mimics†

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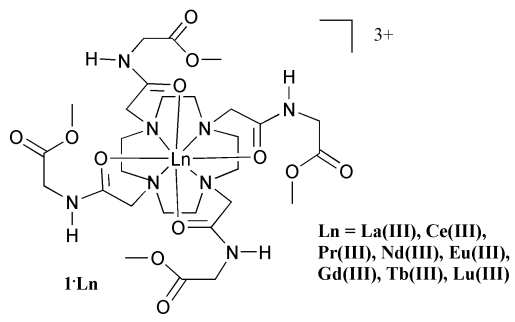
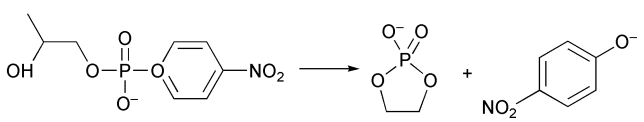
The lanthanide ion based macrocyclic complexes 1·Ln mimic the hydrophobic nature of ribonucleases, where the lanthanide ions induce the formation of a hydrophobic cavity for 1, giving rise to a large order of magnitude enhancement in the hydrolytic cleavage of HPNP.

Currently, there is a great interest in the development of robust catalytic systems that can effectively mimic important enzymatic reactions under physiological conditions.[1] The development of such catalysts with the aim of achieving fast and site-selective hydrolytic cleavage of the phosphate ester bonds of RNA over DNA is of particular interest.[1,2] This is of prime importance for the development of novel antisense drugs,[3] ribonuclease and ribozyme mimics and gene technology. Ribonucleases often possess one or more metal ion centers at their active site,[1] which in combination with basic amino acids such as histidine and lysine accelerate their reactions dramatically. This has encouraged the development of synthetic ribonucleases where transition[4] and lanthanide metal ion complexes have been employed.[1,2,3,5] Such complexes accelerate the rate of hydrolysis *via* Lewis acid or nucleophilic activation, often through the synergic action of several metal centres.[2,4,5] However, the use of cofactors such as amino acids, in conjunction with these metal centres has been less explored.[6] We are interested in developing physiologically stable ribonuclease mimics based on the use of 1,4,7,10-tetraazacyclododecane (cyclen) derived lanthanide complexes where the cofactors are integrated covalently into the cyclen framework as pendant arms.[7] Herein we discuss the synthesis, physical characteristics, and cleavage evaluation towards the RNA model compound 2-hydroxypropyl *p*-nitrophenyl phosphate (HPNP) of **1·Ln**, which incorporates rather simple pseudo 'GlyGly' cofactors, and show that they give rise to a remarkable enhancement in the rate of hydrolysis of HPNP.

Inspired by the work of Morrow and coworkers who used a La(III) complex to hydrolyse HPNP with $k = 5.8 \times 10^{-2}$ h^{-1},[8] and from our recent investigations into the use of amide based cyclen lanthanide complexes as luminescent switches and sensors,[9] we developed **1** and its lanthanide ion complexes **1·Ln**, as ribonuclease mimics. We predicted that the tetraamide-derived cyclen complex, with its concave structure and central lanthanide ion, would be an ideal synthetic platform for the integration of amino acid cofactors. The ligand, **1**, was synthesized in a two step synthesis in 68% overall yield (See ESI†). ‡ The ^{1}H NMR in $CDCl_3$ indicated C_4 symmetry with only five resonances being observed. The corresponding cationic lanthanide complexes **1·La**, **1·Ce**, **1·Pr, 1·Nd, 1·Eu**, **1·Gd**, **1·Tb** and **1·Lu** were formed by reacting an equimolar amount of **1** with the appropriate $Ln(SO_3CF_3)_3$ salt in refluxing dry CH_3CN or MeOH. We were able to grow crystals of **1·Eu** (**1·EuK$_2$(CF$_3$SO$_3$)$_5$**, the K^+ is bonding to the glycine ester, ESI†) from methanol–$CHCl_3$ suitable for crystallographic investigation (See ESI for structure†).§ [10] This showed the Eu^{3+} ion placed in the centre of the cavity, coordinated by the four nitrogens of cyclen and the oxygens of the carboxyamides (average N···Eu and O···Eu bond lengths were 2.661(4) and 2.369(3) Å, respectively). A single triflate molecule occupies the ninth coordination site, giving an overall monocapped square antiprism (CSAP) geometry,[11] with enantiomeric conformation for the ring NC-CN as δδδδ, and the pendant arms arranged in an anticlockwise, Λ fashion.[11] Significant to our design, the four GlyGly moieties (one of which has its amino terminus as a part of the macrocyclic ring) form the walls of a cavity. Importantly, ^{1}H NMR in CD_3OD indicated that the CSAP geometry was also the major isomer (>95%) in solution.[11]



All HPNP cleavage studies were carried out at 37 °C and at pH 7.4 in 50 mM HEPES buffer (in 96 : 4 H_2O–MeOH) with an equimolar amount of catalyst (0.173 mM). The HPNP hydrolysis was monitored by the appearance of a new absorption band at 400 nm, corresponding to the formation of the *p*-nitrophenolate anion (Scheme 1). The cleavage of HPNP by **1·Ln** was in all cases much faster than anticipated, given the fact that glycine lacks the extra cooperative sites found in basic amino acids such as lysine and histidine. Moreover, since the glycine esters extend the size of the cavity, it might have been expected to inhibit the approach of the complex. For **1·La** the rate of hydrolysis was found to display pseudo first order kinetics with $k = 0.41$ h^{-1} and $\tau_{1/2}$ of 1.7 h, Table 1. This is a remarkable 3400-fold rate enhancement (k_{obs}) compared to the uncatalyzed reaction. Moreover, this is seven times faster than the kinetics observed for Morrow's La^{3+} complex. Table 1 summarizes these results for all the **1·Ln** complexes. It is particularly important to note that **1·Eu** shows a significant rate enhancement with k of 0.15 h^{-1}, and $k_{obs} = 1250$. In fact, all the complexes showed remarkable cleavage ability and a clear trend emerges from Table 1: the larger ions are more potent than the smaller. This can in part be explained by higher coordination



Scheme 1 The mechanism for the hydrolytic cleavage of **HPNP**.

† Electronic supplementary information (ESI) available: full experimental details, Fig. S1–S6. See http://www.rsc.org/suppdata/cc/b2/b205349g/

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Table 1 Results of the hydrolysis of HPNP using **Ln·1**

Complex[a]	k/h^{-1}[b,c,g]	$\tau_{½}$ /h	k_{obs}[d]
1·La[e]	0.41	1.69	3417
1·Ce	0.37	1.87	3083
1·Pr	0.30	2.31	2500
1·Nd	0.20	3.46	1667
1·Eu[f]	0.15	4.62	1250
1·Ga	0.12	5.77	1000
1·Tb[f]	0.0935	7.41	779
1·Lu	0.072	9.63	600

[a] Measured using an Agilent 8453 spectrophotometer fitted to circulating temperature controlled water bath, and water driven mechanical stirring, in 50 mM HEPES buffer, at pH 7.4 and at 37 °C. [b] Average over three measurement and three half-lifetimes. [c] k values were determined by fitting the data to first order rate kinetics using Biochemical Analysis Software for Agilent ChemStation. Errors are within ±10%. [d] These enhancement factors are obtained from the ratio of the catalyst *vs.* the uncatalysed reaction using k_{ucat} = 0.00012 h^{-1} (R. Breslow and D-L Huang, *Proc. Natl. Acad. Sci. USA*, 1991, **88**, 4080). [e] The **1·La** complex did not cleave the DNA model compound bis(*p*-nitrophenyl) phosphate (BNPP) over 24 h. [f] We were unable to determine the hydration number q accurately. [g] 20% EDTA affected the rate of hydrolysis slightly but we believe that this is due to binding of one or more of the EDTA carboxylic acids to the metal ion complex rather than metal extraction. **1·Eu** and **1·Tb** were stable to competitive Cu(II) (sulfate) exchange in water over a week at pH 6.5 (measured by UV/VIS). We are currently investigating the stability of the other complexes.

number requirements for the larger ions that are fulfilled by the additional water molecules,[7,11] which are important for both inner and outer sphere catalytic activation modes.[1,2] In contrast, a Cu(II) complex of **1** was found to be inactive, *i.e.* no measurable cleavage of HPNP was observed over 24 h. Potentiometric pH titration for **1·La** revealed that *ca.* 1.5–2 base equivalents were needed to deprotonate the water molecules of **1·La**. We estimated these pK_as to be *ca.* 8.2 and 8.5, respectively, but we were unable to determine them accurately (see ESI†) even when the titrations were repeated at different concentrations. In contrast**,** a single pK_a of 7.38 was measured very accurately for **1·Eu**. For **1·La**, we predict that one of the two water molecules can be rapidly displaced upon binding to the phosphate ester, revealing extra binding sites over that of **1·Eu**. The second water molecule (or bound hydroxide) is then able to carry out a nucleophilic reaction on the phosphodiester.[2,4] Preliminary ^{31}P NMR binding studies in buffered H_2O using **1·Eu** and **1·La**, and diethylphosphate [$(CH_3CH_2O)_2PO_2^-$] (DEP), which lacks the 2-hydroxy group, showed that **1·Eu** binds more strongly to DEP than **1·La** which needed 30 equivalents of DEP *vs. ca.* one for **1·Eu** to obtain saturation for the ^{31}P signal. We predict that similar binding preferences can be expected for HPNP.

It is remarkable that **1·Ln** shows such a high rate enhancement despite the fact that the Lewis acid centre is more shielded from the solvent environment due to the steric effect enforced by the glycine esters. It is our prediction that these rate enhancements are due to hydrophobic effects caused by the arrangement of the amino esters around the metal ion centre, giving rise to the formation of a hydrophobic pocket around the ion. This possibly favours the formation of stronger interactions between the ion and the phosphodiester. Similar observations have previously been seen for example in Collman's 'picket fence' porphyrins,[12] and in mimicking the active site of carbonic anhydrase by Boxwell and Walton.[13] We are currently investigating these features in more detail. Secondly, our assumption that HPNP binds more weakly to **1·La** than **1·Eu** will contribute to the release of the product from the cavity of the lanthanide complexes.

Although the complexes are highly potent at pH 7.4, the pH-rate profile for **1·La** (Fig. S2 in ESI†) shows that the activity (at 37 °C) is strongly influenced by pH, with an optimum rate at *ca.* pH 8.5 of k = 0.807 h^{-1}. This correlates well with the pH titration of **1·La** discussed earlier. This is a remarkable 6725-fold enhancement, and almost twice that seen at pH 7.4. This also implies that the most active form of the catalyst has at least one hydroxy ion bound to the lanthanide ion centre. It is also possible that at higher pH (pH > 8.5), this hydroxy group binds too strongly to the phosphate and this in turn could inhibit further coordination of HPNP. Preliminary investigation has also shown that most of the complexes efficiently cleaved a 24 mer-mRNA sequence from the GAG-HIV gene at pH 7.4 and 37 °C after 4 h of incubation (See ESI†). Complexes such as **1·La** and **1·Eu** induce cleavage at every base pair, whereas upon incubation with **1**, no cleavage is observed, indicating the vital role of the Lewis acid centre in the hydrolysis (this cleavage was not quantified, ESI†). We are currently developing analogue compounds by incorporating other cofactors into the cyclen structure and incorporating these complexes into oligonucleotides as potential antisense agents.

In conclusion, the various lanthanide complexes **1·Ln** show significant rate enhancement in the cleavage of HPNP. To the best of our knowledge, **1·La** displays one of the largest rate accelerations observed for HPNP by trivalent, non-redoxactive lanthanide complexes. We thank Enterprise Ireland, Dublin Corporation (postgraduate scolarship to S. M.), and TCD for financial support, Dr Hazel M. Moncrieff for helpful discussion and Dr John E. O'Brien for assisting with NMR.

Notes and references

‡ **1**: Found for $C_{28}H_{49}N_8O_{12}$ (MH^+): 689.3470. Calc.: 689.3469. Found for $KC_{28}H_{49}N_8O_{12}$: C 46.21; H 6.65; N 15.40; Calc.: C 46.64, H 6.88; N 14.9.

§ Data were collected on a Bruker SMART diffractometer with graphite monochromated Mo-Kα radiation using omega/phi scans. The structure was solved using direct methods and refined with the SHELXTL program package. The two triflate anions are disordered; firstly the anion associated with the K centres has been modelled over two sites with the major component being 76(1)% occupancy. The second anion is bound to the Eu centre and is disordered about a four-fold symmetry element and has been modelled as 25% occupancy for each position. *Crystal data* for $C_{33}H_{48}N_8F_{15}S_5K_2Eu$: M = 1664.25, tetragonal, space group $P4/ncc$, $a = b$ = 17.2721(8), c = 21.0475(14) Å, U = 6279.0(6) Å^3, Z = 4, μ = 1.425 mm^{-1}, $F(000)$ = 3336, D_c = 1.761 g cm^{-3}, R_{int} = 0.0408, transmission range (max., min.) = 1.000, 0.804, crystal size = 0.33 × 0.28 × 0.21 mm. A total of 51944 reflections were measured for the angle range $3 < 2\theta < 58$ and 3844 independent reflections were used in the refinement. The final parameters were $wR2$ = 0.1366 and $R1$ = 0.0479 [$I > 2\sigma I$].CCDC 177867. See http://www.rsc.org/suppdata/cc/b2/b205349g/ for crystallographic data in CIF or other electronic format.

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