An Esterase-Activated Magnetic Resonance Contrast Agent

Marco Giardiello,[a] Mark P. Lowe*[a] and Mauro Botta[b]

[a] Department of Chemistry, University Road, University of Leicester, Leicester, LE1 7RH, UK. E-mail: mplowe@le.ac.uk

[b] Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale "Amedeo Avogadro", Via Bellini 25/G, I-15100, Alessandria, Italy.

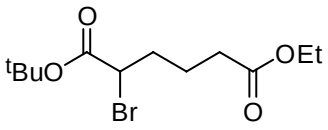
Supporting Information

Experimental

General procedures. NMR spectra were recorded on Brüker ARX250 and DPX300 instruments using standard Brüker software. Low resolution ESMS were recorded on an open access Micromass Quatro (LC) spectrometer. FABMS were recorded on a Kratos Concept spectrometer (xenon gas, 7 kV) with nitrobenzyl alcohol as matrix. Accurate mass ESMS were recorded on a Finnigan MAT 900 XLT high resolution double focussing mass spectrometer with tandem ion trap (polyethylenimine reference compound).

Materials. Reagents are commercially available and were used without further purification. [Eu.**4**] and [Gd.**4**] were prepared according to literature procedures.[1] Porcine liver esterase was purchased from Sigma-Aldrich (E3019) as a lyophilised powder, minimum 15 units/mg solid.

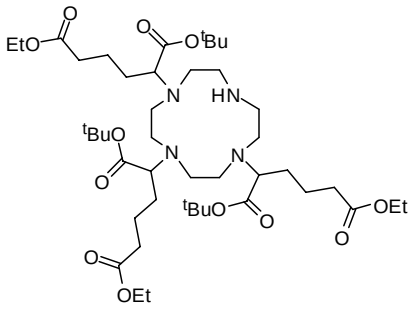
2-Bromoadipic acid 1-tert-butyl ester 6-ethyl ester



Adipic acid monoethyl ester (10.55 g, 60.56 mmol) was added to thionyl chloride (27.25 g, 0.23 mol) and the mixture was heated at reflux for 24 hours. Bromine (9.98 g, 62.44 mmol) was added drop wise over 3 hours. Gentle reflux was maintained throughout and the solution was left at 75^{o}C for 48 hours. Thionyl chloride was then removed under reduced pressure and the residue was dissolved in dry ether (50 ml) and evaporated three times. The residue was then re-dissolved in dry ether (50 ml) and slowly added to dry *tert*-butanol (6.97 g, 94.00) and triethylamine (8.71 g, 86.10 mmol) under nitrogen and at room temperature. The solution was then left to stir for 24 hours. The mixture was then poured into water and extracted with (3 x 100 ml) ether. The combined

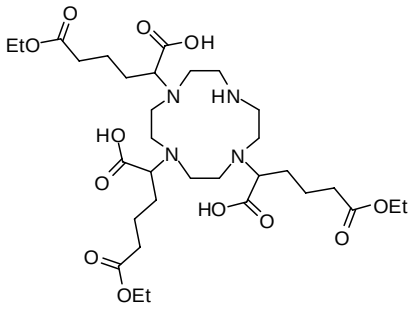
extracts were washed with water, dilute sodium carbonate solution and again with water. After drying over sodium sulfate the ether was removed under reduced pressure. The pure product was collected following distillation under reduced pressure to give the product (7.91 g, 44%) as a pale yellow oil. ν_{max} cm^{-1} 2954 (CH), 1732 (C=O), 1436, 1364, 1258, 1195, 1152 (C-O); δ_H (300 MHz; $CDCl_3$) 1.3 (3 H, t, $^3J_{HH}$ 7.0 Hz, ~$CO_2CH_2CH_3$), 1.5 (9 H, s, ^{t}Bu), 1.7 (2 H, m, ~$CH_2CH_2CH_2$~), 2.0 (2 H, m, ~$CHBrCH_2CH_2$~), 2.4 (2 H, t, $^3J_{HH}$ 7.3 Hz, ~$CH_2CH_2CO_2CH_3$), 4.1 (3 H, m, ~CBr*H* and ~$CO_2CH_2CH_3$); δ_C (75 MHz; $CDCl_3$) 13.2 (~$CO_2CH_2CH_3$), 21.7 (~$CH_2CH_2CH_2$~), 26.7 (~$CO_2C(CH_3)_3$), 32.4 (~$CH_2CH_2CO_2CH_2CH_3$), 33.1 (~$CHBrCH_2CH_2$~), 45.2 (~*C*BrH), 59.4 (~$CO_2CH_2CH_3$), 81.4 (~$CO_2C(CH_3)_3$), 167.6 (carbonyl ~$CHBrCO_2C(CH_3)_3$), 171.8 (carbonyl ~$CH_2CO_2CH_2CH_3$); *m/z* (FAB+) 309 $[M+H]^+$.

1,4,7-tris[(4'-ethoxycarbonyl)-1'-tert-butoxycarbonylbutyl]-1,4,7,10-tetraazacyclododecane **1**



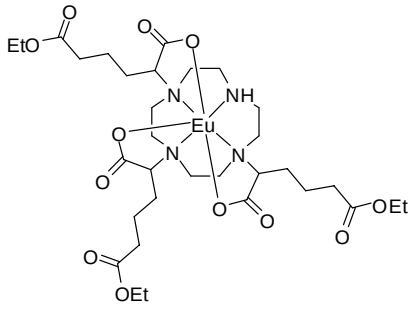
1,4,7,10-tetracyclododecane (0.18 g, 1.05 mmol) and 2-Bromoadipic acid 1-tert-butyl ester 6-ethyl ester (1.01 g, 3.27 mmol) in the presence of K_2CO_3 (0.15 g, 1.09 mmol) were heated in dry MeCN (10 ml) at 60 ^{o}C under nitrogen for 5 days. The solution was then filtered through celite and the solvent was removed under reduced pressure. The product was purified by column chromatography with solvent gradient elution from 100% DCM to 20% THF, 80% DCM. R_f = 0.58 (70% THF, 30% DCM) to give **1** (0.44 mg, 48%) a yellow oil. ν_{max} cm^{-1} 3642, 2964, 1727 (C=O), 1146; δ_H (300 MHz; $CDCl_3$) 1.2 (9 H, t, $^3J_{HH}$ 7.3 Hz, ~$CO_2CH_2CH_3$), 1.4 (27 H, 3 x s, ^{t}Bu), 1.5 – 1.8 (12 H, br, ~$CHNCH_2CH_2$~ and ~$CH_2CH_2CH_2$~), 2.3 (6H, br, ~$CH_2CH_2CO_2CH_2CH_3$), 2.4 –3.3 (16 H, br, ring H), 3.7 (3H, br, ~CN*H*), 4.0 (6 H, q, $^3J_{HH}$ 7.0 Hz, ~$CO_2CH_2CH_3$); δ_C (75 MHz; $CDCl_3$) 13.2 (~$CO_2CH_2CH_3$), 21.3 (~$CH_2CH_2CH_2$~), 24.6 (~$CHNCH_2CH_2$~), 27.2 (~$CO_2C(CH_3)_3$), 29.3 (~$CO_2C(CH_3)_3$), 32.7 (~$CH_2CH_2CO_2CH_2CH_3$), 44.8, 45.5, 47.5, 49.0, 50.2, 50.6, 51.1 (ring CH_2), 59.3 (~$CO_2CH_2CH_3$), 66.9 (~*C*NH), 81.0 (~$CO_2C(CH_3)_3$), 170.5 (carbonyl ~$CHNCO_2C(CH_3)_3$), 172.0 (carbonyl ~$CH_2CO_2CH_2CH_3$); *m/z* (HR-ESMS+) $[M+H]^+$ calcd for $C_{44}H_{81}N_4O_{12}$; 857.5846, found; 857.5846.

1,4,7-tris[(4'-(ethoxycarbonyl)-1'-carboxybutyl]-1,4,7,10-tetraazacyclododecane **2**



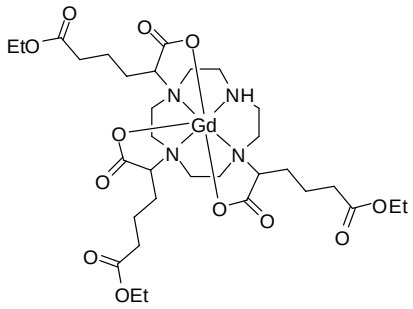
1 (0.451 g, 0.54 mmol) was dissolved in dichloromethane (2.5 ml) and TFA (2.5 ml) was carefully added. The mixture was stirred at room temperature for 3 hours under nitrogen. The solvent was then removed under reduced pressure. Two portions of dichloromethane (5 ml) was added and removed under reduced pressure, followed by 2 portions of ether (5 ml), which was also removed under reduced pressure to yield **2** (0.140 g, 38%) as a white hygroscopic powder. ν_{max} cm^{-1} 2930, 1719 (C=O), 1674, 1375, 1177, 1130; δ_H (300 MHz; CD_3OD) 1.1 (9H, t, $^3J_{HH}$ 7.0 Hz, ~$CO_2CH_2CH_3$), 1.5 – 2.0 (12 H, br, ~$CHNCH_2CH_2$~ and ~$CH_2CH_2CH_2$~), 2.3 – 2.4 (6 H, br, ~$CH_2CH_2CO_2H$), 2.9 – 3.7 (16 H, br, ring H), 3.8 (6 H, br q, $^3J_{HH}$ 6.5 Hz ~$CO_2CH_2CH_3$); *m/z* (FAB+) 689 $[M+H]^+$.

Europium(III) 1,4,7-tris[(4'-(ethoxycarbonyl)-1'-carboxybutyl]-1,4,7,10-tetraazacyclododecane, [Eu.**2**]



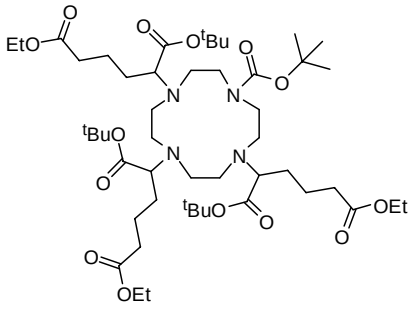
2 (49.9 mg, 72.4 μmol) and $EuCl_3.6H_2O$ (26.5 mg, 72.4 μmol) were dissolved in water (10 ml). The solution was adjusted to pH 5.5 using NaOH and was heated at 90°C for 24 hours. After the solution was cooled the pH was raised to 10 and the solution was filtered through a celite plug to remove any $Eu(OH)_3$. The solution was then lowered to pH 5.5 and added to a suspension of Dowex MAC-3 weak acid-cation exchange resin and stirred for 10 minutes. The solution was then decanted from the resin and lyophilised to give [Eu.**2**] **(**59.2 mg, 97%) as a white hygroscopic powder. ν_{max} cm^{-1} 3244, 1675, 1591, (C=O), 1423, 1198, 1137; *m/z* (HR-ESMS+) $[M+H]^+$ calcd for $C_{32}H_{54}N_4O_{12}Eu$; 837.2931, found; 837.2931 (^{151}Eu).

Gadolinium(III) 1,4,7-tris[(4'-(ethoxycarbonyl)-1'-carboxybutyl]-1,4,7,10-tetraazacyclododecane, [Gd.**2**]



2 (68.5 mg, 99.4 μmol) and $GdCl_3.6H_2O$ (37.4 mg, 10.1 μmol) were dissolved in water (10 ml). The solution was adjusted to pH 5.5 using NaOH and was heated at 90°C for 24 hours. After the solution was cooled the pH was raised to 10 and the solution was filtered through a celite plug to remove any $Gd(OH)_3$. The solution was then lowered to pH 5.5 and added to a suspension of Dowex MAC-3 weak acid-cation exchange resin and stirred for 10 minutes. The solution was then decanted from the resin and lyophilised to give [Gd.**2**] (65.4 mg, 78%) as a white hygroscopic powder. ν_{max} cm^{-1} 3328, 1674, 1579, (C=O), 1425, 1197, 1139; *m/z* (HR-ESMS+) $[M+H]^+$ calcd for $C_{32}H_{54}N_4O_{12}Gd$; 844.2974, found; 844.2968 (^{158}Gd).

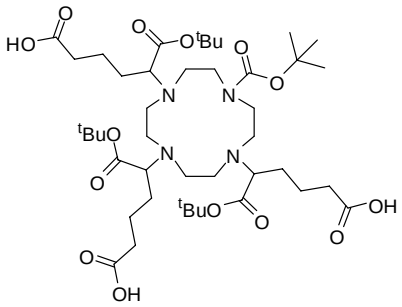
1-tert-butoxycarbonyl-4,7,10-tris[(4'-ethoxycarbonyl)-1'-tert-butoxycarbonylbutyl]-1,4,7,10-tetraazacyclododecane



To a solution of **1** (0.162 g, 0.189 mmol) and triethylamine (0.019 g, 0.189 mmol) in dry DCM (10ml) at 0°C was added di-*tert*-butyl-dicarbonate (0.041, 0.189 mmol) in 10 ml DCM drop wise under nitrogen and was left to stir for 24 hours. The solvent was then removed under reduced pressure and the product was purified by silica column chromatography with solvent gradient elution from 100% DCM to 10% MeOH, 90% DCM. R_f = 0.60 (10% THF, 90% DCM) to give the product (145 mg, 80%) as a yellow oil. ν_{max} cm^{-1} 2976, 2934, 1726 (C=O), 1689 (C=O), 1366, 1143; δ_H (300 MHz; $CDCl_3$) 1.3 (9 H, t, $^3J_{HH}$ 6.7 Hz, ~$CO_2CH_2CH_3$), 1.5 (36 H, 2 x s, ^{t}Bu), 1.5 – 1.9 (12 H, br, ~CHNC$H_2$$CH_2$~ and ~$CH_2CH_2CH_2$~), 2.3 –2.4 (6 H, br, ~$CH_2CH_2CO_2CH_2CH_3$), 2.4 – 3.6 (19 H, br, ring H (16 H) and ~CN*H* (3 H) overlap), 4.1 (6 H, q, $^3J_{HH}$ 7.0 Hz, ~$CO_2CH_2CH_3$); δ_C (75 MHz; $CDCl_3$) 14.2 (~$CO_2OCH_2CH_3$), 22.1 (~$CH_2CH_2CH_2$~), 28.2 (~$CO_2C(CH_3)_3$), 28.4 (~$CO_2C(CH_3)_3$), 29.3 (~CHNCH_2CH_2~), 34.0 (~$CH_2CH_2CO_2CH_2CH_3$), 46.3, 47.2, 47.6, 48.8, 49.5, 50.0, 51.0, 51.4 (ring CH_2), 60.1

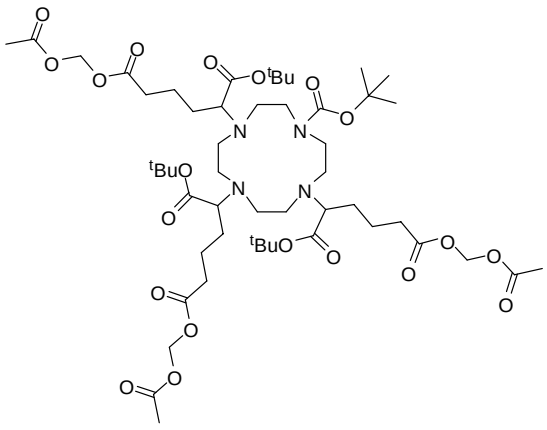
(~$CO_2CH_2CH_3$), 61.7 (~*C*NH), 80.7 (BOC ~$CO_2C(CH_3)_3$), 80.9 (~$CO_2OC(CH_3)_3$), 155.7 (BOC, CO_2), 171.6 (carbonyl ~$CHNCO_2C(CH_3)_3$), 173.1 (carbonyl ~$CH_2CO_2CH_2CH_3$); *m/z* (ESMS+) 957 $[M+H]^+$.

1-tert-butoxycarbonyl-4,7,10-tris[(4'-carboxyl)-1'-tert-butoxycarbonylbutyl]-1,4,7,10-tetraazacyclododecane



The Boc-protected hexa-ester (0.145 g, 0.151 mmol), was dissolved in 10 ml EtOH and 10 ml 1M NaOH and stirred overnight. EtOH was then removed under reduced pressure, the pH of the solution was lowered to 5 and the solution lyophilised. The product was then extracted from the salt residue with 20% dry MeOH/DCM solution which was then removed under reduced pressure to yield a pale oil (0.129 g, 97%). ν_{max} cm^{-1} 2975, 2932, 1719 (C=O), 1367, 1144; δ_H (300 MHz; $CDCl_3$), 1.5 (36 H, 2 x s, ^{t}Bu), 1.5 – 1.9 (18 H, br, ~CHNCH_2CH_2~ and ~CH_2CH_2CH_2~), 2.4 (6 H, br, ~CH_2C$H_2$$CO_2$H), 2.5 – 3.9 (19 H, br, ring H (16 H) and ~CN*H* H (3 H)); *m/z* (ESMS+) 873 $[M+H]^+$.

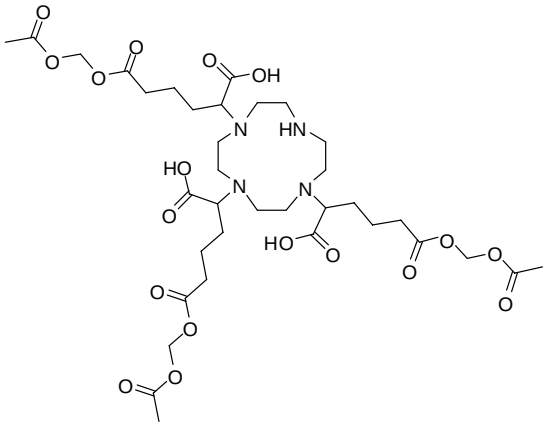
1-tert-butoxycarbonyl-4,7,10-tris[(4'-(acetoxymethoxycarbonyl)-1'-tert-butoxycarbonylbutyl]-1,4,7,10-trtraazacyclododecane



To a solution of the Boc-protected tris-^{t}butylester (0.131 g, 0.106 mmol) in dry DCM was added DIPEA (0.058 g, 0.451 mmol) and bromomethyl acetate (0.072 g, 0.451 mmol) under nitrogen and was left to stir for 24 hours. The solvents were removed under reduced pressure and the product was purified by column chromatography using ethyl acetate as eluent to yield a pale oil (74.9 mg, 45%). R_f = 0.79 (ethyl acetate). ν_{max} cm^{-1} 2975, 1763, 1720, 1688 (C=O), 1367, 1143; δ_H (300 MHz; $CDCl_3$) 1.4 (36 H, s, ^{t}Bu), 1.5 – 1.8 (16 H, br, ~CHNCH_2CH_2~ and ~CH_2CH_2CH_2~), 2.0 (9H, s, ~CO_2CCH_3), 2.2 –2.4 (6 H, br, ~$CH_2CH_2CO_2CH_2$~), 2.5 – 3.7 (19 H, br, ring H

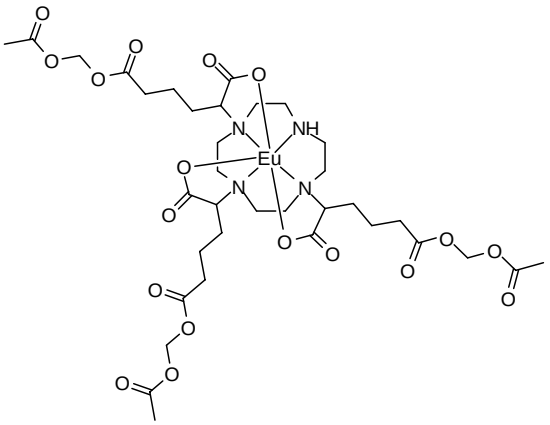
(16 H) and ~CN*H* (3 H)), 5.7 (6H, s, ~OCH_2O~); δ_C (75 MHz; $CDCl_3$) 19.7 (~CO_2CCH_3), 20.7 (~$CH_2CH_2CH_2$~) 27.5 (~$CO_2C(CH_3)_3$), 28.8 (~CHNCH_2CH_2~), 34.0 (~$CH_2CH_2CO_2CH_2$~), 45.4, 46.3, 46.9, 47.4, 49.1, 50.0 (ring CH_2), 60.8 (~*C*NH), 78.1 (~OCH_2O~), 80.1 (~$CO_2C(CH_3)_3$), 154.7 (BOC CO_2), 168.6 (carbonyl ~CHNC$O_2C(CH_3)_3$), 170.9 (carbonyl ~CO_2CCH_3), 171.38 (carbonyl ~CHNCO_2CH_2~); *m/z* (ESMS+) 1089 $[M+H]^+$.

1,4,7-tris[(4'-(acetoxymethoxycarbonyl)-1'-carboxybutyl]-1,4,7,10-tetraazacyclododecane **3**



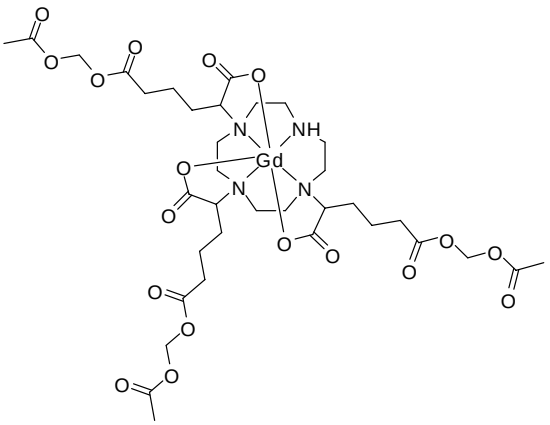
The boc-protected pro-ligand (0.075 g, 0.069 mmol) was dissolved in dichloromethane (2.5ml) and TFA (2.5 ml) was carefully added. The mixture was stirred at room temperature for 3 hours under nitrogen. The solvent was then removed under reduced pressure. Two portions of dichloromethane (5ml) was added and removed under reduced pressure, followed by 2 portions of ether (5ml), which was also removed under reduced pressure to yield **3** (0.057 g, 99%) as a white hygroscopic powder. ν_{max} cm^{-1} 2962, 1755 (C=O), 1668, 1181, 1138, 977; δ_H (300 MHz; CD_3OD); 1.6 – 1.9 (12H, br, ~CHNCH_2CH_2~ and ~$CH_2CH_2CH_2$~), 2.0 (9H, s, ~CO_2CCH_3), 2.3 – 2.5 (6H, br, ~$CH_2CH_2CO_2H$), 2.6 – 3.6 (19 H, br, ring H (16 H) and ~CN*H* (3 H)), 5.7 (6H, s, ~OCH_2O~); *m/z* (ESMS+) 821 $[M+H]^+$.

Europium(III) 1,4,7-tris[(4'-(acetoxymethoxycarbonyl)-1'-carboxybutyl]-1,4,7,10-tetraazacyclododecane, [**Eu.**3**]**



3 (39.1 mg, 47.6 μmol) and $EuCl_3.6H_2O$ (17.5 mg, 47.6 μmol) were dissolved in MeOH (10 ml). The solution was heated at 55^{o}C for 24 hours. The solvent was removed under vacuum and the residue taken up in water and the pH adjusted to 5.5. This was added to a suspension of Dowex MAC-3 weak acid-cation exchange resin and stirred for 10 minutes to remove any unreacted Eu(III). The solution was then decanted from the resin and lyophilised to give [Eu.**3**] (41.8 mg, 90%) as a white hygroscopic powder. ν_{max} cm^{-1} 2969, 1726, (C=O), 1619, 1419, 1368, 1202, 1142, 981; *m/z* (HR-ESMS+) $[M+H]^+$ calcd for $C_{35}H_{54}N_4O_{18}Eu$; 971.2640, found; 971.2630 (^{153}Eu).

Gadolinium(III) 1,4,7-tris[(4'-(acetoxymethoxycarbonyl)-1'-carboxybutyl]-1,4,7,10-tetraazacyclododecane, [Gd.**3**]



3 (28.2 mg, 34.4 μmol) and $GdCl_3.6H_2O$ (12.8 mg, 34.4 μmol) were dissolved in MeOH (10 ml). The solution was heated at 55^{o}C for 24 hours. The solvent was removed under vacuum and the residue taken up in water and the pH adjusted to 5.5. This was added to a suspension of Dowex MAC-3 weak acid-cation exchange resin and stirred for 10 minutes to remove any unreacted Gd(III). The solution was then decanted from the resin and lyophilised to give [Gd.**3**] (21.6 mg, 65%) as a white hygroscopic powder. ν_{max} cm^{-1} 2953, 1734 (C=O), 1619,

1416, 1368, 1202, 1144, 982; *m/z* (HR-ESMS+) $[M+H]^+$ calcd for $C_{35}H_{54}N_4O_{18}Gd$; 976.2669, found; 976.2665 (^{158}Gd).

Europium Lifetime Measurements

Excited state lifetime measurements were made on a Jobin Yvon Horiba Fluoromax-P (using DataMax for Windows v2.2). Lifetimes were measured by excitation (395 nm) of the sample with a short 40 ms pulse of light (500 pulses per point) followed by monitoring the integrated intensity of light (594 nm) emitted during a fixed gate time of 0.1 ms, at a delay time later. Delay times were set at 0.1 ms intervals, covering 4 or more lifetimes. Excitation and emission slits were set to 15 and 5 nm bandpass respectively. The obtained decay curves were fitted to a simple mono-exponential first-order decay curve using Microsoft Excel.

q-values were calculated using the following equation,[2]

$$q = 1.2[k_{H_2O} - k_{D_2O} - 0.25]$$

For [Eu.**2**] and [Eu.**3**] in the presence of 30 mM $NaHCO_3$ at pH 7.4 an 'apparent' *q* is quoted. The data for this gives an excellent fit for a single exponential decay with *q* calculated from this fit as 1.2; however, there are clearly two species in solution (a q =0 and q =2). Fitting to bi-exponential decay gives the same result: for e.g. [Eu.**2**] in the presences of carbonate at pH 7.4; the fit gives a species of k = 2.29 ms^{-1} and another of k = 3.67 ms^{-1} in a ratio of 40:60, i.e. a q = 0 and a q = 2 species with 40% q = 2 (the same ratio obtained from fitting to a single exponential).

^{1}H Relaxation Data

The observed longitudinal water proton relaxation times (T_{1obs}) were measured on a Stelar Spinmaster spectrometer (Stelar, Mede (PV) Italy) operating at 20 MHz, by means of the standard inversion-recovery technique (16 experiments, 2 scans). A typical 90° pulse width was 3.5 μs and the reproducibility of the T_{1obs} data was ±0.5%. Complex concentrations were estimated *via* mineralization with nitric acid.

Esterase Hydrolysis Studies

Four aqueous solutions were prepared at pH 7.4 (0.1 M NaCl): solutions contained either 0.22 mM Gd complex**;** 0.22 mM Gd complex + 10 mM $NaHCO_3$; 0.22 mM Gd complex + 100 units porcine liver esterase; 0.22 mM Gd complex + 10 mM $NaHCO_3$ + 100 units porcine liver esterase. Relaxivities (via T_{1obs} measurements) were recorded prior to (to establish that no ester hydrolysis occurred in the absence of esterase) and following incubation for ~ 2 hours at 37°C.

pH vs. Relaxivity Titrations

pH measurements were recorded using a Jenway 3510 pH meter with a BDH probe, model 309-1025-02 calibrated at pH 4, 7 and 10. The pH titration was carried out in a background of constant ionic strength (I = 0.1

NaCl, 298 K). Aqueous solutions of 1 mM Gd complexes and 30 mM $NaHCO_3$ were raised to pH 10 by addition of 1 M NaOH and titrated to acid pH by addition of small aliquots of 1 M or 0.1 M HCl.

References

1 D. Messeri, M. P. Lowe, D. Parker and M. Botta, *Chem. Commun.*, 2001, 2742.

2 A. Beeby, I. M. Clarkson, R. S. Dickins, S. Faulkner, D. Parker, L. Royle, A. S. de Sousa, J. A. G. Williams and M. Woods, *J. Chem. Soc., Perkin Trans. 2*, **1999**, 493.