ESI

A Ratiometric and Non-Enzymatic Luminescence Assay for Uric Acid: Differential Quenching of Lanthanide Excited States by Anti-Oxidants

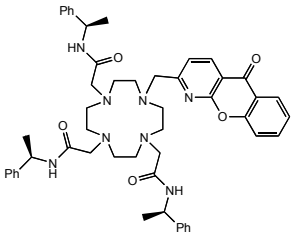
Robert A. Poole, Filip Kielar, Siobhan L. Richardson, Philip A. Stenson and David Parker*

A. Examples of Ligand and Complex Synthesis
B. Details of Urate Analysis in Urine Samples

A. Examples of Ligand and Complex Synthesis

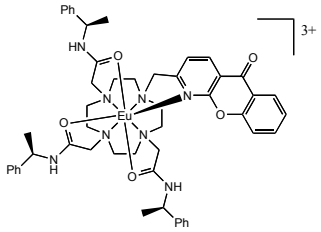
For details of the synthesis of other ligand precursors and intermediates mentioned, see reference 10.

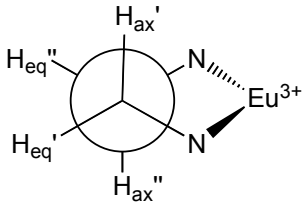
1-(2-Methyl-1-azaxanthone)-4,7,10-tris[(S)-1-(1-phenyl)ethylcarbamoylmethyl]-1,4,7,10-tetraazacyclododecane, 5



2-Methyl-1-azaxanthone-1,4,7,10-tetraazacyclododecane (130 mg, 0.34 mmol), K_2CO_3 (236 mg, 1.70 mmol) and KI (4 mg) were dissolved in acetonitrile (10 ml) and the pale orange solution heated to 60ºC under argon. (S)-N-(1-phenylethyl)chloroacetamide (269 mg, 1.36 mmol, 4 equivalents) in DCM (10 ml) was added, and the reaction mixture refluxed at 60ºC for 60 hours. On cooling the solution was filtered and washed with DCM. The solution was then washed with water and dried (K_2CO_3). Removal of the solvent under reduced pressure yielded a crude brown oil which was purified via column chromatography (alumina, DCM → 0.4% MeOH:DCM) yielding the *title compound* as a pale orange crystalline solid (208 mg, 70%), m.p. 112-113ºC. δ_H ($CDCl_3$) 1.45 (9H, m, 3 x CH_3), 2.91 (22H, m br, 8 x cyclen CH_2 and 3 x NCH_2CO), 3.79 (2H, s br, CH_2–AzaH), 4.74 (1H, m br, PhCH), 5.00 (2H, m br, 2 x PhCH), 7.20 (16H, m, 3 x Ph and H^7), 7.42 (1H, m, H^9), 7.60 (1H, m, H^3), 7.78 (1H, m, H^8), 8.31 (1H, m, H^6), 8.56 (1H, m, H^4). *m/z* ($ESMS^+$) 453 (M + Ca, 20%), 464 (M + K + Na, 40%), 866 (M + H, 40%), 888 (M + Na, 100%). HRMS (ES^+), found: 887.4582 (M + Na); $C_{51}H_{60}N_8O_5Na$ requires 887.4579

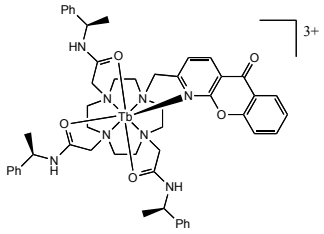
$[Eu.5(OH_2)]^{3+}$





1-(2-Methyl-1-azaxanthone)-4,7,10-tris[(S)-1-(1-phenyl)ethylcarbamoylmethyl]-1,4,7,10-tetraazacyclododecane (30 mg, 0.035 mmol) and $Eu(OTf)_3$ (21 mg, 0.035 mmol) were dissolved in CH_3CN (5 ml). The solution was stirred at 80ºC for 60 hours under argon. The solvent was then removed under reduced pressure, and the brown oil redissolved in a minimum of acetonitrile and added dropwise to diethyl ether (60 ml) yielding a fine precipitate. The product was centrifuged and the process repeated. An ion exchange was then performed on a pre-washed DOWEX resin in the chloride form. The complex was obtained as a colourless solid (24 mg, 69 %), m.p. > 250ºC. δ_H (CD_3OD) partial assignment: -19.96 (1H, s, *NCH_2CO*), -18.05 (1H, s, H^2eq'), -17.72 (1H, s, *NCH_2CO*), -15.13 (2H, s, *2 x NCH_2CO*), -12.97 (1H, s, H^4eq'), -11.80 (1H, s, *H^2eq"*), -10.95 (1H, s, H^4ax"), -10.55 (1H, s, Heq), -10.28 (1H, s, H^1ax"), -9.88 (1H, s, *NCH_2CO*), -8.20 (1H, s, H^4eq"), -6.06 (1H, s, *NCH_2CO*), -5.03 (1H, s, H^3eq"), -4.85 (1H, s, H^2ax"), -4.85 (1H, s, H^1eq"), -2.71 (3H, d, CH_3), -2.67 (1H, s, H^3ax"), -1.92 (6H, d, 2 x CH_3), -0.41 (1H, s, H^3eq'), 7.99 (1H, s, H^1eq'), 5.79-11.86 (26H, AzaH, CH_2-AzaH, 3 x PhCH, 3 x Ph), 13.77 (1H, s, H^4ax'), 20.58 (1H, s, H^3ax'), 21.62 (1H, s, H^2ax'), 31.15 (1H, s, H^1ax'). *m/z* ($ESMS^+$) 517 (M^{3+} + F^-, 100%). HRMS (ES^+), found: 517.1926 (M^{3+} + F^-); $C_{51}H_{60}N_8O_5EuF$ requires 517.1929; λ_{ex} (H_2O): 335 nm; $\tau(H_2O)$: 0.54 ms; $\tau(D_2O)$: 1.73ms; ϕ_{H2O}: 0.08

[Tb.5(OH$_2$)]$^{3+}$



1-(2-Methyl-1-azaxanthone)-4,7,10-tris[(S)-1-(1-phenyl)ethylcarbamoylmethyl]-1,4,7,10-tetraazacyclododecane (50 mg, 0.058 mmol) and Tb(OTf)$_3$ (35 mg, 0.058 mmol) were dissolved in CH_3CN (5 ml). The solution was stirred at 80ºC for 60 hours under argon. The solvent was then removed under reduced pressure, and the brown oil re-dissolved in a minimum of acetonitrile and added dropwise to diethyl ether (60 ml) yielding a fine precipitate. The product was centrifuged and the process repeated. An ion exchange was performed on a pre-washed DOWEX resin in the chloride form. The complex was obtained as a pale yellow solid (35 mg, 59 %), m.p. > 250ºC. *m/z* (ESMS$^+$) 521 (M^{3+} + F^-, 100%). HRMS (ES$^+$), found: 521.1949 (M^{3+} + F^-); $C_{51}H_{60}N_8O_5TbF$ requires 521.1957; λ_{ex} (H_2O): 335 nm; τ (H_2O): 1.65 ms; τ(D_2O): 2.89ms; ϕ_{H2O}: 0.37 .

Synthesis of 2 and its Tb/Eu complexes

Diethyl 7-carboxaldehyde-dipyrido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate

Selenium dioxide (480 mg, 4.33 mmol) was added to the solution of diethyl 7-methyldipyrido[3,2-a:2',3'-c]quinoxaline-2,3-dicarboxylate (810 mg, 2.07 mmol) in dioxane (170 ml). The mixture was boiled under reflux for 4h and was allowed to cool down afterwards. The reaction mixture was filtered through a Celite plug. The solvent was removed under reduced pressure to give the crude product, which was used without further purification. ^{1}H NMR (200 NMR, $CDCl3_3$): δ 1.52 (6H, t, J = 7.2, $OCH_2C\underline{H}_3$), 4.60 (4H, q, J = 7.2, OCH_2), 7.92 (1H, dd, J = 8.6, 4.6, H11), 8.46 (1H, d, J = 8.2, H5), 9.43 (1H, dd, J = 4.4, 1.8, H10), 9.61 (1H, dd, J = 8.2, 1.8, H12), 9.74 (1H, dd, J = 8.4, 0.8, H6), 10.60 (1H, d, J = 1.0, COH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.2 (CH_3), 63.3 (CH_2), 121.7 (C5), 125.0 (C11), 126.8 (q Ar), 129.2 (q Ar), 134.7 (C12), 136.0 (C6), 139.5 (q Ar), 141.2 (q Ar), 144.5 (q Ar), 145.3 (q Ar), 147.8 (q Ar), 148.4 (q Ar), 153.9 (C10), 154.8 (C7), 164.6 (CO ester), 164.7 (CO ester), 193.7 (CO aldehyde).

Diethyl 7-(hydroxymethyl)dipyrido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate

Sodium cyanoborohydride (130 mg, 2.07) was added to a solution of diethyl 7-carboxaldehyde-dipyrido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate (840 mg, 2.07 mmol) $CHCl_3$-EtOH 7 : 1 (105 ml). The reaction mixture was boiled under reflux for 4h. The reaction mixture was poured into concentrated Na_2CO_3 (150 ml). The organic phase was separated and the water layer was extracted with $CHCl_3$ (4x120 ml). The organic layer was dried over K_2CO_3. The solvent was removed under reduced pressure to give the crude product, which was obtained by recrystallisation from $CHCl_3$/Hexane as pale yellow-green solid (400 mg, 0.98 mmol, 48%). ^{1}H NMR (300 MHz, CDCl3): δ 1.51 (6 H, t, J = 7.2, $OCH_2C\underline{H}_3$), 4.59 (4H, q, J = 7.2, OCH_2), 5.24 (2H, s, CH_2OH), 7.83 (1H, dd, J = 8.1, 4.5, H11), 7.94 (1H, d, J = 8.4, H6), 9.30 (1H, dd, J=4.5, 1.8, H10), 9.53 (1H, d, J = 9.3, H5), 9.60 (1H, dd, J = 8.1, 1.8, H12). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.4 (CH_3), 63.1 (OCH_2), 66.0 (CH_2OH), 121.9 (C6), 124.5 (C11), 125.3 (qAr), 126.5 (qAr), 134.6 (C12), 135.1 (C5), 140.0 (q Ar), 140.4 (q Ar), 144.0 (q Ar), 144.4 (q Ar), 147.5 (q Ar), 148.0 (q Ar), 153.4 (C10), 164.7 (C7), 164.9 (CO), 165.0 (CO). m/z (ES^+) 429 (MNa^+).

Diethyl 7-(chloromethyl)dipyrido[3,2-f:2',3'-h]quinoxaline-3,2-dicarboxylate

Phosphorus trichloride (473 mg, 3.44 mmol) was added to a solution diethyl 7-(hydroxymethyl)dipyrido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate (350 mg, 0.86 mmol) in $CHCl_3$ (150 ml). The reaction mixture was heated under reflux for 6 h and allowed to cool down to room temperature afterwards. The reaction was quenched by addition of concentrated Na_2CO_3 solution (150 ml). The layers were separated and the water was extracted with CH_2Cl_2 (3x150ml) and $CHCl_3$ (1x150 ml). The organic layer was dried over K_2CO_3. The solvent was removed under reduced pressure to give the crude product. Purification was achieved by chromatography on alumina (gradient elution: CH_2Cl_2 to 2% CH_3OH-CH_2Cl_2) The product was obtained as a yellow glass (95 mg, 0.22 mmol, 26 %). ^{1}H NMR (300 MHz, CDCl3): δ 1.45 (6H, t, J = 7.2, $OCH_2C\underline{H}_3$), 4.53 (4H, q, J = 7.2, OCH_2), 5.07 (2H, s, CH_2Cl), 7.78 (1H, dd, J = 8.1, 4.5, H11), 8.05 (1H, d, J = 8.4, H6), 9.31 (1H, dd, J = 4.5, 1.8, H10), 9.50 (1H, dd, J = 8.1, 1.8, H12), 9.53 (1H, d, J = 8.7, H5). ^{13}C NMR (75 MHz, $CDCl_3$): δ14.4 (CH_3), 47.3 (CH_2Cl), 63.2 (OCH_2), 123.8 (C11), 124.6 (C6), 125.6 (q Ar), 126.6 (q Ar), 134.5 (C12), 135.7 (C5), 140.1 (q Ar), 140.4 (q Ar), 144.3 (q Ar), 144.4 (q Ar),

147.6 (q Ar), 148.1 (q Ar), 153.8 (C10), 161.0 (C7), 165.0 (CO), 165.1 (CO). m/z (ES^+) 447 (MNa^+).

1-(Diethyl-7'-methyldipyrido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate)-4,7,10-tris-tert-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododecane

Potassium carbonate (11 mg, 0.079 mmol) and a catalytic amount of KI were added to a solution of 1,4,7-tris-*tert*-butoxycarbonylmethyl-1,4,7,10-tetrataacyclododecane (40 mg, 0.077 mmol) and diethyl-7-(chloromethyl)dipyrido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate (35 mg, 0.083 mmol) in acetonitrile (5ml). The mixture was heated under reflux overnight, under argon. The solution was filtered and the salts were washed with CH_2Cl_2. The solvent was removed under reduced pressure to give the crude product. Purification was achieved by chromatography on alumina (gradient elution: CH_2Cl_2 to 2% CH_3OH-CH_2Cl_2). The product was obtained as a yellow glass (40 mg, 0.44 mmol, 56%). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.08 (18 H, s, CH_3), 1.43 (9 H, s, CH_3), 1.50 (3H, t, *J* = 7.2, CH_3), 1.51 (3H, t, *J* = 7.2, CH_3), 2.25-3.58 (24 H, br m, CH_2 ring, NCH_2), 4.58 (2H, q, *J* = 7.2, OCH_2), 4.59 (2H, q, *J* = 7.2, OCH_2), 7.84 (1H, d, J = 8.4, H6), 7.86 (1H, dd, *J* = 8.2, 4.4, H11), 8.98 (1H, dd, *J* = 4.4, 1.8, H 10), 9.53 (1H, d, *J* = 8.4, H5), 9.59 (1H, dd, *J* = 8.2, 1.8, H12). ^{13}C NMR (75 MHz, $CDCl_3$): δ 14.4 ($OCH_2\underline{C}H_3$), 28.0 (CH_3), 28.3 (CH_3), 56.4-60.8 (CH_2 ring), 63.3 (OCH_2), 81.9-82.8 (CH_2CO,CH_2), 124.5 (C6), 124.7 (C11), 125.2 (q Ar), 126.7 (q Ar), 134.9 (C12), 135.2 (C5), 139.9 (q Ar), 140.2 (q Ar), 144.2 (q Ar), 144.7 (q Ar), 147.3 (q Ar), 147.7 (q Ar), 153.3 (C10), 163.2 (C7), 164.8 (COOEt), 164.9 (COOEt), 173.0 (CO). m/z (ES^+) 926 (MNa^+).

$[Tb.2]^{2-}$

A solution of 1-(diethyl-7'-methyldipyrido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate)-4,7,10-tris-tert-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododecane (50 mg, 55 μmol) in 6 M HCl (10 ml) was heated under reflux overnight. The solvent was removed under reduced pressure to give the product. The product was checked by ^{1}H NMR to ensure complete ester hydrolysis, and was used for complexation immediately. ^{1}H NMR (300 MHz, CD_3OD): δ 2.90-4.00 (24 H, br m, CH_2), 8.62 (2H, m, C6, C11), 9.57 (2H, m, C5, C12), 10.34 (H, d, *J* = 8.1, H10). The ligand was dissolved in a mixture of methanol and water (1:1, 6 ml) and the pH was raised to 5.5

by addition of a 1 M solution of aqueous KOH solution. $TbCl_3.6H_2O$ (22 mg, 59 μmol) was added and the mixture heated at 90°C for 48 h. The pH was readjusted to 5.5 after 24 h by 1M solution of KOH. The solvents were removed under reduced pressure. The residue was dissolved water and the pH was adjusted to 7.0 by 1 M solution of KOH and ion exchange was performed on a weakly acidic resin. The solution was freeze-dried to yield a white solid, which was purified by reverse phase HPLC (C-18; MeCN/H_2O). The solvent was removed under reduced pressure to give the product as a white solid (12 mg, 14 μmol, 24 %). $\lambda_{abs}(H_2O)$ 347 nm; τ (H_2O) 1.80 ms. HRMS (ES^-): found: 833.1339 [M-H]; $C_{31}H_{30}N_8O_{10}{}^{159}Tb$ requires 833.1344.

[Eu.2]$^{2-}$

A solution of 1-(Diethyl-7'-methyldyprido[3,2-f:2',3'-h]quinoxaline-2,3-dicarboxylate)-4,7,10-tris-tert-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododecane (50 mg, 55 μmol) in 6 M HCl (10 ml) was heated under reflux overnight. The solvent was removed under reduced pressure to give the product. The product was checked by ^1H NMR to ensure complete ester hydrolysis, and was used for complexation immediately. ^1H NMR (300 MHz, CD_3OD): δ 2.90-4.00 (24 H, br m, CH_2), 8.62 (2H, m, C6, C11), 9.57 (2H, m, C5, C12), 10.34 (H, d, J = 8.1, H10). The ligand was dissolved in a mixture of methanol and water (1:1, 6 ml) and the pH was raised to 5.5 by addition of a 1 M solution of KOH. $EuCl_3.6H_2O$ (22 mg, 60 μmol) was added and the mixture heated at 90°C for 48 h. The pH was readjusted to 5.5 after 24 h by 1M solution of KOH. The solvents were removed under reduced pressure. The residue was dissolved water and the pH was adjusted to 7.0 by 1 M solution of KOH and ion exchange was performed on a weakly acidic resin. The solution was freeze-dried to yield a white solid, which was purified by reverse phase HPLC as above. The solvent was removed under reduced pressure to give the product as a white solid (10 mg, 12 μmol, 22 %). $\lambda_{abs}(H_2O)$ 347 nm; $\tau_{Eu}(H_2O)$ 1.0 ms. HRMS (ES^-): found: 825.1285 [M-H]; $C_{31}H_{30}N_8O_{10}{}^{151}Eu$ requires 825.1289.

Example 3: synthesis of 8 and [Ln.8]

1,4,7-Tris(benzyl-ethyloxyphosphinatomethyl)-10-*tert*-butyoxycarbonyl-1,4,7,10-tetraazacyclododecane

Benzyl diethoxyphosphine (1.4 g, 6.6 mmol) was added to a mixture of 1-*tert*-butoxycarbonyl-1,4,7,10-tetraazacyclododecane (0.4g, 1.47 mmol) and paraformaldehyde (0.3 g) in dry THF (23 ml). The reaction mixture was heated to reflux for 8 hours. The reaction mixture was filtered and the solvent was removed under reduced pressure. The residue was purified by column chromatography on alumina (CH_2Cl_2 to 6% MeOH) to yield the product as an oil (0.3 g, 24%). ^{1}H NMR (300 MHz, $CDCl_3$): δ1.18 (9 H, m, CH_3), 1.39 (9H, s, $C(CH_3)_3$), 2.60-3.80 (28 H, m, NCH_2,PCH_2), 3.95 (6 H, m, OCH_2), 7.30 (15 H, m, phenyl). ^{31}P NMR (120 MHz): δ 50.31 (P). m/z (ES^+) 883.4 ($M+Na^+$).

1,4,7-Tris(benzyl-ethyloxyphosphinatomethyl)-1,4,7,10-tetraazacyclododecane

A solution of 1,4,7-tris(benzyl-ethyloxyphosphinatomethyl)-10-*tert*-butyoxycarbonyl-1,4,7,10-tetraazacyclododecane (450 mg, 0.523 mmol) in CH_2Cl_2 (10 ml) and TFA (20 ml) was stirred at room temperature under argon atmosphere overnight. The solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2 and saturated K_2CO_3 solution. The product was extracted into CH_2Cl_2 (3x50mL) The combined organic extracts were dried over K_2CO_3 and the solvent was removed under reduced pressure to give the product as a pale brown oil (330 mg, 0.434 mmol, 83%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.18 (9 H, m, CH_3), 2.60-3.40 (28 H, m, NCH_2, PCH_2), 3.90 (6 H, m, OCH_2), 7.25 (15 H, m, Phenyl). ^{31}P NMR (120 MHz): δ 51.45 (P). m/z (ES^+) 761.3 (MH^+).

1-(2-Methylazaxanthone)-4,7,10-tris(benzyl-ethyloxyphosphinatomethyl)-1,4,7,10-tetraazacyclododecane

A solution of 1,4,7-tris(benzyl-ethyloxyphosphinatomethyl)-1,4,7,10-tetraazacyclododecane (180 mg, 0.237 mmol), 2-bromomethyl-1-azaxanthone (76 mg, 0.262 mmol) and Cs_2CO_3 (77 mg, 0.236 mmol) in acetonitrile 20 ml was heated to reflux under argon atmosphere for 6 hours. The reaction mixture was filtered and the solvent was removed under reduced pressure. The residue was purified by chromatography on alumina (CH_2Cl_2 to 2% MeOH/CH_2Cl_2) to give the product as a

pale-brown glass (120 mg, 0.124 mmol, 52%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.13 (9H, s, CH_3), 2.4-3.4 (28H, bm, macrocycle, PCH_2, $PhCH_2$), 3.70-4.15 (8H, bm, OCH_2, CH_2), 7.26 (15 H, m, Ph), 7.41 (1H, m, H^7), 7.55 (2 H, m, H^3, H^9), 7.70 (1H, m, H^8), 8.28 (1H, m, H^6), 8.68 (1H, m, H^4). ^{31}P NMR (120 MHz): δ 49.87. m/z (ES^+) 970.4 (MH^+), 992.5 ($M+Na^+$).

[Tb.8]

A solution of 1-(2-methylazaxanthone)-4,7,10-tris(benzyl-ethyloxyphosphinatomethyl)-1,4,7,10-tetraazacyclododecane (35 mg, 36.1 μmol) in 6 M HCl (7 ml) was heated to reflux for 24 hours. The progress of the ester hydrolysis was monitored by ^{1}H and ^{31}P NMR. The solvent was removed under reduced pressure. The residue was dissolved in water (3 ml) and MeOH (3 ml) and $TbCl_3.6H_2O$ (15mg, 40.2 μmol) was added. The pH of the reaction mixture was increased to 5.5 by the addition of 1 M aqueous KOH solution. The reaction mixture was heated to 60^{o}C overnight. The solvents were removed under reduced pressure and the residue was purified by column chromatography on alumina (CH_2Cl_2 to 10% MeOH) to give the product as a light yellow solid (10 mg, 9.6 μmol, 27%).

$\lambda_{abs}(H_2O)$ 335 nm; $\tau_{Tb}(H_2O)$ 3.50 ms, $\tau_{Tb}(D_2O)$ 3.60 ms ϕ_{Tb} (H_2O) 4%; m/z TOF-ESMS: 1043($[M+H]^+$; 1065 ($[M+Na]^+$.

[Eu.8]

A solution of 1-(2-methylazaxanthone)-4,7,10-tris(benzyl-ethyloxyphosphinatomethyl)-1,4,7,10-tetraazacyclododecane (40 mg, 41.2 μmol) in 6 M HCl (6 ml) was heated to reflux for 24 hours. The progress of the ester hydrolysis was monitored by ^{1}H and ^{31}P NMR. The solvent was removed under reduced pressure. The residue was dissolved in water (3 ml) and MeOH (3 ml) and $EuCl_3.6H_2O$ (30 mg, 81.9μmol) was added. The pH of the reaction mixture was increased to 5.5 by the addition of 1 M KOH solution. The reaction mixture was heated to 60^{o}C overnight. The solvents were removed under reduced pressure and the residue was purified by column chromatography on alumina (CH_2Cl_2 to 10% MeOH) to give the product as a pale brown solid (9 mg, 8.7 μmol, 21 %). $\lambda_{abs}(H_2O)$ 335 nm;

$\tau_{Eu}(H_2O)$ 1.20 ms; $\tau_{Eu}(D_2O)$ 1.36 ms ; m/z TOF-ESMS: 1035($[M+H]^+$; 1057 ($[M+Na]^+$.

B Details of Urate Analysis in Urine Samples

In preparing for the assay, three solutions were made up;

1) A stock solution of pH adjusted buffer (0.1m HEPES), to be used for dilution of the assay components and the uric acid containing samples.
2) A 1:1 mixture of europium(III) and terbium(III) complexes of ligand **3** (each typically giving rise a to a solution that is 5 μM concentration in each complex; R = CO_2^-, R' = $CH_2CH_2CO_2^-$) bearing a sensitising moiety and whose absorbance in a buffered aqueous solution was typically 0.1
3) A quantity of uric acid sufficient to make up a 1 mM solution in pH adjusted buffer: this is to be diluted in order to provide a series of solutions from which a calibration plot can be obtained.

The complexes were dissolved separately in the buffer solution and their concentrations adjusted to give absorbances suitable for the sensitivity of the fluorescence multi-well plate reader or fluorimeter to be used; an absorbance of 0.1 allowed a range of detection from 500 nM – 50 μM urate. The two solutions of equal absorbance were combined in equal volumes.

A series of uric acid containing solutions were prepared by dilution of the 1mM stock with reaction buffer. A suitable range of concentrations based on a complex solution with an absorbance of 0.1 were 2, 4, 6, 8, 10, 20, 30, 40, 50 and 100 μM; n.b. the final concentrations in the assay will be 50 % lower.

The uric acid containing samples (in urine or serum) was diluted with buffer; typical dilutions were 500x, 100x and 10x.

The assay is preferably performed using a multiwell plate reader (the following values assume a 96-well plate is used and that the volume in each well is 200 μL; if a fluorimeter or other plate size is used scalings must be made accordingly). The

calibration was performed by adding 100 μL of each of the uric acid solutions to separate wells on the plate followed by 100 μL of the complex solution; each was performed in triplicate to minimise error. Further wells were then filled with 100 μL of each of the uric acid containing samples to be measured and 100 μL of the complex mixture.

Measurements of emission intensity were performed with a fluorimeter or multi-plate reader fitted with an emission analyser, using excitation in a range suitable for the chromophore and for the case of a combined europium(III) and terbium(III) system at emission wavelengths of 546 (Tb $\Delta J = 5$; 616 (Eu $\Delta J = 2$ + Tb $\Delta J = 3$); and 700 nm (Eu $\Delta J = 4$). The inherent differences in sensitivity to quenching of the pair of complexes allows for analysis to be made ratiometrically, each complex effectively acts as an internal reference against which the other is compared.

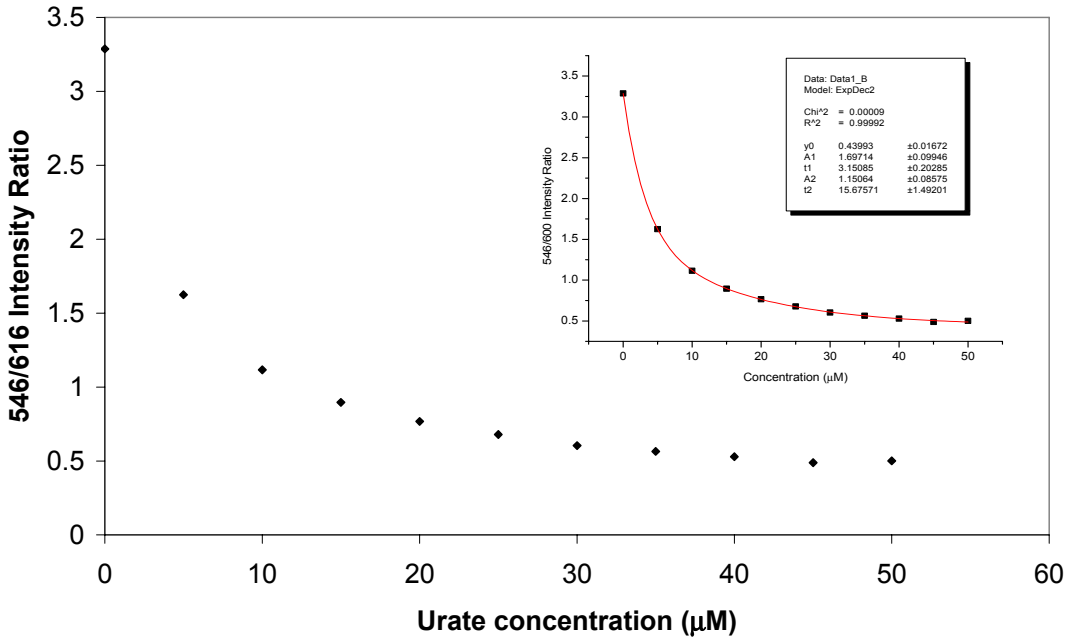
The calibration curve takes the form of a bi-exponential decay generated by plotting urate concentration against either the 546/616 or 546/700 fluorescence intensity ratios. Determination of the sample urate concentration was made by comparison of the appropriate ratio (i.e. 546/616 or 546/700) with the calibration curve, or by fitting the curve mathematically and solving the equation for the ratio.

Values obtained by this method using diluted urine samples from healthy volunteers, (containing varying quantities of ascorbate) were compared to those using a commercial enzymatic kit (Invitrogen, Amplex Red). Each set of data was compared to a standardised solution of sodium urate (assayed by accurate weighing and checked by measuring the absorbance of the solution at 290nm ($\varepsilon = 1.22 \times 10^4$ $M^{-1}cm^{-1}$)). For the calibration of the luminescence assay, intensity ratio measurements were made in triplicate for ten different dilutions of a standardised sodium urate solution. Each value recorded was the mean of ten repetitions, using a 96 well plate and an Analytik Flash Scan 530 (excitation at 313 nm). The measured intensity ratio varied by less than 1% in each case.

In analysing samples of urine of unknown uric acid concentration, the urate concentration obtained by the luminescence method was found to be within 10% of the value estimated by the independent enzymatic assay.

Examples of data obtained and its analysis are given in the two Figures below:

Figure 1: Variation of the emission intensity of the 546 (Tb) / 616 or 700 (Eu) bands as a function of added sodium urate concentration (pH 7.4, 298 K, 0.1 M HEPES buffer). Insets show the fit to the observed decay curves.



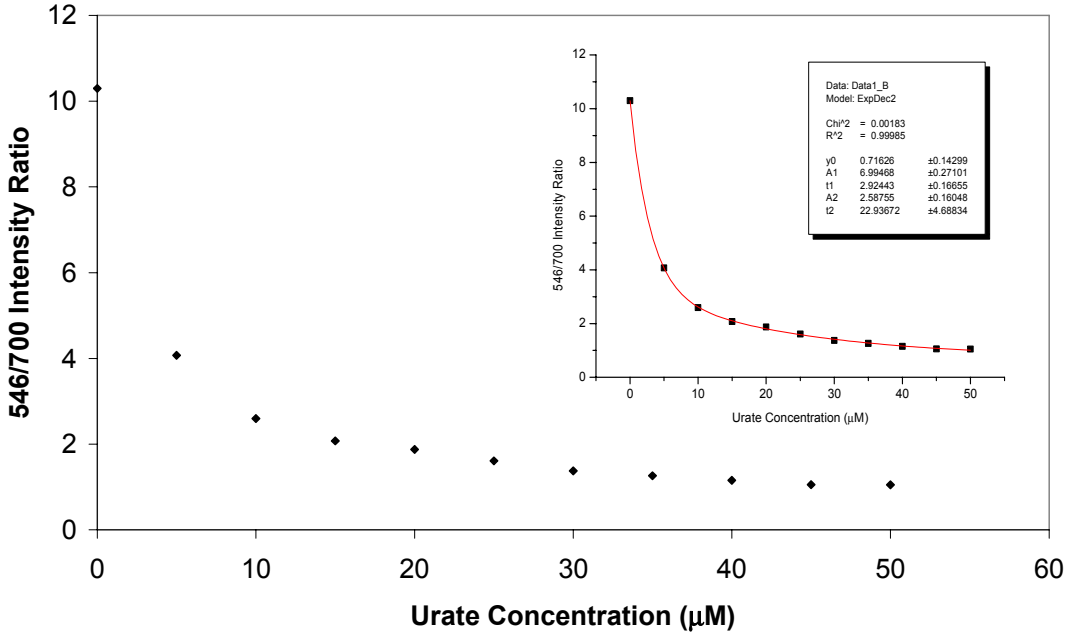


Figure 2: Total emission spectrum for a 1:1 mixture of [Eu.**3**]$^{3-}$ and [Tb.**3**]$^{3-}$ in the presence of increasing concentrations of sodium urate (λ_{ex} 313 nm, pH 7.4, 298 K). The vertical axis refers to relative emission intensity, in each case. Spectral data were recorded on an Analytik Flash Scan 530 with samples in the wells of the multiwell plate reader (excitation at 313 nm in this case)