A model system using modulation of lanthanide luminescence to signal Zn^{2+} in competitive aqueous media †

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Two pentadentate tribasic ligand systems containing aniline or benzylamine nitrogens covalently linked to a proximate kinetically stable Eu or Tb complex are described. The affinity of these complexes and their non-conjugated analogues for Zn^{2+}, Ca^{2+} and Mg^{2+} ions has been measured at ambient pH in a high salt background. Apparent binding constants for the parent ligands (L^1: Zn^{2+} log β_{ML} 5.04, Ca^{2+} 3.91, Mg^{2+} 2.1, L^3: Zn^{2+} 5.93, Ca^{2+} 5.00, Mg^{2+} 3.60) were slightly lowered in the aniline-based terbium conjugate [TbL^4], and were the same for the benzylamine-based conjugate [LnL^2], except for zinc binding for which a slightly enhanced affinity was observed. Changes in the form of ligand absorption and emission spectra and in the intensity of delayed lanthanide luminescence characterised metal ion binding. With [LnL^2], a 42 and 26% increase in emission at 700 nm (Eu) and 545 nm (Tb) accompanied zinc binding in a simulated extracellular background, with an apparent dissociation constant of 0.6 μM (295 K).

Zinc, calcium and magnesium ions play a vital role in cellular processes. The important role of 'non-enzymatic' zinc is starting to be unravelled;[1] examples of zinc transporting proteins are emerging[2] and Zn^{2+} is also implicated in aspects of neurotransmission in the brain.[3] The total zinc ion concentration in serum is of the order of 10 μM[4] and in mammalian cells this rises towards millimolar levels, although the concentration of unbound, or 'available' zinc ions is believed to be much lower than this. Several zinc-selective complexing agents have been developed bearing fluorescent reporter groups including proteins,[5] peptides[6] and macrocycles.[7] The most important zinc probes, however, are based on fluorescent 8-tosylamidoquinoline derivatives which exhibit high zinc selectivity and good cell permeability.[1,8] They operate on large changes in fluorescence emission intensity at *ca.* 490 nm, following excitation at 365 nm. The pre-eminent role of intracellular Ca^{2+} ions as secondary messengers has been revealed primarily through the work of Tsien *et al*. who developed first selective intensity[9] and then ratiometric probes[10] operating in the sub-micromolar range. Changes in cellular Mg^{2+} ion concentrations are believed to be less marked than for Zn^{2+} and the leading methods that seek to allow the signalling of free Mg^{2+} concentrations in the millimolar range are based on London's complexing agent.[11,12] More recently, Kimura and co-workers[7a,13a] have defined robust dansyl-pendant 12-N_3 and 12-N_4 complexing agents which bind Zn^{2+} with high affinity (K_d of 5×10^{-13} M at pH 7.8) and with emission intensity changes of up to a factor of 10. A similar approach has also been adopted using a fluorescein–12-N_4 conjugate,[13b] while earlier approaches with N-functionalised 12-N_4 ligands had used anthryl or naphthyl fluorophores.[7a,13c]

With this background in mind, we have set out to develop systems in which the delayed luminescence from a lanthanide reporter group serves to signal changes in metal ion concentration. Related approaches have been devised for signalling changes in pH (range 5.5 to 7.5),[14a] pO_2[14b] and pX (X = HCO_3^-, Cl^-)[15,16] in which the analyte modulates the emission intensity, lifetime or polarisation from a terbium or europium ion. Such an approach requires the definition of a kinetically stable lanthanide complex linked to an appropriate metal-binding complexing agent. Accordingly, we set out to prepare the ligand L^1 and its lanthanide conjugate [LnL^2], in which it was assumed that the benzylic N donor would enhance Zn *vs.* Mg/Ca selectivity. For purposes of comparison, the modified 'London'-type ligand L^3 was also prepared (*cf.* L^5)[11,12] with its lanthanide conjugate [LnL^4]. In each case, the connecting amide group is bound to the Ln^{3+} ion bringing the aryl chromophore close to the Ln ion, allowing its use as a sensitising antenna. At the outset, it was evident that such conjugates would only be expected to function *via* changes in the delayed emission intensity, as no significant change in the lanthanide ion coordination environment on ion binding is likely. Furthermore the nature of the ligand centred and LMCT charge transfer bands in the target complexes restricts the excitation wavelength to below 320 nm, which obviously precludes the direct application of such systems. Therefore, the work set out to establish the principle of time-delayed signalling of metal ion concentrations in competitive aqueous media; adaptation to excitation in the range 350→400 nm with variation of the form of the emission constitutes the next step.[16]

Ligand and complex synthesis

Stepwise alkylation of *o*-hydroxybenzylamine, **1**,[17] with ethyl bromoacetate in acetonitrile afforded the triester **2** (Scheme 1), which was nitrated at −20 °C with conc. nitric acid in acetic acid to give the 5-nitro product, **3**, in 83% yield. Reduction over palladium on carbon gave the amine **4**, from which the α-chloramide **5** was prepared by acylation with chloroacetyl chloride. Ester hydrolysis and purification of the acid on a Dowex ion-exchange resin yielded L^1. Alkylation of 1,4,7-tris-(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (MeCN, Cs_2CO_3) gave the hexa-ester **7** in moderate yield. This compound was also prepared in a two-step process involving mono-alkylation of cyclen (Scheme 1) in THF followed by alkylation of the three secondary amine sites ($BrCH_2CO_2{}^tBu$, MeCN, Cs_2CO_3) in a higher overall yield, but with formation of

† Examples of spectral changes and of data analysis are available as supplementary data. For direct electronic access see http://www.rsc.org/suppdata/p2/b0/b003963m/

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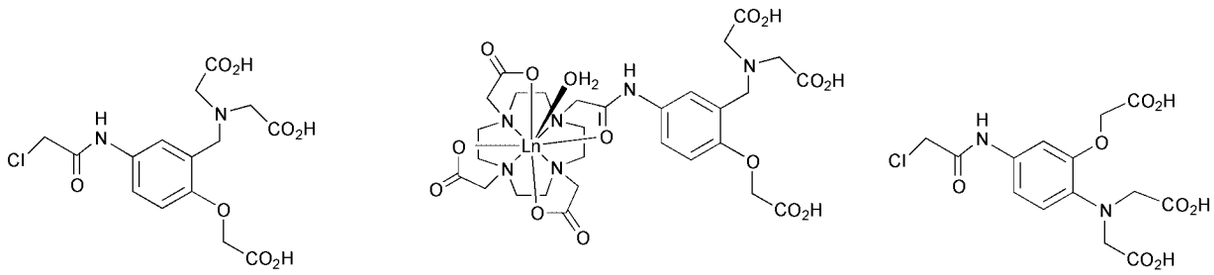
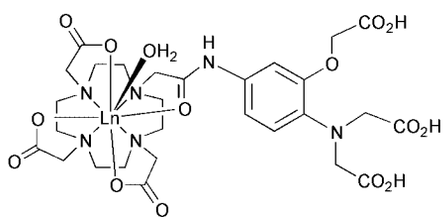


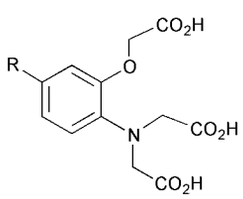
L^1

LnL^2 *Ln = Eu, Tb*

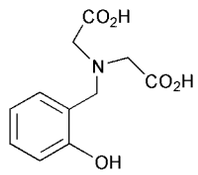
L^3



LnL^4 *Ln = Eu, Tb*



L^5 **a**) R = F
b) R = H
c) R = CH_2-9-anthryl



L^6

the tertiary amide **8** as a by-product. Selective removal of the *tert*-butyl esters (CF_3CO_2H–CH_2Cl_2) gave the tri-ester **9** and lanthanide complexation ($Ln(OAc)_3$–H_2O) followed by ester hydrolysis (pH 13/20 °C) afforded the desired complexes [LnL^2] which eluted from a weak-acid ion-exchange column with aqueous ammonia solution.

The synthesis of the related ligand L^3 also relied upon controlled nitration and selective deprotection steps. Alkylation of *o*-nitrophenol with *N*-methylchloroacetamide gave the ether **11** which was hydrogenated over Pd/C to yield the amine **12**. *N*,*N*-Dialkylation with ethyl bromoacetate (MeCN, Na_2HPO_4, KI, 4 Å sieves) proceeded in 93% yield and reaction at −20 °C with nitronium tetrafluoroborate in MeCN gave rise to nitration *para* to the anilino-nitrogen in 70% isolated yield. Protection of the secondary amide (Scheme 2) followed by hydrogenation of the nitro group afforded the amine **16**. *N*-Acylation with chloroacetyl chloride followed by basic hydrolysis gave L^3, which was purified as the ammonium salt by ion-exchange chromatography. Formation of the penta-ester **19** was undertaken by two different methods as in Scheme 1, and BOC deprotection followed by lanthanide ion complexation and controlled ester hydrolysis yielded the desired complexes [LnL^4].

pH dependence of absorption and emission spectra

Absorption spectra for L^1 and [LnL^2] (Ln = Eu or Tb) were similar in form with λ_{max} = 255 nm and a broad band at 295 nm tailing out to 335 nm (Fig. 1). The lower energy band was half as intense for L^1 and both bands increased in intensity (*ca.* 100%) on protonation without shifting position. The variation of absorbance at 255 nm with pH was monitored over the range 2.5 to 11, revealing a pK_a at 7.35 (±0.05) (Table 1), associated with *N*-protonation. Beyond pH 9, a further reduction in absorbance is probably associated with amide deprotonation (pK_a *ca.* 10.5).

In emission, two broad bands were observed for L^1 at 370 and 440 nm which may be ascribed to locally excited and charge transfer excited states respectively. The 370 nm band did not change in intensity over the pH range 6 to 11 (λ_{exc} = 250 or 262 nm). For [EuL^2], very weak ligand fluorescence was observed, consistent with quenching of the singlet excited state by Eu^{3+} involving photoinduced electron transfer. Sensitised emission from the Eu ion was also weak (Fig. 2), remaining relatively

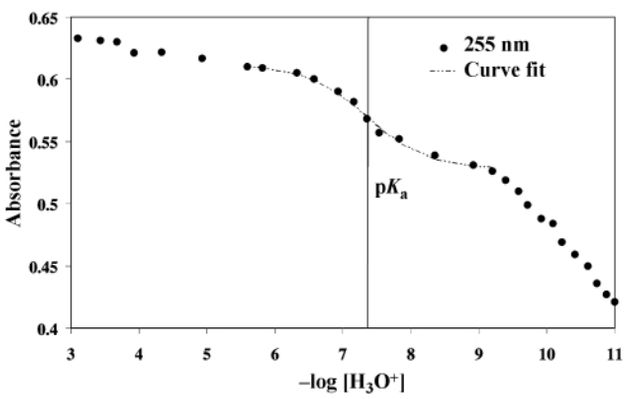


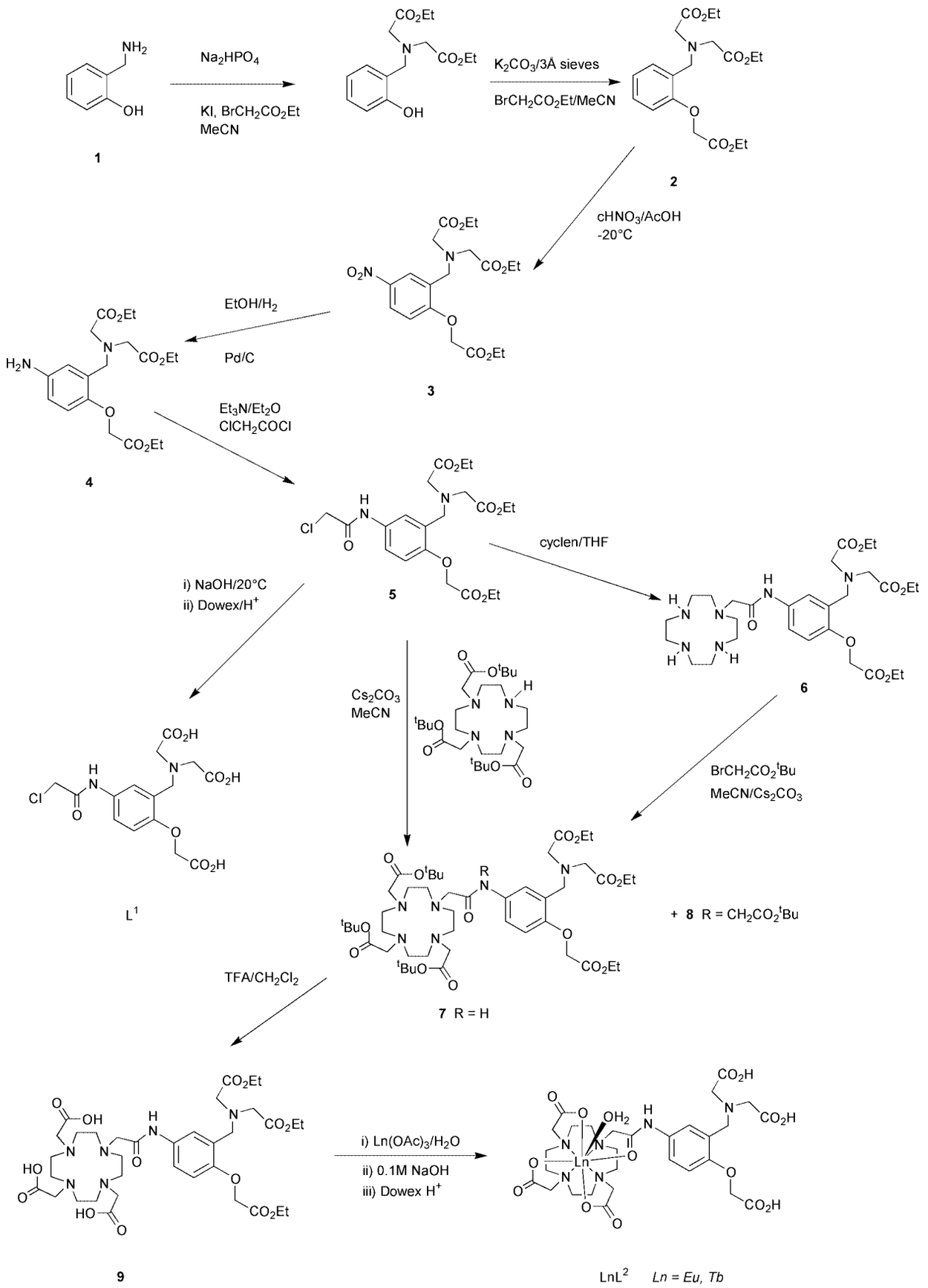
Fig. 1 UV–visible spectra of [EuL^2] at 255 nm as a function of pH. Evaluation of pK_a value was determined by non-linear least-squares fit.

Table 1 Ligand and complex protonation constants (estimated error is ±0.1 p*K* units, 295 K, *I* = 0.1 M NaCl)

Ligand/ complex	Ground state pK_a	Excited state pK_a
L^1	7.35	6.3 [a]
[EuL^2]	7.34	[f]
[TbL^2]	7.38	[f]
L^3	7.17	~6.8 [b]
[EuL^4] [c]	6.4	[f]
[TbL^4]	6.4	[d]
L^{5a}/L^{5b} [e]	5.5	

[a] Estimated by observing the variation in fluorescence emission intensity at 440 and 370 nm. [b] Examining the variation of fluorescence emission at 347 nm; the band at 415 nm increased by less than 10% from pH 6 to 8.5. [c] Eu emission intensity changed by less than 5% over the pH range 5 to 9. [d] Terbium emission intensity decreased by less than 10% over the pH range 5.5 to 8.5. [e] The 9-anthrylmethyl derivative L^{5c} has pK_a = 6.3. [f] Not determined.

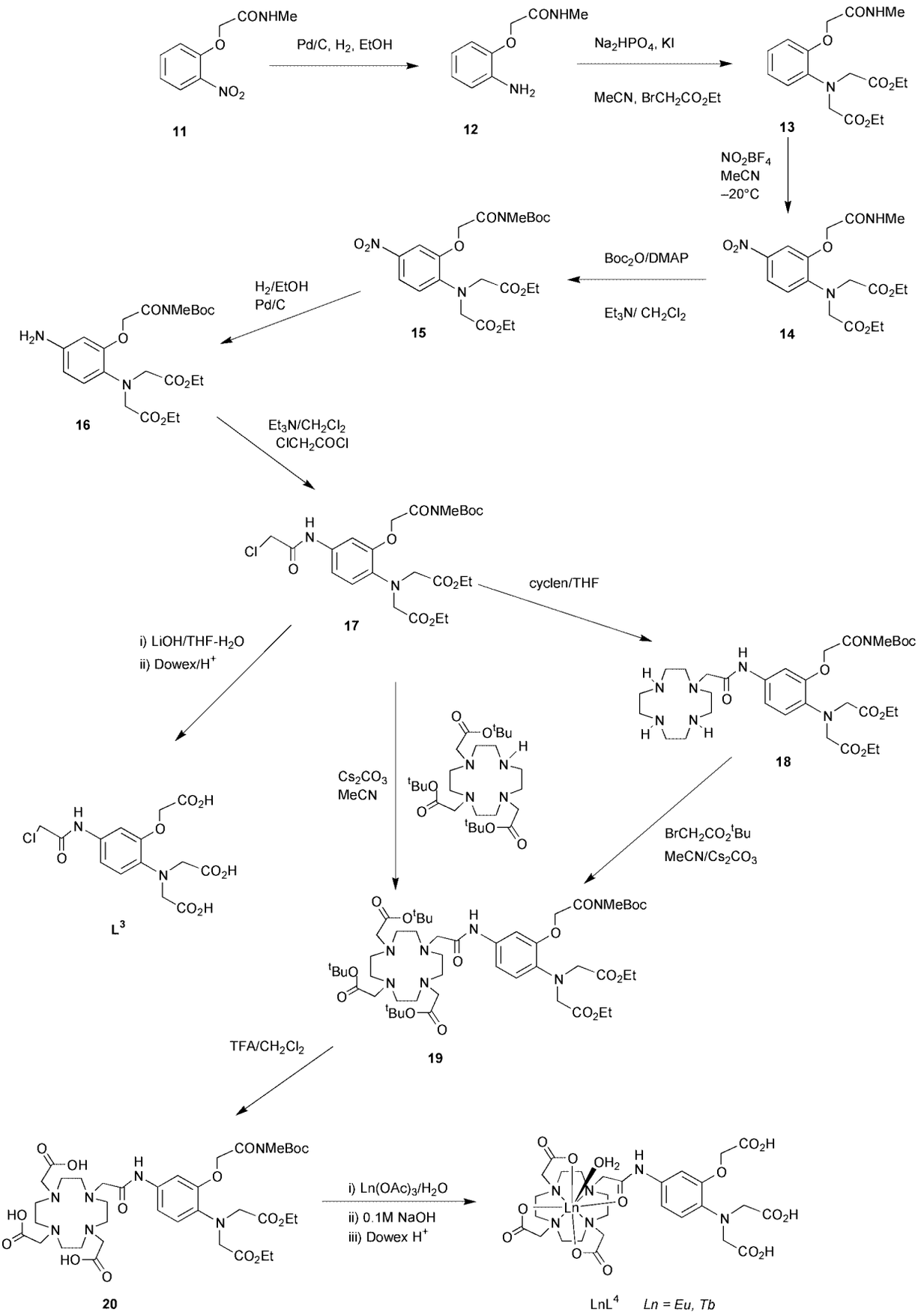
constant over the pH range 2–8 (λ_{exc} = 262 or 247 nm) before reducing by a factor of 7 between pH 8 and 11. The emission from the terbium complex was also weak at 370 nm and the metal based emission (λ_{exc} = 247 nm) was constant from pH 3 to 8 and dropped by *ca.* 50% from 8 to 11. The reduction in lanthanide luminescence in [LnL^2] and the 440 nm ligand



Scheme 1

fluorescence in L^1 is presumably associated with amide deprotonation, enhancing the facility of PET quenching of the excited state. The consistent ground state pK_a values (7.35) for *N*-protonation of L^1 and [LnL^2] (Table 1), may be compared to values of 8.07 and 8.11 reported for *o*-hydroxybenzyldiacetic acid.[18,19] The lower pK_a here may reflect the lesser stabilisation of the conjugate acid afforded by hydrogen bonding to the aryl ether oxygen.

The behaviour of L^3 was more complex and successive deprotonation of the carboxylic acid, aniline nitrogen and



Scheme 2

amide NH gave rise to marked changes in the absorption spectrum (Fig. 3), each with its characteristic isosbestic point. Two of the equilibria were analysed in detail; protonation of the carboxylate was characterised by an isosbestic point at 273 nm so that by monitoring the variation of absorption with pH in the range 6 to 9 at 274 nm the pK_a for aniline protonation could be determined. It was found to be 7.17 (Table 1). By a similar approach, an apparent pK_a of 4.25 was associated with

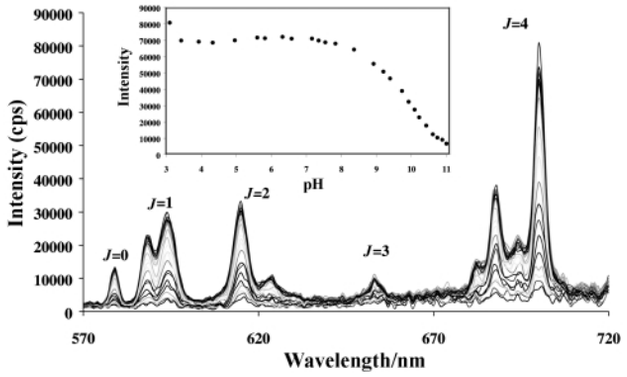


Fig. 2 Effect of pH on the intensity of europium luminescence in EuL^2 (λ_{exc} = 262 nm; $^5D_0 \rightarrow {}^7F_J$). *Inset*: The effect at 700 nm.

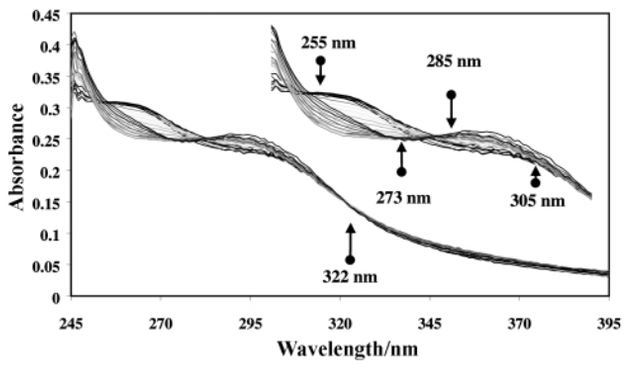


Fig. 3 UV–visible absorbance spectra of L^3 as a function of pH (295 K, I = 0.1 M Me_4NNO_3). *Inset*: Four of the five isosbestic points in the range 247 to 317 nm.

carboxylate protonation. For $[TbL^4]$, a single isosbestic point at 263 nm characterised the pH-dependent absorption spectra (Fig. 4), with a pK_a of 6.4. With $[EuL^4]$, an isosbestic point at 328 nm was revealed and the apparent pK_a was also 6.4 (295 K, I = 0.1 M NMe_4NO_3). These aniline protonation constants are slightly higher than those reported for 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (5.5)[20] and L^{5a} (5.5)[11] but similar to the 9-anthrylmethyl derivative L^{5c} (6.3).[12] The lower pK_a values in the lanthanide complexes suggest that complexation to Eu or Tb enhances the extent of nitrogen lone-pair conjugation. The emission spectrum of L^3 (λ_{exc} 294 nm) revealed a single broad band at 410 nm which reduced in intensity by over 50% as the pH dropped from 8 to 3.5 and developed a shoulder at *ca.* 350 nm, rising in intensity fourfold over the range 8 to 5.5, before falling again as carboxylate protonation occurred below pH 4.5. An approximate pK_a for the excited state of 6.8 was calculated by fitting the variation of emission intensity with pH. For $[EuL^4]$, emission spectra were very weak but in common with $[TbL^4]$, a less than 10% reduction in lanthanide emission intensity was apparent over the pH range 5 to 8, following excitation at the isosbestic wavelength.

Effect of metal ions on absorption and emission spectra

a) L^3 and $[TbL^4]$

The absorption spectral perturbations accompanying addition of $CaCl_2$, $Mg(ClO_4)_2$ and $ZnCl_2$ were monitored at 295 K in a high salt aqueous background buffered to pH 7.4. Representative behaviour is shown in Fig. 5 for the binding of L^3 with $CaCl_2$. Changes were analysed by non-linear least squares fitting to a 1 : 1 binding model (Fig. 5) and by a Hill plot (see supplementary information for examples). Reasonable agreement between the two methods of analysis is revealed in the

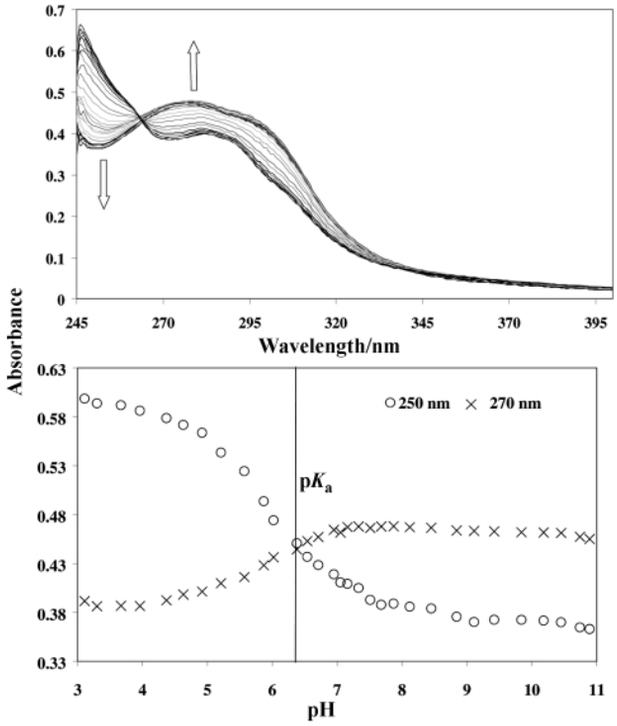


Fig. 4 *Upper*: UV–visible absorbance spectra of $[TbL^4]$ as function of pH (295 K, I = 0.1 M Me_4NNO_3) with an isosbestic point at 263 nm. *Lower*: UV–visible absorbance monitored at 250 and 270 nm and determination of pK_a value from the intercept.

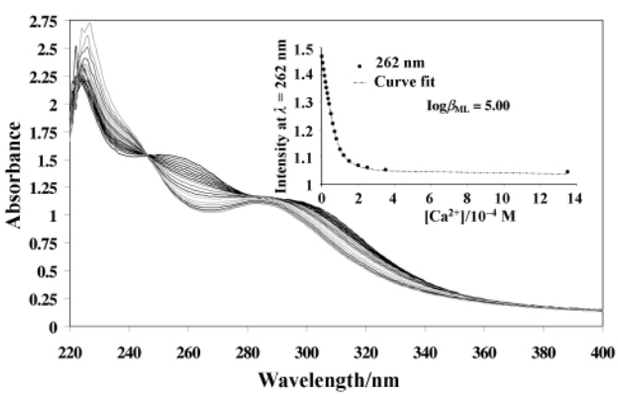


Fig. 5 Effect of adding $CaCl_2$ on L^3 absorption spectra (295 K, 10 mM HEPES, 20 mM NaCl, 115 mM KCl, pH 7.4). *Inset*: Iterative least-squares fitting to a 1 : 1 model of Ca(II)–L^3 complex.

latter comparison and the errors quoted in Table 2 reflect the experimental variance rather than the nature of the statistical analysis. Similar methods were used to examine the behaviour of $[TbL^4]$ (Table 2), although $[EuL^4]$ was not examined as it had been shown not to give rise to significant luminescence—even in the presence of excess added metal ions. With $[TbL^4]$, lower metal binding affinities were revealed, *e.g.* log β = 4.98 *cf.* 5.93 for L^3 consistent with the reduced pK_a of the aniline donor in the lanthanide complexes (Table 1).

The effect of adding Zn, Mg and Ca ions on the total luminescence spectrum was also monitored. For example, addition of $ZnCl_2$ to $[TbL^4]$ gave rise to a 40% increase in the emission band at 440 nm and a 60% increase in terbium emission at 545 nm—due to the strong, magnetic-dipole allowed $\Delta J = -1$ transition. Binding affinities echoed the apparent stability sequence that had been revealed by the absorption spectral changes (Table 2) and the binding affinities derived from the fluorescence emission intensity changes were within 10% of those assessed by examining variation of lanthanide luminescence.

Table 2 Apparent metal ion binding affinities (log K_{ML})[a] and luminescence enhancement factors (LE)[b] (295 K; pH 7.2; 10 mM HEPES, 20 mM NaCl, 115 mM KCl). The values in parentheses are derived from Ln luminescence

Ligand/ complex	Mg^{2+}	%LE	Ca^{2+}	%LE	Zn^{2+}	%LE
L^3	3.60		5.00		5.93	
$[TbL^4]$	3.17		4.27		4.98	
	(3.0)	10	(4.14)	39	(4.60)	60
L^{5a} [c]	3.1		4.6		N.d.	
L^{5b} [c]	3.1		5.1		N.d.	
L^1	2.11		3.91		5.04	
$[TbL^2]$	2.0		3.84		5.48	
	(2.5)	7	(4.37)[d]	11	(6.07)[e]	26
$[EuL^2]$	2.1		3.90		5.38	
	(1.9)		(4.06)	7	(5.99)	42

[a] Binding constants were obtained by iterative least-squares fitting to a 1:1 model and have an estimated error (mean of two independent titrations) of ±0.15 log units. [b] For corrected Eu emission, this refers to the $\Delta J = 4$ band at 700 nm; for Tb, the $\Delta J = -1$ band at 545 nm was monitored. [c] Data from ref. 11. [d] Excitation was at 320 nm in this case and observation of the changes in ligand fluorescence at 404 nm gave log $K_a = 4.27$. For remaining lanthanide complexes, $\lambda_{exc} = 262$ nm (or a wavelength at which minimal changes in absorbance occurred on metal ion binding). Absorption spectral changes were monitored at 240 or 250 nm (294 nm for L^1). [e] A value of 6.35 was obtained from a Hill plot.

Although the original and subsequent work on London's ligand[11,13] highlights the advantages of its use for monitoring Mg^{2+} concentrations, it is only in the absence of Ca^{2+} that this is possible, *e.g.* for intracellular measurements. However free Zn^{2+}ions—if present in concentrations around the micromolar level—may seriously compromise such measurements especially as the kinetics of Mg^{2+} binding are generally considerably slower than those of Ca^{2+} and Zn^{2+}.[11,21] It was considered worthwhile to examine Zn^{2+} binding, in a simulated extracellular ionic background at pH 7.25, *i.e.* with 1.16 mM $MgCl_2$ and 2.3 mM $CaCl_2$ present. The effect of adding $ZnCl_2$ was monitored by absorption and Tb luminescence changes. Binding isotherms in each case were slightly unusual in form and the corresponding Hill plot showed two distinct linear regions. Such behaviour could be regarded as a two-step binding process leading to formation of a 1:1 zinc complex. The intermediate complex could be a mixed Ca–Zn species. Analysis of the data suggested apparent log β values for 1:1 zinc complexation of 4.4 and 5.2 for this stepwise process: thus the complex $[TbL^4]$ at a concentration of 80 μM may respond to changes in $[Zn^{2+}]$ in the range 10 to 100 μM under the chosen simulated conditions, with a variation in Tb luminescence of 40%, but may be subject to interference from calcium ions if the Ca concentration is relatively high.

b) L^1 and $[LnL^2]$

The addition of $ZnCl_2$, $CaCl_2$ or $Mg(ClO_4)_2$ to L^1 at pH 7.4 caused no shift in the position of the primary absorption band at 250 nm; only a small decrease in the intensity of the tail at 292 nm was evident. With $[TbL^2]$ and $[EuL^2]$, a small blue-shift was evident with *ca.* 10% hyperchromism at 250 nm when $ZnCl_2$ was added. Smaller changes were observed following addition of $CaCl_2$ (≤3 nm shift in λ_{abs}) and no significant changes in absorption occurred following addition of $Mg(ClO_4)_2$, consistent with little or no aryl ether oxygen participation in ion binding. In fluorescence emission with $[TbL^2]$, two bands were observed at 365 nm and at 440 nm—the lower energy band being twice as intense. These may also tentatively be ascribed to locally excited and charge transfer states respectively. Addition of $ZnCl_2$ caused the former band to increase slightly in intensity while the 440 nm band shifted to the blue by *ca.* 13 nm and reduced in intensity by *ca.* 20%, following excitation at 262 nm (Fig. 6). For $[EuL^2]$ fluorescence emission spectra were *ca.* 20 times weaker, as noted above, owing to quenching of the singlet excited state by electron transfer to Eu^{3+}.[16]

Changes in emission from the lanthanide excited state also accompanied metal ion binding. An enhancement of 26 and 42% was observed for $[TbL^2]$ at 545 nm and for $[EuL^2]$ at

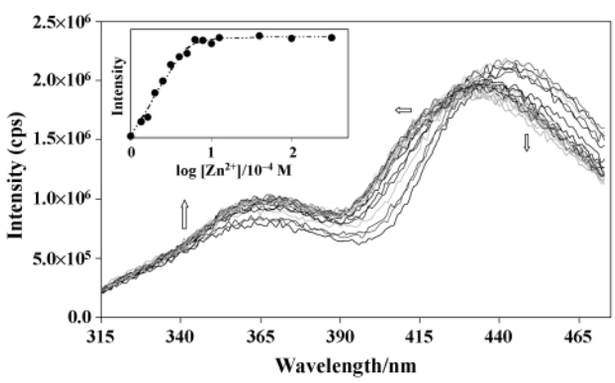


Fig. 6 Effect of adding $ZnCl_2$ on chromophore emission spectra ($\lambda_{exc} = 262$ nm) in $[TbL^2]$ (295 K, 10 mM HEPES, pH 7.4, 20 mM NaCl, 115 mM KCl). *Inset*: Iterative least-squares fitting to a 1:1 model of Zn(II)–TbL^2 ($\lambda_{em} = 404$ nm; $[TbL^2] = 0.08$ mM, log $\beta_{ML} = 6.00$).

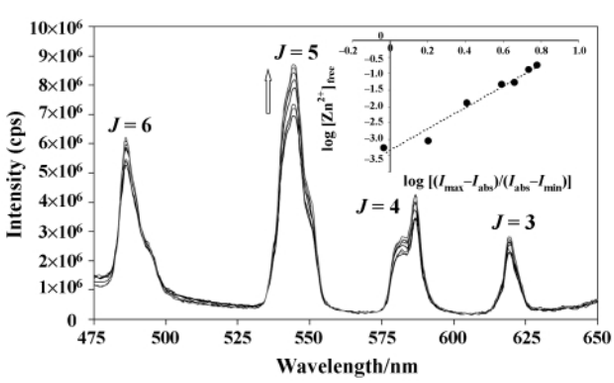


Fig. 7 Effect of adding $ZnCl_2$ on terbium emission spectra ($\lambda_{exc} = 262$ nm; $^5D_4 \rightarrow {}^7F_J$) in $[TbL^2]$ (295 K, 10 mM HEPES, pH 7.4, 20 mM NaCl, 115 mM KCl). *Inset*: Hill plot analysis as a function of pZn^{2+} ($\lambda_{em} = 545$ nm; $[TbL^2] = 0.08$ mM, log $\beta_{ML} = 6.35$).

700 nm following addition of $ZnCl_2$ (Figs. 7 and 8 and Table 2). No change in the form of the spectrum was observed, consistent with a constant local coordination environment for the lanthanide ion. Measurements of the rate constants for decay of the Eu or Tb excited state were carried out in H_2O and D_2O, in the absence and presence of $ZnCl_2$ and $CaCl_2$. The values for $[EuL^2]$ ($k_{H_2O} = 1.61$, $k_{D_2O} = 0.45$ ms^{-1}) were unchanged in the presence of added ions and indicate a lanthanide hydration state of one ($q = 1.0$).[22] For $[TbL^2]$, the radiative rate constants for decay of the $^5D_4$ excited state were similarly unaffected

by addition of $ZnCl_2$ or $CaCl_2$ ($k_{H_2O} = 0.55$; $k_{D_2O} = 0.29$ ms^{-1}; $q = 1.0$).

The relatively high affinity of [TbL^2] for zinc ions (Table 2) suggested that changes in zinc ion concentration in the micromolar range might be observed by the enhancement of the terbium emission intensity. In the presence of an ionic background of 1.16 mM $MgCl_2$, 2.3 mM $CaCl_2$, 140 mM NaCl and 4 mM KCl at pH 7.3, the terbium emission intensity at 545 nm was modulated by 25%, with an apparent dissociation constant $K_d = 0.6$ μM (295 K, $\lambda_{exc} = 262$ nm).

Discussion and conclusions

The simple photochemical scheme (Scheme 3) highlights the different ways in which lanthanide luminescence may be modulated following excitation into a proximate antenna group.[16] The intermediate aryl singlet and triplet or the lanthanide excited state itself may be perturbed, for example by variations in the energy of the singlet or triplet, by changes in the rate of intermolecular (*e.g.* collisional) quenching or by variations in the rate of quenching electron transfer processes. In this case, emission from the lanthanide complex simply echoes changes in the locally excited and internal charge transfer states, induced by metal ion binding to the aniline N in L^3 and [LnL^4] and to the aryl oxygen and benzylic nitrogen in the case of L^1 and [LnL^2]. The observed intensity enhancement for lanthanide luminescence, following metal ion binding or *N*-protonation is likely to be related to suppression of photo-induced electron transfer from or to the intermediate aryl singlet excited state.[14,23,24]

With L^1 and [LnL^2] the zinc ion is bound more strongly than either Ca^{2+} or Mg^{2+} consistent with zinc's preference for a tertiary N donor.[25] Ion-binding affinities are higher in [LnL^2] than L^1, but for zinc only. The enhanced discrimination in favour of Zn^{2+} may be related to the observed changes in the ligand absorption and emission spectra. Evidence for aryl ether coordination was found with Zn^{2+} only with each of the [LnL^2] complexes; the lanthanide ion is bound to the amide carbonyl group thereby promoting intramolecular charge transfer in both the ground and excited states, involving the conjugation of the *p*-substituted ether oxygen lone pair. The orientation of this oxygen lone pair is important as it helps to determine the strength of the chelating interaction with the zinc ion.

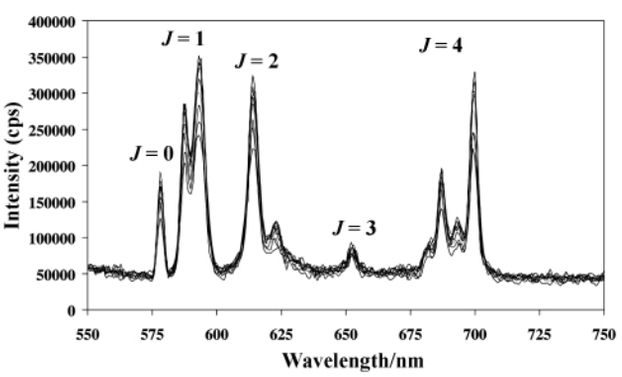


Fig. 8 Effect of adding $ZnCl_2$ on europium emission spectra ($\lambda_{exc} = 262$ nm; $^5D_0 \rightarrow {}^7F_J$) in [EuL^2] ([EuL^2] = 0.1 mM, [$ZnCl_2$] = 0.02 to 1.0 mM; 295 K, 10 mM HEPES, pH 7.4, 20 mM NaCl, 115 mM KCl).

With L^3 and [LnL^4], the aniline N is more strongly conjugated into the π system and metal ion binding perturbs the orientation of the nitrogen lone pair giving rise to significant changes in absorption and emission spectra. The nitrogen is less strongly basic in [LnL^4] compared to L^3 and this also lowers the apparent metal ion binding affinities to a similar extent (Tables 1 and 2). Binding affinities follow a very similar pattern to those reported for the parent complexing agent, L^5, with $Zn^{2+} > Ca^{2+} > Mg^{2+}$. This study highlights the possible strong interference effect of Zn^{2+} ions on measurements of intracellular Mg^{2+}—a point which may have been overlooked in the earlier reports of the utility of this system.[11,12]

The observed zinc ion selectivity and sensitivity with [TbL^2] are promising but practicable analyses are not within reach with this system; future developments based on this model must embrace the need for longer wavelength excitation (≥350 nm) and the requirement for intracellular localisation. In addition, a system is required in which modulation of the lifetime or emission spectrum of the lanthanide reporter occurs, involving either a change in the lanthanide coordination environment or a perturbation in the rate of quenching of the lanthanide excited state.

Experimental

Reagents and solvents

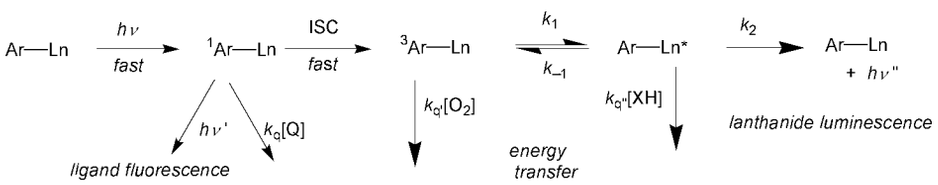
Reagents and solvents were purified using standard techniques. Solvents were dried over an appropriate drying agent before use: acetonitrile and triethylamine over calcium hydride, tetrahydrofuran (THF) over sodium with benzophenone as an indicator, and *N*,*N*-dimethylformamide (DMF) was used directly from a "sure-seal" bottle. The water used was high purity water with a conductivity ≤0.04 μS cm^{-1}, obtained from the PURITE™ purification system. 1,4,7,11-Tetraazacyclododecane is commercially available from Strem Chemicals and was used as received.

Chromatography

Column chromatography was carried out using "gravity" silica (Merck) or neutral alumina (Merck) which was pretreated with ethyl acetate. Cation-exchange chromatography was performed using Dowex 50W (H^+ form) strong ion-exchange resin, which had been pre-treated with an aqueous solution of 1 M HCl.

Spectroscopy

^{1}H NMR spectra were recorded at 65.15 MHz on a 1.53 T magnet connected to a Varian VXR400 console, at 199.99 MHz on a Varian Mercury-200, at 299.91 MHz on a Varian Unity-300, at 399.96 MHz on a Varian VXR400 and 499.79 MHz on a Varian Unity Inova-500 spectrometer. ^{13}C NMR spectra were recorded on the Varian Mercury-200 at 50.2 MHz, Varian Unity-300 at 75.4 MHz, Varian VXR400 at 100.58 MHz, and Varian Unity Inova-500 at 125.6 MHz. Mass spectra were recorded using a VG Platform II electrospray mass spectrometer with methanol or water as a carrier solvent. Melting points were determined on a Reichert Kofler Block and are uncorrected. Elemental analyses were determined using a Carlo ERBA 1106 instrument. Accurate masses were measured



Scheme 3

by the EPSRC National MS Service at the University of Swansea.

Absorbance and luminescence measurements

UV absorption spectra were recorded at 25 °C using a UNICAM (UV2) spectrometer. Fluorescence and lanthanide emission spectra were obtained using a Fluorolog 3–11 fluorimeter equipped with a Spex 1934D3 phosphorimeter. In both cases, emission spectra were acquired following excitation at the given wavelength and were corrected for the wavelength dependence of the photomultiplier tube. Throughout the titration experiments, the ligand solutions were stirred and the solution concentration range was 5–10 × 10^{-5} M. Aliquots of 1 or 3 cm^3 were taken for absorbance and luminescence measurements in standard quartz cuvettes with a 1 cm path length. Experiments were repeated and the mean value is given in the tables.

(a) Determination of p*K* values. The pH was monitored using a Corning Semi-Micro Combination electrode operated through a Jenway 3020 pH meter, calibrated prior to use using standard buffer solutions of pH 4.00 and 10.00 at 25 °C. Solutions contained 0.1 M $Me_4N^+NO_3^-$ to maintain an approximately constant ionic strength during the titration. Titrations were run from alkaline to acid pH and were shown in each case to be fully reversible. In a typical pH titration study, a known mass—*ca.* 0.9 mg of the complexing agent was dissolved in water (10 cm^3) containing 0.1 M $Me_4N^+NO_3^-$ and adjusted to pH 11.0 with 1 M NaOH stock solution (approximately 2 μl). A constant volume (1 μl) from 1 M or 0.1 M HNO_3 stock solution was added sequentially and the pH was measured before and after recording the absorbance, fluorescence and lanthanide emission spectra. A plot of the variation in intensity as a function of pH was made. For example, $-\log [H_3O^+]$ was plotted against the absorbance at 255 or 274 nm (L^1, EuL^2 and TbL^2) and 250 or 270 nm (L^3, EuL^4, and TbL^4). Similarly, plots of the variation in emission intensity as a function of pH were made, plotting against: (i) fluorescence at 370 or 440 nm (L^1; λ_{exc} = 262, 294 and 320 nm) and 347 or 415 nm (L^3), and (ii) lanthanide emission at 700 nm (EuL^2 and EuL^4) and 545 nm (TbL^2 and TbL^4). These curves were fitted by a simple iterative least-squares analysis procedure operating under the Microsoft Excel program. Eqn. (1) was applied to compute the corresponding p*K* values.

$$I_{obs} = (I_{max}(10^{(pH - pK)}) + I_{min})/(1 + 10^{(pH - pK)}) \quad (1)$$

(b) Binding constant determination. For metal ion titration experiments, solutions were made up to resemble the intracellular milieu of human cells. Thus, a typical solution contained 115 mM KCl, 20 mM NaCl, and 10 mM HEPES buffered with Tris base to pH 7.2–7.4. The effect of metal ions was investigated by addition of a solution of a metal chloride to a 10 mL solution of the ligand in the same solvent. The metal chloride was at a concentration of 0.1 or 1 M (as appropriate to the effective association constant for the system under investigation) and typically 1 μL increments of these stock solutions were added to the ligand solution, using a Gilson 2 μL pipette. These high concentration stock solutions ensured that a dilution of only 1–3% occurred, with a negligible effect on both the absorbance and emission spectra. In a typical binding study, a known mass—*ca.* 0.7 mg of the ligand was dissolved in buffered (pH 7.2 or 7.4) aqueous solution (10 cm^3). A constant volume (1 μL) from 1 M or 0.1 M MCl_2 (M = Zn^{2+}, Ca^{2+} or Mg^{2+}) solution was added sequentially and absorbance, fluorescence and lanthanide emission spectra were recorded. Following the changes in absorbance, fluorescence and metal emission intensity, which occurred as zinc or calcium or magnesium was added, the K_{ML} was determined from two separate types of plot. A Hill plot of log $[M^{2+}]_{free}$ *vs.* log $[(I_{max} - I_{obs})/(I_{obs} - I_{min})]$ gives $1/K_{ML}$ as the slope of the curve. $[M^{2+}]_{free}$ was calculated from eqn. (2).

$$[M^{2+}]_{free} = [M^{2+}]_{total} - \{((I_{max} - I_{obs})/(I_{max} - I_{min})) \times [chelator]\} \quad (2)$$

K_{ML} was also calculated directly from an iterative least-squares procedure, curve fitting to a 1:1 binding model, as expressed in eqn. (3), where $A = ([M^{2+}] + [ML] + K_{ML}^{-1})$, I_{int}

$$I_{obs} = I_{int} + ((I_{ML} - I_{int})/(2K_{ML}[M^{2+}])) \times \{A - (A^2 - 4 \times [M^{2+}] \times [ML])^{0.5}\} \quad (3)$$

and I_{ML} are the intensities in the absence and excess of metal ion respectively; $[M^{2+}]$ and [ML] are the concentrations of the total and bound metal ion respectively. Plotting the intensity observed against the total metal ion concentration for each sequential addition gives the value of K_{ML}, [ML], I_{int} and I_{ML}.

(c) Determination of Zn^{2+} stability constants under extracellular media. A known mass—*ca.* 0.6 mg of TbL^2 or TbL^4 was dissolved in an aqueous solution (10 mL) containing 140 mM NaCl, 4.6 mM KCl, 1.16 mM $MgCl_2$, 2.3 mM $CaCl_2$ and 10 mM HEPES buffered with Tris base to pH 7.3. A constant volume (0.5 μL) from a 0.2 M $ZnCl_2$ solution was added sequentially and absorbance, fluorescence and lanthanide emission spectra were recorded. Excitation was effected near the isosbestic wavelength (λ_{exc} = 262 nm) and at λ_{exc} = 320 nm, while absorption spectra were monitored at 250 nm.

o-Hydroxybenzylamine[17] (1)

To a stirred suspension of lithium aluminium hydride (14.2 g, 0.375 mol) in tetrahydrofuran (150 mL) under argon was added, dropwise, a solution of salicylamide (30 g, 0.22 mol) in THF (15 mL). The reaction was highly exothermic and the flask was kept in an ice–salt bath at −10 to −15 °C. After the addition was complete, the mixture was stirred overnight at ambient temperature and then refluxed for two more days. To the cooled reaction mixture, saturated sodium sulfate solution was added slowly while stirring vigorously. The thick inorganic precipitate was filtered and the filtrate was concentrated to dryness. The remaining crude was dissolved in dichloromethane (100 mL) and then washed with water. After drying with $CaCl_2$, filtering and removing solvent under reduced pressure, an off-white powder was obtained. The inorganic precipitate was continuously extracted with THF and product isolated as above. The combined aqueous phases were also continuously extracted with CH_2Cl_2 and a colourless solid was obtained (14.2 g, 0.115 mol, 53%), mp 97–99 °C. The literature reports only the hydrate.[17]

^{1}H NMR (300 MHz, $CDCl_3$) 4.14 (s, 2H, $PhCH_2N$), 6.78 (td, *J* = 7.2, 7.8 and 1.0 Hz, 1H, H-5′), 6.86 (dd, *J* = 7.8, 1.0 Hz, 1H, H-3′), 6.98 (dd, *J* = 7.2, 1.5 Hz, 1H, H-6′), 7.16 (td, *J* = 7.8, 7.2, 1.5 Hz, 1H, H-4′); ^{13}C NMR (75.4 MHz, $CDCl_3$) 45.6 ($PhCH_2N$), 116 (C-3′), 118 (C-1′), 119 (C-5′), 128.1 (C-6′), 128.9 (C-4′), 158.5 (C-2′); MS (*ES*$^+$) *m*/*z* 124 (MH^+), 106 ($M^+ - NH_3$). Anal.: C_7H_9NO requires: C, 68.3; H, 7.36; N, 11.3%. Found: C, 67.9; H, 7.62; N, 11.7%.

N,*N*-Bis(ethoxycarbonylmethyl)-2-(ethoxycarbonylmethoxy)-benzylamine (2)

A mixture of *o*-hydroxybenzylamine **1** (2.75 g, 0.022 mol), potassium iodide (14.8 g, 0.089 mol), disodium hydrogen phosphate (12.66 g, 0.089 mol) and ethyl bromoacetate (9.9 mL, 0.089 mol) in acetonitrile (100 mL), in an atmosphere of dry argon, was boiled under reflux overnight. At this point, only *N*,*N*-bis(ethoxycarbonylmethyl)-2-hydroxybenzylamine was observed to be formed.

^{1}H NMR (300 MHz, $CDCl_3$) 1.29 (t, 6H), 3.54 (s, 4H, 2 × NCH_2), 3.99 (s, 2H, $PhCH_2N$), 4.21 (q, 4H), 6.76 (td, J = 7.5, 8.1, 1.2 Hz, 1H, H-5′), 6.87 (dd, J = 8.1, 1.2 Hz, 1H, H-3′), 6.98 (dd, J = 7.5, 1.2 Hz, 1H, H-6′), 7.16 (td, J = 8.1, 7.5, 1.2 Hz, 1H, H-4′); ^{13}C NMR (75.4 MHz, $CDCl_3$) 14.2 (OCH_2CH_3), 53.9 ($PhCH_2N$), 56.2 (OCH_2CH_3), 61.1 (2 × NCH_2), 116.6 (C-3′), 117.5 (C-1′), 119.2 (C-5′), 129.4 (C-6′), 129.5 (C-4′), 157.4 (C-2′), 170.8 (2CO); MS (ES^+) m/z 296 (MH^+), 318 (MNa^+).

An additional amount of stronger base, *viz.*, potassium carbonate (6.6 g, 0.045 mol) and freshly dried molecular sieves (3 Å) were added, and the mixture boiled under reflux under argon for 24 h. The insoluble materials were filtered off and the solvent was removed under reduced pressure. The remaining crude was placed in high vacuum to remove traces of ethyl bromoacetate. The product was obtained as a dark oil (6.8 g, 0.018 mol, 80%) and was used without further purification.

^{1}H NMR (200 MHz, $CDCl_3$) 1.28 (t, 9H), 3.61 (s, 4H, 2 × NCH_2), 4.01 (s, 2H, $PhCH_2N$), 4.17 (q, 4H), 4.23 (q, 2H), 4.65 (s, 2H, OCH_2CO), 6.76 (dd, J = 7.4, 1.0 Hz, 1H, H-3′), 6.99 (td, J = 7.4, 1.0 Hz, 1H, H-5′), 7.22 (td, J = 7.4, 1.52 Hz, 1H, H-4′), 7.45 (dd, J = 7.4, 1.5 Hz, 1H, H-6′); ^{13}C NMR (75.4 MHz, $CDCl_3$) 14.1 (3 × OCH_2CH_3), 51.8 ($PhCH_2N$), 54.6 (2 × NCH_2), 60.3 (3 × OCH_2CH_3), 65.7 (OCH_2CO), 111.6 (C-3′), 121.6 (C-5′), 127.0 (C-1′), 128.3 (C-6′), 131 (C-4′), 156 (C-2′), 168.8 (CO), 171.3 (2CO); MS (ES^+) m/z 404 (MNa^+).

N,*N*-Bis(ethoxycarbonylmethyl)-2-(ethoxycarbonylmethoxy)-5-nitrobenzylamine (3)

To a stirred suspension of **2** (1.6 g, 4.24 mmol) in acetic acid (1 mL) at −20 °C, concentrated nitric acid (8 mL) was added dropwise, maintaining the bath temperature below −10 °C. After the addition was complete and the reaction mixture reached 0 °C, water (40 mL) was added. The aqueous acidic solution was continuously extracted with chloroform for 24 h using a liquid extractor apparatus. The organic phase was then dried over $CaCl_2$, filtered and removed under reduced pressure to give **3** as a red oily product (1.5 g, 3.5 mmol, 83%). Crystallisation from ethanol occurred upon standing at 4 °C to yield a fine white powder (1.3 g, 72%) mp 67–68 °C.

^{1}H NMR (500 MHz, $CDCl_3$) 1.28 (t, 6H), 1.29 (t, 3H), 3.62 (s, 4H, 2 × NCH_2), 4.01 (s, 2H, $PhCH_2N$), 4.17 (q, 4H), 4.23 (q, 2H), 4.74 (s, 2H, OCH_2CO), 6.77 (d, J = 9.0 Hz, 1H, H-3′), 8.14 (dd, J = 9.0, 3.0 Hz, 1H, H-4′), 8.49 (d, J = 3.0 Hz, 1H, H-6′); ^{13}C NMR (125.7 MHz, $CDCl_3$) 14.1 (OCH_2CH_3), 14.2 (2 × OCH_2CH_3), 51.8 ($PhCH_2N$), 55.0 (2 × NCH_2), 60.6 (2 × OCH_2CH_3), 61.7 (OCH_2CH_3), 65.5 (OCH_2CO), 110.7 (C-3′), 124.4 (C-4′), 126.0 (C-6′), 129.2 (C-1′), 142.3 (C-5′), 160.6 (C-2′), 167.6 (CO), 171.2 (2CO); MS (ES^+) m/z 427 (MH^+). Anal.: $C_{19}H_{26}N_2O_9$ requires: C, 53.5; H, 6.14; N, 6.57%. Found: C, 53.4; H, 6.16; N, 6.58%.

N,*N*-Bis(ethoxycarbonylmethyl)-2-(ethoxycarbonylmethoxy)-5-aminobenzylamine (4)

Catalytic hydrogenation of **3** (4.9 g, 0.011 mol) in ethanol (140 mL) over 10% Pd/C (10% w/w) was carried out at medium pressure (50–60 psi). After the uptake of hydrogen ceased, the catalyst was filtered off (Celite), and the solvent was removed under reduced pressure. The crude material was purified by column chromatography on silica gel, eluting with ethyl acetate–petroleum ether (2:1) to give **4** as a pale yellow oil (4.1 g, 94%).

^{1}H NMR (500 MHz, $CDCl_3$) 1.27 (t, 6H), 1.28 (t, 3H), 3.59 (s, 4H, 2 × NCH_2), 3.94 (s, 2H, $PhCH_2N$), 4.15 (q, 4H), 4.23 (q, 2H), 4.54 (s, 2H, OCH_2CO), 6.55 (dd, J = 8.5, 2.5 Hz, 1H, H-4′), 6.63 (d, J = 8.5 Hz, 1H, H-3′), 6.87 (d, J = 2.5 Hz, 1H, H-6′); ^{13}C NMR (125.7 MHz, $CDCl_3$) 14.1 (OCH_2CH_3), 14.2 (2 × OCH_2CH_3), 51.8 ($PhCH_2N$), 54.5 (2 × NCH_2), 60.4 (2 × OCH_2CH_3), 61.1 (OCH_2CH_3), 67.1 (OCH_2CO), 114.3 (C-4′), 114.8 (C-6′), 118.0 (C-3′), 128.5 (C-1′), 141 (C-5′), 149.6 (C-2′), 169.4 (CO), 171.4 (2CO); MS (ES^+) m/z 397 (MH^+), 419 (MNa^+). Anal.: $C_{19}H_{28}N_2O_7$ requires: C, 57.6; H, 7.12; N, 7.07%. Found: C, 57.3; H, 7.11; N, 7.06%.

N,*N*-Bis(ethoxycarbonylmethyl)-2-(ethoxycarbonylmethoxy)-5-(β-chloroethanamido)benzylamine (5)

A mixture of **4** (0.58 g, 1.45 mmol) and triethylamine (0.26 mL, 1.45 mmol) in diethyl ether (80 mL) was cooled to −20 °C and a solution of chloroacetyl chloride (0.15 mL, 1.8 mmol) in diethyl ether (40 mL) was added dropwise, maintaining the temperature of the bath at −10 °C. Once the addition was complete, the reaction was stirred under argon, at room temperature, overnight. Insoluble salts were filtered and the filtrate was washed with 1 M aqueous acid chloride (50 mL) followed by 10% sodium bicarbonate solution (100 mL). The ethereal phase was dried with $CaCl_2$, filtered and solvent was removed under reduced pressure to give an oily crude product. The remaining aqueous layer was washed with chloroform (2 × 30 mL) and material isolated as above. The combined oily crude was purified by column chromatography on silica gel eluting with ethyl acetate–petroleum ether (4:6) (R_f = 0.3) to give **5** as a yellow oil (0.54 g, 1.14 mmol, 80%).

^{1}H NMR (500 MHz, $CDCl_3$) 1.25 (t, 6H), 1.26 (t, 3H), 3.58 (s, 4H, 2 × NCH_2), 3.99 (s, 2H, $PhCH_2N$), 4.15 (q, 4H), 4.16 (s, 2H, $ClCH_2CO$), 4.23 (q, 2H), 4.60 (s, 2H, OCH_2CO), 6.72 (d, J = 9.0 Hz, 1H, H-3′), 7.44 (d, J = 3.0 Hz, 1H, H-6′), 7.66 (dd, J = 9.0, 3.0 Hz, 1H, H-4′), 8.25 (bs, 1H, NH); ^{13}C NMR (125.7 MHz, $CDCl_3$) 14.4 (OCH_2CH_3), 14.5 (2 × OCH_2CH_3), 43.1 ($ClCH_2CO$), 51.7 ($PhCH_2N$), 54.9 (2 × NCH_2), 60.7 (2 × OCH_2CH_3), 61.5 (OCH_2CH_3), 66.2 (OCH_2CO), 112.5 (C-3′), 120.9 (C-4′), 123.2 (C-6′), 128.4 (C-1′), 131.1 (C-5′), 153.7 (C-2′), 164.0 (NHCO), 167.0 (CO), 171.6 (2CO); MS (ES^+) m/z 473 (MH^+), 495 (MNa^+). Anal.: $C_{21}H_{29}N_2O_8Cl\cdot\frac{1}{2}H_2O$ requires: C, 52.3; H, 6.27; N, 5.81%. Found: C, 52.2; H, 6.09; N, 5.48%.

N,*N*-Bis(carboxymethyl)-2-(carboxymethoxy)-5-(2-chloroethanamido)benzylamine (L^1)

A suspension of **5** (0.38 g, 0.8 mmol) in aqueous sodium hydroxide solution (10 mL, 0.22 M) was stirred under argon for 24 h. The clear solution was then washed with chloroform (2 × 20 mL), which gave recovery of 80 mg of **5**, and concentrated. Purification using ion-exchange column chromatography over Dowex 50W resin, in strong acid form, gave L^1 (eluted with 1% aqueous ammonia) as an off-white solid (0.24 g, 0.62 mmol, 80%), mp 161–163 °C.

^{1}H NMR (500 MHz, D_2O) 3.58 (s, 4H, 2 × NCH_2), 3.99 (s, 2H, $PhCH_2N$), 4.15 (q, 4H), 4.16 (s, 2H, $ClCH_2CO$), 4.23 (q, 2H), 4.60 (s, 2H, OCH_2CO), 6.72 (d, J = 9.0 Hz, 1H, H-3′), 7.44 (d, J = 3.0 Hz, 1H, H-6′), 7.66 (dd, J = 9.0, 3.0 Hz, 1H, H-4′), 8.25 (bs, 1H, NH); ^{13}C NMR (125.7 MHz, D_2O) 42.9 ($ClCH_2CO$), 54.7 ($PhCH_2N$), 56.5 and 56.7 (2 × NCH_2), 67.4 (OCH_2CO), 112.8 (C-3′), 119.1 (C-4′), 123.8 (C-6′), 124.9 (C-1′), 141.0 (C-5′), 145.3 (C-2′), 155.3 (NHCO), 170.6 and 170.7 (3 × CO); MS (ES^-) m/z 387 ($M - H^+$), 331 ($M - CO_2H$). Anal.: found (as bis(ammonium) salt dihydrate): C, 39.2; H, 6.34; N, 12.3%; $C_{15}H_{27}N_4O_{10}Cl$ requires: C, 39.3; H, 5.93; N, 12.2%.

1-{3-[*N*,*N*-Bis(ethoxycarbonylmethyl)aminomethyl]-4-(ethoxycarbonylmethoxy)phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecane (6)

1,4,7,10-Tetraazacyclododecane (0.37 g, 2.17 mmol), **5** (0.4 g, 0.865 mmol) and potassium iodide (0.144 g, 0.865 mmol) in dry THF (10 mL) were heated (40 °C) under argon for 8 h.

The reaction was stirred until TLC analysis (silica gel, ethyl acetate–petroleum ether 1:1 v/v) showed complete consumption of **5** (disappearance of $R_f = 0.65$). Potassium iodide (0.05 g) was added to accelerate reaction completion. Insoluble precipitates were filtered and the solvent was removed under reduced pressure. The semi-solid crude was dissolved in chloroform, washed with water, dried (K_2CO_3), filtered and solvent removed under reduced pressure. An oily crude material was obtained (0.5 g, 0.8 mmol, 90%) which was used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) 1.25 (t, 6H), 1.26 (t, 3H), 3.58 (s, 4H, $2NCH_2$), 3.99 (s, 2H, $PhCH_2N$), 4.15 (q, 4H), 4.16 (s, 2H, $ClCH_2CO$), 4.23 (q, 2H), 4.60 (s, 2H, OCH_2CO), 6.72 (d, $J = 9.0$ Hz, 1H, H-3′), 7.44 (d, $J = 3.0$ Hz, 1H, H-6′), 7.66 (dd, $J = 9.0$, 3.0 Hz, 1H, H-4′), 8.25 (bs, 1H, NH); ^{13}C NMR (125.7 MHz, $CDCl_3$) 14.4 (OCH_2CH_3), 14.5 ($2 \times OCH_2CH_3$), 43.1 ($ClCH_2CO$), 51.7 ($PhCH_2N$), 54.9 ($2 \times NCH_2$), 60.7 ($2 \times OCH_2CH_3$), 61.5 (OCH_2CH_3), 66.2 (OCH_2CO), 112.5 (C-3′), 120.9 (C-4′), 123.2 (C-6′), 128.4 (C-1′), 131.1 (C-5′), 153.7 (C-2′), 164.0 (NHCO), 167.0 (CO), 171.6 (2CO); MS (ES^+) m/z 473 (MH^+), 495 (MNa^+).

1,4,7-Tris(*tert*-butoxycarbonylmethyl)-10-{3-[*N*,*N*-bis(ethoxycarbonylmethyl)aminomethyl]-4-(ethoxycarbonylmethoxy)-phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecane (7)

Method A. To a solution of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane[2] (0.25 g, 0.48 mmol) in acetonitrile (10 mL), dry Cs_2CO_3 (0.2 g, 0.6 mmol) and a solution of *N*,*N*-bis(ethoxycarbonylmethyl)-2-(ethoxycarbonylmethoxy)-5-(β-chloroethanamido)benzylamine **5** (0.25 g, 0.54 mmol) in acetonitrile (5 mL) were added. The reaction mixture was heated to 70 °C for 72 h under argon and the reaction was monitored by TLC (silica gel, CH_2Cl_2–THF 3:7). After cooling, insoluble material was filtered off, solvent was removed under reduced pressure and the remaining gum was washed with water–CH_2Cl_2 mixture (100 mL; 50:50 v/v). The organic layer was separated, dried over $CaCl_2$, filtered and concentrated to dryness under reduced pressure. The resulting crude was purified by column chromatography (silica gel) eluted with CH_2Cl_2–THF–MeOH–NH_3 (3:7:0.5:0.5) (R_f = 0.85) to give **7** as an oil (0.15 g, 0.05 mmol, 10%).

^{1}H NMR (300 MHz, $CDCl_3$) 1.25 (t, 9H), 1.45 (s, 27H), 2.89 (m, 12H, $3 \times NCH_2CH_2N$), 3.11 (m, 4H, NCH_2CH_2N), 3.29 (s, 2H, $NCH_2CO_2{}^t$Bu), 3.39 (s, 4H, $2 \times NCH_2CO_2{}^t$Bu), 3.58 (s, 4H, $2 \times NCH_2$), 4.00 (s, 2H, $PhCH_2N$), 4.17 (s, 2H, NCH_2CON), 4.20 (q, 6H), 4.63 (s, 2H, OCH_2CO), 6.68 (dd, $J = 6.0$, 2.6 Hz, 1H, H-4′), 7.44 (m, 1H, H-6′), 7.66 (m, 1H, H-4′), 8.2 (bs, 1H, NH); ^{13}C NMR (125.7 MHz, $CDCl_3$) 13.2 ($3 \times OCH_2CH_3$), 27.2 and 29.3 ($3 \times O^t$Bu), 42.0, 46.3, 48.2 and 50.3 ($8 \times CH_2$), 46.3 (NCH_2CON), 50.4 ($PhCH_2N$), 53.8, 55.0 and 55.5 ($5 \times NCH_2CO_2R$), 59.5 and 60.3 ($2 \times OCH_2CH_3$), 65.0 (OCH_2CO), 80.9 ($3 \times O^t$Bu), 112.0 (C-4′), 120.0 (C-3′), 122 (C-6′), 124 (C-5′), 127.5 (C-1′), 134.7 (C-2′), 150.0 (NCH_2CON), 168.6, 169.5 and 170.3 ($6 \times CO_2R$); MS (ES^+) m/z 973 (MNa^+).

Method B. To a solution of 1-{3-[*N*,*N*-bis(ethoxycarbonylmethyl)aminomethyl]-4-(ethoxycarbonylmethoxy)phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecane **6** (0.5 g, 0.082 mmol) in acetonitrile (40 mL), dry Cs_2CO_3 (0.9 g, 2.7 mmol) and *tert*-butyl bromoacetate (0.5 g, 2.7 mmol) were added. The reaction mixture was heated to reflux for 48 h under argon and the reaction monitored by TLC (silica gel, CH_2Cl_2–THF 3:7). After cooling, insoluble material was filtered off, solvent removed under reduced pressure and the remaining gum was washed with water and CH_2Cl_2 (50 mL, 50:50). The organic phase was separated, dried over $CaCl_2$, filtered and concentrated to dryness under reduced pressure. The resulting crude was purified by column chromatography on silica gel, eluting with CH_2Cl_2–THF–MeOH–NH_3 (3:7:0.5:0.5) mixture to give **7** (0.1 g, 0.03 mmol, 25%).

1,4,7-Tris(*tert*-butoxycarbonylmethyl)-10-{3-[*N*,*N*-bis(ethoxycarbonylmethyl)aminomethyl]-4-(ethoxycarbonylmethoxy)-phenyl-*N*-(*tert*-butoxycarbonylmethyl)carbamoylmethyl-1,4,7,10-tetraazacyclododecane (8)

The resulting crude from Method B (as above) also gave compound **8** as a by-product, which was isolated from the column in the less polar fractions (eluted with CH_2Cl_2–THF–MeOH 65:30:5) ($R_f = 0.92$). Approximately 60 mg of pure material were obtained as a pale yellow oil, and additional material (40 mg) was present in mixtures.

^{1}H NMR (400 MHz, $CDCl_3$, −50 °C) 1.25 (t, 9H), 1.45 (s, 36H), 2.0–3.0 (m, 16H, $4 \times NCH_2CH_2N$), 3.0–3.5 (m, 10H, $4 \times NCH_2CO_2{}^t$Bu, NCH_2CON), 3.54 (s, 4H, $2 \times NCH_2CO_2Et$), 3.96 (s, 2H, $PhCH_2N$), 4.11 (q, 2H), 4.25 (q, 6H), 4.64 (s, 2H, OCH_2CO), 6.71 (d, $J = 8.7$ Hz, 1H, H-3′), 7.12 (dd, $J = 8.7$, 2.6 Hz, 1H, H-4′), 7.42 (d, $J = 2.6$ Hz, 1H, H-6′); ^{13}C NMR (125.7 MHz, $CDCl_3$) 15.0 ($3 \times OCH_2CH_3$), 28.2 and 31.3 ($4 \times O^t$Bu), 52.1, 52.8, and 53.7 ($4 \times NCH_2CH_2N$), 52.1 ($PhCH_2N$), 55.1 (NCH_2CON), 56.1 and 56.3 ($6 \times NCH_2CO_2R$), 60.6 and 61.9 ($2 \times OCH_2CH_3$), 65.8 (OCH_2CO), 81.9 ($4 \times O^t$Bu), 112.9 (C-4′), 127.4 (C-3′), 129.7 (C-5′), 130.5 (C-6′), 135.6 (C-1′), 156.1 (C-2′), 152.0 (NCH_2CON and CO_2R), 168.6, 169.5, 171.3, 172.1 and 173.5 ($5 \times CO_2R$); MS (ES^+) m/z 1087 (MNa^+).

General method for the synthesis of lanthanide(III) complexes [EuL^2] and [TbL^2]

1,4,7-Tris(*tert*-butoxycarbonylmethyl)-10-{3-[*N*,*N*-bis(ethoxycarbonylmethyl)aminomethyl]-4-(ethoxycarbonylmethoxy)-phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecane **7** (0.1 g, 0.11 mmol) was dissolved in an 80% solution of trifluoroacetic acid (TFA) in dichloromethane (3 mL) and stirred at room temperature under argon overnight. After removal of volatiles under reduced pressure, the remaining crude was washed with CH_2Cl_2 (2×10 mL) followed by diethyl ether (2×10 mL). The resulting off-white gum was dissolved in water, frozen and solvent removed by lyophilisation to give **9** as a colourless solid (75 mg, 96 μmol, 87%), which was used without further purification.

^{1}H NMR (300 MHz, D_2O) 1.04 (t, 9H), 2.7–3.3 (m, 16H, $4 \times NCH_2CH_2N$), 3.37 (s, 2H, NCH_2CON), 3.40 (s, 2H, NCH_2CO_2Et), 3.42 and 3.44 ($2 \times$ s, 4H + 2H, $3 \times NCH_2CO_2H$), 3.97 (s, 2H, $PhCH_2N$), 4.10 (q, 6H), 4.20 (s, 2H, OCH_2CO), 6.9 (d, $J = 6.0$ Hz, 1H, H-3′), 7.4 (dd, $J = 6.0$, 2.5 Hz, 1H, H-6′), 7.5 (bs, 1H, H-4′); MS (ES^+) m/z 392 ($M + 2H^+$), 783 (MH^+).

A solution of either europium(III) acetate trihydrate (40 mg, 100 μmol) or terbium nitrate pentahydrate (45 mg, 100 μmol) in water (10 mL) was added to 1,4,7-tris(carboxymethyl)-10-{3-[*N*,*N*-bis(ethoxycarbonylmethyl)aminomethyl]-4-(ethoxycarbonylmethoxy)phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecane **9** (75 mg, 96 μmol). The pH was raised to 5.5 by addition of sodium hydroxide solution at which point the ligand slowly dissolved. The solution was heated at 90 °C for 24 h. The lanthanide complex formation was monitored by electrospray mass spectrometry (ES^-) which gave m/z values with the correct isotope pattern for M^- and $(M - H)^-$ (M = triethyl ester of EuL^2 or TbL^2). The reaction mixture was cooled and NaOH pellets were added (approx. 0.1 M) to allow hydrolysis of the remaining ester groups, at room temperature (24 h). Following neutralisation of the reaction solution to pH 7, the lanthanide complexes were purified by ion-exchange column chromatography using Dowex 50W cation resin in strong acid form, eluting with diluted aqueous ammonium (1%) solution. The solvent of the appropriate fraction (determined from UV absorption of the hydrolysed lanthanide

complex spotted on a silica TLC plate) was frozen and water removed by lyophilisation. The complexes [EuL^2] or [TbL^2] were obtained as pale yellow solids in 85–90% isolated yield.

1,4,7-Tris(carboxylatomethyl)-10-{3-[*N*,*N*-bis(carboxymethyl)aminomethyl]-4-(carboxymethoxy)phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecaneeuropium(III) (EuL^2). MS (*ES*$^-$) *m*/*z* 423 ($M - 2H^+$), 847 ($M - H^+$); HRMS: $C_{29}H_{39}N_6O_{14}Eu$ requires 847.1659. Found: 847.1660.

1,4,7-Tris(carboxylatomethyl)-10-{3-[*N*,*N*-bis(carboxymethyl)aminomethyl]-4-(carboxymethoxy)phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecaneterbium(III) (TbL^2). MS (*ES*$^-$) *m*/*z* 426 ($M - 2H^+$), 853 ($M - H^+$); HRMS: $C_{29}H_{39}N_6O_{14}Tb$ requires 853.1700. Found: 853.1692.

N-Methylchloroethanamide (10)

The reagent was prepared following a published procedure[7] using *N*-methylammonium chloride and chloroacetyl chloride. A major improvement in yield (85% *vs.* 35% reported) was obtained by repeatedly extracting the aqueous phase with chloroform; mp 43–44 °C.

^{1}H NMR (300 MHz, $CDCl_3$) 2.88 (d, $J = 4.8$ Hz, 3H), 4.08 (s, 2H), 6.73 (bs, NH).

2-Nitro-1-(*N*-methylcarbamoylmethoxy)benzene (11)

A mixture of 2-nitrophenol (13 g, 0.094 mol), potassium iodide (15.6 g, 0.094 mol), potassium carbonate (13 g, 0.094 mol) and *N*-methylchloroacetamide (10.1 g, 0.094 mol) in acetone (100 mL) was refluxed under argon for two days. The cooled reaction was concentrated under reduced pressure and the remaining crude was dissolved in CH_2Cl_2 (100 mL). After washing with water, the organic layer was dried over $CaCl_2$, filtered and solvent removed under reduced pressure. A pale yellow powder was obtained (18 g, 0.086 mol, 93%) which was used without further purification, mp 93–94 °C.

^{1}H NMR (300 MHz, $CDCl_3$) 2.96 (d, $J = 4.8$ Hz, 3H), 4.63 (s, 2H), 7.06 (dd, $J = 8.4$, 1.2 Hz, 1H, H-6′), 7.16 (td, $J = 8.4$, 7.5, 1.2 Hz, 1H, H-4′), 7.61 (td, $J = 8.4$, 7.5, 1.2 Hz, 1H, H-5′), 8.04 (dd, $J = 7.5$, 1.2 Hz, 1H, H-3′); ^{13}C NMR (75.7 MHz, $CDCl_3$) 26.0 (N*C*H$_3$), 67.7 (OCH$_2$), 114.5 (C-6′), 121.7 (C-4′), 126.6 (C-3′), 135.3 (C-2′, C-5′), 150.9 (C-1′), 167.2 (NH*C*O); ^{1}H NMR (300 MHz, D_2O) 2.62 (s, 3H), 4.64 (s, 2H), 6.90 (m, 2H$_{aryl}$), 7.17 (m, 2H$_{aryl}$); ^{13}C NMR (50.3 MHz, D_2O) 25.8 (N*C*H$_3$), 67.3 (OCH$_2$), 113.5 (C-6′), 119.5 (C-3′), 122.7 (C-5′), 124.3 (C-3′), 130.7 (C-2′), 150.5 (C-1′), 171.0 (NH*C*O); Anal.: $C_9H_{10}N_2O_4$ requires: C, 51.4; H, 4.79; N, 13.3%. Found: C, 51.3; H, 4.74, N, 13.1%.

2-Amino-1-(*N*-methylcarbamoylmethoxy)benzene (12)

Catalytic hydrogenation of **11** (7.8 g, 0.0371 mol) in ethanol (180 mL) over 10% Pd/C (10% w/w) was carried out at medium pressure (50–60 psi). After the uptake of hydrogen ceased, the catalyst was filtered through Celite and solvent was removed under reduced pressure to give **12** as red oily crude material in quantitative yield. Upon standing the oily product partially crystallised to a semi-solid form.

^{1}H NMR (300 MHz, D_2O) 2.62 (s, 3H), 4.64 (s, 2H), 6.90 (m, 2H$_{aryl}$), 7.17 (m, 2H$_{aryl}$); ^{13}C NMR (50.3 MHz, D_2O) 25.8 (N*C*H$_3$), 67.3 (OCH$_2$), 113.5 (C-6′), 119.5 (C-3′), 122.7 (C-5′), 124.3 (C-3′), 130.7 (C-2′), 150.5 (C-1′), 171.0 (NH*C*O); MS (*ES*$^+$) *m*/*z* 181 (*MH*$^+$), 203 (*MNa*$^+$). Anal.: $C_9H_{12}N_2O_2$ requires: C, 60.0; H, 6.71; N, 15.5%. Found: C, 59.6; H, 6.66, N, 15.2%.

2-[*N*,*N*-Bis(ethoxycarbonylmethyl)amino]-1-(*N*-methylcarbamoylmethoxy)benzene (13)

A mixture of **12** (10.8 g, 0.06 mol), potassium iodide (20 g, 0.12 mol), disodium hydrogen phosphate (17 g, 0.12 mol) and ethyl bromoacetate (13.3 mL, 0.12 mol) in acetonitrile (100 mL) was refluxed under argon until TLC analysis showed the consumption of **12**. When necessary, disodium hydrogen phosphate and freshly dried molecular sieves (4 Å) were added to accelerate reaction completion. Insoluble material was filtered off and solvent removed under reduced pressure. The remaining crude was dissolved in CH_2Cl_2 (50 mL), washed with water, and dried ($CaCl_2$). After filtration and solvent removal, **13** was obtained as a colourless oil (19.6 g, 0.055 mol, 93%). Upon standing, the compound crystallised to give colourless needles, mp 58–60 °C.

^{1}H NMR (300 MHz, $CDCl_3$) 1.26 (t, $J = 7.2$ Hz, 6H), 2.86 (d, $J = 5.1$ Hz, 3H), 4.10 (s, 4H, 2 × NCH$_2$), 4.18 (q, $J = 7.2$ Hz, 4H), 4.60 (s, 2H), 6.90 (td, $J = 7.8$, 1.5 Hz, 1H, H-5′), 6.93 (dd, $J = 7.5$, 1.8 Hz, 1H, H-3′), 7.00 (dd, $J = 7.8$, 1.8 Hz, 1H, H-6′), 7.05 (dd, $J = 7.5$, 1.8 Hz, 1H, H-4′); ^{13}C NMR (75.7 MHz, $CDCl_3$) 14.4 (2 × OCH$_2$*C*H$_3$), 26.2 (N*C*H$_3$), 54.1 (2 × NCH$_2$), 61.2 (2 × O*C*H$_2$CH$_3$), 69.2 (OCH$_2$), 114.7 (C-5′), 122.0 (C-4′), 122.8 (C-3′), 124.4 (C-6′), 139.7 (C-2′), 151.1 (C-1′), 169.6 (NH*C*O), 171.6 (2 × *C*O$_2$R); MS (*ES*$^+$) *m*/*z* 352 (*MH*$^+$), 375 (*MNa*$^+$). Anal.: $C_{17}H_{24}N_2O_6 \cdot H_2O$ requires: C, 55.1; H, 7.02; N, 7.57%. Found: C, 55.3; H, 6.82, N, 7.50%.

5-Nitro-2-[*N*,*N*-bis(ethoxycarbonylmethyl)amino]-1-(*N*-methylcarbamoylmethoxy)benzene (14)

A solution of **13** (10.6 g, 0.03 mol) in acetonitrile (50 mL) was cooled to −20 °C before nitronium tetrafluoroborate (3.96 g, 0.03 mol) in acetonitrile (100 mL) was added dropwise, maintaining the temperature of the bath below −10 °C. After the addition was complete, the reaction mixture was stirred at ambient temperature overnight. Water (150 mL) was added to quench any remaining nitronium salts and after an hour, the entire aqueous–acetonitrile mixture was concentrated. The concentrated mixture solution was washed with chloroform (5 × 100 mL), dried ($CaCl_2$), filtered, and solvents removed under reduced pressure. The crude product was then purified by column chromatography on silica gel eluting with ethyl acetate–petroleum ether (1 : 1) ($R_f = 0.38$) to give **14** as a pale yellow oil that crystallised upon standing (8.4 g, 0.021 mol, 70%), mp 78–80 °C.

^{1}H NMR (400 MHz, $CDCl_3$) 1.27 (t, $J = 7.2$ Hz, 6H), 2.83 (d, $J = 4.8$ Hz, 3H), 4.14 (s, 4H, 2 × NCH$_2$), 4.20 (q, $J = 7.2$ Hz, 4H), 4.60 (s, 2H), 6.90 (d, $J = 8.9$ Hz, 1H, H-3′), 7.05 (bs, NH), 7.76 (d, $J = 2.4$ Hz, 1H, H-6′), 7.88 (dd, $J = 8.9$, 2.4 Hz, 1H, H-4′); ^{13}C NMR (100.6 MHz, $CDCl_3$) 14.1 (2 × OCH$_2$*C*H$_3$), 25.8 (N*C*H$_3$), 53.9 (2 × NCH$_2$), 61.4 (2 × O*C*H$_2$CH$_3$), 68.7 (OCH$_2$), 109.3 (C-6′), 118.5 (C-3′), 118.8 (C-4′), 142.0 (C-5′), 145.6 (C-2′), 148.5 (C-1′), 167.5 (NH*C*O), 170.6 (2 × *C*O$_2$R); MS (*ES*$^+$) *m*/*z* 398 (*MH*$^+$), 420 (*MNa*$^+$). Anal.: $C_{17}H_{23}N_3O_8$ requires: C, 51.4; H, 5.83; N, 10.6%. Found: C, 51.7; H, 5.96, N, 10.3%.

5-Nitro-2-[*N*,*N*-bis(ethoxycarbonylmethyl)amino]-1-(*N*-methyl-*N*-*tert*-butoxycarbonylcarbamoylmethoxy)benzene (15)

A mixture of **14** (11.5 g, 0.029 mol), triethylamine (4 mL, 0.029 mol), 4-(*N*,*N*-dimethylamino)pyridine (3.5 g, 0.029 mol) and di-*tert*-butyl carboxy anhydride (12.6 g, 0.058 mol) in CH_2Cl_2 (120 mL) was stirred at room temperature under argon overnight. The mixture was washed with HCl (100 mL, 1 M) followed by 10% sodium bicarbonate solution (200 mL). After the usual work-up, an oil was obtained which crystallised upon standing (12.5 g, 0.025 mol, 88%), mp 93–95 °C.

^{1}H NMR (400 MHz, $CDCl_3$) 1.23 (t, $J = 7.2$ Hz, 6H), 1.56 (s, 9H), 3.18 (s, NMe), 4.23 (q, $J = 7.2$ Hz, 4H), 4.31 (s, 4H, 2 × NCH$_2$), 5.21 (s, 2H), 6.70 (d, $J = 9.0$ Hz, 1H, H-3′), 7.56 (d, $J = 2.5$ Hz, 1H, H-6′), 7.82 (dd, $J = 9.0$, 2.54 Hz, 1H, H-4′); ^{13}C NMR (100.6 MHz, $CDCl_3$) 14.1 (2 × OCH$_2$*C*H$_3$), 27.8 (3 × Me), 31.0 (N*C*H$_3$), 54.0 (2 × NCH$_2$), 60.9 (2 × O*C*H$_2$-

CH_3), 69.5 (OCH_2), 84.1 (CMe_3), 108.0 (C-6′), 115.9 (C-3′), 118.4 (C-4′), 140.5 (C-5′), 145.4 (C-2′), 147.7 (C-1′), 152.7 (N*C*OO), 169.9 (NH*C*O), 170.4 (2 × CO_2R); MS (ES^+) *m*/*z* 520 (MNa^+), 536 (MK^+). Anal.: $C_{22}H_{31}N_3O_{10}$ requires C, 53.1; H, 6.28; N, 8.45%. Found: C, 52.8; H, 6.29, N, 8.30%.

5-Amino-2-[*N*,*N*-bis(ethoxycarbonylmethyl)amino]-1-(*N*-methyl-*N*-*tert*-butoxycarbonylcarbamoylmethoxy)benzene (16)

Catalytic hydrogenation of **15** (12.5 g, 0.025 mol) in ethanol (120 mL) and chloroform (30 mL) over 10% Pd/C (10% w/w) was carried out at medium pressure (50–60 psi). After the uptake of hydrogen had ceased, the catalyst was filtered through Celite, and the solvent was removed under reduced pressure. The oily crude was then purified by column chromatography on silica gel eluting with ethyl acetate ($R_f = 0.4$) to give **16** as a yellow oil (8.75 g, 0.019 mol, 75%). Upon standing the compound crystallised, mp 90–92 °C.

^{1}H NMR (500 MHz, $CDCl_3$) 1.21 (t, $J = 7$ Hz, 6H), 1.54 (s, 9H), 3.16 (s, NMe), 4.12 (s, 4H, 2 × NCH_2), 4.13 (q, $J = 7$ Hz, 4H), 5.13 (s, 2H), 6.18 (d, $J = 2.0$ Hz, 1H, H-6′), 6.22 (dd, $J = 8.5$, 2.5 Hz, 1H, H-4′), 6.84 (d, $J = 8.5$ Hz, 1H, H-3′); ^{13}C NMR (125.7 MHz, $CDCl_3$) 14.4 (2 × OCH_2CH_3), 28.3 (3 × Me), 31.4 (NCH_3), 54.4 (2 × NCH_2), 60.6 (2 × OCH_2CH_3), 70.5 (OCH_2), 83.9 (CMe_3), 103.2 (C-6′), 108.8 (C-4′), 122.9 (C-3′), 131.6 (C-2′), 142.8 (C-5′), 152.2 (C-1′), 153.2 (N*C*OO), 171.6 (NH*C*O), 171.8 (2 × CO_2R); MS (ES^+) *m*/*z* 490 (MNa^+), 506 (MK^+).

5-(β-Chloroethanamido)-2-[*N*,*N*-bis(ethoxycarbonylmethyl)-amino]-1-(*N*-methyl-*N*-*tert*-butoxycarbonylcarbamoylmethoxy)-benzene (17)

To a solution of **16** (0.35 g, 0.75 mmol) in diethyl ether (50 mL), triethylamine (0.125 mL, 0.9 mmol) was added. The solution was cooled to −20 °C and a solution of chloroacetyl chloride (0.075 mL, 0.9 mmol) in diethyl ether (20 mL) was added dropwise, maintaining the temperature of the bath below −10 °C. After the addition was complete, the reaction mixture was maintained at ambient temperature overnight. Insoluble salts were filtered off and the filtrate was washed with hydrochloric acid (30 mL, 1 M) followed by 10% sodium bicarbonate solution (60 mL). The ethereal phase was dried over $CaCl_2$, filtered and solvent removed under reduced pressure to give a red oily crude that was purified by column chromatography on silica gel eluting with ethyl acetate–petroleum ether (1 : 6 to 4 : 6) ($R_f = 0.65$); compound **17** was obtained as a yellow oil (0.26 g, 0.52 mmol, 80%).

^{1}H NMR (500 MHz, $CDCl_3$) 1.24 (t, $J = 7$ Hz, 6H), 1.55 (s, 9H), 3.17 (s, NMe), 4.14 (s, 2H, $ClCH_2$), 4.16 (q, $J = 7$ Hz, 4H), 4.20 (s, 4H, 2 × NCH_2), 5.16 (s, 2H), 6.87 (d, $J = 8.6$ Hz, 1H, H-3′), 6.98 (dd, $J = 8.6$, 2.4 Hz, 1H, H-4′), 7.13 (d, $J = 2.4$ Hz, 1H, H-6′), 8.12 (bs, NH); ^{13}C NMR (125.7 MHz, $CDCl_3$) 14.2 (2 × OCH_2CH_3), 28.0 (3 × Me), 31.1 (NCH_3), 42.8 ($ClCH_2$), 53.7 (2 × NCH_2), 60.6 (2 × OCH_2CH_3), 70.0 (OCH_2), 83.89 (CMe_3), 107.0 (C-6′), 113.5 (C-4′), 120.0 (C-3′), 131.5 (C-5′), 136.6 (C-2′), 150.1 (C-1′), 152.8 (N*C*OO), 163.5 ($ClCH_2CO$), 171.0 (CO_2R), 171.3 (2 × CO_2R); MS (ES^+) *m*/*z* 566 (MNa^+), 1109 (M_2Na^+).

5-(β-Chloroethanamido)-2-[*N*,*N*-bis(carboxymethyl)amino]-1-(carboxymethoxy)benzene (L^3)

To a solution of **17** (0.125 g, 0.22 mmol) in THF (2.5 mL), aqueous lithium hydroxide solution (1 mL, 1 M) was added. The reaction mixture was stirred at ambient temperature under argon overnight. Reaction was monitored by the disappearance of the starting material (silica TLC plate; ethyl acetate–petroleum ether 1 : 1, $R_f = 0.6$) and then THF was removed under reduced pressure. The remaining concentrated aqueous crude was neutralised to pH = 7 and loaded on top of an H^+ cation-exchange column. The product L^3 was collected by elution with diluted aqueous ammonia solution (1%); removal of the solvent by lyophilisation gave L^3 (identified from UV absorption of the hydrolysed compound **17** spotted on silica TLC plate as a dark solid (0.053 g, 1.4 mmol, 60%), mp > 260 °C (dec.).

^{1}H NMR (300 MHz, D_2O, pD = 10) 3.45 (s, 4H, 2 × NCH_2), 3.89 (s, 2H, $ClCH_2CO$), 4.60 (s, 2H, OCH_2CO), 6.26 (d, $J = 2.1$ Hz, 1H, H-6′), 6.30 (dd, $J = 8.4$, 2.1 Hz, 1H, H-4′), 7.05 (d, $J = 8.4$ Hz, 1H, H-3′); ^{13}C NMR (75.3 MHz, D_2O, pD = 10) 43.9 ($ClCH_2CO$), 61.2 (2 × NCH_2), 66.9 (OCH_2CO), 101.1 (C-3′), 109.6 (C-4′), 125.8 (C-6′), 131.9 (C-1′), 145.4 (C-5′), 152.8 (C-2′), 175.1 (NH*C*O), 176.6 and 180.6 (3 × CO); MS (ES^-) *m*/*z* 297 ($M - ClCH_2CO^+$), 335 ($M - CO_2$). Anal.: found (as tris(ammonium) salt monohydrate): C, 41.4; H, 6.05; N, 15.3%; $C_{14}H_{26}N_5O_9Cl$ requires: C, 41.00; H, 5.91; N, 15.8%.

1-{3-(*N*-Methyl-*N*-*tert*-butoxycarbonylcarbamoylmethoxy)-4-[*N*,*N*-bis(ethoxycarbonylmethyl)amino]phenylcarbamoyl-methyl}-1,4,7,10-tetraazacyclododecane (18)

A solution of 1,4,7,10-tetraazacyclododecane (0.24 g, 1.39 mmol), **17** (0.3 g, 0.55 mmol) and potassium iodide (0.12 g, 0.74 mmol) in dry THF (60 mL) was heated (40 °C) under argon overnight. The reaction was monitored by TLC (silica gel, ethyl acetate 100%) (disappearance of $R_f = 0.85$). The insoluble precipitate was filtered and solvent was removed under reduced pressure. The semi-solid crude was dissolved in chloroform (20 mL), washed with water, dried over K_2CO_3, filtered and solvent removed under reduced pressure. An oily gum was obtained (0.27 g, 0.53 mmol, 73%) which was used without further purification.

^{1}H NMR (300 MHz, $CDCl_3$) 1.21 (t, 6H), 1.54 (s, 9H, O′Bu), 2.69 (bs, 12H, 3 × NCH_2CH_2N), 2.72 (bs, 4H, CH_2NCH_2), 3.16 (s, 3H, NCH_3), 3.22 (s, 2H, NCH_2CON), 4.14 (q, 4H), 4.17 (s, 4H, 2 × NCH_2), 5.17 (s, 2H, OCH_2CO), 6.88 (d, $J = 8.7$ Hz, 1H, H-3′), 7.22 (dd, $J = 8.7$, 2.4 Hz, 1H, H-3′), 7.35 (d, $J = 2.4$ Hz, 1H, H-6′), 9.96 (bs, NH); ^{13}C NMR (75.3 MHz, $CDCl_3$) 14.5 (OEt), 28.3 (O′Bu), 31 (N-Me), 45.8 and 48.1 (8 × NCH_2), 53.9 (NCH_2CO and 5 × NCH_2CO_2), 61.0 (OEt), 72.7 (OCH_2-CO), 84.0 (O′Bu), 107 (C-6′), 111 (C-3′), 120.5 (C-4′), 129 (C-2′), 145.4 (C-5′), 152.8 (C-1′), 153.1 (NH*C*O), 172 (5 × CO).

1,4,7-Tris(*tert*-butoxycarbonylmethyl)-10-{3-(*N*-methyl-*N*-*tert*-butoxycarbonylcarbamoylmethoxy)-4-[*N*,*N*-bis(ethoxycarbonyl-methyl)amino]phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclo-dodecane (19)

Method A. To a solution of 1,4,7-tris(*tert*-butoxycarbonyl-methyl)-1,4,7,10-tetraazacyclododecane (0.16 g, 0.32 mmol) in MeCN (45 mL), dry Cs_2CO_3 (0.24 g, 0.74 mmol) and a solution of 5-(β-chloroethanamido)-2-[*N*,*N*-bis(ethoxycarbonylmethyl)-amino]-1-(*N*-methyl-*N*-*tert*-butoxycarbonylcarbamoylmethoxy)-benzene **17** (0.2 g, 0.37 mmol) in MeCN (15 mL) were added. The reaction mixture was heated to 40 °C for 72 h under argon and reaction monitored by TLC (silica gel, CH_2Cl_2–THF 3 : 7). After cooling, insoluble material was filtered off and solvent removed under reduced pressure; the resultant gum was washed with a water–CH_2Cl_2 mixture (30 mL; 50 : 50 v/v). The organic layer was separated, dried over $CaCl_2$, filtered and concentrated to dryness under reduced pressure. The resulting crude was purified by column chromatography on neutral alumina eluting with CH_2Cl_2–MeOH 95 : 5, to give **19** as an oil (0.14 g, 0.13 mmol, 35%).

^{1}H NMR (300 MHz, $CDCl_3$) 1.18 (t, 6H), 1.46 (s, 27H), 1.48 (s, 9H), 2.75–3.4 (m, 16H, 3 × NCH_2CH_2N), 3.10 (s, 3H, NCH_3), 3.45 (s, 2H, NCH_2CON), 3.64 (3 × s, 6H, 3 × NCH_2-$CO_2{}^t$Bu), 4.09 (q, 4H), 4.45 (s, 4H, 2 × NCH_2), 5.16 (s, 2H, OCH_2CO), 6.75 (d, $J = 7.0$ Hz, 1H, H-3′), 7.22 (t, $J = 6.0$ Hz, 1H, H-4′), 7.32 (m, 1H, H-6′), 9.10 (bs, NH); MS (ES^+) *m*/*z* 1022 (MH^+), 1044 (MNa^+).

Method B. Compound **19** was also prepared by alkylation of **18** with *tert*-butyl bromoacetate in acetonitrile in the presence of dry Cs_2CO_3. The method followed was that used for the conversion of **6** to **7** and the product was obtained in 31% overall, 0.03 mmol, 25% yield, giving the same physicochemical data to that obtained in Method A, above.

General method for the synthesis of lanthanide(III) complexes [EuL^4] and [TbL^4]

The ester **19** (0.24 g, 0.23 mmol) was dissolved in 80% solution of trifluoroacetic acid (TFA) in CH_2Cl_2 (5 mL) and the solution stirred at room temperature under argon overnight. After removal of solvent under reduced pressure, the remaining crude was washed with CH_2Cl_2 (2 × 10 mL) followed by diethyl ether (2 × 10 mL). The resulting off-white gum was dissolved in water, frozen and water removed by lyophilisation to give **20** as a dark solid (0.2 g, 0.23 mmol), which was used without further purification.

A solution of either europium(III) acetate trihydrate (80 mg, 200 μmol) or terbium nitrate pentahydrate (130 mg, 300 μmol) in water (10 mL) was added to 1,4,7-tris(carboxymethyl)-10-{3-(*N*-methyl-*N*-*tert*-butoxycarbonylcarbamoylmethoxy)-4-[*N*,*N*-bis(ethoxycarbonylmethyl)amino]phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecane **20** (1 equivalent). The pH was raised to 5.5 by addition of sodium hydroxide solution at which point the ligand slowly dissolved. The solution was heated at 90 °C for 48 h. The lanthanide complex formation was monitored by electrospray mass spectrometry (ES^-) which revealed *m*/*z* values with the correct isotope patterns for M^- and $(M - H)^-$ (M = EuL^4 or TbL^4). The reaction mixture was cooled and aqueous lithium hydroxide solution (1 mL, 1 M) was added to allow hydrolysis of the remaining ester groups under argon at room temperature overnight. Following neutralisation of the reaction solution to pH 7, the lanthanide complexes were purified by ion-exchange column chromatography using a Dowex 50W cation resin in the strong acid form, eluting with dilute aqueous ammonium (1%) solution. The solvent of the appropriate fraction (determined from UV absorption of the hydrolysed lanthanide complex spotted on silica TLC plate) was frozen and water removed by lyophilisation to give the complexes [EuL^4] or [TbL^4] as pale yellow–brown solids in 75–80% yield.

1,4,7-Tris(carboxylatomethyl)-10-{3-(carboxymethoxy)-4-[*N*,*N*-bis(carboxymethyl)amino]phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecaneeuropium(III) (EuL^4). MS (ES^-) *m*/*z* 838/841 (M *as mono lithium salt* $- H)^+$. Anal.: found (as tris(ammonium) salt trihdyrate): C 35.9, H 6.23, N 13.3%. $C_{28}H_{52}N_9O_{17}Eu$ requires: C 35.8, H 5.58, N 13.5%.

1,4,7-Tris(carboxylatomethyl)-10-{3-(carboxymethoxy)-4-[*N*,*N*-bis(carboxymethyl)amino]phenylcarbamoylmethyl}-1,4,7,10-tetraazacyclododecaneterbium(III) (TbL^4). MS (ES^-) *m*/*z* 397 ($M - CO_2H - H$), 795 ($M - CO_2H$); HRMS: $C_{27}H_{36}N_6O_{12}Tb$ requires 795.1645 ($M - CO_2H$). Found: 795.1641.

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