Effective and efficient sensitisation of terbium luminescence at 355 nm with cell permeable pyrazoyl-1-azaxanthone macrocyclic complexes†

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Emissive terbium complexes, suitable for protein conjugation, incorporating a pyrazoyl-1-aza-xanthone chromophore have been prepared; they exhibit cellular uptake and possess a much lower sensitivity to excited state quenching.

Emissive lanthanide complexes that can be sensitised efficiently are being studied[1,2] as components of bioassays,[3] spatially localised sensors,[4] or as donors in time-resolved energy transfer systems.[5,6] There is a need to find long-lived emissive probes suitable for application in living cells.[7,8] This requires that they be non-toxic and cell permeable, resist photobleaching and photofading, exhibit kinetic stability with respect to degradation and be immune to quenching of the excited state of the lanthanide(III) ion.[9] Well-established ligands have been studied that present 8 or 9 donors and incorporate a heterocyclic sensitising moiety that harvests incident light efficiently (large ε) and transfers its energy in an intramolecular process to generate the lanthanide excited state.[10] The ligand is designed to inhibit vibrational deactivation of the lanthanide(III) excited state, which is particularly problematic with proximate OH and NH oscillators.[11]

Recently, we introduced substituted 1-azaxanthone and azathiaxanthones as effective sensitisers for Eu and Tb emission in aqueous media.[7a,9] In extending their utility, we set out to incorporate an added donor atom, extend the conjugation length and increase the excitation wavelength. A 2-pyrazoyl group is suitable for this purpose, as 2-pyrazoylpyridines constitute a well-studied class of ligand in coordination chemistry[12] and are effective donors in Ln^{III} complexes.[13] Such a group, *e.g.* **1a**, could then be incorporated into a nonadentate ligand, such as $\mathbf{L^1}$ or $\mathbf{L^2}$, with the expectation that the derived Tb(III) complex excludes water from the immediate coordination environment.

Reaction of 7-*tert*-butyl-1-azaxanthone, **2a**, with methyl triflate followed by oxidative hydrolysis ($Fe(CN)_6^{3-}$, H_2O) and chlorination ($POCl_3$) of the intermediate *N*-methylpyridone yielded the 2-chloro derivative, **2b**, in 40% overall yield after chromatographic purification. Reaction with 2-methylpyrazole (NaH/THF) followed by selective bromination of the methyl group (NBS, CCL_4, 0.2% (^{t}BuO)$_2$O$_2$) gave the bromomethyl derivative, **1b**, in 56% yield.† Stepwise reaction of **1b** with 1,4,7-*tris*(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (MeCN, Cs_2CO_3) followed by treatment with trifluoroacetic acid (50% in CH_2Cl_2, 20 °C) afforded L^1 as the trifluoroacetate salt. The enantiopure ligand $\mathbf{L^2}$ was prepared in a different sequence: reaction of 1,4,7-*tris*(*tert*-butoxycarbonyl)-1,4,7,10-tetraazacyclododecane with **1b** (MeCN, K_2CO_3, 20 °C) gave the 10-alkyl derivative and treatment with TFA (50% in CH_2Cl_2, 20 °C) followed by *N*-alkylation with 3 equiv. of (*S*)-**3a**(CH_3CN, Cs_2CO_3, 1% KI) yielded (*SSS*)-$\mathbf{L^{2a}}$. Similarly, reaction of (*S*)-**3b** gave the *p*-carboxymethyl analogue, (*SSS*)-$\mathbf{L^{2b}}$. The Tb(III) complexes of $\mathbf{L^1}$ and $\mathbf{L^2}$ were prepared by reaction in aqueous methanol ($TbCl_3$) and MeCN ($Tb(CF_3SO_3)_3$) respectively. The complex [Tb.$\mathbf{L^2}$]$(CF_3SO_3)_3$) was converted into the chloride salt by anion exchange chromatography to enhance water solubility. Controlled base hydrolysis of [Tb.$\mathbf{L^{2b}}$]Cl_3 (pH 10.5, 20 °C) gave the acid, offering a route to the preparation of protein conjugates.

Absorption, emission and triplet energy data for [Tb.$\mathbf{L^1}$] and [Tb.$\mathbf{L^2}$]$^{3+}$ are collated in Table 1, compared to the mono-aqua azaxanthone analogues [Tb.$\mathbf{L^3}$]/[Tb.$\mathbf{L^4}$]$^{3+}$ and the related tetraaza-triphenylene complexes [Tb.$\mathbf{L^5}$]/[Tb.$\mathbf{L^6}$]$^{3+}$ that lack a bound water molecule. The absorption spectrum of [Tb.$\mathbf{L^1}$] shows a moderately intense long wavelength band at 348 nm (ε 15,050 $M^{-1}cm^{-1}$); it absorbs well at 355 nm, a common laser excitation wavelength. The molar extinction coefficient for [Tb.$\mathbf{L^1}$] is about twice that of the [Tb.$\mathbf{L^3}$]/[Tb.$\mathbf{L^5}$] analogues. The total emission spectrum, (Fig. 1), reveals the expected Tb spectral fingerprint (τ_{H_2O} 2.24 ms for [Tb.$\mathbf{L^1}$], and also shows azaxanthone fluorescence (ϕ_{em}^{f} ~15%) centred at 445 nm. This ligand-based emission, whilst limiting the metal-based quantum yield, provides an observable band for luminescence microscopy and facilitates flow cytometric studies. In the terbium complex [Tb.$\mathbf{L^{2b}}$]$^{3+}$, this ligand emission comprised only 7% of the total observed emission and the terbium quantum yield was 61% (295 K, H_2O), a high value for such systems.[1,2]

Circularly polarised emission spectra (ESI†) for the Δ(*SSS*) and Λ(*RRR*) terbium(III) complexes of ligand $\mathbf{L^{2a}}$ were recorded (295 K, pH 5.5). Emission dissymmetry factors (*g* values) for the (*SSS*)-isomer are +0.02 (489 nm), −0.13 (540 nm), +0.01 (583 nm); mirror image CPL spectra were recorded for the (*RRR*) isomer. The sign and sequence of the transitions observed are the opposite to those observed for (*SSS*)-[Tb.$\mathbf{L^6}$]$^{3+}$. This complex has been established (by correlation to the behaviour of the related (*SSSS*)-tetra-amide system, examined by X-ray crystallography[9,14]) to possess a $\Delta(\lambda\lambda\lambda\lambda)$ chirality for the lay out of the ring pendant arms and the NCCN chelates of the ring respectively. Thus, the Tb complexes of $\mathbf{L^2}$ adopt the alternate square-antiprismatic geometry, *i.e.* (*SSS*)-Λ-($\delta\delta\delta\delta$). Such stereocontrol has been noted in detailed analyses of amino-acid adducts of lanthanide(III) complexes, with analogous heptadentate ligands.[14]

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† Electronic supplementary information (ESI) available: Experimental details for the synthesis of key intermediates, **1b**, L^1, L^{2a} L^{2b} and their Tb(III) complexes, as well as selected total emission and circularly polarised spectra. See DOI: 10.1039/b709805g

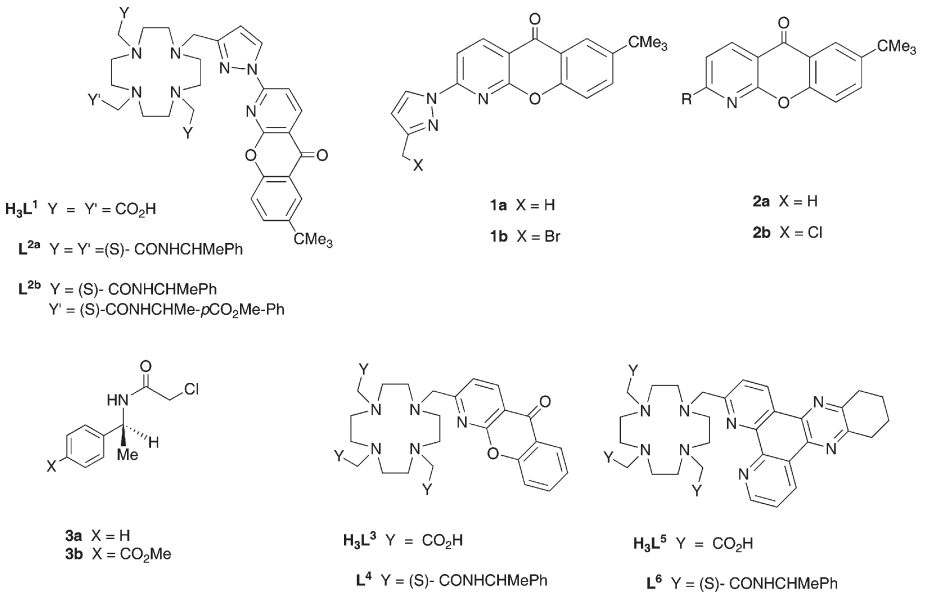

Table 1 Absorption, emission, lifetime (± 10%) triplet energies[a,b,c] (77 K) and quantum yield (± 15%) data for Tb(III) complexes of $\mathbf{L^1}$–$\mathbf{L^5}$

Complex	λ_{max}/nm	ε/M^{-1} cm^{-1}	$k^{Tb}_{H_2O}$/ms^{-1}	$k^{Tb}_{D_2O}$/ms^{-1}	θ^{Tb}_{em} (H_2O)	E^{Gd}_T /cm^{-1} (77 K)
[Tb$\mathbf{L^1}$]	348	15,050	0.44	0.37	15	23,450
[Tb$\mathbf{L^{2a}}$]$^{3+}$	348	15,050	0.50	0.42	18	23,470
[Tb$\mathbf{L^{2b}}$]$^{3+}$	349	15,050	0.45	0.36	61	23,470
[Tb$\mathbf{L^3}$]	336	6,900	0.55	0.37	24	24,800
[Tb$\mathbf{L^4}$]$^{3+}$	335	6,900	0.61	0.35	37	24,800
[Tb$\mathbf{L^5}$]	347	8,300	0.68	0.60	33	23,800
[Tb$\mathbf{L^6}$]$^{3+}$	348	8,300	0.64	0.58	40	23,800

[a] Triplet energies were measured on Gd analogues in a frozen glass of Et_2O/isopentane/ethanol or EtOH/MeOH (2 : 1). [b] A broad azaxanthone ligand fluorescence was observed at 445 nm (*ca.* 15% ϕ_f) for complexes of $\mathbf{L^1}$/$\mathbf{L^{2a}}$; this was less intense than for $\mathbf{L^3}$/$\mathbf{L^4}$; no ligand fluorescence is observed with $\mathbf{L^5}$/$\mathbf{L^6}$, and only about 5% for the Tb complex of $\mathbf{L^{2b}}$. [c] q values of zero for the terbium complexes of $\mathbf{L^1}$ and $\mathbf{L^{2a}}$, were estimated by applying a well-established method, based on analysis of radiative decay constants in H_2O and D_2O.[15]

A key issue in assessing intracellular applications is their sensitivity towards quenching by endogenous electron-rich species (urate/ascorbate) either when the complexes are free or protein bound (covalent or non-covalent).[9] Accordingly, the Stern–Volmer quenching constants (K_{sv}^{-1}/mM) defining their relative sensitivity to dynamic quenching (the variation of emission intensity mirrored the measured lifetime change) have been assessed (Table 2). The quenching process with urate is believed to involve intermediate exciplex formation. This is disfavoured as the sensitising chromophore becomes less susceptible to accept electron density and is reflected in the ligand reduction potential. For **1a**, this was −1.52 V (*vs* NHE, 298 K, 0.1 M NBu_4ClO_4, MeCN), compared to −1.07 V for the cyclohexyl tetraazatriphenylene and −1.60 V for the 1-aza-xanthone **2a**. Terbium complexes of $\mathbf{L^1}$ and $\mathbf{L^2}$ resisted urate and ascorbate quenching more effectively than the comparator complexes. Non-covalent protein binding of the Tb complex by serum albumin inhibits this process.[9] Incremental addition of bovine serum albumin to [Tb.$\mathbf{L^{2a}}$]$^{3+}$ resulted in less than a 7% decrease in the terbium emission lifetime over the range 0.01 to 0.7 mM added protein. In the presence of 0.4 mM human serum albumin, 0.1 mM urate and 0.2 mM ascorbate, (pH 7.4, 298 K, 0.1 M HEPES), the emission lifetime of [Tb.$\mathbf{L^{2a}}$]$^{3+}$ was

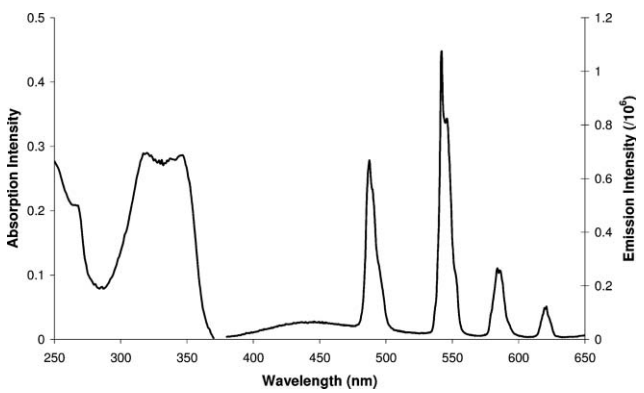

Fig. 1 Absorption (*left*) and total emission spectrum for [Tb.$\mathbf{L^{2a}}$]$^{3+}$ (pH 7.4 295 K, 0.1 M HEPES).

Table 2 Sensitivity of terbium complexes to excited state quenching by urate, ascorbate or iodide (pH 7.4, 0.1 M HEPES, 298 K)

Complex	K_{sv}^{-1} (urate)/mM	K_{sv}^{-1} (ascorbate)/mM	K_{sv}^{-1} (I^-)/mM
[Tb$\mathbf{L^1}$]	0.03	1.39	5.35
[Tb$\mathbf{L^2}$]$^{3+}$	0.055	0.36	13.8
[Tb$\mathbf{L^3}$]	0.012	0.57	53.5
[Tb$\mathbf{L^4}$]$^{3+}$	0.04	0.37	9.2
[Tb$\mathbf{L^5}$]	0.006	0.38	2.1
[Tb $\mathbf{L^6}$]$^{3+}$	0.025	0.18	0.9

[a] One electron oxidation potentials for ascorbate, iodide and urate follow the sequence: 0.30, 0.54 and 0.59 V (*vs* NHE) respectively.

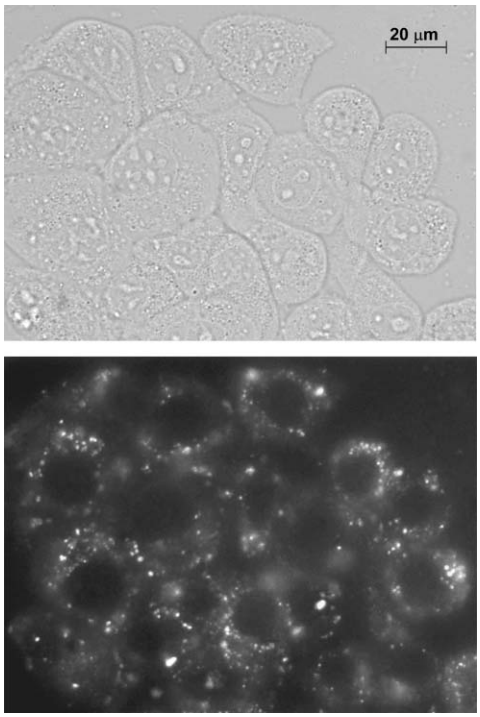

Fig. 2 Luminescence microscopy images of $[Tb.\mathbf{L}^{2a}]^{3+}$ in CHO cells following incubation for 4 h (50 μM complex; observing ligand (445 nm) or Tb (545 nm) emission gave super-imposable images).

within 10% of its value in water, *i.e.* 2.1 ms, compared to 0.7 ms and 0.5 ms for $[Tb.\mathbf{L}^{6}]^{3+}$ and $[Tb.\mathbf{L}^{4}]^{3+}$ respectively, under the same conditions. Thus, protein association does not quench the excited state of $[Tb.\mathbf{L}^{2}]^{3+}$, and the presence of protein shields the complex from quenching.

The complex $[Tb.\mathbf{L}^{2a}]^{3+}$ was incubated for varying periods of time (1 to 12 h; 50 or 100 μM complex) with CHO (Chinese hamster ovarian) or NIH-3T3 (mouse skin fibroblast) cells, under standard conditions used previously to examine $[Tb.\mathbf{L}^{4}]^{3+}$ and $[Tb.\mathbf{L}^{6}]^{3+}$. Examination of the loaded cells by luminescence microscopy revealed complex uptake, (Fig. 2), and localisation, presumably following receptor mediated endocytosis within endosomes in the cytoplasm, but without the tendency for nuclear localisation observed with $[Tb.\mathbf{L}^{6}]^{3+}$ and $[Tb.\mathbf{L}^{4}]^{3+}$. Such behaviour augurs well for the application of such complexes as emissive probes or components of intracellular assays.

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Notes and references

1 S. Pandya, J. Yu and D. Parker, *Dalton Trans.*, 2006, 2757; T. Gunnlaugsson and J. P. Leonard, *Chem. Commun.*, 2005, 3114; R. J. Aarons, J. K. Notta, M. M. Melani, R. Vidyasagar, J. Narvainen, S. Allan, N. Spencer, R. A. Kauppinen, J. S. Snaith and S. Faulkner, *Chem. Commun.*, 2006, 909; D. Parker, *Chem. Soc. Rev.*, 2004, **33**, 156.

2 T. Gunnlaugsson, J. P. Leonard, K. Senechal and A. K. Harte, *Chem. Commun.*, 2004, 782; S. Petoud, E. Muller, E. G. Moore, J. D. Xu, J. Sokolnicki, J. P. Riehl, U. N. Le, S. M. Cohen and K. N. Raymond, *J. Am. Chem. Soc.*, 2007, **128**, 77; R. S. Dickins, J. A. K. Howard, J. M. Moloney, D. Parker and R. D. Peacock, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 521.

3 M. Gabourdes, V. Bourgine, G. Mathis, H. Bazin and B. Alpha-Bazin, *Anal. Biochem.*, 2004, **333**, 105; D. Maurel, J. Kriazeff, G. Mathis, E. Trinquet, J. P. Pin and H. Ansanay, *Anal. Biochem.*, 2004, **329**, 253.

4 T. Gunnlaugsson, C. P. McCoy and F. Sorneo, *Tetrahedron Lett.*, 2004, **45**, 8403; S. Blair, M. P. Lowe, C. E. Mathieu, D. Parker, P. K. Senanayake and R. Kataky, *Inorg. Chem.*, 2001, **40**, 5860; D. Parker and J. A. G. Williams, *Chem. Commun.*, 1998, 245; O. Reany, T. Gunnlaugsson and D. Parker, *J. Chem. Soc., Perkin Trans. 2*, 2000, 1819.

5 H. Bazin, E. Trinquet and G. Mathis, *Rev. Mol. Biotechnol.*, 2002, **82**, 233.

6 P. R. Selvin, *Annu. Rev. Biophys. Biomol. Struct.*, 2002, **31**, 275; I. Hemmila and V.-M. Mukkala, *Crit. Rev. Clin. Lab. Sci.*, 2001, **38**, 441.

7 J. Yu, D. Parker, R. Pal, R. A. Poole and M. J. Cann, *J. Am. Chem. Soc.*, 2006, **128**, 2294; K. Hanoaka, K. Kikuchi, H. Kojima, Y. Urano and T. Nagano, *J. Am. Chem. Soc.*, 2004, **126**, 12470; G. Bobba, J.-C. Frias and D. Parker, *Chem. Commun.*, 2002, 890; H. C. Manning, S. M. Smith, M. Sexton, S. Haviland, M. F. Bai, K. Cederquist, N. Stella and D. J. Bornhop, *Bioconjugate Chem.*, 2006, **17**, 735.

8 A.-S. Chauvin, C. Vandevyver, S. Comby and J.-C. G. Bunzli, *Chem. Commun.*, 2007, 1716; D. Parker and R. Pal, *Chem. Commun.*, 2007, 474; J.-C. Frias, G. Bobba, M. J. Cann, D. Parker and C. J. Hutchison, *Org. Biomol. Chem.*, 2003, **1**, 905.

9 R. A. Poole, F. Kielar, S. L. Richardson, P. A. Stenson and D. Parker, *Chem. Commun.*, 2006, 4084; R. A. Poole, G. Bobba, M. J. Cann, J.-C. Frias, D. Parker and R. D. Peacock, *Org. Biomol. Chem.*, 2005, **3**, 1013; R. A. Poole, C. P. Montgomery, E. J. New, A. Congreve and D. Parker, *Org. Biomol. Chem.*, 2007, **5**, 2055.

10 R. Ziessel, N. Weibel, L. J. Charbonniere, M. Guardigli and A. Roda, *J. Am. Chem. Soc.*, 2004, **126**, 4888; B. Song, E. Wang and J. Yuan, *Chem. Commun.*, 2005, 3553; M. Xiao and P. R. Selvin, *J. Am. Chem. Soc.*, 2001, **123**, 7067.

11 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; R. S. Dickins, A. S. de Sousa, D. Parker and J. A. G. Williams, *Chem. Commun.*, 1996, 697.

12 H. Adams, W. Z. Alsindi, G. M. Davies, M. B. Duriska, T. L. Easun, H. E. Fenton, J. M. Herrera, M. W. George, K. L. Ronayne, X. L. Sun, M. Towrie and M. D. Ward, *Dalton Trans.*, 2006, 39; Y. Mulyana, C. J. Kepert, C. F. Lindoy, A. Parkin and P. Turner, *Dalton Trans.*, 2005, 1598.

13 E. Brunet, O. Juanes, R. Sedano and J.-C. Rodriguez-Ubis, *Org. Lett.*, 2002, **4**, 213; J. C. Rodriguez-Ubis, R. Sedano, G. Barroso, O. Juanes and E. Brunet, *Helv. Chim. Acta*, 1997, **80**, 86; E. Brunet, O. Juanes, R. Sedano and J. C. Rodriguez-Ubis, *Tetrahedron*, 2005, **61**, 6757.

14 R. S. Dickins, A. S. Batsanov, J. A. K. Howard, D. Parker, H. Puschmann and S. Salamano, *Dalton Trans.*, 2004, 70.

15 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.